ORIGIN BINDING PROTEINS AND THEIR ROLE IN MAMMALIAN DNA REPLICATION

by

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To my parents,

Georgia and Jim Dimitrios

&

To my grandmother,

Sophia

ABSTRACT

A 186-bp fragment of *ors*8, a mammalian DNA replication origin, was previously identified as the minimal sequence required for origin function *in vivo* and *in vitro*. The 186-bp origin contains, among other features, an octamer motif, serving as the binding site for the transcription factor Oct-1, and an A3/4-homologous region, serving as the binding site for the origin binding protein, OBA. The research objective of this thesis is to investigate the role of Oct-1 and OBA in mammalian *in vitro* DNA replication.

Depletion of the Oct-1 protein inhibited *in vitro* DNA replication to basal levels. The inhibition was partially reversed upon the exogenous addition of Oct-1 POU protein. Site-directed mutagenesis of the octamer motif in the origin resulted in a mutant clone that lacked Oct-1 binding but replicated efficiently. The results suggest that Oct-1 is an enhancing component in DNA replication but its effect is not caused through direct DNA binding, but rather through protein-protein interactions.

OBA was purified from HeLa cells through its ability to interact with the 186-bp origin. OBA binds to A3/4, a 36-bp mammalian origin sequence. The DNA binding subunit of OBA is identical to the 86 kDa subunit of Ku antigen. Depletion of OBA/Ku, by inclusion of anti-Ku antibodies to the *in vitro* replication reaction of p186, inhibited DNA replication. Furthermore, addition of the A3/4 oligonucleotide to the replication reaction of mammalian origin-containing plasmids (p186, p*ors*12, and pX24) inhibited replication. This inhibition was reversed upon addition of OBA. In contrast, SV40 *in vitro* replication remained unaffected. OBA also possessed DNA helicase activity. By co-immunoprecipitation analyses, OBA was found to associate with DNA polymerases δ and ε, PCNA, topoisomerase II, RF-C, RP-A, DNA-PKcs, and Oct-1.

The replication activity of *xrs*-5, a derivative of Chinese hamster ovary (CHO) K1 cell line defective in Ku86, was also examined. Total and cytoplasmic extracts from *xrs*-5 cells replicated p186 DNA as efficiently as CHO K1 extracts. In contrast, *xrs*-5 nuclear cell extracts lacked DNA replication activity, which was restored upon addition of OBA.

The data implicate OBA/Ku in a direct role in the initiation of mammalian DNA replication as an origin-specific binding protein with helicase activity. The physical association of OBA/Ku with Oct-1 and the replication machinery reveals a possible mechanism by which these proteins are recruited to DNA replication origins. A cytoplasmic Ku-like origin-specific binding protein likely compensates for the absence of the endogenous OBA/Ku in *xrs*-5 cells.

These studies provide a better understanding of the proteins that bind to mammalian origins, thereby aiding in understanding the mechanisms that regulate the initiation of DNA replication.

RÉSUMÉ

Auparavant, nous avons demontré qu'un fragment de 186-pb de *ors*8, une origine de réplication d'ADN de mammifère, était la séquence minimale requise pour la fonction de l'origine *in vivo* et *in vitro*. Le fragment de 186-pb contient un motif octamer qui sert comme site de liaison pour le facteur de transcription Oct-1 et un site homologue à A3/4, qui sert comme un site de liaison pour OBA. L'objective de recherche de cette thèse est de caractériser le rôle d'Oct-1 et d'OBA dans la réplication mammifère *in vitro*.

L'épuisement d'Oct-1 a inhibé *in vitro* la réplication de l'ADN à un niveau basale. Cette inhibition a été partiellement inversée par l'addition exogéne de la protéine POU d'Oct-1. La mutagenèse dirigée au site de liaison d'Oct-1 dans l'origine a produit un clone muté qui n'intéragit pas avec Oct-1 mais qui possède une activité de réplication comparable au clone sauvage. Les résultats suggérent qu'Oct-1 est une composante amplificateur dans la réplication de l'ADN, cependant ces effets ne sont pas causés par la liaison directe de l'ADN mais par l'intéraction protéines-protéines.

OBA a été purifié des cellules HeLa en utilisant un plan de purification basé sur le fait que OBA intéragit avec l'origine de 186-pb. OBA se lie aussi à A3/4, une séquence d'origine de 36-pb. Le polypèptide d'OBA qui se lie à l'ADN est identique à Ku86. L'épuisement d'OBA par l'utilization d'un anticorps à la réaction *in vitro* a inhibié la réplication de l'ADN de p186. En outre, l'addition d'un oligonucleotide comprenant la séquence A3/4 dans la réplication *in vitro* de plasmides contenant des origines mammifères a aussi inhibé la réplication. L'inhibition a été reversée par l'addition de la protéine OBA. Par contre, la réplication *in vitro* de SV40 n'a pas été affecté. OBA possède une activité d'helicase. De plus, nous avons demontré une interaction avec OBA et des protéines requises pour la réplication par co-immunoprecipitation, incluant Oct-1.

L'activité de réplication de *xrs*-5, une lignée dérivée des cellules CHO, manquante Ku86, a été aussi étudiée. Les extraits cellulaires cytoplsmiques et totaux de *xrs*-5 avaient une activité de réplication semblable aux cellules CHO avec le phénotype sauvage, tandis que les extraits nucléaires ne possédait pas d'activité de réplication. Lorsque OBA a été ajouté aux extraits nucléaires, leur activité de réplication a été rétablie.

Les résultats impliquent OBA/Ku dans la réplication de l'ADN mammifère comme une protéine qui se lie à l'origine de réplication avec une activité d'helicase. L'interaction de OBA/Ku avec Oct-1 et les protéines de réplication suggère un mécanisme où ces protéines sont recrutées à l'origine de réplication. Une protéine cytoplasmique semblable a Ku probablement remplace la protéine endogène OBA/Ku dans les cellules *xrs*-5.

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Dia Matheos, December 2000

PREFACE

This doctoral thesis was written in accordance to the Guidelines for Submitting a Doctoral or Master's Thesis, by the Faculty of Graduate Studies and Research, McGill University. The guidelines state:

"As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following:

(a) a table of contents; (b) an abstract in English and French; (c) an introduction which clearly states the rational and objectives of the research; (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); (e) a final conclusion and summary;

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis."

CONTRIBUTION OF AUTHORS

Chapter Two: **Matheos, D.,** Ruiz, M.T., Price, G.B., and Zannis-Hadjopoulos, M. "Oct-1 enhances the *in vitro* replication of a mammalian autonomously replicating DNA sequence." 1998 *J. Cell. Biochem.* **68:** 309-327.

I carried out all the experiments of this manuscript, with the exception of the bandshift reaction presented in Figure 2B, which was performed by Marcia Ruiz, who is co-author. I also wrote and revised the manuscript. Maria Zannis-Hadjopoulos and Gerald B. Price supervised and co-supervised, respectively, the project and are co-authors.

Chapter Three: Ruiz, M.T., **Matheos, D.,** Price, G.B., and Zannis-Hadjopoulos, M. "OBA/Ku86: DNA binding specificity and involvement in mammalian DNA replication". 1999 *Mol. Biol. Cell* **10:**567-580.

I performed the *in vitro* replication assays and wrote the corresponding sections of the manuscript. Marcia Ruiz performed the purification of OBA, the Western blot, and the bandshift reactions. Maria Zannis-Hadjopoulos and Gerald B. Price supervised and co-supervised, respectively, the project and are co-authors.

Chapter Four: **Matheos, D.,** Ruiz, M.T., Price, G.B., and Zannis-Hadjopoulos, M. " OBA/Ku: An origin-specific binding protein that associates with replication proteins, is required for mammalian DNA replication". Submitted *Mol. Biol. Cell*

I performed all the experiments and wrote the manuscript. Marcia Ruiz purified OBA and is co-author. Maria Zannis-Hadjopoulos and Gerald B. Price supervised and co-supervised, respectively, the project and are co-authors.

Chapter Five: **Matheos, D.,** Price, G.B., and Zannis-Hadjopoulos, M. "Analysis of the replication competence of the *xrs*-5 mutant cells defective in Ku86". Submitted *Biochim. Biophys. Acta.*

I performed all the experiments and wrote the manuscript. Maria Zannis-Hadjopoulos and Gerald B. Price supervised and co-supervised, respectively, the project and are co-authors.

Additional publications by the author can be found at the end of this thesis (page 162).

CLAIMS TO ORIGINALITY

The following results, presented in this thesis and constituting my own work, are original:

- The Oct-1 transcription factor enhances mammalian *in vitro* DNA replication.
- Oct-1 does not enhance replication by direct binding to the octamer motif in the origin, but rather through protein-protein interactions.
- OBA is Ku antigen.
- OBA/Ku is involved in mammalian *in vitro* replication, at the level of initiation, as an origin specific binding protein that requires an A3/4 homologous region.
- OBA is not involved in DNA elongation.
- DNA-PKcs and the GATA transcription factor are not involved in mammalian DNA replication.
- OBA/Ku interacts with: proteins of the replication machinery including DNA polymerases ε and δ, DNA topoisomerase II, RF-C, PCNA, RP-A, DNA-PKcs, and Oct-1.
- OBA has DNA helicase activity.
- Total and cytoplasmic extracts from *xrs*-5 cells support replication as efficiently as extracts from wild-type CHO K1 cells.
- The defective replication ability of the *xrs*-5 nuclear cell extracts is restored upon addition of OBA.
- *Xrs*-5 cells have an A3/4 sequence-specific binding protein in the cytoplasm that allows for efficient DNA replication.

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LIST OF ABBREVIATIONS

aa	amino acids
ABF-1	ARS binding factor 1
Ad pol	Adenovirus polymerase
ARS	Autonomously replicating sequence
apOBA	affinity-purified OBA
Aux	Auxiliary element
bp	base pairs
BPV	bovine papilloma virus
BrdUTP	bromodeoxyuridine
BSA	bovine serum albumin
CBP	cruciform binding protein
Cdc6/Cdc45	cell division cycle protein
CHO	Chinese hamster ovary
CHO K1 C	CHO K1 cytoplamic extracts
CHO K1 N	CHO K1 nuclear extracts
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
DNase	DNA nuclease
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	catalytic subunit of DNA-PK
Dnmt-1	DNA methyltransferases
ds	double-stranded
DSB	double strand break
ddTTP	didioxy-TTP
DUE	DNA unwinding element
EBV	Epstein-Barr virus
EMSA	electrophoretic mobility assay
FEN-1	Flap endonuclease 1
FT	flowthrough
G1 or G2	gap 1 or gap 2 phase of the cell cycle
HDH II	Human DNA helicase II
HSV	Herpes simplex virus
IR	inverted repeat
kbp	kilobase pair
kDa	kilodalton
M	mitotic phase
MAR	matrix attachment region
MCM	mini chromosome maintenance
MMTV	minute virus of mouse
NC	nuclear and cytoplasmic cell extracts
NF-1	nuclear factor 1
NRE-1	negative regulatory element 1
NRS	normal rabbit serum
NSF	normal skin fibroblast
nt	nucleotide
NTP	nucleoside triphosphates
OBA	Origin binding activity
ORC	ori recognition complex
ori	origin
ors	origin enriched sequences
ORE	origin recognition element
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
pTP	pre-terminal protein of adenovirus
RF-C	replication factor C
RI	replicative intermediates
RNA	ribonucleic acid
RNase	RNA nuclease
RP-A	replication protein A
S	DNA synthesis phase
SCID	severe combined immunodeficiency
Sir	silence information regulatory proteins
ss	single-stranded
SV40	simian virus 40
T-Ag	T antigen
TP	Adenovirus terminal protein
V(D)J	variable, diversity, joining genes
WRN	Werner protein
UV	ultra violet light
YAC	yeast artificial chromosome

CHAPTER ONE

Introduction

Mammalian DNA replication:
Origins, proteins, model systems and regulatory mechanisms

I. OVERVIEW AND RESEARCH OBJECTIVES

Cellular proliferation is a central event in a variety of medical research fields, including cancer. The regulatory mechanisms that govern mammalian DNA replication must be well understood in order to develop a complete knowledge of growth and control at the cellular level. A distinguishing characteristic of cancer cells is their abnormal response to regulatory mechanisms that control the division of normal cells, thus allowing then to divide in an uncontrolled manner.

DNA synthesis is a multistep process that is highly regulated through protein-protein and protein-DNA interactions at the origin of replication during the initiation step. In mammalian cells, DNA replication initiates at approximately 10^4-10^6 replication origins and proceeds bidirectionally. Clusters of these origins are activated and replicated in a defined spatial and temporal order. In normal mammalian cells, each origin is activated only once per cell cycle. Although evidence for specific chromosomal origins in mammalian cells exists, it remains to be established what DNA sequences serve as cellular replication origins, how their activation is coordinated, what are their distinguishing characteristics, and which proteins activate them.

The research objectives of this thesis were:

- To investigate the role in DNA replication of a transcription factor binding site, the octamer motif, present in a mammalian origin.
- To study the involvement of the transcription factor Oct-1 in mammalian DNA replication.
- To characterize an origin binding protein (OBA/Ku).
- To determine the binding specificity of OBA/Ku to a mammalian origin consensus sequence termed A3/4.
- To study the involvement of OBA/Ku in mammalian DNA replication.
- To investigate the interaction of OBA/Ku with the DNA replication machinery.
- To determine the DNA replication activity of *xrs*-5 mutant cells that are defective in OBA/Ku.

II. STRUCTURAL FEATURES OF REPLICATION ORIGINS

Almost half a century ago, Jacob, Brenner and Cuzin (1963) proposed the replicon model to describe bacterial DNA replication. In this model, a cis-acting replicator is bound by a trans-acting initiator, and initiation of DNA replication occurs. The replicon was defined as a genetic element that replicates as a unit with a single origin. A replication origin is defined either as a genetic origin or replicator, which serves as the binding site for the initiator protein, or as a functional origin, which represents the actual site where replication begins. The replicator and the initiation site coincide in bacteria, bacterial plasmids, bacteriophages, viruses, yeast and mitochondria. Metazoan origins, however, appear more complex and are determined by multiple parameters.

Nevertheless, several mammalian origins of DNA replication have been identified (reviewed in Boulikas; 1996, Todovoric et al., 1999). The mapped origins are quite heterogeneous in size, chromosomal localization and activation time in S phase. A good understanding of the function and sequence characteristics of origins of DNA replication is required to gain knowledge on the mechanism of differential gene expression. The study of origins in model systems has revealed several DNA sequence and structural elements that are common to prokaryotes and to lower and higher eukaryotes (reviewed in DePamphilis, 1993; Boulikas, 1996; DePamphilis, 1996; Pearson et al., 1996; Zannis-Hadjopoulos and Price, 1998; Zannis-Hadjopoulos and Price, 1999 and references therein).

Origins of DNA replication can be subdivided in two basic components, the core and the auxiliary element (Figure 1). The core element represents the minimal essential sequence required for initiation of DNA replication that is indispensable for origin function. The origin auxiliary element represents the flanking sequence of the core element and is dispensable. The auxiliary sequences increase the activity of the core origin and usually represent binding sites for transcription factors.

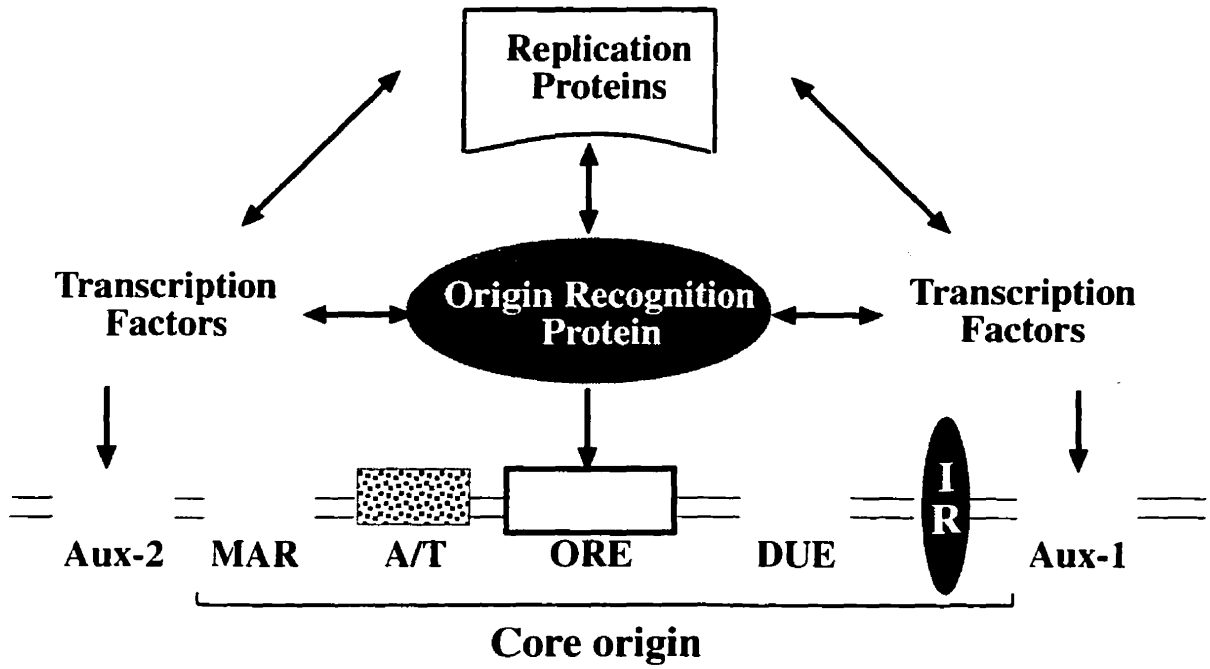


Figure 1. Structure of an eukaryotic origin of DNA replication. The origin recognition element (ORE), DNA unwinding element (DUE), matrix attachment region (MAR), AT-rich, inverted repeat (IR) extruded into a cruciform, and the auxiliary sequences are shown (see text for detail).

i) Core element

The core origin consists of several elements including the origin recognition element (ORE), the DNA unwinding element (DUE), an AT-rich sequence, bent DNA, nuclear matrix attachment regions (MAR) and inverted repeats (IR).

A. Origin recognition element (ORE)

The ORE represents the binding site of the origin recognition protein, which is required to initiate DNA replication. In simple genomes, the ORE represents a consensus binding sequence for the initiator protein. For instance, Simian virus 40 (SV40) and polyoma T-Antigen (T-Ag), the adenovirus pre-terminal protein (pTP) and the Epstein-Barr virus (EBV) EBNA-1 recognize their respective binding sequence in the ORE, unwind the DNA and recruit other replication proteins at the origin site.

B. DNA unwinding element (DUE)

The DUE is an easily unwound DNA region. It is determined by base-stacking interactions and thus depends on nucleotide sequence and not only on AT content (Natale et al., 1992). When mutated, the DUE decreases the ability of the DNA to be unwound, consequently decreasing the efficiency of DNA replication (Umek and Kowalski, 1988). The spacing between ORE and DUE is critical, suggesting that protein interactions between these elements may be required for DNA replication (reviewed in DePamphilis, 1996). Moreover, in viral systems, including SV40 and polyoma, the DUE overlaps with the region where T-Ag mediated DNA unwinding starts, suggesting that the DUE determines the site for DNA unwinding and entry of the replication machinery (reviewed in DePamphilis, 1993).

C. AT-rich sequences

The AT-rich sequences consist of an A-rich and a T-rich strand, the length of which can affect origin function (Gerard and Gluzman, 1986; Koff et al., 1991). They usually are present in the core origin, as is the case for Herpes simplex virus (HSV),

SV40 and polyoma or they can flank the core origin, as in EBV. The AT-rich sequences are primarily believed to facilitate DUE activity. Other functions that have been assigned to AT-rich regions include aiding in the attachment of the origin to the nuclear matrix, since the majority of matrix attachment regions have AT-rich tracts, representing binding sites for a specific class of regulatory proteins (reviewed in Boulikas, 1996), and facilitating cruciform extrusion (reviewed in Pearson et al., 1996).

D. Bent DNA

Bent DNA is a sequence-directed curvature of the DNA helix (Koo et al., 1986; reviewed in Zannis-Hadjopoulos and Price, 1999), which arises from a short tract of 3-6-bp, which deflect from the helical axis. Bent DNA is thought to be involved in the unwinding of double-stranded DNA and in aiding in the initiation of replication and/or protein-DNA interactions (reviewed in DePamphilis, 1996; Zannis-Hadjopoulos and Price, 1999 and references therein). Bent DNA has been found in the SV40 origin (Deb et al., 1986b,c), yeast ARS elements (Snyder et al., 1986), Chinese hamster dihydrofolate reductase (DHFR) amplicon (Caddle et al., 1990), the monkey minimal origin of *ors*8 (Todd et al., 1995), and many others putative replication origins (reviewed in Boulikas et al., 1996).

E. Nuclear matrix attachment regions (MAR)

The nuclear matrix is the subcellular nuclear fraction composed of a nucleoprotein fibrillary network that remains after sequential extraction of chromatin with a non-ionic detergent and nuclease digestion (Berezney and Coffey, 1974; Georgiev et al., 1991; reviewed in Malkas, 1998). It organizes the DNA into specific domains, each one corresponding to a replication unit of 10-200-kbp, and provides sites for nucleic acid control in transcription and replication.

The DNA attachment sites to the matrix are referred to as matrix attachment regions (MAR). MAR are short, AT-rich DNA sequences that contain the ATTTA and ATTA sequence motifs (Boulikas, 1992). They have been found in the DHFR replication origin (Dijkwel and Hamlin, 1988), the mouse IgH enhancer origin (Ariizumi et al.,

1993), the human 343 origin (Wu et al., 1993), the β-globin origin (Boulikas, 1993), and in several monkey origin-rich-sequences, *ors*, (Rao et al., 1990; Mah et al., 1993).

Approximately 100, 000 MAR are present in the nucleus of a mammalian cell. Interestingly, the majority of MAR include DNA replication origins (Razin et al., 1990; Georgiev et al., 1991; reviewed in Zannis-Hadjopoulos and Price, 1999) and initiation of DNA replication takes place on the nuclear matrix (reviewed in Boulikas, 1996). Also, elongation of nascent DNA proceeds by reeling the old strands through the matrix where the replication forks are anchored (reviewed in Boulikas, 1996). Recently, it was demonstrated that initiation sites in the mammalian DHFR chromosomal locus are not defined by attachment to the nucleoskeleton (Ortega and DePamphilis, 1998). Also, an intact nuclear structure and the packaging of DNA into chromatin are required to carry out initiation of DNA replication in cell extracts from *Xenopus* eggs (Wu et al., 1997). It is believed that the nuclear matrix is involved in the assembly or maintenance of cellular replication factors (reviewed in DePamphilis, 1998). Nevertheless, the role of the nuclear matrix seems to be a facultative rather than an essential one since: 1) Origin recognition complex (ORC)-dependent DNA replication in *Xenopus* egg extracts can occur in the absence of nuclear structure (Walter et al., 1998), 2) SV40 chromosomes can replicate outside the nucleus (Su and DePamphilis, 1976), and 3) DNA replication can be achieved in a reconstituted system using soluble factors (Stillman, 1989). Other reports implicating the nuclear matrix in DNA replication have been published (Berezney and Coffey, 1974; Jackson and Cook, 1986b; Dijkwel et al., 1986; Tubo and Berezney, 1987; reviewed in Boulikas, 1996; Malkas, 1998; Zannis-Hadjopoulos and Price, 1999 and references therein).

F. Inverted repeats (IR)

Inverted repeats (IR) (reviewed in Pearson et al., 1996; Boulikas, 1996; Zannis-Hadjopoulos and Price, 1998; 1999 and references therein), or palindromic sequences, are DNA elements that are widely distributed along the DNA of many organisms such as plants (Bazetoux et al., 1978), yeast (Klein et al., 1980), Physarum (Hardman and Jack, 1977), *Drosophila* (Wilson and Thomas, 1974), mouse (Cech and Pardue, 1975), *Xenopus* and human (Dott et al., 1976). IR are present in a nonrandom fashion along the genome

(Klein and Welch, 1980) and are commonly found in DNA replication origins of prokaryotic (Zyskind et al., 1983; Hiasa et al., 1990), viral (Muller and Fitch, 1982) and eukaryotic organisms (Campbell, 1986; Hand, 1978; Zannis-Hadjopoulos et al., 1984; Zannis-Hadjopoulos et al., 1985; Landry and Zannis-Hadjopoulos, 1991; Nielsen et al., 1994).

IR can extrude into cruciform structures when the DNA is torsionally strained, which occurs during the transcription and replication processes. A cruciform is a four-branched DNA secondary structure that arises through intrastrand base pairing of the two complementary strands of the IR (reviewed in Pearson et al., 1996). Cruciforms are thermodynamically favourable structures and they are stable upon formation (Bell et al., 1991).

Several lines of evidence suggest that cruciform formation occurs during origin activation and that DNA replication may be regulated by cruciform binding proteins. Monoclonal antibodies raised against cruciform DNA were able to enhance DNA replication in permeabilized mammalian (CV-1 monkey) cells through stabilization of the cruciforms (Zannis-Hadjopoulos et al., 1988); using these anti-cruciform antibodies, it was shown that there exists a dynamic distribution of cruciforms in mammalian nuclei (monkey and human adenocarcinoma SW48 cells) that is maximum at the G1/S boundary (approximately $3x10^5$ cruciforms/nucleus), and non-existent during G0, G2/M, or in metaphase chromosomes (Ward et al., 1990; Ward et al., 1991). The anti-cruciform antibodies were also used to affinity-purify mammalian origins of DNA replication (Nielsen et al., 1994). Finally, the abundance of cruciforms in MAR, believed to be the sites of DNA replication, further supports a role of inverted repeats in origin function (Boulikas and Kong, 1993). Thus, cruciforms alter the degree of supercoiling of the DNA, the positioning of nucleosomes, and might also interact with replication proteins (reviewed in Pearson et al., 1996; Zannis-Hadjopoulos and Price, 1998; Zannis-Hadjopoulos and Price, 1999).

A human cruciform binding protein, CBP, has been purified (Pearson et al., 1994) and identified as a member of the 14-3-3 family of proteins (Todd et al., 1998). CBP is a structure-dependent protein that does not bind to either linear double-stranded or single-

stranded DNA (Pearson et al., 1994a). A role for CBP in the initiation of DNA replication has been suggested (Todd et al., 1998).

ii) Auxiliary elements

The auxiliary elements of replication origins generally consist of binding sites for transcription factors. These auxiliary elements affect the efficiency but not the mechanism of replication. The involvement of transcription factors in DNA replication has been clearly shown in adenovirus, papovaviruses (SV40 and polyoma), papillomavirus, and in *Saccharomyces cerevisiae* (reviewed in DePamphilis, 1993; Boulikas, 1996; van der Vliet, 1996; Murakami and Ho, 1999 and references therein). Furthermore, many of the mapped mammalian replication origins also contain binding sites for transcription factors whose involvement in DNA replication is only starting to be looked at.

Several mechanisms have been proposed to describe how transcription factors stimulate core origin activity (reviewed in DePamphilis, 1993; van der Vliet, 1996; Murakami and Ho, 1999). Four mechanisms are described below.

1. Recruitment of the replication machinery through protein-protein interactions

Transcription factors stimulate DNA replication by binding to replication proteins and targeting them to the replication origins. Furthermore, transcription factors can facilitate the assembly of a multiprotein preinitiation complex and stabilize it. For example, in adenovirus replication, the transcription factor NF-1 interacts directly with the viral DNA polymerase (Ad pol), while the transcription factor Oct-1 interacts with the pre-terminal protein (pTP). Together, they stabilize the pTP-pol complex, direct it to the origin and increase the binding of the initiator complex to the origin, thus stimulating replication up to ten-fold (reviewed in van der Vliet, 1996). Furthermore, bovine papillomavirus type-1 (BPV) replication is enhanced by the E2 protein. In the presence of E2, the initiator E1 helicase binds to the core origin cooperatively with E2, with higher affinity and specificity, resulting in enhanced origin unwinding by E1 (Seo et al., 1993b,c).

2. Modulation of chromatin structure

Chromatin structure can act as a general repressor of origin activity by interfering with the binding of initiation factors to replication origins. Binding of transcription factors is thought to release replication from the repressive effect of chromatin by modulating chromatin structure. For instance, assembly of the SV40 origin into nucleosomes interferes with subsequent DNA replication. However, when NF-1 is bound to the auxiliary region of SV40, the origin region remains nucleosome-free, and DNA replication is not repressed following nucleosome assembly (Cheng and Kelly, 1989).

3. Induction of structural changes in the origin

Binding of transcription factors to replication origins might lead to structural DNA changes, such as DNA bending and unwinding. This could in turn facilitate the formation of a preinitiation complex by positioning initiation proteins closer together or by unwinding DNA on the surface of a multiprotein complex. Binding of the ARS binding factor 1, ABF-1, to the *Saccharomyces cerevisiae* origin and of NF-1 and Oct-1 to the adenovirus origin results in DNA bending.

4. Transcriptional activation of DNA replication

In bacteriophages and bacteria, transcription of the DNA template in the vicinity of a replication origin activates DNA synthesis (Baker and Kornberg, 1988). In viral systems, such as polyoma and papillomaviruses, replication origins are situated upstream of the early promoter. However, replication levels are not affected upon deletion of the promoter (Prives et al., 1987). In higher eukaryotes, transcription is not required for DNA replication, since the first S phase in mammalian embryos and the first few S phases in the embryos of frogs, flies and fish occur in the absence of transcription. Nevertheless, transcription may be required in selecting initiation sites in adult tissue cells since active genes are replicated early and inactive genes are replicated late (reviewed in

DePamphilis, 1999). Furthermore, about 50% of mammalian origins map to regions containing transcription promoters or enhancers and transcription was shown to induce replication from origins near the promoter region of the human c-myc gene (Ohba et al., 1996).

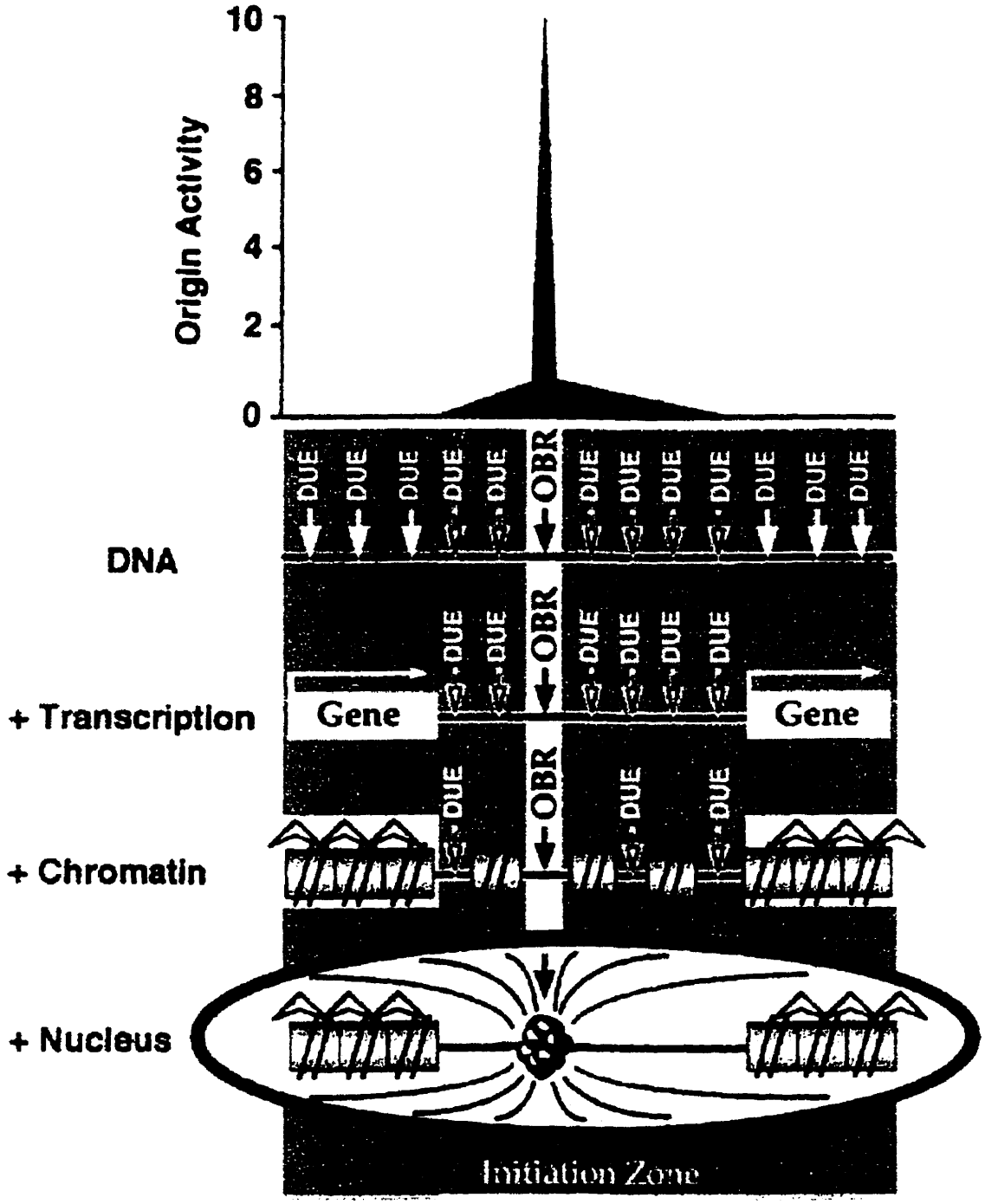
III. FACTORS THAT DETERMINE DNA REPLICATION INITIATION SITES

In order to account for results suggesting that multiple, nonrandom sites exist in differentiated cells, and that multiple, random sites exist in embryonic cells on naked DNA, the Jesuit model (Figure 2) for metazoan origins has been proposed (DePamphilis, 1996). This model suggests that there are many more potential initiation sites in the genome than are required. Therefore, various factors must exist to determine which initiation sites are chosen. These factors include DNA sequence, nuclear structure, chromatin structure, transcription, and methylation.

i) DNA sequence

Origins of DNA replication in metazoan cells, similar to viruses and yeast, are determined by specific DNA sequences. Many studies have shown that there exist specific initiation sites in broad regions. These initiation sites can function in many different cell types of the same species (Kumar et al., 1996), and can also exhibit origin activity when transferred to ectopic chromosomal locations (Handeli et al., 1989; Aladjem et al., 1998; Malott and Leffak, 1999; Altman et al., 2000; Koleman et al., 2000). Furthermore, a DNA consensus sequence (see section IV. ii) E. f) Mammalian origin consensus sequence) for mammalian origins of DNA replication was recently found (Price et al., submitted), further supporting the notion that origins are determined by sequence.

Figure 2. The Jesuit model for site specific initiation in the chromosome of metazoan cells. The DNA genome contains many potential sites of initation, which may correspond to DUE regions. However, transcription, chromatin structure, and nuclear structure can suppress initiation at some of these sites and activate initation at other sites. See text for further detail. (Reproduced from DePamphilis, 1998).


10
8
6
4
2
0
Origin Activity
DUE
OBR
DNA
+ Transcription
Gene
+ Chromatin
+ Nucleus
Initiation Zone

ii) Nuclear structure

Unlike prokaryotic and viral genomes that can replicate in the presence of purified soluble factors, initiation of metazoan DNA replication requires a nuclear structure. Replication of DNA introduced into *Xenopus* eggs or egg extracts does not occur unless DNA is assembled into chromatin, and organized into a nuclear structure (Wu et al., 1997; Dimitrova and Gilbert, 1998). An intact nucleus is also required for initiation of DNA replication in human (Krude et al, 1997) and yeast cells (Pasero et al., 1997).

One role of the nuclear structure is in establishing initiation sites. When sperm chromatin or naked DNA is added to *Xenopus* egg extracts, DNA replication is initiated randomly and at many sites along the DNA. However, if intact nuclei are used, site-specific initiation occurs (Gilbert et al., 1995). The nuclei must be isolated from late G1. If early G1 nuclei are used, then initiation also occurs randomly. Thus, specific initiation of DNA replication requires a nuclear structure and a cell cycle dependent event (Wu and Gilbert, 1996). Nevertheless, it has been suggested that the role of the nuclear structure is facilitative rather than obligatory (DePamphilis, 1999), since ORC-dependent DNA replication has been achieved in *Xenopus* extracts in the absence of nuclear structure by substituting with a concentrated nuclear extract (Walter et al., 1998). Additional reasons are presented in section II.i) E. Nuclear matrix attachment regions (MAR).

iii) Chromatin structure

Chromatin consists of long arrays of nucleosomal DNA interspersed with specific regulatory nucleoprotein complexes. Chromatin structure has been suggested to be involved in DNA replication by limiting the accessibility of initiation factors to origins of DNA replication. In early development, chromatin structure is absent and initiation can occur anywhere along the genome. Changes in chromatin structure also determine which initiation sites can be used (Lawlis et al., 1996). For example, when condensed chromosomes of metaphase-arrested Chinese hamster ovary (CHO) cells are incubated with *Xenopus* egg extracts, initiation does not occur at the preferred oriβ site but rather at a new site, oriδ.

iv) Transcription and transcription factors

Transcription is another parameter that determines initiation sites. First, it has been observed that active genes replicate early in S phase, while inactive genes replicate late (Hatton et al., 1988). For example, the β-globin and the cystic fibrosis gene loci are early replicating in cells that express them, but become late replicating in cells that do not express them (Epner et al., 1983; Selig et al., 1992). Second, many origins of DNA replication have been mapped close to or at gene promoters, including the c-myc (Vassilev and Johnson, 1990), the β-globin (Kitsberg et al., 1993) and the rat aldolase origins (Zhao et al., 1994). Third, CpG islands serve both as transcription and replication initiation sites (Delgado et al., 1998). Fourth, transcription factor binding sites are present in many origins of DNA replication such as the *S. cerevisiae* ARS (Marahrens and Stillman, 1992), the Chinese hamster DHFR (DePamphilis, 1993), the hamster rhodopsin (Gale et al., 1992), the murine IgH intronic enhancer Eμ (Staudt and Lenardo, 1991), and the mammalian *ors* sequences (Rao et al., 1990; Wu et al., 1993; Todd et al., 1995; Matheos et al., 1998; [see chapter two of this thesis]). Also, a human origin, clone 343, has been mapped to a transcribed region of the genome (Wu et al., 1993). Mechanisms by which transcription factors enhance DNA replication are described above. However, the activity of some replication origins may not be affected by local transcription units, such as the β-globin origin (Aladjem et al., 1995). Furthermore, transcription may not be required for DNA replication, since the first S phase in mammalian embryos and the first few S phases in frogs, flies, and fish embryos occur in the absence of transcription (reviewed in DePamphilis, 1999).

v) DNA methylation

DNA modification by methylation is another factor that can influence activity of initiation sites. In prokaryotes, DNA methylation regulates replication origins by increasing their rate of reinitiation; the assembly of the *Escherichia coli* replication complex is delayed when the origin is in a hemimethylated state (Campbell and Kleckner, 1990). It has been suggested that differential methylation of replication origins during the cell cycle can regulate origin activation (Szyf, 1996). However, recent evidence argues

against this idea, since most origins examined do not have a similar methylation pattern (Araujo et al., 1998). For example, some origins, such as the lamin B2, are partially methylated, while others, like the c-myc origin, are unmethylated. Heavily methylated origins, including the DHFR oriβ, have also been reported (Rein et al., 1997a,b; Araujo et al., 1998). Furthermore, unlike *E. coli*, a lag time between replication and methylation, that may regulate initiation of DNA replication, does not exist in mammalian cells (Araujo et al., 1998). In contrast, concurrent replication and methylation occurs. It has been suggested that a cluster of methylated CpGs can contribute to the assembly of prereplication complexes at specific sites (DePamphilis, 1999).

IV. REPLICATION MODEL SYSTEMS

Two types of DNA synthesis exist: continuous and semidiscontinuous (Figure 3). In continuous DNA replication, the complementary DNA strand is synthesized continuously from the origin to the end of the genome by a single priming event per template strand. Two complementary DNA strands are synthesized independently and asynchronously. Continuous DNA replication is the mechanism used to duplicate mitochondrial DNA and the DNA of viruses with linear genomes. In contrast, eukaryotic chromosomal DNA replication occurs by the semidiscontinuous mechanism. One strand, referred to as the leading strand, is synthesized continuously from a single primer, whereas the other strand, the lagging strand, is synthesized as short DNA fragments, termed Okazaki fragments, which are elongated by many different RNA primers. Synthesis of the leading strand proceeds in the 5'-3' direction in contrast to lagging strand polymerization that occurs in the opposite, 3'-5', direction.

Due to the complexity of the mammalian genome and existence of multiple (10^4-10^6) replication origins, investigators have turned to viral and yeast systems to study the mechanisms of cellular replication.

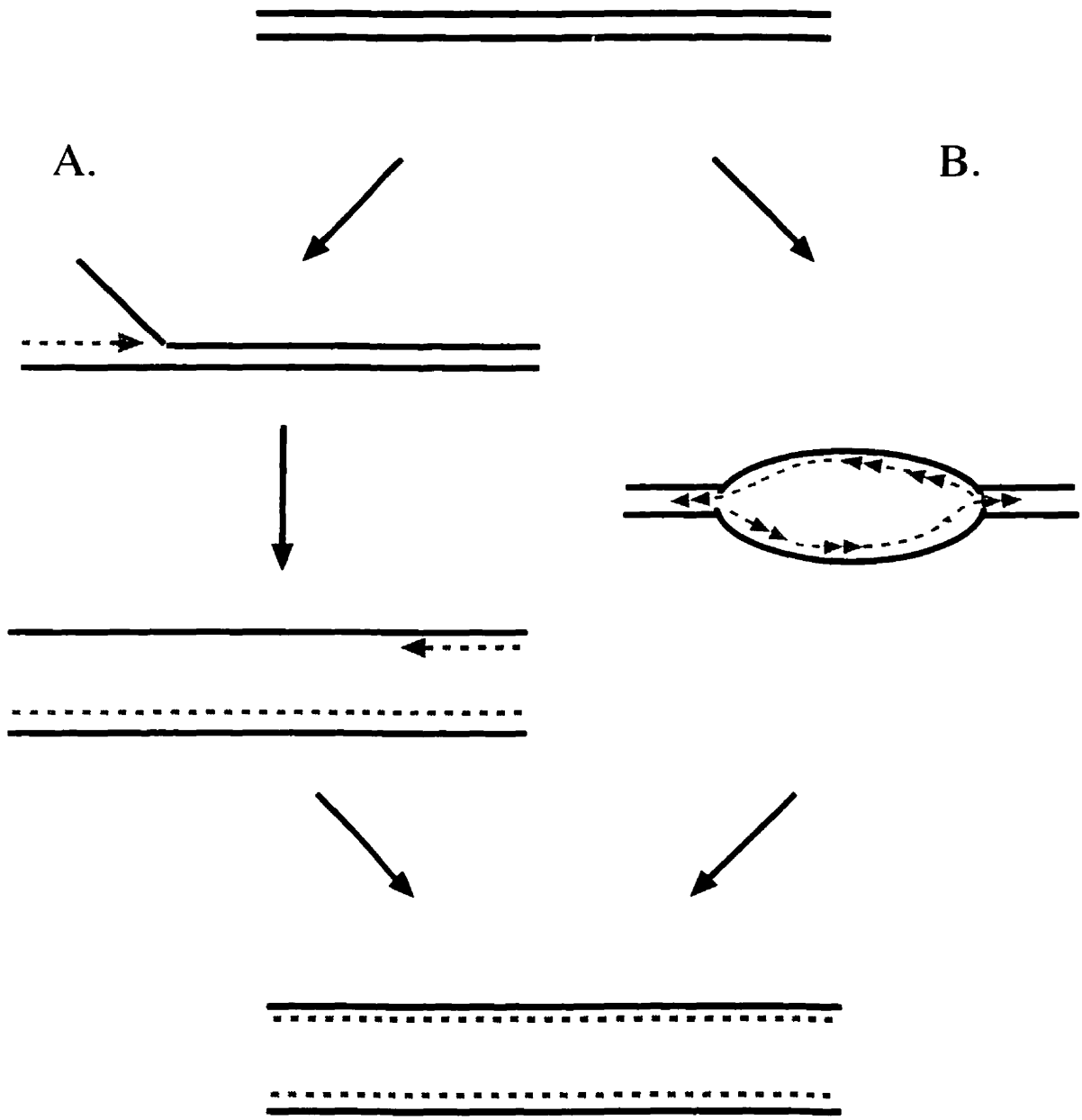


Figure 3. Continuous (A) versus semidiscontinuous (B) DNA replication. The two basic mechanisms for replicating DNA are shown with nascent DNA (broken/red line) synthesized from primers in the direction of the arrows. (Adapted from Brush and Kelly, 1996).

i) Continuous DNA replication

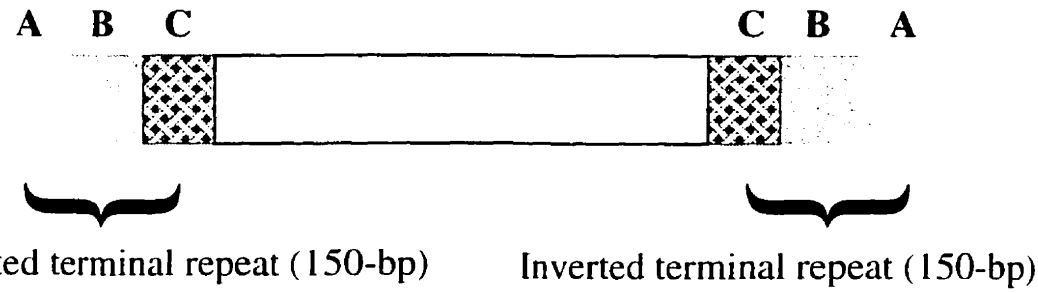
A. Adenovirus

Adenovirus contains a blunt-ended linear double-stranded DNA genome of approximately 36-kbp. The study of adenovirus replication provides a good model system to study continuous DNA replication.

The two origins of DNA replication of approximately 50-bp each are located at either 3' end of the linear genome, within the inverted repeat sequence of 100-150-bp (Figure 4). Three DNA domains, A, B, and C, are necessary for DNA replication. The 18-bp AT-rich domain A constitutes the minimal or core origin. The 20-bp domain B represents the binding site for the host cell protein NF-1, while the 12-bp domain C is the binding site for NF-III, or Oct-1. Domains B and C, referred to as the auxiliary sequences, stimulate initiation of DNA replication *in vivo* (Hay, 1985) and *in vitro* (Harris and Hay, 1988). The 5' end of each DNA strand is covalently linked to a virally encoded protein, the 55 kDa terminal protein, TP. Four virally encoded proteins are also required for replication (reviewed in Stillman, 1989). These include: 1) the 80 kDa precursor terminal protein (pTP), required for initiation priming (Challberg and Kelly, 1989), 2) the 140 kDa DNA polymerase (pol), required for DNA synthesis (Lichy et al., 1982), which forms a complex with pTP, 3) the single-stranded DNA binding protein (DBP), required for the stabilization of single-stranded regions (Lindenbaum et al., 1986), and 4) the adenovirus protease which cleaves the pTP. The viral proteins sustain a limited level of initiation and two cellular nuclear factors, NF-1 and Oct-1, are required to stimulate initiation (O'Neill and Kelly, 1988), by tethering the pTP-pol complex to the core origin (van Leeuwen et al., 1997).

Adenovirus DNA replication is initiated *via* a protein-primed, rather than RNA-primed, mechanism followed by elongation *via* a strand displacement mechanism (Figure 5). Virus replication initiates when the pTP-pol complex binds the origin at the end of the genome and a phosphodiester bond is formed between the first nucleotide, dCMP, and pTP (Challberg et al., 1980). Adenovirus DNA polymerase initiates replication in the form of a tight heterodimer with pTP-dCMP. Shortly after initiation, DNA polymerase dissociates from the primer protein allowing the polymerase to proceed with chain

A. Adenovirus genome (36-Kbp) organization



B. Adenovirus minimal origin (50-bp)

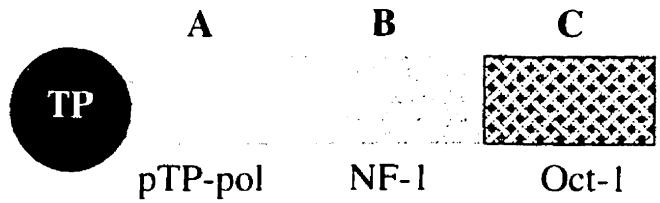


Figure 4. Organization of the adenovirus origin. A. The 36-kbp linear double-stranded DNA genome contains two origins of DNA replication, located within the 150-bp inverted terminal repeats. B. The 50-bp minimal origin is divided into domain A, B, and C, which serve as binding sites for pTP-pol, NF-1 and Oct-1, respectively. The TP protein is attached to the 5' end of the DNA.

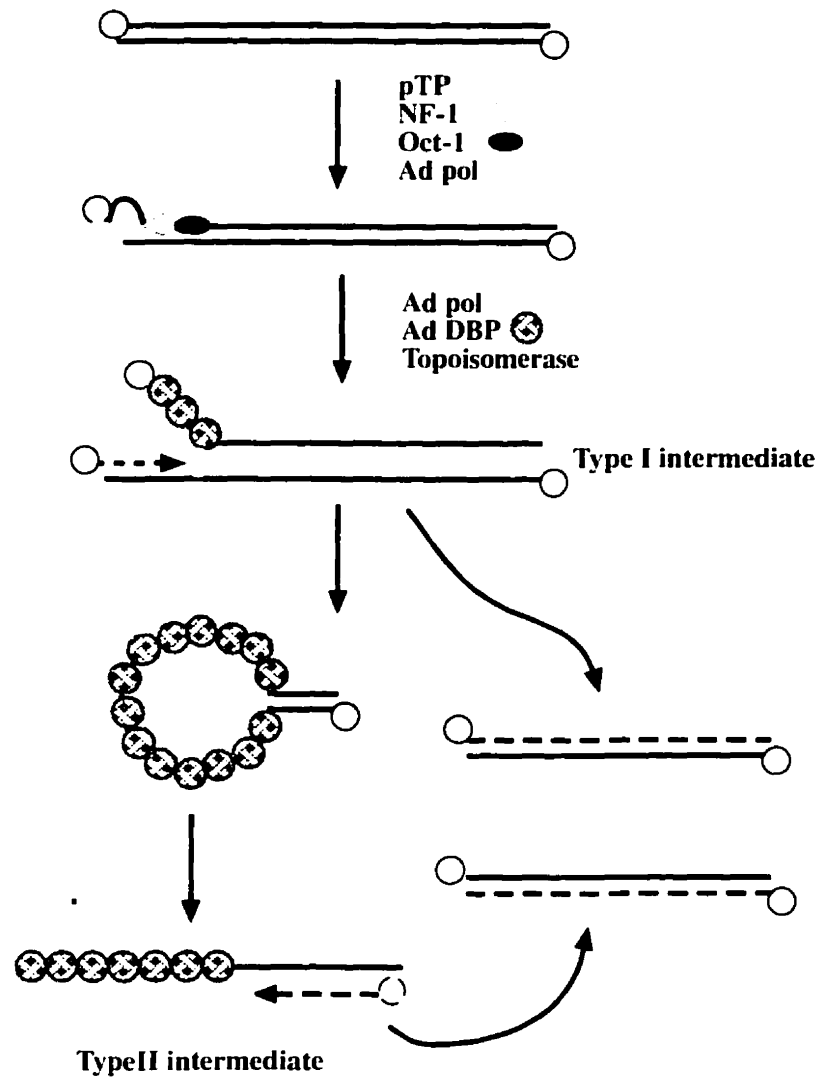


Figure 5. Model for adenovirus DNA replication. The 5' ends of the adenovirus genome are covalently linked to the terminal protein (TP). Initiation of replication requires the preterminal protein (pTP), the adenovirus polymerase (Ad pol), and the cellular proteins NF-1 and Oct-1. Initiation is facilitated by the DNA-binding protein (DBP). Initiation requires the formation of a phosphodiester bond between a serine residue in pTP and the 5' phopshoryl group of dCMP. Elongation requires the Ad pol, DBP, and a cellular topoisomerase (NF-II). Displacement synthesis on type I intermediate leads to a progeny duplex and a displaced single-strand. This displaced single-strand circularizes via self-complementary ends and serves as a substrate for a second initation event, identical to the first one. Completion of DNA synthesis on type II intermediates results in the formation of a second progeny duplex. (Reproduced from Brush and Kelly, 1996).

elongation and proofreading (King et al., 1997; Mendez et al., 1997). Unlike other systems, adenovirus replication does not require a DNA helicase to unwind the double-stranded genome. Instead, elongation is dependent on the DBP protein, which has helix stabilizing properties. Multimerization of DBP is the driving force for ATP-independent DNA unwinding during strand displacement synthesis (Dekker et al., 1998). Following initiation of replication at one end of the linear genome, DNA elongation produces a progeny duplex strand and a free single strand. This free strand can form a panhandle structure *via* its complementary DNA ends, and since the ends are identical to the original genome, it can replicate *via* the same mechanism. Elongation of adenovirus DNA replication requires an additional cellular factor, NF-II, which functions as a topoisomerase.

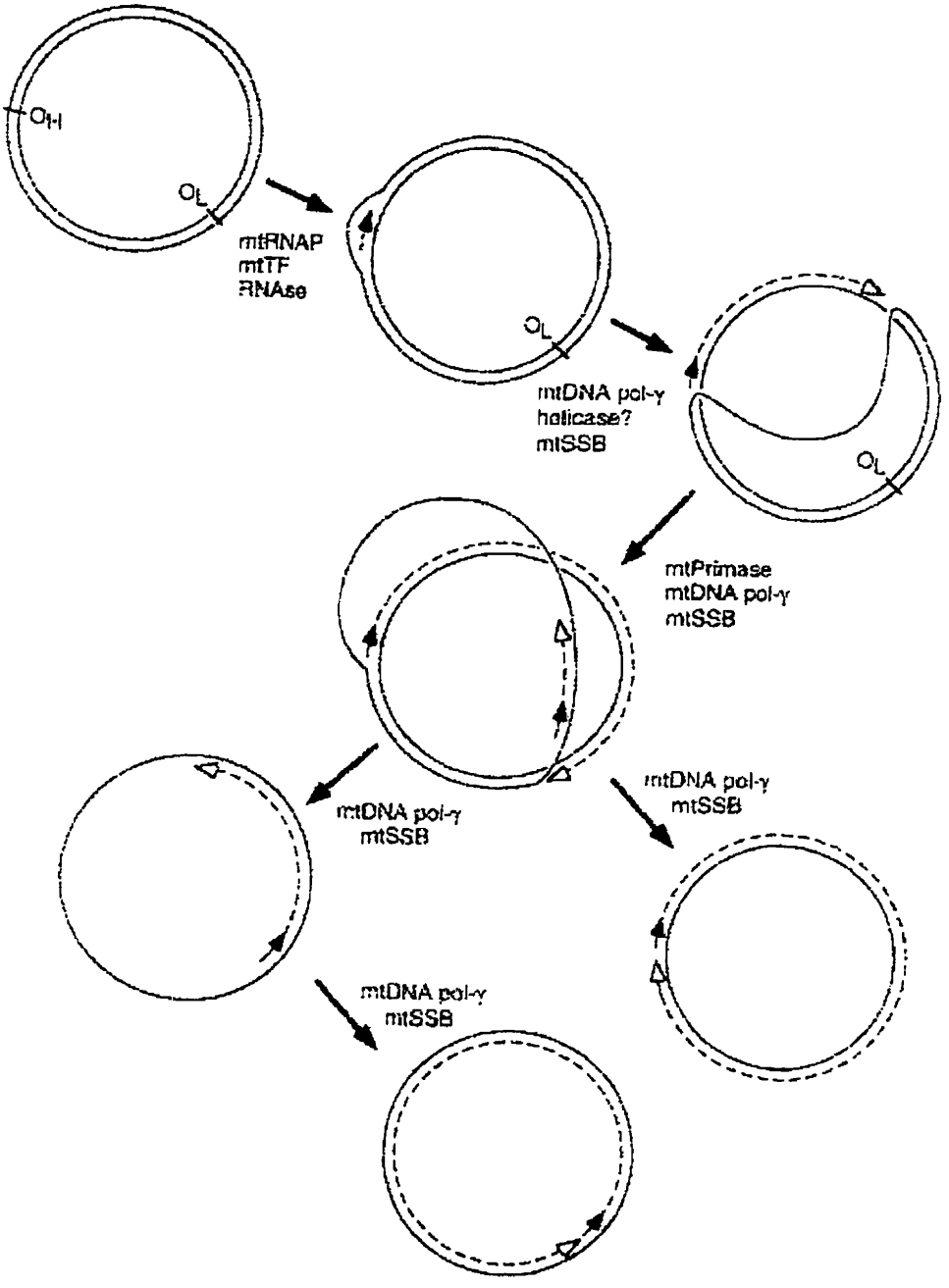
As stated above, adenovirus replication is strongly enhanced by two cellular transcription factors, NF-1 and Oct-1. NF-1 and Oct-1 interact with different regions of the preinitiation complex and dissociate independently once initiation has occurred. NF-1 directly contacts the DNA polymerase, while Oct-1 interacts with pTP (van Leeuwen et al., 1997). The transcription factors, thus, recruit the initiator proteins to the core origin and stabilize the preinitiation complex.

B. Mitochondria

The mammalian mitochondrial genome consists of a closed double-stranded 16-kbp DNA molecule. Cells have 10^3-10^4 mitochondrial genomes with an average of 5-10 per organelle. The mitochondrial genome is replicated throughout the cell cycle. Furthermore, some genomes are replicated several times per cell cycle while others are not replicated at all (reviewed in Clayton, 1992; Shadel and Clayton, 1997).

The mitochondria genome contains two origins of replication, O_H and O_L, one on each of the two strands, referred to as H and L (Clayton, 1982) (Figure 6). At both of these origins, DNA synthesis is primed by RNA molecules. The O_H origin is activated first, resulting in the formation of a unidirectional replication fork (Chang and Clayton, 1985). At this replication fork, the parental H strand is displaced while the nascent H strand is continuously elongated. The O_L origin, located 11-kbp downstream of O_H, is activated when it is rendered single-stranded by the passage of the H strand replication

Figure 6. Model for mitochondrial DNA replication. O_H and O_L represent the two mitochondrial origins which are located about two-thirds of the genome apart. Initiation of DNA replication begins with the synthesis of a transcript by the mitochondrial RNA polymerase (mtRNAP) and a transcription factor (mtTF). The transcripts are processed by the RNase MRP (RNase) to form a primer for initiation of DNA replication. If cleavage occurs, a nascent H strand (broken line) is synthesized by the DNA polymerase γ. Other proteins likely participate in this process, such as a single-stranded DNA binding protein (SSB). Synthesis of the L strand is initiated by the mitochondrial primase (mtPrimase) after the displacement fork has passed O_L. (Reproduced from Brush and Kelly, 1996).


O_{H}
O_L
mtRNAP
mtTF
RNAse
O_L
mtDNA pol-γ
helicase?
mtSSB
O_L
mtPrimase
mtDNA pol-γ
mtSSB
mtDNA pol-γ
mtSSB
mtDNA pol-γ
mtSSB
mtDNA pol-γ
mtSSB

fork (Wong and Clayton, 1985). The L strand is also continuously elongated from the RNA primer, yet asynchronously from the H strand, since the two origins are spaced by two thirds of the genome. Once the synthesis of the H strand is complete, two products are generated: one consisting of the full length H strand progeny while the other is an incomplete progeny that then gets extended to full length (Figure 6).

The mitochondrial genome contains a D-loop, in the vicinity of O_H origin, which consists of short H DNA strands whose 5' end is located at the origin. Within the D-loop region, there exist two promoters that serve to prime H strand synthesis (Chang and Clayton, 1985). The mitochondrial RNA polymerase and a transcription factor, mtTFA, are needed to synthesize these primers that are elongated by the mitochondrial DNA polymerase γ. The primers are removed by the action of RNase MRP (Mitochondrial RNA Processing) (Chang and Clayton, 1987). In contrast, priming of the L strand is mediated by a mitochondrial DNA primase (Hixson et al., 1986). Mitochondrial single-stranded DNA binding proteins (Tiranti et al., 1993) and DNA helicases (Helman and Hauswirth, 1992) have also been identified.

ii) Semi-discontinuous DNA replication

A. Papovaviruses

a) Simian virus 40 (SV40)

Simian virus 40 (SV40) (reviewed in Bullock, 1997), a papovavirus family member, has a double-stranded circular genome of 5243-bp. It contains a single, fixed origin located in a noncoding region of the genome that overlaps with transcriptional promoters of early and late genes. The origin is subdivided into a 64-bp core element and adjacent auxiliary regions (Aux-1 and Aux-2), each one spanning approximately 40-bp. The core origin is both necessary and sufficient for initiation of SV40 replication *in vivo* and *in vitro*, while the flanking auxiliary elements increase the efficiency and cell type specificity of replication (Hassell and Brinton, 1986).

The SV40 core origin (Figure 7) contains three functional regions: 1) a 17-bp adenine-thymine (AT) rich region shown to assume a naturally bent conformation (Deb et

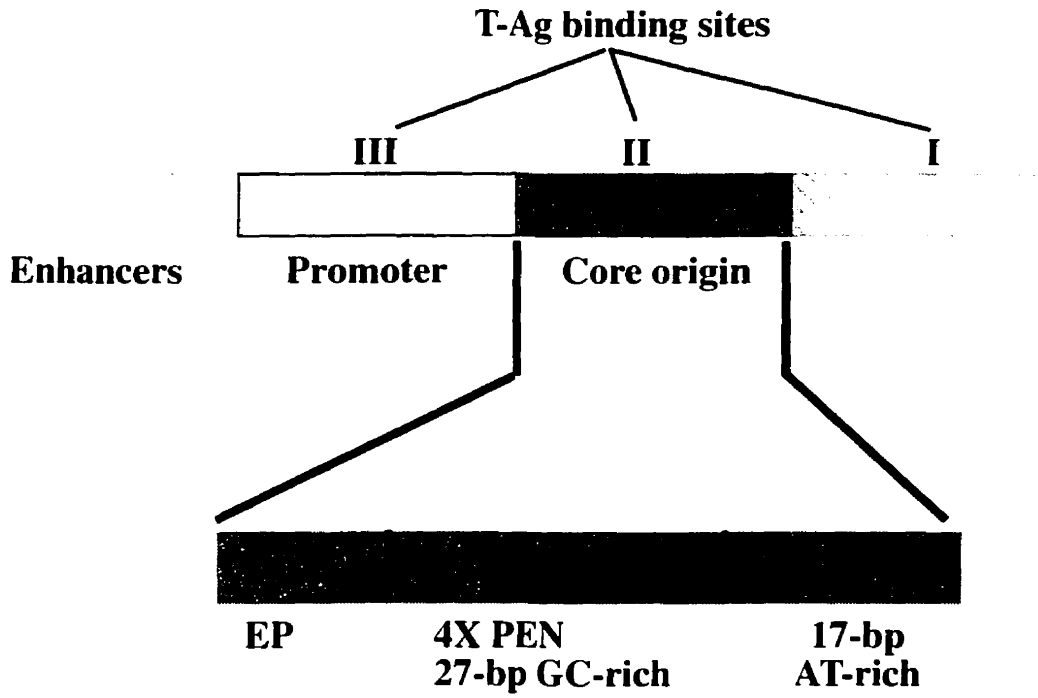


Figure 7. Organization of the SV40 origin of DNA replication. See text for details.

al., 1986b,c), 2) a core region which contains a cluster of four GAGGC pentanucleotides, referred to as binding site II or pentanucleotide palindrome (PEN) and a 27-bp GC rich palindrome (DeLucia et al., 1983), and 3) a region referred to as the early palindrome, EP, which is the site of the origin of bidirectional replication where the first RNA priming occurs (Hay and DePamphilis, 1982; Hay et al., 1984). The GAGGC pentanucleotides in the PEN are arranged as two pairs that are inverted relative to each other. The individual pentanucleotides serve as binding sites for T-Antigen (T-Ag), the SV40 initiator protein (DeLucia et al., 1983; Tegtmeyer et al., 1983) and binding to all four pentanucleotides is required for initiation of DNA replication (Deb et al., 1987). The EP region is a two-part domain. First, it includes an 8-bp DNA unwinding element (DUE). The DUE is melted after T-Ag binding to the core origin (Borowiec et al., 1991). It represents a highly conserved region in papovaviruses in which mutations result in a significant loss of origin activity (Dean et al., 1992). The other domain of EP, proximal to the PEN, functions as a spacer whose mutation does not affect replication (Deb et al., 1986a). Finally, the AT-rich region is necessary for DNA replication (Gerard and Gluzman, 1986) and can undergo protein-independent DNA bending (Deb et al., 1986b, c). Also, the AT- rich region becomes structurally distorted subsequent to T-Ag binding to site II and sequences from within the AT-rich region activate the unwinding activity of T-Ag. The AT-rich region also serves as the binding site for various cellular factors, such as the Oct-1 transcription factor. The three functional regions of the SV40 origin are required for SV40 replication, as demonstrated by experiments in which single base substitutions resulted in a decrease in DNA replication (Dean et al., 1987). Furthermore, the spacing between these three regions is also critical for replication activity (Parsons and Tegtmeyer, 1992) suggesting that origin structure is critical for efficient replication.

T-Ag, the 82 kDa SV40 initiator protein, is the only virally encoded protein required for DNA replication. Numerous posttranslational modifications of T-Ag have been described, including phosphorylation, O-glycosylation, adenylation, ADP ribosylation and acylation (reviewed in DePamphilis and Bradley, 1986; Fanning and Knippers, 1992). With the exception of the phosphorylation events, little is known about the functional consequences of the other modifications. T-Ag possesses intrinsic ATPase and DNA helicase activities. Binding of T-Ag to the SV40 core element leads to origin

unwinding, thus allowing for initiation of replication to occur. Two hexamers of T-Ag bind specifically to the SV40 core origin (Jones and Tjian, 1984). DNA replication proceeds bidirectionally through the replication fork mechanism where the DNA is synthesized continuously on the leading strand and discontinuously, *via* Okazaki fragments, on the lagging strand (DePamphilis, 1993). Binding of transcription factors to the origin auxiliary elements increases the frequency of initiation up to 100-fold (Guo et al., 1989). To carry out its multiple biological functions, T-Ag associates with many cellular proteins, including DNA topoisomerase I, RP-A, and DNA polymerase α/ primase complex (reviewed in Bullock et al., 1997). These proteins are reviewed in section VII. of this thesis. Termination of DNA replication occurs when the two replication forks meet and the daughter molecules segregate into monomeric circles.

SV40 replicates its DNA in a similar fashion to that used for replicating chromosomes of the host cells, primarily through the action of host cellular enzymes (DePamphilis and Wassarman, 1982). Similar to cellular DNA, upon infection of suitable host cells, the SV40 genome is transported to the nucleus, where the DNA is packaged into chromatin and DNA replication occurs during the S phase of the cell cycle. However, unlike cellular DNA, SV40 DNA replication can undergo several rounds of replication during a single S phase (DePamphilis and Bradley, 1986).

b) Polyoma virus

Polyoma, another member of the papovavirus family, is closely related to SV40 except that it replicates in murine instead of monkey cells. The polyoma origin consists of a 72-bp core origin, serving as the T-Ag binding site, and two flanking auxiliary elements, Aux-1 and Aux-2. Aux-1 contains T-Ag binding sites whereas Aux-2 comprises the transcriptional enhancer, consisting of transcription factor binding sites (Guo and DePamphilis 1992; Prives et al., 1987). Like SV40, T-Ag binds to the core origin as a hexamer, has DNA-dependent ATPase and helicase activities, and can bind DNA polymerase α/primase complex. The interaction of T-Ag with DNA polymerase α/primase complex determines the host range specificity of the virus (Stadlbauer et al., 1996). Polyoma T-Ag, however, binds weakly to the DNA and further relies on auxiliary

factors for efficient DNA replication, which can enhance replication up to 1000-fold (reviewed in van der Vliet, 1996; Murakami and Ito, 1999).

B. Papillomaviruses

Most of the information on viral DNA replication of the papillomaviruses has been derived from the bovine papillomavirus type 1, BPV. BPV is an attractive model system because its viral genome is packaged into chromatin, forming a double-stranded circular minichromosome, like cellular DNA, SV40 and polyoma. Furthermore, the genome of BPV is stably maintained as an episome throughout replication. However, unlike SV40 and polyoma, BPV replicates its genome only once per cell cycle (Gilbert and Cohen, 1987; Nallaseth and DePamphilis, 1994), thus making it an attractive model to study higher eukaryotic DNA replication.

The genome of BPV is a double-stranded circular DNA molecule of 7.9-kbp. It has an origin of bidirectional replication of approximately 60-bp (Figure 8). The core origin consists of a 1) 23-bp AT-rich region, 2) an imperfect inverted repeat, and 3) an 18-bp binding site for the 68 kDa viral initiator protein, E1 (Ustav et al., 1991). E1 has similar properties to T-Ag, including origin binding, and DNA-dependent ATPase and helicase activities (Thorner et al., 1993; Yang et al., 1993). Two short auxiliary regions flank the core origin. Unlike SV40 and polyoma, the auxiliary sequences serve as binding sites for a virally encoded, and not cellular, transcription factor, E2. E2 is a 48 kDa viral transcriptional enhancer and replication enhancer that binds as a dimer to the auxiliary sequence, bends DNA and considerably enhances DNA replication (McShan and Wilson, 1997). Enhancement of the initiation of DNA replication by the E2 transcription factor is related to its ability to bind to the E1 initiator protein. The interaction between the two virally-encoded proteins is cooperative and results in a higher affinity of E1 for its binding sites in the origin (Mohr et al., 1990; Blitz and Laimins, 1991). In other words, cooperative binding of E1 and E2 to the origin results in the formation of an origin recognition complex. The E1-E2 complex remains replication inactive until additional replication proteins are recruited. For instance, E2 can recruit RP-A (Li and Botchan, 1993). Then, in an ATP-dependent step, E2 is displaced, possibly through structural changes induced in the origin and to the activation of E1's helicase activity, allowing the

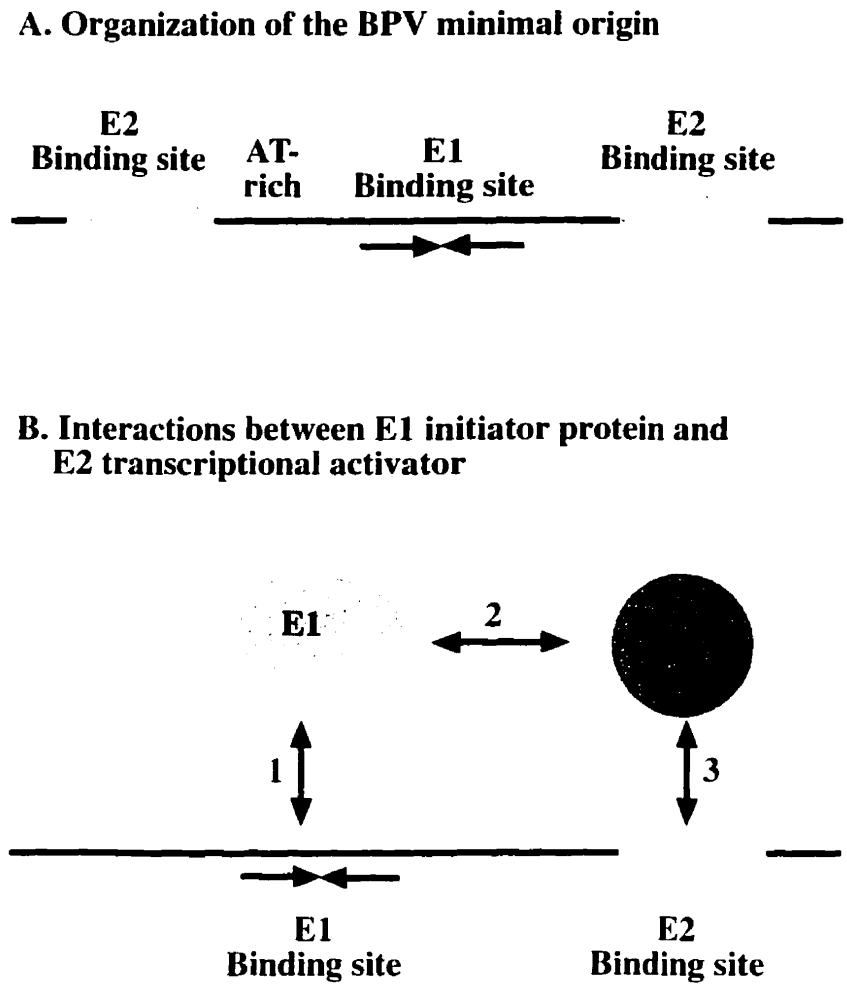


Figure 8. Structure of the minimal origin of BPV. A. Schematic representation of the minimal origin indicating the binding sites for E1 and E2. B. Schematic model illustrating the interactions between E1 and DNA, E2 and DNA, and E1 and E2, that are required for the formation of a replication-competent complex (Adapted from Stenlund, 1996).

binding of additional E1 (Sanders and Stenlund, 1998). E1 multimers subsequently attract further replication proteins such as DNA polymerase α/primase and form a prereplication complex.

C. Herpes virus

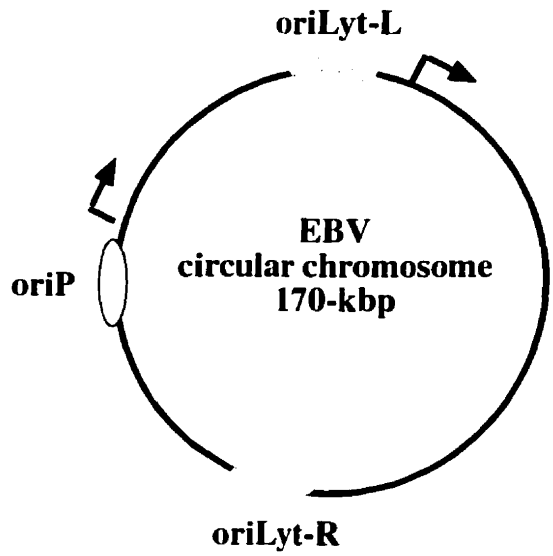
Epstein-Barr virus (EBV) is a member of the herpes family and is specific for B lymphocytes. It consists of a 170-kbp linear, supercoiled DNA that is replicated once per cell cycle. EBV replicates its DNA at the same rate as cellular chromosomes (Yates and Guan, 1991) and provides a good model system for the regulation of DNA copy number in mammalian cells. EBV possesses two origins of DNA replication, oriP and oriLyt. OriP is responsible for extrachromosomal maintenance during the latent state, while oriLyt is needed for genome amplification during lytic growth (Yates et al., 1984; Sugden et al., 1987).

OriP contains two noncontiguous elements, the dyad symmetry (DS) and the family of repeats (FR) elements (Figure 9). These elements are separated by 1-kbp. DS coincides with the functional origin. It contains four binding sites for EBNA-1, the only virally encoded protein required for replication (Reisman et al., 1985). FR contains 20 tandemly repeated copies of a 30-bp sequence, each one containing a 12-bp palindrome that serves as an EBNA-1 binding site (Rawlins et al., 1985).

EBNA-1 dimers initially bind to the FR sites, followed by the DS. Binding of EBNA-1 to either of these sites alone is relatively weak, and the bound EBNA-1 complexes at both sites are needed to interact. This interaction leads to a looping out of the DNA, allowing saturation at lower concentrations *via* cooporativity (Frappier and O'Donnell, 1991). EBNA-1 does not possess any enzymatic activity. Its main role is to provide torsional stress to the origin, allowing unwinding. The crystal structure of EBNA-1 bound to DNA suggests that indeed it facilitates DNA bending and unwinding (Edwards et al., 1998). Furthermore, EBNA-1 can recruit RP-A to the origin, and this further facilitates origin unwinding (Zhang et al., 1998).

The EBNA-1 protein and the FR sequences are essential for EBV replication. Interestingly, the DS element can be deleted and EBV episomes are still recovered. However, initiation in these mutated viruses occurs outside oriP and is limited to only a

A. EBV chromosome



B. Structure of oriP

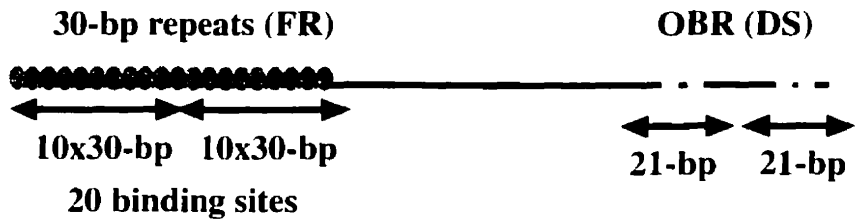


Figure 9. EBV origin structure. A. The positions of oriP and oriLyt on the circularized EBV chromosome are shown. B. The bipartite structure of oriP with the family of repeats (FR) and the dyad symmetry (DS) region are indicated. Refer to text for detail. (Adapted from Yates, 1996).

few cell cycles (Kirchmaier and Sugden, 1998). Thus, it is likely that initiation at oriP is required for ordered episomal replication in synchrony with the cell cycle, but not for viral replication, per se (reviewed in DePamphilis, 1999).

In the lytic phase of EBV, oriLyt is required for replication. Two copies of oriLyt exist, DS-L and DS-R (Hammerschmidt and Sugden, 1988). The DS-L origin contains a 225-bp AT-rich region flanked by a 320-bp and 370-bp elements. The flanking elements contain binding sites for the viral transcription factors BZLF1, which enhances viral replication.

D. Yeast

a) Saccharomyces cerevisiae

The budding yeast, *S. cerevisiae*, represents another model for studying DNA replication. It is particularly relevant because it replicates its genome from approximately 300 origins distributed over several chromosomes, thus closely resembling higher eukaryotes that contain 10^4-10^6 origins. Furthermore, unlike viral origins and like higher eukaryotic origins, yeast origins do not exhibit rigid modular anatomy in which the sequence elements require a specific spacing and orientation, though they are dependent on the DNA sequence.

S. cerevisiae origins coincide with autonomously replicating sequences, ARS elements, which were initially identified as sequences that allowed plasmids to be maintained extrachromosomally (reviewed in Campbell and Newlon, 1991; Toone et al., 1997). Two-dimensional origin mapping techniques showed that the majority of the ARS functioned as origins on yeast chromosomes (Linskens and Huberman, 1988; Dubey et al., 1991). The activation of the origin function of the ARS was influenced by their surrounding chromosomal structure (Held and Heintz, 1992). The ARS are usually situated in AT-rich regions of the genome with an average spacing of 100-kbp.

The ARS elements of *S. cerevisiae* (reviewed in Marahrens and Stillman, 1996) consist of a DNA sequence of 100-150-bp and contain two essential elements, A and B (Figure 10). The A element corresponds to an AT-rich region with an ARS consensus sequence, ACS. The ACS, 5'(A/T)TTTAT(A/G)3' (Broach et al., 1982), is the only

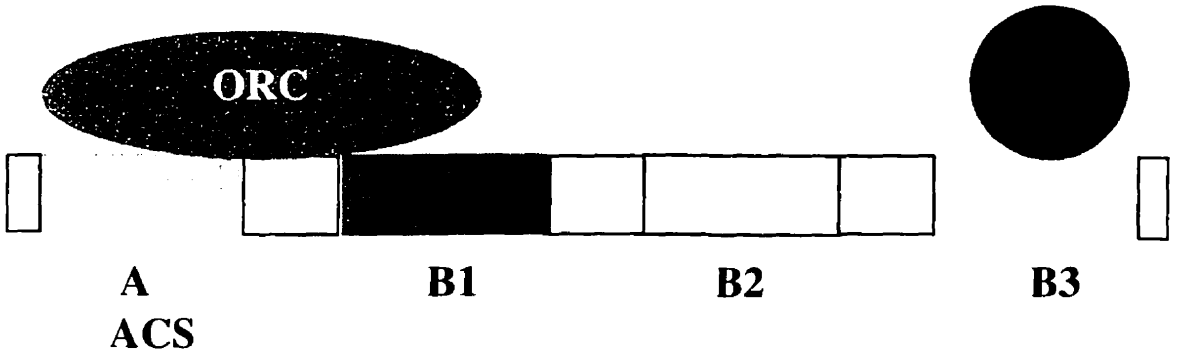


Figure 10. Orgnization of the *S. cerevisiae* origin, the ARS element. Domains A and B and the binding sites for ORC and ABF-1 are shown. Domain A contains the perfect match to the ARS consensus sequence (ACS).

region common to all budding yeast origins. It also serves as the binding site for the yeast initiator protein, the six-subunit origin recognition complex, ORC (Bell and Stillman, 1992; Diffley and Cocker, 1992). The A element is essential but not sufficient for ARS function. Thus, the B element, composed of three functional domains (B1, B2 and B3), is also needed (Marahrens and Stillman, 1992). The B1 element also serves as an ORC binding site. The B2 element contains a DUE of 50-100-bp and may affect replication efficiency as a cis-acting regulator (Lin and Kowalski, 1997). The B3 element serves as the binding site for the 81 kDa ABF-1 (ARS binding factor) transcription factor (Diffley and Stillman, 1988; Diffley and Cocker, 1992). The B3 domain has been identified in many ARS in variable positions, up to 1-kbp from the A element, implying that it can function in a position and orientation independent manner to stimulate replication. Furthermore, it can efficiently be substituted by other transcription factor binding sites (Marahrens and Stillman, 1992). Recently, the nucleotide position at which DNA synthesis initiates within ARS1, one of the *S. cerevisiae* origins, was determined (Bielinski and Gerbi, 1998; Bielinski and Gerbi, 1999). The start site spanned a region of 18-bp and was found within element B1 toward B2, adjacent to the binding site of the initiator, ORC.

The ACS and part of the B1 element, representing only 15% of budding yeast origin sequence, are the only sequences that are conserved in *S. cerevisiae* origins (Huang and Kowalski, 1996). Yeast origins also vary in the frequency and the order in which they are activated (Friedman et al., 1996; Yamashita et al., 1997). The surrounding DNA context greatly influences the ability of the ARS to function as a replication origin (Friedman et al., 1996; Raghuraman et al., 1997), which is not only limited to ORC binding since not all ORC-bound origins fire (Santocanale and Diffley, 1996).

b) Schizosaccharomyces pombe

The fission yeast, *S. pombe*, provides an even better model system to study mammalian DNA replication. In addition to the *S. cerevisiae* advantages over the viral systems, *S. pombe* resembles higher eukaryotes in the structure of unprocessed mRNAs, the organization of small nuclear U2RNA, and the cell division cycle (Allshire et al., 1987). Furthermore, *S. pombe* divides symmetrically, unlike *S. cerevisiae* that divide by

budding. Also, *S. pombe*, like higher eukaryotes, possesses large centromeres with multiple repeated sequences/elements.

S. pombe replication origins range from 500-800-bp and lack an identified consensus sequence that is essential for replication, although they do contain ARS elements (Sakaguchi and Yamamoto, 1982; Caddle and Calos, 1994). The ARS elements contain large AT-rich regions composed of several copies of short stretches of asymmetric AT-rich DNA (Zhu et al., 1994) that can bind proteins, like Abp2, with high affinity for AT-rich sequences (Sanchez et al., 1998).

Mutational studies on *S. pombe* origins have revealed some minimal sequences required for DNA replication. In the *ars*1 origin, for instance, a 782-bp fragment, containing an essential 30-bp AT-rich sequence, was sufficient for ARS activity (Clyne and Kelly, 1995). Another chromosomal origin, *ars*3002, was shown to contain two essential 30-55-bp domains in addition to numerous stimulatory elements within a 600-bp sequence that exhibited ARS activity (Dubey et al., 1996). Nevertheless, a consensus sequence has not been published yet.

E. Mammalian origins

Initial attempts to identify mammalian origins of DNA replication, as sequences that function as ARS elements, showed that any large (> 10-kbp) DNA fragment from a mammalian chromosome was able to provide ARS activity to plasmids transfected into human or rodent cells (Heinzel et al., 1991; Caddle and Calos, 1992; Krysan et al., 1993; Krysan and Calos, 1993). Thus, DNA length appeared more critical than actual DNA sequence. This was further supported by studies showing that initiation occurred at any single randomly chosen site on any DNA molecule following injection of DNA into the eggs of frogs, sea urchins, or fish, or to extracts of *Xenopus* eggs and *Drosophila* embryos (Shinomiya and Ina, 1991; Hyrien and Mechali, 1993; Coverley and Laskey, 1994). However, *in situ* mapping of initiation sites of DNA replication and subsequent physical and genetic analyses of the loci have indeed revealed that DNA replication in metazoan cells occurs at specific sites (reviewed in Zannis-Hadjopoulos and Price, 1998 and references therein). However, DNA replication may initiate nonrandomly at multiple sites within a replicon (Waltz et al., 1996; Kobayashi et al., 1998) and the activity of initiation

Table I. Mammalian DNA replication origins.

Origin	Organism	Main conclusion
β-globin	Human	Mapped within 2-kbp
Lamin B2	Human	Mapped within 500-bp at a site with cell-cycle regulated protein-DNA interactions
HSP70	Human	Mapped to 400-bp in promoter region
rRNA	Human Mouse Rat	Multiple initiation sites in 31-kbp nontranscribed region, with preferred region upstream of rDNA transcription start site
DNA methyltransferase	Human	Mapped within dnmt-1 gene
c-myc	Human	Mapped within a 2.5-kbp region upstream of gene
343	Human	Mapped within a 2.2-kbp region at the 3' end of an actively transcribed region
DHFR	Hamster	Mapped within initiation zone of 55-kbp with three preferred origin localized at 0.5-8-kbp resolution
Ribosomal protein S14	Hamster	Mapped in 2.5-kbp region overlapping with gene
Rhodopsin	Hamster	Mapped in 10-kbp region overlapping with gene
CAD	Hamster	Mapped in 5-kbp region in the CAD transctiptional unit
GADD and TK	Hamster	Mapped in promoter region including a CpG island
Aldolase B	Rat	Mapped in a 1-kbp region containing the AldB promoter
Ig heavy chain locus	Mouse	Mapped within a 600-bp region within enhancer
Ors	Monkey	Mapped to 0.5-2-kbp

*Table adapted from Todorovic et al., 1999.

sites is regulated during development (Walter and Newport, 1997) and may be affected by nuclear, chromatin or DNA structure (reviewed in DePamphilis, 1999).

From the estimated 100, 000 mammalian origins, less than twenty have been identified, thus far (Table I) (reviewed in Todorovic et al., 1999). Even more, only a limited few have been characterized in great detail.

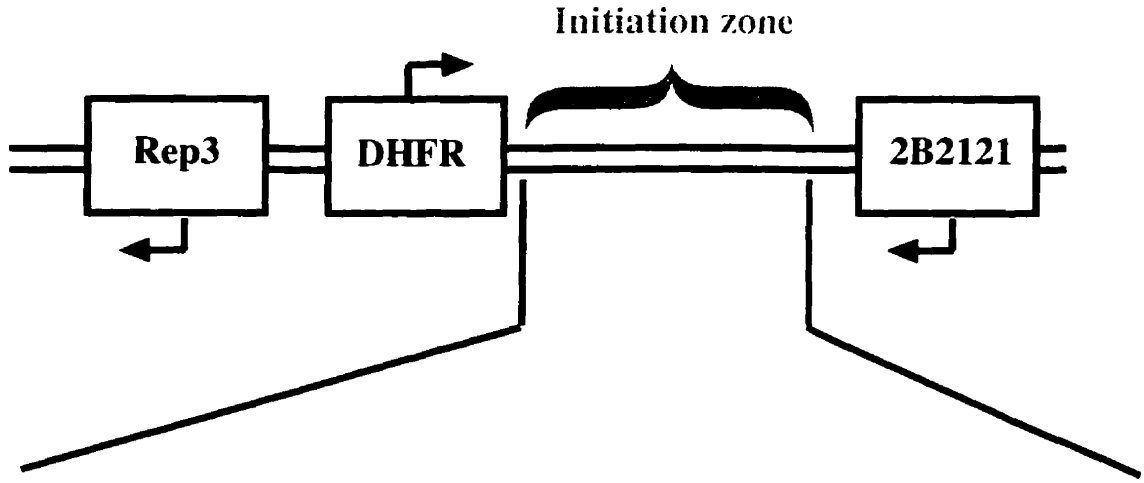
a) Hamster dihydrofolate reductase (DHFR) locus origins

The replication initiation region of the dihydrofolate reductase gene in the Chinese hamster ovary (CHO) cells has been extensively studied over the last few years, and attempts have been made to map the origin with every existing technique, in numerous laboratories (Anachkova and Hamlin, 1989; Leu and Hamlin, 1989; Handeli et al., 1989; Burhans et al., 1990; Burhans et al., 1991; Gilbert et al., 1995; Pelizon et al., 1996; Wu an Gilbert, 1997; Wang et al., 1998; Kobayashi et al., 1998, and references therein). To date, the results are still quite controversial.

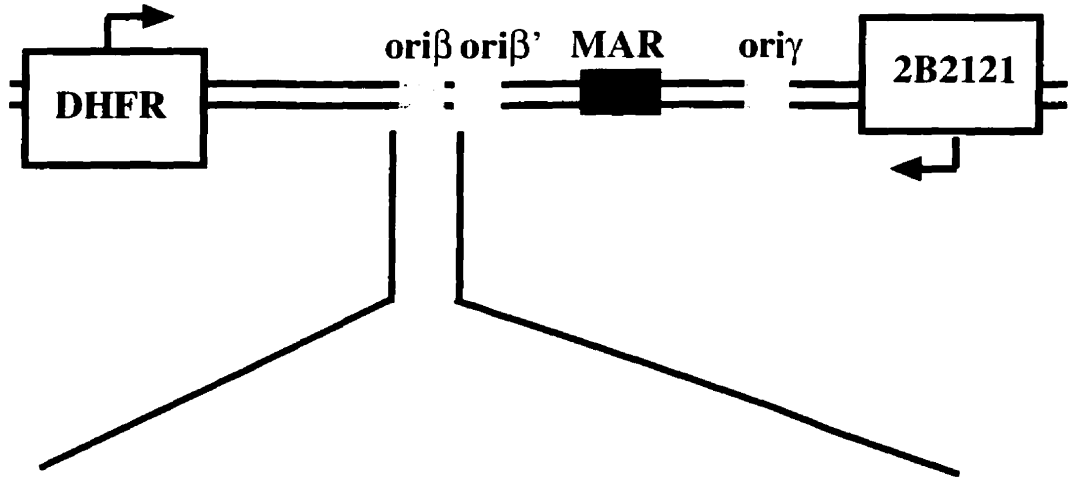
Initial mapping techniques, using two dimensional gel electrophoresis mapping techniques to detect replication bubbles, indicated that initiation events were spread over a 55-kbp region between the DHFR and 2BE2121 genes (Figure 11) (Vaughn et al., 1990; Dijkwel and Hamlin, 1995). Other techniques, measuring the abundance of nascent DNA strands, identifying the earliest labelled nascent fragments or determining the replication fork polarity, have indicated only two preferred initiation sites within this 55-kbp region (Kobayashi et al., 1998, and references therein). These preferred sites include oriβ, the strongest replication site, located within a 2-kbp locus 17-kbp downstream of the DHFR gene, and oriγ, located 23-kbp downstream of oriβ. Furthermore, careful analysis of 21 different genomic regions surrounding oriβ, identified yet another initiation site, oriβ', that is located 5-kbp downstream of oriβ. Oriβ' is used at a much lower frequency that the other two sites (Kobayashi et al., 1998). Taken together, the results suggest that a broad initiation zone could be composed of several individual origins, some of which are preferentially activated (DePamphilis, 1999).

Examination of the sequence elements at, and surrounding the preferred 450-bp oriβ site has revealed that it contains several typical origin features similar to viral and

A. The 250-kbp DHFR amplicon



B. The 55-kbp initiation zone



C. Sequence elements of oriβ

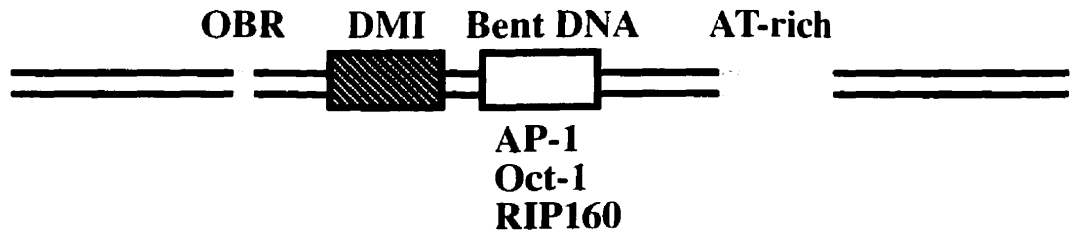


Figure 11. Organization of the Chinese hamster DHFR amplicon. A. The direction of transcription through the DHFR, Rep3, and 2BE2121 transcription units within the 250-kbp amplicon is indicated. B. The 55-kbp initiation zone has three preferred initiation sites, oriβ, oriβ', and oriγ (see text for details). C. The preferred initiation site, oriβ, contains several sequence elements such as bent DNA, AT-rich region, a densely methylated island (DMI), and binding sites for Oct-1, AP-1 and RIP160. The origin of bidirectional replication (OBR) is also shown. (Adapted from Heintz, 1996).

lower eukaryotic origins (Figure 11). These include an AT-rich region, bent DNA and binding sites for several transcription factors, such as AP-1 and Oct-1 (reviewed in Heinz, 1996). Also, several repeated sequence motifs, such as ATT, which are binding sites for the RIP160 protein, also exist. The region surrounding oriβ also has DUE and MAR elements. Two specific protein-DNA interactions have been detected as micrococcal nuclease hypersensitive sites, one at oriβ, and the other at oriγ (DePamphilis, 1999). Recently, deletions studies of the DHFR locus were performed to determine the genetic elements required for mammalian origin activity (Kalejta et al., 1998). Deletion of a 5' intergenic spacer and the 3' end of the DHFR gene abolished origin firing from oriβ, indicating that additional cis-acting sequences are required for efficient replication. Reports from other laboratories have recently shown that a 5.7-kbp fragment of the DHFR locus containing the oriβ initiation region was able to initiate DNA replication when stably transfected into random chromosomal locations in a hamster cell line lacking the endogenous DHFR locus (Altman et al., 2000; Kolman et al., 2000). Furthermore, specific DNA sequences within this fragment, consisting of an AT-rich stretch and a GA dinucleotide repeat, were required for efficient DNA replication.

b) Human β-globin origin

The human β-globin locus has been extensively studied because it is linked to many genetic defects, including haemoglobin Lepore syndrome, Hispanic thalassemia, and other β-thalassemias. In this 200-kbp β-globin locus, containing 5 linked genes (ε, γA, γG, δ and β), a single origin has been identified in a 2-kbp region upstream of the β-globin gene, between the δ and β genes (Kitsherg et al., 1993). This origin is active in a variety of different cell types and is independent of the expression of the β-globin gene. Furthermore, when a region containing this origin is deleted, replication at this site is abolished and the region is passively replicated by another unidentified upstream origin (Kitsherg et al., 1993).

An important regulatory element of the β-globin origin is present in the 5' upstream locus control region (LCR). Deletion of this region prevents β-globin expression, abolishes origin function and shifts replication timing from early to late S phase (Aladjem

et al., 1995). Recently, an 8-kbp DNA sequence encompassing the β-globin origin initiated DNA replication when targeted to a new chromosomal location using the CRE/LOX recombination system (Aladjem et al., 1998). Deletions within this sequence suggested the presence of a core replicator sequence and two flanking auxiliary sequences. Interestingly, the LCR was not needed in this ectopic site (Aladjem et al., 1998). It is postulated that the LCR is required at the chromosomal location to decondense chromatin structure (Aladjem et al., 1995).

c) Human lamin B2 origin

The human lamin B2 origin is another mammalian origin that has been studied in detail. It was initially isolated as a nascent DNA fragment synthesized in synchronized HL-60 cells immediately after release into S phase (Triboli et al., 1987) but has been shown to be functional in all cell types (Kumar et al., 1996). By competitive polymerase chain reaction (PCR) mapping techniques, the initiation site has been located to a 474-bp region corresponding to the 5' end of the lamin B2 gene and the 3' portion of the intergenic spacer separating the lamin B2 gene from the adjacent gene, ppv1 (Giacca et al., 1994). More recently, the start sites, at a single nucleotide level reflecting the transition point between continuous and discontinuous DNA synthesis for upper and lower nascent DNA, has been identified for the lamin B2 origin (Abdurashidova et al., 2000). The initiation event occurs within an AT-rich region. *In vivo* genomic footprinting experiments have detected a 78-nt protected region coinciding with the initiation site (Dimitrova et al., 1996; Abdurashidova et al., 1998). The footprint is highly asymmetric on the two strands, similar to that observed for the ORC in yeast and T-Ag in SV40. The presence and the size of this footprint change in a cell cycle-dependent fashion, suggesting the assembly of a preinitiation complex that changes to a postreplication complex and then disappears during mitosis. Recently, ORC2, Cdc6, and MCM2 were identified as members of the footprinted complex in the lamin B2 origin (Todorovic et al., 2000).

d) Human c-myc origin

Another human origin of DNA replication has been identified approximately 2-kbp upstream of the c-myc gene, within a 2.4-kbp DNA fragment (Iguchi-Ariga et al., 1988; McWhinney and Leffak, 1990; Dobbs et al., 1994). The 2.4-kbp fragment contains characteristic origins features such as a DUE, a unique nucleosome organization, and matches to the *S. cerevisiae* ARS consensus (Malott and Leffak, 1999). It is able to confer autonomous replication to plasmids when transfected into HeLa cells (Iguchi-Ariga et al., 1988; McWhinney and Leffak, 1988; 1990; McWhinney et al., 1995), and in a cell free *in vitro* DNA replication system (Berberich et al., 1995; Ishimi et al., 1995). Interestingly, the activity of the 2.4-kbp origin fragment was two-fold higher in HeLa cells when compared to normal human skin fibroblasts (NSF) (Tao et al., 1997). Recently, the 2.4-kbp fragment was shown to possess chromosomal replicator activity since it initiated DNA replication when integrated at an ectopic site in the HeLa genome (Malott and Leffak, 1999). Even though the majority of the c-myc origin characterization has been performed with the 2.4-kbp fragment, multiple, nonrandom initiation sites have been reported in the c-myc locus (Berberich et al., 1995; McWhinney et al., 1995; Waltz et al., 1996; Trivedi et al., 1998). When the activity of these multiple initiation sites, spanning a region of over 12-kbp, was compared in HeLa and NSF cells, no difference in the c-myc locus was observed (Tao et al., 2000). However, the origin activity of all the sites was two-fold lower in NSF, as previously reported for the 2.4-kbp fragment (Tao et al., 1997). This suggests that regulators, that are likely present in higher concentrations in transformed cells, influence the activity of the initiation sites in the c-myc locus.

e) Origin enriched sequences (ors)

Origins enriched sequences, *ors*, represent early replicating DNA sequences that were isolated by extrusion of African green monkey kidney nascent DNA from active replication bubbles at the onset of S phase (Zannis-Hadjopoulos et al., 1981; 1983; 1985; Kaufmann et al., 1985). The nascent strand extrusion method is based on the instability of replication bubbles leading to the isolation of double-stranded DNA *via* extrusion (Figure 12). The rationale behind the method is that short nascent DNA should contain an origin

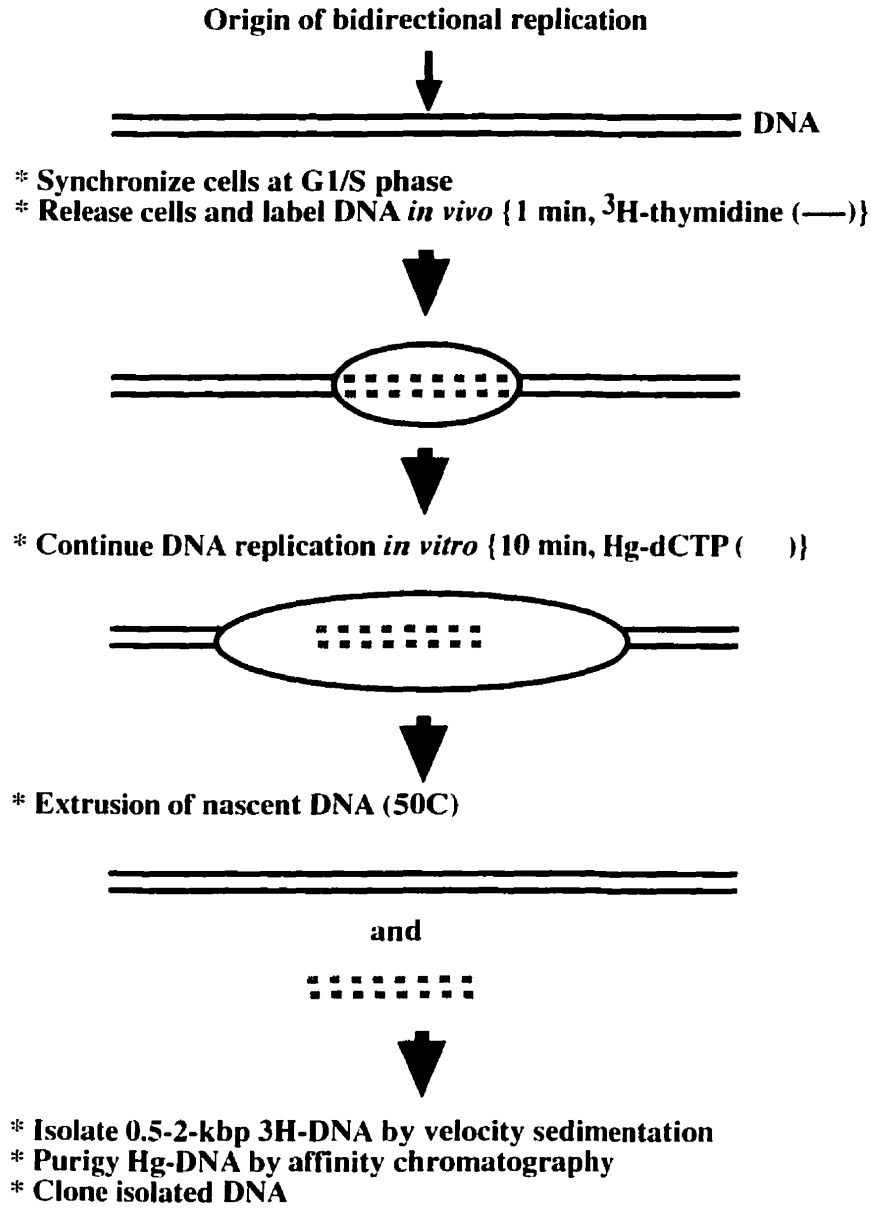


Figure 12. Nascent strand extrusion method. The nascent strand extrusion method was used to purify the *ors* origins from early replicating CV-1 DNA. The cells are synchronized at G1/S, released into S phase, and the DNA is labeled *in vivo* with ^{3}H-thymidine. Nuclei are then isolated and the DNA is replicated *in vitro* in the presence of Hg-dCTP. The nascent strands are extruded, purified, and cloned into pBR322. The resulting clones are tested for autonomous replication, as described in the text.

of bidirectional replication at or near its centre, and was proven using the SV40 replication system (Zannis-Hadjopoulos et al., 1984). The nascent fragments that were isolated, ranging in size from several hundred base pairs to 2-kbp, were cloned into plasmid vectors, and assayed for their ability to replicate.

Over 50% of the *ors* clones tested were able to replicate autonomously *in vivo*, upon transfection in CV-1, COS or HeLa cells, as assayed by the *Dpn*I resistance and bromodeoxyuridine substitution assays (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991). Furthermore, at least four of the *ors*, *ors*3, 8, 9, and 12, were able to replicate *in vitro*, in a cell free system that uses HeLa cell extracts (Pearson et al., 1991). The ability of the *ors* to serve as initiation sites was further confirmed by electron microscopic examination of the replicative intermediates (Pearson et al., 1994b), and by *in vivo* chromosomal mapping techniques (Wu et al., 1993b; Pelletier et al., 1999). Furthermore, when cloned in YAC vectors, the autonomously replicating DNA sequences were maintained under G418 selection as HeLa episomes (Nielsen et al., 2000).

Initiation of autonomous replication from the *ors* is site-specific and sequence-dependent, as mapped by earliest labelled fragment analysis. Furthermore, replication is semiconservative and bidirectional. DNA replication from the *ors* is dependent on the replicative DNA polymerases α and/or δ, as demonstrated by sensitivity to aphidicolin, an inhibitor of DNA polymerases α and/or δ, and resistance to ddTTP, an inhibitor of DNA polymerases β and γ.

Nucleotide sequence analysis revealed that the *ors* contained unique or low copy, middle repetitive (O family) or highly repetitive (Alu-like or α-satellite) sequences (Rao et al., 1990). Also, AT-rich regions, IR sequences, bent DNA, ARS consensus sequence of yeast, and MAR were also present (Rao et al., 1990). They also contained transcriptional regulatory elements, such as CTF/NF-1 and related sequences, the β-globin transcriptional control region (CACCC), as well as the Oct-1 binding sequence (Rao et al., 1994; Landry and Zannis-Hadjopoulos, 1991; Wu et al., 1993).

Among the functional *ors*, *ors*8 has been analyzed in detail, both *in vivo* (Landry and Zannis-Hadjopoulos, 1991; Zannis-Hadjopoulos et al., 1992) and *in vitro* (Pearson et al., 1991; Zannis-Hadjopoulos et al., 1992). *Ors*8 is 483-bp, present in less than 5 copies/ haploid CV-1 genome, and is replicated in the early part of S phase (Zannis-Hadjopoulos

et al., 1985; 1988). Replication of *ors*8 is semiconservative, as shown both *in vivo*, by the incorporation of bromodeoxyuridine (BrdUTP) (Frappier and Zannis-Hadjopoulos, 1987), and *in vitro,* by the incorporation of BrdUTP and separation of BrdUTP-substituted DNA on both neutral and alkaline cesium chloride (CsCl) density gradients (Pearson et al., 1991; Zannis-Hadjopoulos et al., 1992). Time-course and electron microscopy mapping of the *ors*8 origin of replication showed that replication starts within the *ors* and proceeds bidirectionally (Pearson et al., 1991; 1994). The replicating genomic *ors*8 is enriched on the nuclear matrix in early S phase (Mah et al., 1993).

By deletion analysis, a 186-bp DNA fragment of *ors*8 was identified as the minimal sequence required for origin function *in vivo* and *in vitro* (Todd et al., 1995). This minimal replicating sequence contains an imperfect inverted repeat capable of extruding into a cruciform, a measurable sequence-directed DNA bent, and several repeated sequence motifs, such as ATTA and ATTTA, which occur frequently in the matrix attachment sites of several genes and in the DHFR oriβ (Boulikas, 1992). It also has two 7-bp stretches that are identical to A3/4 (Ruiz et al., 1999), a 36-bp mammalian origin consensus sequence (Price et al., submitted) (see section IV. ii), E. f) Mammalian origin consensus sequence). The 186-bp fragment also contains an imperfect binding site for the Oct-1 transcription factor and several binding sites for the GATA family of transcription factors.

Oct-1 has been shown to enhance the *in vitro* DNA replication of p186, a pBR322-based plasmid that contains the minimal origin of *ors*8 (Matheos et al., 1998; [see chapter two of this thesis]). Although Oct-1 was able to bind to the imperfect consensus sequence in the 186-bp fragment, its effect on replication was not mediated by direct binding to the octamer motif but rather through protein-protein interactions (Matheos et al., 1998; Matheos et al., submitted; [see chapter two and four of this thesis, respectively]). The GATA transcription factors, however, were shown not to be involved in DNA replication (Matheos et al., submitted; [see chapter four of this thesis]).

The186-bp fragment was also used as a probe for the purification of an origin specific binding activity, OBA (Ruiz et al., 1995). OBA was, subsequently, demonstrated to possess sequence-specific binding to A3/4, and its DNA binding subunit has been identified as the 86 kDa subunit of Ku antigen (Ruiz et al., 1999; [see chapter three of this

thesis]). Ku antigen, a heterodimer of 70 kDa and 86 kDa, serves as the DNA binding subunit of the DNA-dependent protein kinase (DNA-PK), and is described in detail in section X of this thesis. A role of OBA/Ku antigen in DNA replication has been demonstrated (Ruiz et al., 1999; Matheos et al., submitted; Matheos et al., submitted; [see chapter three, four, and five, respectively, of this thesis]).

f) Mammalian origin consensus sequence

A degenerate consensus sequence for mammalian replication origins has been previously published (Dobbs et al., 1994). However, no functional test for this consensus sequence was reported. Another mammalian consensus sequence, with homologies in the human lamin B2 and the β-globin origins, ACE origin of *Drosophila* chorion genes, and the yeast *S. cerevisiae* and *S. pombe* ARS elements has also been described (Todorovic et al., 2000). The sequence of this consensus is 5' TTTATTG 3'. *In vivo* genomic footprints indicated that the region around this area binds ORC2, Cdc6, and MCM5. However, this consensus sequence is highly AT-rich and may simply represent an AT-rich stretch, a common feature of origins of DNA replication.

Recently, a mammalian consensus sequence, capable of supporting autonomous replication *in vivo* and *in vitro*, has been identified (Price et al., submitted). The consensus sequence was derived from mammalian African green monkey *ors* (see above) with autonomously replicating activity (Landry and Zannis-Hadjopoulos, 1991) or from such sequences isolated from normal human skin fibroblast (Nielson et al., 1994) cells, using a reiterative process between pairs of monkey and human sequences. The derived consensus is 36-bp and 46% GC-rich, 5'CCTCAAATGGTCTCCAATTTTCCTTTGG CAAATTCC 3', and has homologies to the mammalian *ors*, c-myc, lamin B2, β-globin, IgM μ chain enhancer, heat shock protein 70, DHFR, NOA3, rodent RPS14 origins and to CpG islands. One version of the consensus, termed A3/4, has been studied extensively. A3/4 is able to confer autonomous replication to plasmids *in vitro* and *in vivo*, upon transfection into HeLa, bovine and chicken embryo fibroblasts, in normal (WI38 lung fibroblasts) and immortal (mouse 3T3) cells, and in fruit fly (*Drosophila* S2) cells. Also, an A3/4-containing plasmid can be maintained as an episome in HeLa cells, under G418 selection.

The A3/4 sequence has been shown to be a sequence-specific binding site for OBA/Ku antigen (Ruiz et al., 1999; Schild-Poulter et al., submitted; [see chapter three of this thesis]). Furthermore, addition of an oligonucleotide comprising the A3/4 sequence to an *in vitro* DNA replication system abolished DNA replication (Ruiz et al., 1999; Matheos et al., submitted; [see chapter three and four, respectively, of this thesis]). The replication level was, however, restored upon the addition of OBA/Ku antigen to the inhibited reaction (Matheos et al., submitted; [see chapter four of this thesis]). Thus, A3/4 represents a functional mammalian consensus sequence.

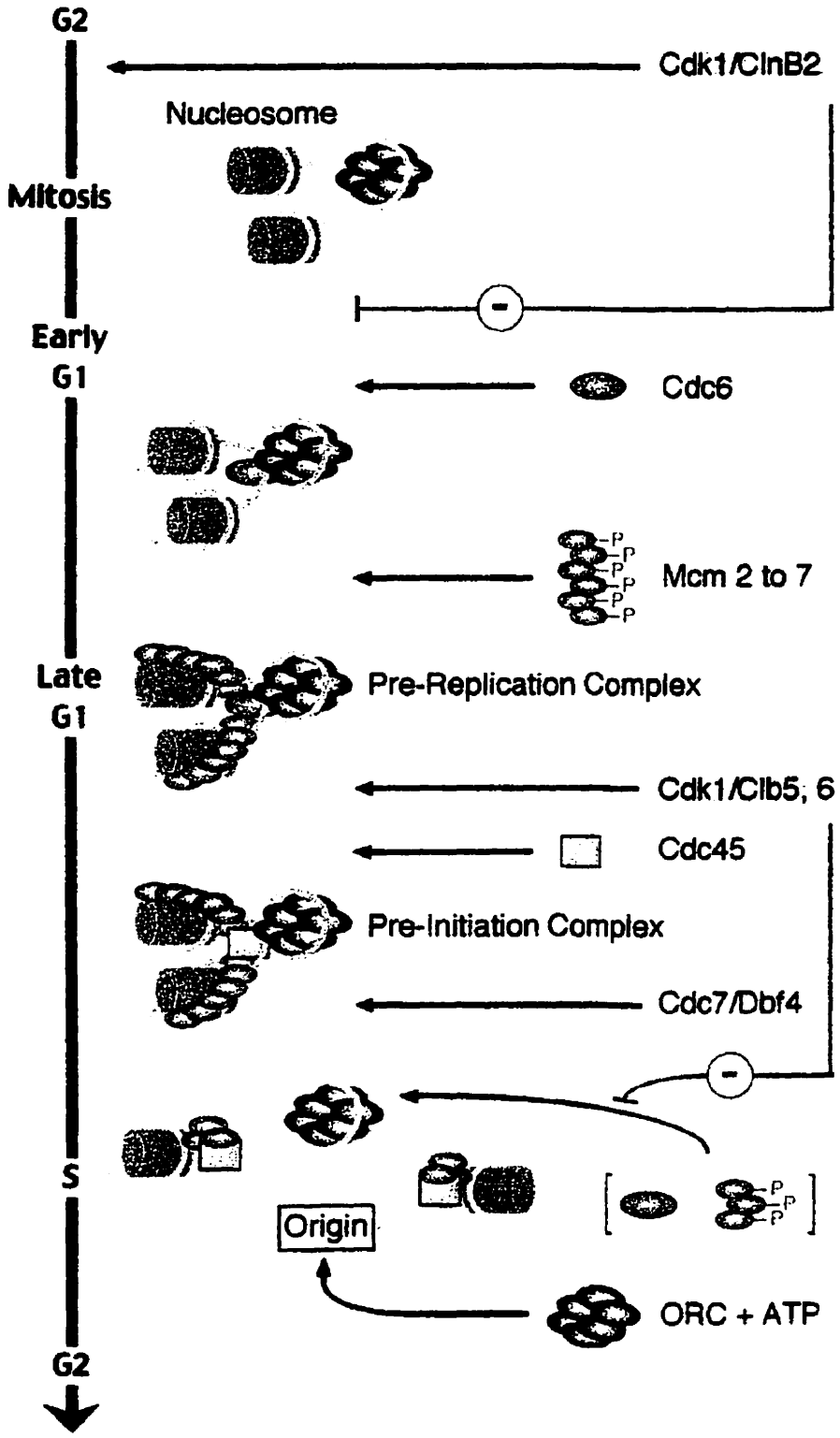
V. ESSENTIAL FACTORS FOR THE REGULATION OF INITIATION OF DNA REPLICATION

In eukaryotic cells, the DNA is organized into chromosomes and replication initiates from multiple origins on these chromosomes that fire in a highly regulated manner. For the faithful transmission of genetic information to progeny cells, the entire DNA genome must be duplicated only once during a single cell cycle. Mechanisms exist to clearly differentiate replicated from unreplicated DNA and to mark unfired origins. This mechanism for regulation of initiation of DNA replication relies on an initiator protein and licensing factors.

Several factors that are essential for the regulation of initiation of DNA replication have been identified and they include the origin recognition complex (ORC), the cell division cycle protein 6 (Cdc6), and the mini chromosome maintenance (MCM) proteins. Most of the present knowledge relies on findings from the budding yeast, *S. cerevisiae.* However, homologues of these proteins have been found in early embryonic and mammalian somatic cells (reviewed in Fujita, 1999).

In the budding yeast, ORC, in an ATP-dependent step, binds to specific DNA sequences in the origin (Figure 13). Upon formation, the ORC/DNA complex is stable throughout the cell cycle. In early G1 phase, Cdc6 binds to the ORC/DNA complex. Then, the MCM proteins are recruited and bind to chromatin at or near a replication origin. Subsequently, Cdc45 replaces Cdc6 in a step that requires CdK1/Ckb5,6, a cyclin-

Figure 13. Assembly and activation of the prereplication complex. See text for detail. (Reproduced from DePamphilis, 1998).


G2
Mitosis
Early G1
Late G1
S
G2
Cdk1/ClnB2
Nucleosome
Cdc6
Mcm 2 to 7
Pre-Replication Complex
Cdk1/Clb5, 6
Cdc45
Pre-Initiation Complex
Cdc7/Dbf4
Origin
ORC + ATP

dependent protein kinase. This represents the preinitiation complex that is activated by the protein kinase Cdc7/Dbf4. Upon activation, initiation of DNA replication occurs. MCM4/6/7 remain associated with the DNA and migrate with the replication fork, perhaps serving as the replicative DNA helicases (Figure 13). Following the formation and activation of the prereplication complex, the DNA replication machinery is recruited to the initiation site and DNA synthesis occurs (see section VII. Mammalian DNA replication proteins).

i) Origin recognition complex (ORC)

The origin recognition complex, ORC (reviewed in Quintana and Dutta, 1999), serves as the yeast initiator protein and binds to the origin of DNA replication. It is composed of six subunits of 120, 72, 62, 56, 53, and 50 kDa (Bell and Stillman, 1992). It was initially identified as binding to yeast ARS sequences *in vitro* (Bell and Stillman, 1992). Genomic footprinting assays and immunoprecipitation of crosslinked chromatin have demonstrated that ORC also binds to ARS *in vivo*, throughout the cell cycle (Diffley et al., 1994; Cocker et al., 1996; Santocanale and Diffley, 1996; Tanaka et al., 1997; Aparicio et al., 1997). The ORC footprint includes the A element and extends into the B1 element of the yeast ARS.

Genetic analyses of yeast ORC mutants have provided evidence that ORC is involved in the initiation of DNA replication. Mutations in ORC2 and ORC5 cause plasmid maintenance defects (Loo et al., 1995). Furthermore, ORC2 mutants are defective at the G1/S phase transition (Bell et al., 1993), and have reduced initiation at chromosomal origins (Fox et al., 1995). Finally, mutations in ARS elements that abolish ORC binding inhibit DNA replication (Rao and Stillman, 1995).

ORC-like complexes, containing all six subunits, have been isolated from *Xenopus* egg extracts and *Drosophila* embryos (Gossen et al., 1995; Carpenter et al., 1996; Rowles et al., 1996). *Xenopus* ORC is essential for chromatin binding of Cdc6 and DNA replication (Carpenter et al., 1996; Rowles et al., 1996). Immunodepletion of ORC from *Xenopus* extracts abolishes *in vitro* DNA replication (Carpenter et al., 1996; Romanowski et al., 1996). *Xenopus* ORC is not bound to chromatin throughout the cell cycle but rather associates with it during the G1 phase and dissociates from it during

mitosis (Coleman et al., 1996). Also, in *Xenopus* cells, ORC immunostaining overlaps with sites of DNA replication during early S phase (Romanowski et al., 1996).

All six ORC subunits of the mammalian ORC complex have now been identified and isolated (Reviewed in Quintana and Dutta, 1999; Fujita, 1999; Dhar et al., 2000), and physical interactions do occur between them, although not in the form of a tight complex (Quintana et al., 1997; 1998; Dhar et al., 2000). ORC2-5 are tightly associated whereas ORC1 and ORC6 are not part of the complex (Wolf et al., 1996; Dhar et al., 2000). To date, no direct evidence of the involvement of ORC in the initiation of mammalian DNA replication has been presented (Quintana and Dutta, 1999). ORC footprints at mammalian origins have been demonstrated for the lamin B2 origin (Abdurashidova et al., 1998; Todorovic et al., 2000). The ORC footprint enlarges along G1 phase, shrinks during S phase, remains unchanged in G2 phase, and disappears at M phase (Abdurashidova et al., 1998; Abdurashidova et al., 2000).

ii) Cell division cycle 6 protein (Cdc6)

Cell division cycle 6 protein, Cdc6, is a 58 kDa protein with a purine nucleotide binding site (Zhou et al., 1989) that is essential for the formation of the prereplication complex and recruitment of the MCM proteins (Cocker et al., 1996; Donovan et al., 1997).

In the budding yeast, Cdc6 is associated with ARS elements *in vivo*, likely through a direct interaction with ORC (Liang et al., 1995; Tanaka et al., 1997). Upon depletion of Cdc6, yeast cells undergo a transient arrest at the G1/S phase boundary, and aberrant mitosis in the absence of DNA replication, resulting in cell death (Kelly et al., 1993; Piatti et al., 1995). Thus, Cdc6 is likely involved in the initiation of DNA replication. Cdc6 is expressed primarily during late M phase and late G1 phase (Piatti et al. 1995). At the G1/S phase transition, Cdc6 is completely degraded in yeast cells (Piatti et al., 1995).

Cdc6 is also essential for DNA replication in *Xenopus* egg extracts, as demonstrated by immunodepletion experiments (Coleman et al., 1996). However, the levels of *Xenopus* Cdc6 remain constant throughout the cell cycle. Instead, control of the availability of Cdc6 is affected by its subcellular localization (Coleman et al., 1996).

In vivo immunodepletion of mammalian Cdc6, by microinjection of Cdc6 antibodies into human cell nuclei, inhibits entry into S phase (Hateboer et al., 1998; Yan et al., 1998). Thus, Cdc6 has an essential role in mammalian DNA replication. Furthermore, the competence to initiate mammalian DNA replication coincides with the time of maximum Cdc6 accumulation (reviewed in Quintana and Dutta, 1999).

iii) Mini chromosome maintenance (MCM) proteins

The mini chromosome maintenance proteins, MCM (reviewed in Kearsey and Labib, 1998; Tye, 1999; Tye and Sawyer, 2000 and references therein), are nuclear proteins that are essential replication initiation factors. They were initially revealed to be involved in DNA replication as the result of a yeast genetic screen for mutants defective in mini chromosome maintenance (Maine et al., 1984). The most popular among them are the MCM2-7, a family of six proteins that are components of the initiation replication machinery (Chong et al. 1995; Todorov et al., 1995; Labib et al., 2000). MCM1 is a transcription factor whose role in DNA replication is unclear (Passmore et al., 1989) while MCM10 functions as an initiator factor that interacts with MCM2-7 (Merchant et al., 1997). Other MCM proteins are involved in chromosome segregation.

In yeast, MCM proteins are loaded around ARS elements to form prereplication complexes in the G1 phase, as demonstrated by chromatin immunoprecipitation assays and MCM formaldehyde crosslinking (Tanaka et al., 1997; Aparicio et al., 1997). However, the crosslinked region moves away from the ARS element with the progression into S phase suggesting that some MCM proteins associate with the ARS element and then move with the replication forks.

MCM2-7 have also been identified in mammals as essential components for the initiation of DNA replication. Injection of MCM antibodies or treatment with antisense oligonucleotides inhibits entry into S phase (Todorov et al., 1994). The total amount of MCM proteins remains constant throughout the cell cycle. However, the amount of MCM proteins bound to chromatin decreases with S phase progression (Todorov et al., 1994; reviewed in Fujita, 1999). Binding of mammalian MCM proteins to chromatin requires the replication-licensing factor B, RLF-B. Phosphorylation of the MCM2-7 complex is likely an important regulatory mechanism for initiation by restricting DNA replication to

once in a cell cycle (Ishimi et al., 2000). However, the phosphorylation of MCM is quite complicated, occurs in S and G2/M phase, and is not well understood (reviewed in Fujita, 1999; Tye, 1999).

A subcomplex of human MCM proteins, MCM4/6/7, have a weak, ATP-dependent helicase activity and can unwind 30-bp of DNA (Ishimi, 1997). MCM4/6/7 exhibit ATP-dependent single-stranded DNA binding, single-stranded DNA stimulated ATPase activity and a weak 3'-5' helicase activity (reviewed in Tye and Sawyer, 2000). The phosphorylation of MCM4/6/7 by a cyclin-dependent kinase (cyclin A/Cdk2) inactivates the helicase activity, thus providing another regulatory mechanism (Ishimi et al., 2000).

iv) Cell division cycle 45 protein (Cdc45)

Cell division cycle 45 protein, Cdc45, is another protein of the prereplication complex that is also essential for DNA replication. Cdc45 directly interacts with the MCM proteins, suggesting that its role is coupled to the action of the MCM complex. Cdc45 is associated with chromatin during late G1 and S phase, and its binding is dependent on the Cdc28-Clb kinase, Cdc6 and MCM2 (Zhou and Stillman, 1998). Also, Cdc45 yeast mutants are defective in initiation of DNA replication, as determined by two-dimensional gel electrophoresis, consistent with a role in initiation (Zou et al., 1997). Loss of Cdc45 in G1 phase prevents DNA replication without preventing subsequent entry into M phase, while loss of Cdc45 during S phase blocks S phase progression. Recently, a role of Cdc45 in elongation of DNA replication was suggested (Tercero et al., 2000), since Cdc45 inactivation prevented progression of individual replication forks. Thus, Cdc45 plays a key role in initiation and elongation of DNA replication.

Cdc45 homologues have been identified in *Xenopus* and human cells (Mimura and Takiwasa, 1998). *Xenopus* Cdc45 is essential for DNA replication, since its depletion abolishes replication. Furthermore, *Xenopus* Cdc45 directly interacts with DNA polymerase α and likely plays an important role in loading DNA polymerase α to the chromatin.

v) Cdc7/Dbf4 kinase

Cdc7/Dbf4 is a serine/threonine protein kinase whose activity is required at the onset of DNA replication (Hollingsworth et al., 1992). Cdc7 is the catalytic subunit and is expressed at a constant level throughout the cell cycle. On the other hand, Dbf4, the regulatory subunit, is expressed periodically from G1 throughout S phase (Jackson et al., 1993; Yoon et al., 1993). The activity of Cdc7/Dbf4 peaks at G1/S phase. At this point, Cdc7/Dbf4 triggers a series of reactions that lead to initiation of DNA replication. The target of the Cdc7/Dbf4 kinase is likely the MCM complex. Cdc7/Dbf4 can phosphorylate MCM proteins *in vitro* (Lei et al., 1997) and this phosphorylation event likely releases the MCM helicase from the inactive prereplication complex (Leatherwood, 1998).

VI. REGULATORY MECHANISMS LIMITING ORIGIN FIRING TO ONCE PER CELL CYCLE

Several regulatory mechanisms have been proposed that ensure that origins fire once and only once per cell cycle (reviewed in DePamphilis, 1998; 1999; Davey and O'Donnell, 2000).

First, Cdc6 is degraded at the onset of S phase and does not reappear until early G1 phase, thus ensuring that the formation of the prereplication complex does not occur until the following G1 phase. Second, metazoan MCM proteins remain within the nucleus during S phase, but RLF-B is absent, thus preventing binding of MCM to chromatin. RLF-B enters the nucleus during M phase. In yeast cells, MCM proteins are secreted to the cytoplasm during S phase and thus are not available for prereplication complex formation. Third, the same cyclin-dependent protein kinase required for mitosis also prevents the assembly of the prereplication complex, ensuring that DNA replication does not occur until M phase is complete.

VII. MAMMALIAN DNA REPLICATION PROTEINS

Replication of the two template strands at the eukaryotic DNA replication fork is a highly coordinated process that does not occur by random collisions between soluble enzymes and DNA. Instead, replication is orchestrated by organized multiprotein complexes (reviewed in Malkas, 1998).

A significant advance in the study of mammalian DNA replication came almost twenty years ago with the development of the first cell free *in vitro* DNA replication system using SV40 (Li and Kelly, 1984). The only requirements for this system include primate cell extracts and the virally encoded initiator protein, T-Ag. Because this virus replicates in the nucleus of its permissive host and its replication is almost entirely dependent on the host cell machinery, it has been an invaluable tool to study DNA replication. Fractionation of the cell extracts and reconstitution of replication function assays identified the cellular proteins required for DNA replication.

Most, if not all, of the proteins required for DNA replication have now been identified and characterized biochemically and genetically. It has become obvious that the sequence of events at the DNA replication fork and the functions of the replication proteins are highly conserved in different organisms.

The principal activities exhibited by replication proteins in prokaryotes and eukaryotes are (reviewed in Waga and Stillman, 1998; DePamphilis, 1999): DNA helicase, DNA primase, DNA polymerase, proofreading exonuclease, sliding clamp, sliding clamp assembly factor, single-strand DNA binding protein, RNA primer excision, DNA ligase, and DNA topoisomerase (Table II).

i) DNA helicases

DNA helicases are enzymes that promote the processive unwinding of the two strands of the DNA duplex to yield a transient single-stranded DNA intermediate required for replication (reviewed in Lohman and Bjornson, 1996). DNA helicases translocate unidirectionally along the DNA strand, displacing the other strand and allowing the

Table II. Mammalian DNA replication proteins.

Protein	Replicative function	Subunit (kDa)
DNA polymerase α/primase	RNA-DNA primer synthesis	186, 70, 58, 48
DNA polymerase δ	DNA polymerase; 3'-5' exonuclease	125, 48, 25, 12
RP-A	Single-stranded DNA binding protein; stimulates polymerases; facilitates helicase loading	70, 29, 14
PCNA	Polymerase α/δ processivity factor; stimulates polymerases and RF-C ATPase	36
RF-C	DNA-dependent ATPase; primer-template DNA binding; stimulates polymerases;PCNA loading	128, 39, 40.5, 39.6, 38.5
RNase HI	Nuclease for removal of RNA primers; Okazaki fragment maturation	89
FEN-1	Nuclease for removal of RNA primers; Okazaki fragment maturation	46
DNA ligase I	Ligation of DNA; joining of Okazaki fragments	125
DNA topoisomerase I	Unlinks parental strand	100
DNA topoisomerase II	Unlinks parental strands and progeny duplexes	172
DNA helicase (candidates include MCM proteins and Ku antigen)	Unwinds the DNA	

*Table modified from Brush and Kelly (1996) and Waga and Stillman (1998).

separated strands to serve as DNA templates for leading and lagging strand synthesis. The helicase reaction is coupled to the binding and hydrolysis of 5' nucleoside triphosphates.

The identified and well-characterized *E. coli* DnaB helicase, the SV40 T-Ag helicase and the helicases of the T7 and T4 bacteriophages share a conserved hexameric structure with similar properties of ATP-dependence, high processivity, and interaction with the replicative DNA polymerase (Baker and Bell, 1998). It is likely that several distinct helicases could function in eukaryotic chromosomal replication and that the role of an individual helicase may be required at a specific step in the replication process (reviewed in Waga and Stillman, 1998). Several eukaryotic DNA helicases have been isolated and studied extensively (reviewed in Borowiec, 1996). However, the DNA helicase used at eukaryotic replication forks is still not identified. At present, the favourite candidates are the MCM proteins, because they appear to travel with the replication fork and they exist as a hexameric complex (reviewed in Tye, 1999; Tye and Sawyer, 2000). Recently, human DNA helicases I and IV (Tuteja et al., 1990; Tuteja et al., 1991) were identified in a mammalian multiprotein replication complex (Sekowski et al., 1998). Furthermore, another component of the mammalian multiprotein DNA replication complex, the DNA-dependent ATPase identified as Ku antigen (Cao et al., 1994), also possesses DNA helicase activity (Tuteja et al., 1994; Matheos et al., submitted [see chapter four of this thesis]) and is involved in DNA replication (Ruiz et al., 1999; Matheos et al., submitted; Matheos et al., submitted; [see chapter three, four, and five of this thesis, respectively]).

ii) DNA primase

DNA primase is an oligoribonucleotide polymerase required to initiate RNA primer synthesis (reviewed in Arezi and Kuchta, 2000). RNA primers are needed for the initiation of leading strand and Okazaki fragment synthesis and are subsequently extended by DNA polymerase α (Lehman and Kaguni, 1988). In eukaryotes, DNA primase consists of two polypeptides of 58 kDa and 48 kDa and is always tightly associated with DNA polymerase α. The p48 subunit contains the primase catalytic subunit whereas the p58 subunit is necessary for the stability and activity of p48. The primase initiation sites are affected by the nucleoside triphosphate (NTP) concentrations:

at low NTP concentration, primers are synthesized at pyrimidine-rich sites, whereas at high, physiological, NTP concentrations, primers are synthesized at all regions (Kirk et al., 1997).

iii) DNA polymerases

DNA synthesis is required to duplicate the genetic information prior to cell division. It is also required during DNA repair, recombination, and bypassing DNA lesions when DNA is damaged. DNA synthesis is achieved through the action of DNA polymerases (reviewed in Burgers, 1998; Hubscher et al., 2000). DNA polymerases are, thus, required for the initiation of DNA synthesis, the elongation of nascent DNA chains and filling in the gaps left by the excision of the RNA primer. The identified DNA polymerases are shown in Table III.

A. DNA polymerase α

DNA polymerase α, the most abundant cellular polymerase, consists of a 186 kDa catalytic subunit containing the polymerase activity and a 70 kDa polypeptide with no associated enzymatic activity (reviewed in Waga and Stillman, 1998). It is responsible for initiating DNA synthesis by adding deoxyribonucleotides onto the RNA primer synthesized by the primase. DNA polymerase α /primase synthesize an RNA-DNA primer of approximately 40-nt in length, including 10-nt of RNA primer. The low processivity and lack of proofreading exonuclease activities further suggest that DNA polymerase α serves primarily in the initiation of DNA synthesis. DNA polymerase α/primase dissociates from the template following primer synthesis (Murakami and Hurwitz, 1993). Furthermore, cyclin-dependent kinases phosphorylate and regulate DNA polymerase α /primase during the cell cycle.

B. DNA polymerase δ

Human DNA polymerase δ was believed to be a heterodimeric protein composed of a 125 kDa and 48 kDa subunit with the main catalytic activity residing in the amino-

Table III. Mammalian DNA polymerases.

Polymerase	Subunits (kDa)	Enzymatic activity	Functional task
α	186 70 58 primase 48 primase	DNA polymerase	Initiation of DNA replication; DSB repair; telomere length regulation; cell-cycle regulation
β	39	DNA polymerase 5' phosphatase	DSB reapir; base-excision repair; meiosis
γ	125 35	DNA polymerase 3'-5' exonuclease	Mitochondrial DNA replication
δ	125 48 25 12	DNA polymerase 3'-5' exonuclease	Leading and lagging strand DNA replication; mismatch repair; DSB repair; base-excision repair; translesion DNA synthesis; cell-cycle regulation
ε	256 55	DNA polymerase 3'-5' exonuclease	Lagging strand DNA replication; DSB repair; nucleotide excision repair; base excision repair; cell-cycle regulation
ζ	353	DNA polymerase	Error-prone tranlesion DNA synthesis
η	78	DNA polymerase	Error-free translesion DNA synthesis
θ	198	DNA polymerase DNA helicase (?)	Repair of interstrand crosslinks
ι	?	DNA polymerase	Error-prone tranlesion DNA synthesis
κ	?	DNA polymerase	Sister chromatid cohesion

*Table adapted from Hubscher et al., 2000.

terminus region of the 125 kDa subunit (Schumacher et al., 2000). Recently, two additional subunits, p12 and p25, have been isolated (Liu et al., 2000) but their function remains to be determined. DNA polymerase δ is a highly processive enzyme that is able to incorporate thousands of nucleotides to the DNA strand in one binding event, without dissociating from the DNA template (Kornberg and Baker, 1992). As a consequence, DNA polymerase δ is responsible for leading strand synthesis at the elongation step. Furthermore, DNA polymerase δ was initially identified and distinguished from other polymerases because it possesses an intrinsic 3'-5' exonuclease activity (Byrnes et al., 1976), allowing DNA polymerase δ to proofread replication errors and to maintain replication fidelity (Lee et al., 1981). DNA polymerase δ associates with RF-C and PCNA to form the processive holoenzyme.

C. DNA polymerase ε

DNA polymerase ε is composed of a 256 kDa and 55 kDa subunit. Its precise role in DNA replication remains unclear. Like DNA polymerase δ, DNA polymerase ε has an intrinsic 3'-5' exonuclease activity (Nishida et al., 1988) and is highly processive. In contrast to DNA polymerase δ, DNA polymerase ε's processivity is not dependent on the interaction with PCNA (Syvaoja and Linn, 1989). Although it can associate with PCNA and RF-C, DNA polymerase ε does not substitute for DNA polymerase δ in the SV40 *in vitro* DNA replication system (Lee et al., 1991). It has been proposed that DNA polymerase ε may mediate the conversion of DNA primers into Okazaki fragments (Nethanel and Kaufmann, 1990) or act as a sensor for DNA damage in eukaryotic cells (Zlotkin et al., 1996). DNA polymerase ε is required for yeast DNA replication where it fills in the gaps that result from RNA primer excision (Mozzherin and Fisher, 1996). Furthermore, this polymerase has been crosslinked to yeast and mammalian replication forks, but has not been found at the forks in SV40 chromosomes, thus suggesting that it may be dispensable for SV40 but not cell replication (Aparicio et al., 1997). Recently, a primary role for DNA polymerase ε in chromosomal replication of murine B lymphocytes was reported (Winter et al., 2000).

Evidence also exists for a role of DNA polymerase ε in DNA repair. DNA polymerase ε has been implicated in the S phase checkpoint pathway (Navas et al., 1995), a feedback mechanism that arrests cell cycle progression upon DNA damage, and in nucleotide excision repair (Shivji et al., 1995).

D. DNA polymerase β

DNA polymerase β, consisting of a single polypeptide of 39 kDa, is a non-processive DNA polymerase that is induced upon DNA damage. Moreover, it does not possess any proofreading exonuclease activity. DNA polymerase β catalyzes the release of 5' terminal deoxyribose phosphate residues from incised apurinic-apyrimidinic sites, which are intermediates in base excision repair (Matsumoto and Kim, 1995; reviewed in Burgers, 1998). Thus, DNA polymerase β is proposed to function in DNA repair, filling in very small gaps (Mullen and Wilson, 1997).

E. DNA polymerase γ

DNA polymerase γ is responsible for the replication of mitochondrial DNA. It consists of two subunits of 125 kDa and 35 kDa. DNA polymerase γ has a 3'-5' exonuclease activity, and its polymerase activity is greatly increased in the presence of PCNA (Taguchi et al., 1995).

F. Other DNA polymerases

Upon a DNA lesion, the replicative DNA polymerases stop, thus blocking leading strand synthesis and interrupting lagging strand synthesis. Cells, however, contain specialized proteins to overcome such replication blocking, thus synthesizing across DNA damage, achieving translession synthesis or lesion bypass (reviewed in Johnson et al., 1999; Woodgate, 1999). Recently, the mammalian DNA polymerase ζ (Nelson et al., 1996), DNA polymerase η (Masutani et al., 1999) and DNA polymerase ι (Johnson et al., 2000) were identified. These DNA polymerases are conserved from yeast to mammals, but are nonessential. DNA polymerases ι and ζ act sequentially to bypass

DNA lesions, with high errors (Johnson et al., 2000). DNA polymerase η efficiently bypasses thymine-thymine cyclobutane dimers with high fidelity (Johnson et al., 1999). DNA polymerase θ, another recently identified DNA polymerase, is involved in the repair of interstrand crosslinks (Sharief et al., 1999). DNA polymerase κ, the latest identified DNA polymerase, is believed to act in sister chromatid cohesion (Wang et al., 2000).

iv) Proofreading exonuclease

A proofreading protein with a 3'-5' exonuclease activity is required for DNA replication. This proofreading exonuclease functions to excise the mismatched nucleotides, ensuring the fidelity of DNA replication. In eukaryotic systems, this activity is associated with DNA polymerases δ and ε.

Mutation rates in eukaryotic cells are estimated at $<10^{-10}$ mutation/base pair replicated per generation, suggesting that eukaryotic replication fidelity is extremely high. The fidelity of leading and lagging strand replication machinery seems to be comparable for some errors, but different for others (reviewed in Kunkel and Beberek, 2000). These differences likely arise from the enzymologically asymmetric replication that occurs on each strand. In other words, lagging strand synthesis requires discontinuous synthesis of Okazaki fragments, more than one polymerase, several switches between polymerases, and synthesis and removal of RNA primers, which may provide opportunities for mistakes.

v) Sliding clamp

Proliferating cell nuclear antigen, or PCNA, is a donut or toroid-shaped homotrimeric polypeptide composed of 36 kDa subunits that acts as a sliding clamp (reviewed in Tsurimoto, 1998). PCNA was initially identified as a nuclear protein present in the sera of patients with systemic lupus erythrymatosus (Miyachi et al., 1978) and later described as an acidic protein found in actively proliferating cells (Bravo and Celis, 1980). PCNA exists as a stable trimer that forms a closed ring with a hole in the centre. PCNA acts by encircling and sliding along the DNA (Schurtenberger et al., 1998).

Table IV. PCNA binding proteins.

PCNA binding protein	Function	Effects of interaction with PCNA
DNA ligase I	Ligation of nicked DNA; DNA repair and joining of Okazaki fragments	Affect DNA synthesis by DNA polymerase δ and ε
Cyclin D	G1 cyclin; activation of CDK	Inhibition of DNA replication
XP-G	Structure-specific repair endonuclease; nucleotide excision repair	Link between excision and repair resynthesis
DNA polymerase δ	DNA synthesis; leading and lagging strand synthesis	Processive DNA synthesis
DNA polymerase ε	DNA synthesis; repair DNA synthesis; checkpoint control	Processive DNA synthesis at high salt concentrations
FEN-1	Flap endonuclease; processing of primer DNA and Okazaki fragment	Stimulation of endonuclease activity
MSH2	Mismatch repair	Stimulation of mismatch repair DNA synthesis
RF-C	Clamp loader; ATPase; primer 3' end-binding	Stimulation of RF-C ATPase; complex formation at 3' end of primer DNA
DNA methyltransferase	Methylation of cytosines in DNA	Targeting to newly replicated DNA

*Table adapted from Tsurimoto, 1998.

Following interaction with PCNA, DNA polymerases δ and ε attach to the DNA template and become highly processive (Eissenberg et al., 1997). Thus, the role of PCNA in DNA replication is mostly as a processivity factor for DNA polymerase δ, as are the *E. coli* B and the T4 45 proteins (Byrnes et al., 1976; Bravo et al., 1987). PCNA is also able to stimulate the ATPase activity of RF-C (Tsurimoto and Stillman, 1990). PCNA does not have binding activity on its own, but rather is loaded onto the DNA by RF-C (Cai et al., 1996). PCNA is capable of binding to many proteins (Table IV), including FEN-1, DNA ligase I, DNA methyltransferase, the nucleotide excision repair protein XPG, and the mismatch repair proteins MLH1 and MSH2 (reviewed in Waga and Stillman, 1998). Therefore, PCNA is thought to be a central player in the coordination of DNA replication, DNA repair, epigenetic inheritance and cell cycle control (reviewed in Tsurimoto, 1998).

vi) Sliding clamp assembly factor

Replication factor C, or RF-C, facilitates coordinated leading and lagging strand synthesis by serving as a molecular hinge between DNA polymerases α and δ (Nethanel et al., 1992; reviewed in Mossi and Hubscher, 1998). It was initially identified in human 293 cells as an essential component of the SV40 *in vitro* DNA replication system (Tsurimoto and Stillman, 1989) and all of its five subunits have now been cloned and assembled into an active complex (Table V) (Ellison and Stillman, 1998). RF-C is required to load the sliding clamp, PCNA, onto replication forks or internal repair sites, *via* the carboxyl-terminus of PCNA (Mossi et al., 1997). RF-C also possesses an intrinsic DNA-dependent ATPase activity that is stimulated by PCNA (Tsurimoto and Stillman, 1990). It preferentially binds to template-primer junctions on the DNA molecule and the function of RF-C resembles that of the *E. coli* γ complex and the λ phage 44/62 proteins, which also serve as a brace (reviewed in Stillman, 1994). Phosphorylation of the PCNA binding domain in RF-C greatly reduces the binding of RF-C to the sliding clamp, thus inhibiting DNA synthesis from DNA polymerases δ and ε. Finally, the interaction of RF-C with PCNA, RP-A, and ATP inhibits DNA polymerase α and stimulates DNA polymerases δ and ε (Maga et al., 1997).

Table V. Biochemical properties of RF-C subunits.

Human RF-C subunit	Size (kDa)	Proposed function
RF-C 140	128.3	DNA binding; PCNA binding
RF-C 40	39	ATP binding; PCNA binding; interaction with DNA polymerase δ; interaction with RF-C 37; ATP-independent PCNA unloading
RF-C 38	40.5	PCNA binding; interaction with RF-C 140
RF-C 37	39.6	DNA binding at primer ends; interaction with DNA polymerase ε; interaction with RF-C 40
RF-C 36	38.5	PCNA binding

*Table adapted from Mossi and Hubschier, 1998.

vii) Single-stranded DNA binding protein

Replication protein A, or RP-A, is a heterotrimeric phosphoprotein composed of a 70 kDa, 29 kDa and 14 kDa subunits (reviewed in Iftode et al., 1999). It binds the melted, single-stranded DNA, facilitates DNA unwinding and stabilizes the opened duplex DNA structure, thereby allowing the entry of other replication proteins (reviewed in Wold, 1997). RP-A binds DNA through two domains in the 70 kDa subunit and another domain in the 32 kDa and 14 kDa subunits (Bochkareva et al., 1998). All the RP-A subunits are essential for DNA replication (Erdile et al., 1991). RP-A is a phosphorylation target for DNA-dependent protein kinase, DNA-PK, and likely the ataxia telangiectasia mutated gene (ATM) protein kinase (Brush et al., 1994; Blackwell et al., 1996; Gately et al., 1998). The p70 and p29 subunits of RP-A are phosphorylated in a cell cycle dependent manner and increased levels of phosphorylated RP-A are seen during DNA damage. RP-A phosphorylation seems to play an important modulatory role in eukaryotic DNA metabolism (reviewed in Iftode et al., 1999). Phosphorylation also increases RP-A's single-stranded DNA binding activity and modulates its ability to participate in protein-protein interactions, as its interactions with T-Ag and DNA polymerase α are reduced (reviewed in DePamphilis, 1999). RP-A can stimulate the activity of DNA polymerase α, and in association with PCNA and RF-C can also stimulate DNA polymerases δ and ε activities (Kenny et al., 1989). In addition to its role in DNA replication, RP-A is also involved in nucleotide excision repair and homologous recombination (reviewed in Wold, 1997; Iftode et al., 1999).

viii) RNA primer excision

The activities of two nucleases, RNA nuclease (RNase) HI and flap endonuclease-1 (FEN-1), are necessary to excise the RNA primers that are synthesized during DNA replication (Figure 14) (Waga et al., 1994; Bambara et al., 1997; reviewed in Waga and Stillman, 1998). The activity of the two nucleases is triggered either by the upstream DNA polymerase complex or the newly synthesized DNA.

RNase HI, an 89 kDa protein, cleaves the RNA primer from the Okazaki fragment at the RNA-DNA heteroduplex junction, recognizing the transition from RNA to DNA on

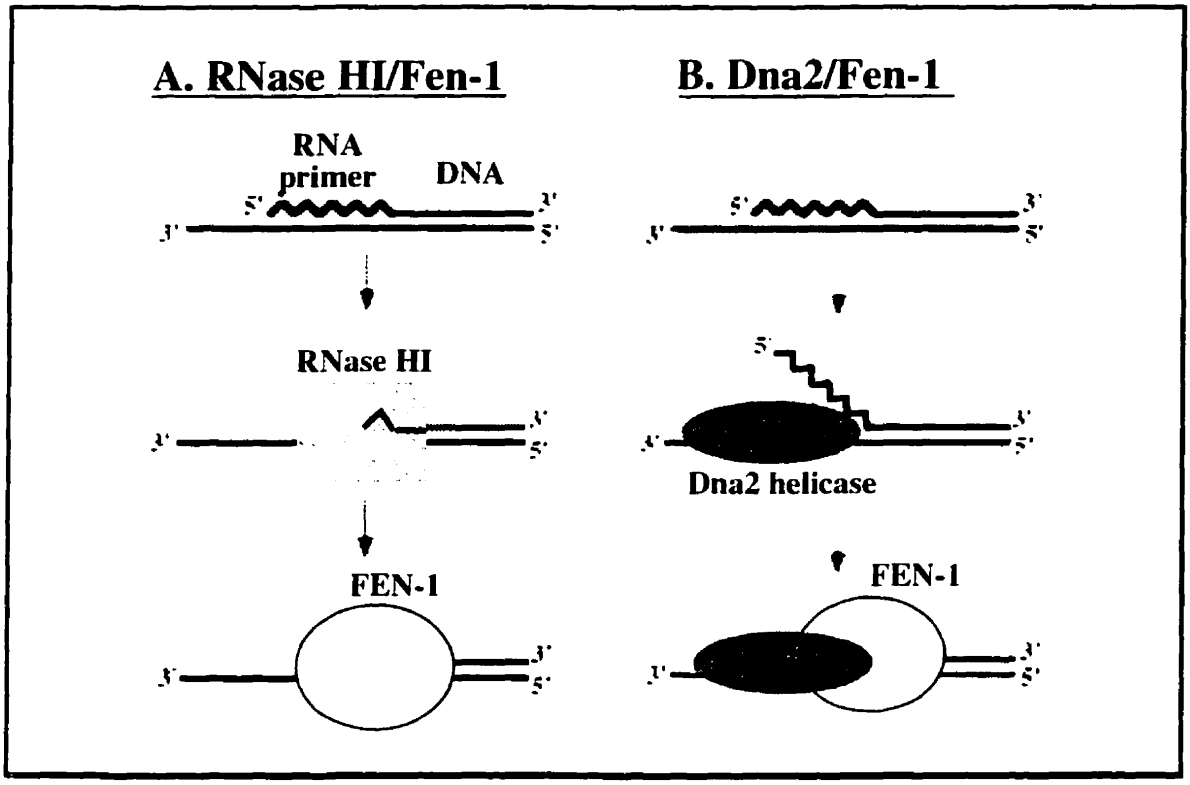


Figure 14. Two mechanisms for the removal of RNA primers. A. RNase HI cleaves the RNA attached to the 5' of the Okazaki fragment, leaving a single ribonucleotide adjascent to the RNA-DNA junction. FEN-1 removes the remaining ribonucleotide. B. Dna2 helicase displaces the RNA segment. FEN-1 cleaves the branch point, releasing the RNA. (Reproduced from Waga and Stillman, 1998).

a single-stranded substrate (Murante et al., 1998). The single ribonucleotide that remains attached to the 5' end of the Okazaki fragment following RNase HI cleavage is removed by FEN-1. FEN-1, flap enoduclease 1, is a 46 kDa, double-stranded specific, 5'-3' exonuclease/endonuclease (reviewed in Lieber, 1997). It degrades double-stranded DNA of the RNA moiety of the DNA/RNA duplex (Turchi et al., 1994; Qui et al., 2000). Another mechanism for removal of the RNA primers, involving Dna2 helicase, has also been proposed (Figure 14) (Budd and Campbell, 1997; reviewed in Waga and Stillman, 1998). Dna2 could displace the RNA primer, creating a flap-like substrate for FEN-1 endonuclease, which will in turn endonucleotically cleave the branch point. This mechanism of RNA primer excision has not yet been proven.

ix) DNA ligases

DNA ligases (reviewed in Tomkinson and Mackey, 1998) catalyze the formation of a phosphodiester bond between adjacent 3' OH and 5' PO_4 termini of nucleotides hydrogen-bonded to a complementary strand. Five mammalian DNA ligase activities have been purified to date and three DNA ligase genes have been cloned (Table VI) (Lindhahl et al., 1997). DNA ligase I is responsible for the joining of adjacent Okazaki fragments to complete lagging strand DNA synthesis (Turchi and Bambara, 1993; Levin et al., 1997). The amino-terminus of DNA ligase I interacts with PCNA, and thus this ligase activity cannot be replaced by another one (Waga et al., 1994). DNA ligase II is a proteolytic fragment of DNA ligase III. DNA ligase III is present in two spliced forms, α and β. DNA ligase III and IV interact with proteins involved in the repair of single-stranded DNA breaks. The joining of double-stranded DNA breaks by these DNA ligases in enhanced by Ku antigen (reviewed in DePamphilis, 1999 and references therein). DNA ligase IIIβ functions in the completion of meiotic recombination or in post meiosis DNA repair pathway. At present, not much is known about DNA ligase V, not even if it is derived from one of the known ligase genes.

Table VI. Mammalian DNA ligases: genes, proteins and interacting proteins.

Gene	Gene product	Size (kDa)	Associated proteins	Cellular process
LIG1	DNA ligase I	125	PCNA	DNA replication; Mismatch repair?
LIG3	DNA ligase II	70	?	?
	DNA ligase IIIα	100	XRCC1	Single-strand DNA break repair; Base excision repair
	DNA ligase IIIβ	100	?	DNA repair; Meiotic recombination
LIG4	DNA ligase IV	100	XRCC4	Non-homologous end joining; V(D)J recombination
?	DNA ligase V	44	?	?

*Table adapted from Tomkinson and Mackey, 1998.

x) DNA topoisomerases

During DNA replication, the unwinding of the two strands can result in the generation of positive supercoiling in front of the replication fork, which can in turn prevent semiconservative DNA replication and chromosome segregation. DNA topoisomerases rapidly relax the positive supercoils and assure that the DNA strands are completely unlinked such that the replicated chromosomes can be segregated to the daughter cells.

Three types of DNA topoisomerases have been identified in eukaryotic cells (reviewed in Nitiss et al., 1998). DNA topoisomerase I, a 100 kDa protein, introduces a single-stranded break in the DNA, thereby relaxing either negatively or positively supercoiled DNA. Topoisomerase II, a 172 kDa protein, introduces a transient double-stranded break and can also release concatenated DNA molecules. Mammalian cells have two types of DNA topoisomerase II, IIα and IIβ. The two differ in their pattern of expression with IIα preferentially expressed in proliferating cells and IIβ expressed at equal levels in both proliferating and quiescent cells. The enzymatic properties of the two are indistinguishable. DNA topoisomerases play a role in SV40 *in vitro* DNA replication, as addition of purified DNA topoisomerase I and II to depleted cell extracts restores DNA replication (Yang et al., 1987). In yeast cells devoid of DNA topoisomerase I and II, DNA replication does take place, although it is very defective and only very short DNA is synthesized, indicating that DNA topoisomerases I and II are not required for initiation (Kim and Wang, 1989). Either of the two types of DNA topoisomerases could function in the progression of the replication fork. However, only DNA topoisomerase II is required for the segregation of newly replicated daughter molecules, where two adjacent replication forks converge (Ishimi et al., 1992). Eukaryotic DNA topoisomerase III was first identified as a gene required to suppress recombination in yeast (Wallis et al., 1989). It acts on negatively, but not positively, supercoiled DNA (Kim et al., 1992).

VIII. MULTIPROTEIN REPLICATION COMPLEXES

DNA replication is a highly organized and controlled process that occurs through a nonrandom association of many enzymatic activities and substrates. Evidence for multiprotein complexes playing a role in DNA replication has been growing over the last decade (reviewed in Malkas, 1998).

Numerous reports have described the isolation of large multiprotein complexes consisting of eukaryotic replication proteins (Jackson and Cook, 1986; Vishwanatha et al., 1986; Mathews and Slabaugh, 1986; Tubo and Berezney, 1987; Reddy and Fager, 1993; Maga and Hubscher, 1996). Although these complexes have shown polymerases activities, they have not shown the ability to support DNA replication.

The first successful purification and characterization of a mammalian multiprotein DNA replication complex, the DNA synthesome, capable of supporting *in vitro* DNA replication of SV40 or polyoma DNA, has been described (Malkas et al., 1990; Wu et al., 1994; Applegren et al., 1995; Coll et al., 1996; Tom et al., 1996; Lin et al., 1997; Jiang et al., 1998). The DNA synthesome has been extensively purified, approximately 5200-fold, using a series of centrifugation, polyethylene glycol precipitations, ion-exchange chromatography, density gradient sedimentation and native polyacrylamide gel electrophoretic steps. It has been isolated from numerous mammalian cells, including HeLa cells (Malkas et al., 1990; Applegren et al., 1995), HL-60 leukemia cells (Lin et al., 1997) and murine mammary carcinoma FM3A cells (Wu et al., 1994). The integrity of the complex is maintained even after treatment with detergents, salts, RNase and DNase digestions, demonstrating that the associations between the proteins in not dependent on random or nonspecific interactions with other cellular proteins. The DNA synthesome isolated from human cells has a sedimentation coefficient of 18S and is capable of supporting SV40 *in vitro* DNA replication, while the murine complex has a sedimentation coefficient of 17S and fully supports polyoma virus *in vitro* DNA replication.

The DNA synthesome consists of at least 30 polypeptides, with molecular masses ranging from 15 kDa to 300 kDa. The DNA replication proteins identified to copurify with the DNA synthesome include (reviewed in Malkas, 1998): DNA polymerase α/primase, DNA polymerases δ and ε, DNA ligase I, RP-A, RF-C, PCNA, DNA

topoisomerases I and II, RNase HI (Baril et al., 1988; Malkas et al., 1990; Wu et al., 1994; Applegren et al., 1995; Coll et al., 1996; Tom et al., 1996; Lin et al., 1997), human DNA helicases I and IV (Sekowski et al., 1998), poly (ADP-ribose) polymerase (PARP) (Simbulan-Rosenthal et al., 1996), and a DNA-dependent ATPase, identified as Ku antigen (Cao et al., 1994). Based on the fractionation, chromatographic, and sedimentation profiles of the individual proteins that copurify with the DNA synthesome, a model has been proposed demonstrating the various interactions (reviewed in Malkas, 1998).

IX. EUKARYOTIC MODEL OF DNA REPLICATION

The mechanism of leading and lagging strand synthesis in mammalian cells has emerged primarily from studies with the SV40 *in vitro* DNA replication system. The current view of the mechanism of DNA synthesis is illustrated in Figure 15 (reviewed in Waga and Stillman, 1994; 1998).

To initiate DNA replication, the DNA double helix is melted at the origin of replication. This initial step is catalyzed by an undefined mammalian helicase, which then proceeds to separate the two DNA strands, thus creating the two replication forks. Subsequently, RP-A binds the single-stranded DNA, maintains the opened duplex and promotes further unwinding. The opened duplex structure facilitates the entry of other replication proteins.

Leading strand DNA replication begins with the synthesis of a short RNA primer by primase. Then, DNA polymerase α, which is tightly associated with primase, adds deoxyribonucleotides to the RNA primer. Once DNA polymerase α has incorporated 30–300 nucleotides, RF-C, in an ATP-dependent step, facilitates the dissociation of DNA polymerase α from the leading strand. Then, the loading of the trimeric PCNA molecule, as a clamp around the DNA occurs. RF-C mediates the DNA polymerase switching process (Waga and Stillman, 1994). Next, DNA polymerase δ associates with PCNA and proceeds with the synthesis of the leading strand, adding thousand of deoxynucleotides in a highly processive fashion.

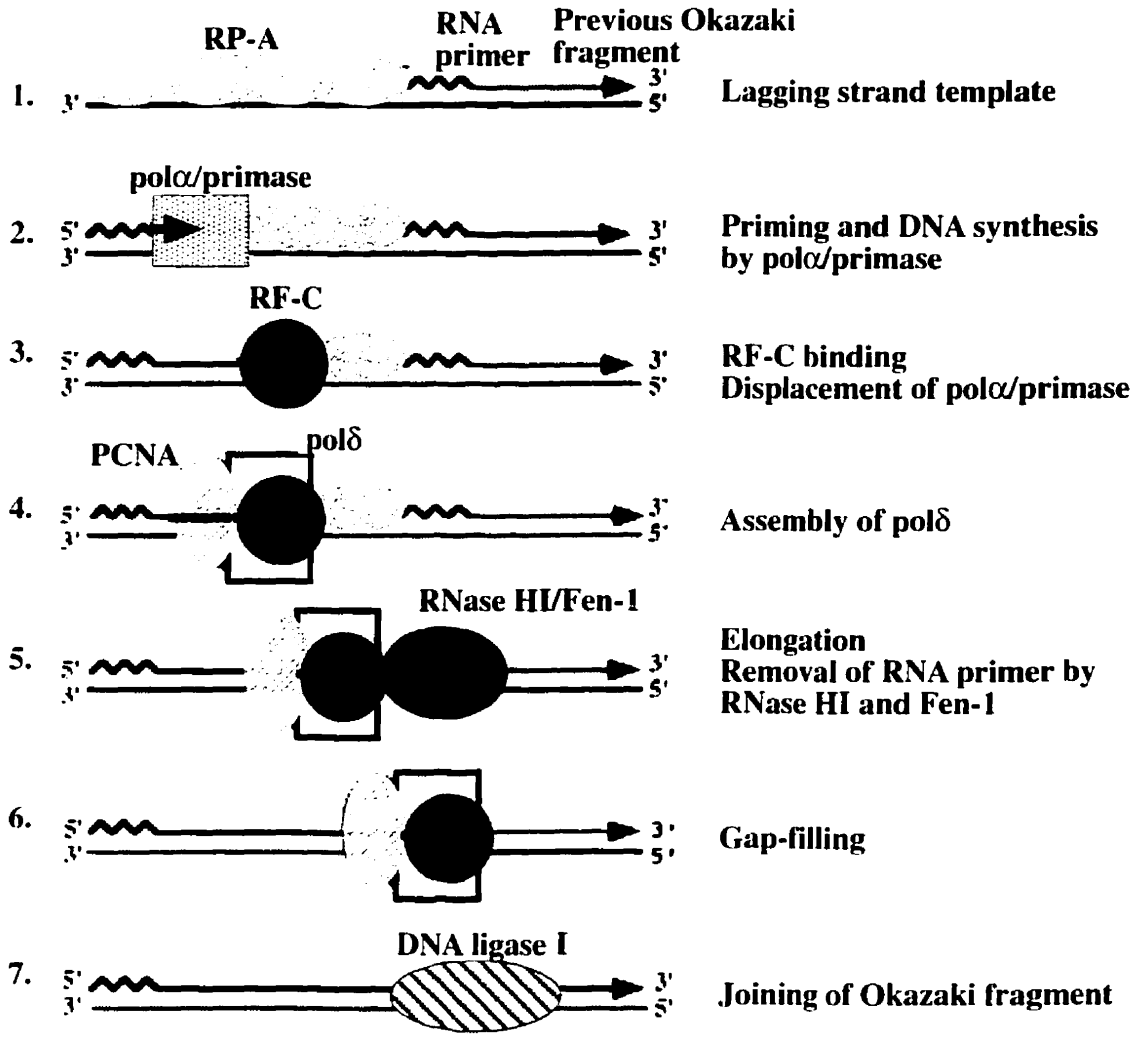


Figure 15. Lagging strand synthesis and polymerase switching. See text for details. (Reproduced from Waga and Stillman, 1998).

Lagging strand DNA replication occurs *via* the discontinuous synthesis of RNA-primed Okazaki fragments (Figure 15). Once again, DNA primase synthesizes short RNA primers that are extended into initiator DNA (approximately 34-nt) by DNA polymerase α. Once the initiator DNA, iDNA, has been synthesized, RF-C binds to the 3' end of the iDNA and loads PCNA, followed by the binding of DNA polymerase δ. DNA polymerase α/primase complex is displaced from the DNA and is prevented from completing DNA replication up to the previously synthesized Okazaki fragment. As the Okazaki fragments are elongated by DNA polymerase δ, the RNA primers are removed by RNase HI and FEN-1. The resulting gap is filled in by DNA polymerase δ, that uses the adjacent Okazaki fragment as a primer, and the nick is sealed by DNA ligase I (Figure 15). Finally, DNA topoisomerase II functions in the segregation of the newly replicated daughter molecules.

DNA replication in the leading and lagging strands seems to be coordinated (Waga and Stillman, 1994). In this coordinated model, the lagging strand is postulated to be looped around in such a manner allowing the leading and lagging strand polymerase complexes to move in concert. One molecule of DNA polymerase α/primase, synthesizing the iDNA-RNA on the lagging, and two molecules of DNA polymerase δ, one elongating the iDNA on the lagging strand and the other one continuously synthesizing the leading strand, are postulated to cooperate at the replication fork. Recently, this DNA loop has been observed by electron microscopy at the replication forks generated by the T7 bacteriophage proteins (Park et al., 1998).

X. KU ANTIGEN

Ku antigen is a heterodimeric protein initially identified in 1981 as an autoantigen recognized by the sera of patients with autoimmune diseases such as systemic lupus erythematosus, Sjorgen's syndrome, scleroderma and polymyositis (Mimori et al., 1981; Reeves, 1985; Mimori et al., 1986; Reeves, 1992). The term Ku derives from the two first letters of the original patient's name. Over the years, many different terms have been used to refer to Ku antigen including nuclear factor IV, transferrin receptor promoter element

binding factor (TREF), proximal sequence element 1 (PSE-1), end binding protein (EBP-80), human DNA helicase II (HDH II), and Ku-2 (de Vries et al., 1989; Roberts et al., 1989; Stuiver et al., 1990; Knuth et al., 1990; May et al., 1991; Falzon et al., 1993; Tuteja et al., 1994).

Ku is an abundant protein ($5x10^5$ copies/nucleus) that has been found in all human cells (Francouer et al., 1986). Moreover, homologues of Ku have been identified in monkeys (Paillard and Strauss, 1991), *Xenopus* (Higashiura et al., 1993), yeast (Feldmann et al., 1993), *Drosophila* (Jacoby and Wensink, 1994) and rodents (Porges et al., 1990; Lee et al., 1996).

The subcellular localization of Ku has been controversial, as discussed in Koike et al. (1999a). Reports of purely nuclear (Koike et al., 1998), cytoplasmic (Bakalkin et al., 1998), membrane (Dalziel et al., 1992), and both nuclear and cytoplamic (Fewell and Kuff, 1996) Ku have been published, probably due to differences in detection methods or to a change in Ku's subcellular localization during the cell cycle. Using immunofluorescence and confocal laser scanning microscopy, Ku70 and Ku86 have been detected in the nuclei of interphase cells (Koike et al., 1999b). In contrast, in mitotic cells, most of the Ku complex was diffused in the cytoplasm with a small fraction present at the periphery of condensed chromosomes. DNA-PKcs, the catalytic subunit with which Ku associates to form the DNA-PK holoenzyme, was found in the nucleus throughout the cell cycle, thus suggesting that Ku is not always associated with the catalytic subunit.

i) Ku antigen: A heterodimer

Ku from all species is composed of two subunits of ~ 70 kDa and ~ 80 kDa and referred to as Ku70 and Ku80 or Ku86, respectively. The cDNA of both subunits have been cloned (Dynan et al., 1998). Ku70 consists of 609 amino acids with an estimated molecular weight of 69 581, and Ku86 consists of 732 amino acids with an estimated molecular weight of 81 914. Both Ku subunits have periodic leucine repeats or leucines alternating with serines, similar to the leucine zipper motif described for a family of proteins involved in transcription (Landshulz et al., 1988; Reeves and Sthoeger, 1989; Yaneva et al., 1989; Wu and Lieber, 1996).

The careful examination of the Ku subunits in lower eukaryotes has suggested that homology exists between Ku70 and Ku86 and they likely arose through duplication and divergence of a single polypeptide, which presumably functioned as a homodimer (Dynan et al., 1998). Ku70 is part of a family of related proteins encoded by multiple genes (Griffith et al., 1992; Oderwald et al., 1996). Ku86 is the product of a single gene, XRCC5 (Taccioli et al., 1994). Three different charge variants of Ku86 exist that occur at the G1/S phase and are dependent on the cell proliferation state (Stuiver et al., 1991). The nature of the modification that distinguishes these variants is not known. Site-specific proteolytic cleavage of Ku86, by a leupeptin-sensitive protease, resulting in a 69 kDa and 18 kDa subunit, has also been reported (Paillard and Strauss, 1993; Han et al., 1996; Jeng et al., 1999). The truncated Ku86 can dimerize with Ku70, and the resulting heterodimer has similar binding properties to Ku (Paillard and Strauss, 1993). KARP-1 is a 9 kDa amino-terminally extended derivative of Ku86 (Myung et al., 1997). It is expressed from the Ku86 locus but utilizes an upstream promoter and additional exons resulting in a longer Ku86. KARP-1 is primate-specific and regulates DNA-PK (Muyung et al., 1997; Myung et al., 1998). The DNA-binding abilities of KARP-1 have not been described.

Although the Ku subunits are quite conserved in size across species, the amino acid sequence of each can differ substantially. The region of the Ku subunits that is required for dimerization and interaction with DNA has been mapped to 150 amino acid residues at the carboxyl-terminal region of both Ku70 and Ku86. Both Ku subunits are required for protein stability (Errami et al., 1996; Singleton et al., 1997), and Ku86 regulates Ku70 levels posttranscriptionally (Chen et al., 1996). However, some recent reports have shown that the Ku subunits can exist as stable monomers. For instance, truncated stable forms of Ku86 have been detected without Ku70, perhaps due to protein conformational changes or loss of degradation signal sequences (Singleton et al., 1997). Also, only Ku86 is present in the nuclear periphery of *xrs*-5 cells (Yasui et al., 1999). Likewise, a small amount of Ku86 remaining in Ku70 knockout cells is believed to promote DNA end joining by functioning either as a monomer or as a homodimer (reviewed in Featherstone and Jackson, 1999).

ii) Ku antigen activities

Ku possesses several enzymatic activities and many different cellular functions have been attributed to Ku (Table VII).

A. DNA binding activities

a) DNA-end binding activities

The initial studies on Ku indicated that it bound to DNA termini regardless of the structure or sequence of the DNA end (Mimori et al., 1986; Paillard and Strauss, 1991; Falzon et al., 1993). Ku is able to bind to blunt ends, to ends with 5' or 3' overhangs, and to ends with hairpin loops (Mimori and Hardin, 1986; Griffith et al., 1992). Furthermore, Ku recognizes single-to double-stranded transitions in the DNA, gapped/nicked molecules and other DNA structures (Blier et al., 1993; Falzon et al., 1993; Tuteja et al., 1994).

When Ku binds to DNA ends or structures, it makes contact with 13-21-bp of DNA (de Vries et al., 1989). Subsequently, Ku can translocate along the DNA with a 25-30-bp periodicity, and additional Ku molecules can load onto the DNA, reminiscent of beads on a string. The recruitment of DNA-PK induces an inward translocation of Ku (Yoo and Dynan, 1999a,b). This process of translocation occurs in the absence of an energy requirement (Mimori et al, 1986; Toth et al., 1993; Blier et al., 1993). The multiple Ku proteins on the DNA have been visualized by electrophoretic mobility shift assays, by DNA protection assays, and by electron microscopy. Ku can also transfer between linear DNA molecules with compatible ends. A few reports have claimed that Ku can also bind to RNA molecules (Yoo and Dynan, 1998). Ku binds to the RNA containing the HIV transactivation response element (Kaczmarski and Khan, 1993). Ku, however, does not bind to total HeLa RNA or to tRNA (Mimori and Hardin, 1986).

DNA end-binding activity is mediated by Ku70, as demonstrated by UV crosslinking experiments (Zhang and Yaneva, 1992; Chou et al., 1992; Giffin et al., 1999)

Table VII. Functions/activities associated with the Ku70 and Ku86 subunits.

Functions/Activities	Ku70	Ku86
DNA-end binding	+	-
ds NRE-1 and related sequences sequence-specific binding	+	- + in presence of Mg+2 and ATP
ss NRE-1 binding	+	+
ds A3/4 binding	-	+
ss A3/4 binding	-	-
Phophorylation by DNA-PK	+	+
Activation of DNA-PK	-	+
ATPase activity	+	+
Helicase activity	+	-
DSB repair and V(D)J recombination	+	+
Transcription	+	+
Telomere maintenance	+	?
Replicative senescence/ aging	?	+
DNA replication	+	+

* see text for details and references

and binding of recombinant Ku70 to DNA attached to nitrocellulose filters (Allaway et al., 1990). Other reports have suggested that both Ku subunits are essential for DNA-end binding (Ono et al., 1994; Wu and Lieber, 1996; Ochem et al., 1997). It is likely that both Ku70 and Ku86 can bind DNA, although Ku70 can interact more intimately (reviewed in Tuteja and Tuteja, 2000). Using *in vitro* translated proteins, it has been shown that the 40 kDa carboxyl-terminus of Ku70 and the 45 kDa carboxyl-terminus of Ku86 are required for DNA end binding (Wu and Lieber, 1996). A core region in Ku70 of 73 amino acids in the carboxyl-terminus (Chou et al., 1992) and a 320 amino acid region in Ku86 (Osipovich et al., 1999) have also been identified as required for end binding.

b) Sequence-specific binding

Over the years, Ku has been shown to bind sequence-specifically to many, unrelated sequences, including to the mouse retroviral-like element (Falzon et al., 1989), transferrin receptor (Roberts et al., 1994), proximal sequence element of the U1 gene (Knuth et al., 1990), T cell receptor B chain (Messier et al., 1993), collagen IV (Genersch et al., 1995), octamer motif (May et al., 1991), AP-1 element (Quinn and Farina, 1991), heat shock element of the hsp70 promoter (Kim et al., 1995), P-element transposition sites (Beall et al., 1994), to transcription factor CTCBF binding site (Genersch et al., 1995) and the GATA sequence of the glycophorin B promoter (Camara-Clayette et al., 1999). Ku has been shown to accumulate over these sequences and affect gene transcription. NRE-1 and A3/4 are two of the strongest sequence-specific binding sites for Ku, and are described below.

1. NRE-1 sequence

The most convincing evidence for sequence-specific binding of Ku was initially described for the negative regulatory element 1 (NRE-1) in the long terminal repeat (LTR) of the mouse mammary tumour virus (MMTV) (Giffin et al., 1996). By well-controlled competition bandshift assays and bandshift assays using microcircles, Ku was shown to bind to NRE-1 with higher affinity than DNA ends (Giffin et al., 1996; Giffin et al., 1999). Furthermore, Ku was able to bind specifically to the upper, single-stranded and

unstructured NRE-1 (Torrance et al., 1998; Giffin et al., 1999) with an affinity intermediate between double-stranded NRE-1 and DNA ends. Two classes of Ku binding sites have previously been reported (Giffin et al., 1997): sequences with similarity to NRE-1 and sequences without similarity to NRE-1. The sequences with similarity to NRE-1 were preferred to DNA ends, while the sequences without similarity were not.

UV crosslinking experiments have indicated that binding to double-stranded NRE-1 is mediated by Ku70 (Giffin et al., 1999). However, Ku86 can also crosslink to the upper strand of NRE-1 in the presence of magnesium and ATP, under ideal conditions for Ku helicase activity and formation of structural transitions (Torrance et al., 1998; Giffin et al., 1999). Interestingly, Ku86 does not become crosslinked in the presence of magnesium or ATP when Ku is bound to DNA ends (Giffin et al., 1999). Also, binding to the upper single-stranded NRE-1 strand occurs and requires both Ku70 and Ku86.

2. A3/4 sequence

A3/4, a 36-bp origin consensus sequence (described in section IV.E. f. of this thesis), has also been found to be a sequence-specific binding site for Ku antigen (Ruiz et al., 1999; [see chapter three of this thesis]). A3/4 is a DNA sequence that is present in mammalian DNA replication origins (Price et al., submitted). In bandshift competition assays, A3/4 was able to compete for Ku binding, whereas other linear oligonucleotides competed with low efficiency. Furthermore, a closed, circular A3/4 containing plasmid strongly competed for OBA binding, whereas the parental vector alone did not, demonstrating that A3/4 is an internal, sequence-specific binding site that is preferred to DNA ends (Ruiz et al., 1999; [see chapter three of this thesis]). In addition, Ku binding was only competed by the double-stranded and neither of the single-strands of A3/4 (Ruiz et al., 1999), and Ku only bound to the double-stranded A3/4 (Schild-Poulter et al., submitted).

Southwestern (Ruiz et al., 1999) and UV crosslinking (Schild-Poulter et al., submitted) experiments have revealed that the Ku86 subunit mediates binding to A3/4, and that the binding is independent of magnesium and ATP. In UV crosslinking experiments, low levels of Ku70 were also crosslinked. Furthermore, the results were reproducible with either HeLa cell purified Ku (OBA) or with recombinant Ku protein.

A3/4 and NRE-1 do not share any sequence homology and represent distinct classes of Ku binding sites. A3/4 represents an intermediate binding site between NRE-1 and DNA ends (Schild-Poulter et al., submitted). Furthermore, although A3/4 is a sequence-specific binding site for Ku antigen, it is not recognized by Ku in the form of a microcircle, whereas NRE-1 is. Thus, additional factors may be involved that stabilize Ku binding to A3/4. Interestingly, although both A3/4 and NRE-1 compete for Ku binding, only A3/4 affects mammalian *in vitro* DNA replication (Ruiz et al., 1999; Matheos et al., submitted; Schild-Poulter et al., submitted; [see chapter three and four of this thesis, respectively]).

B. ATPase and helicase activities

Ku antigen has been identified as the ATPase present in a HeLa cell DNA polymerase α multiprotein replication complex that is able to support SV40 *in vitro* DNA replication (Vishwanatha and Baril, 1990; Cao et al., 1994). ATPases usually catalyze the hydrolysis of ATP in a DNA-dependent manner. The ATPase/Ku stimulated DNA polymerase α/ primase activity by reducing the lag time in the initiation of synthesis on primed, single-stranded DNA templates. Interestingly, mutations in the ATP-binding domains in Ku70 and Ku86 do not appear to affect their function (Singleton et al., 1997; Jin et al., 1997). The ATPase activity of Ku resides in both the Ku70 and Ku86 subunits (Cao et al., 1994; Tuteja et al., 1994; Ochem et al., 1997). The ATPase activity of Ku is unregulated upon its phosphorylation by the DNA-dependent protein kinase (DNA-PK) (Cao et al., 1994).

Ku has also been identified as the human DNA helicase II (HDH II), an ATP-dependent DNA unwinding activity from HeLa cells (Tuteja et al., 1994). Ku unwinds DNA duplexes and not DNA-RNA or RNA-RNA hybrids. It prefers duplexes with fork-like structures rather than without a fork or hanging tail. Moreover, it prefers a 5' tailed fork. Ku does not unwind DNA from blunt ends or nicks, although it does bind to them. The direction of translocation is 3' to 5'. The efficiency of unwinding is drastically decreased if the length of the duplex is increased to 40-bp, and it preferentially unwinds

less than 20-bp. The DNA helicase activity of Ku resides in the 70 kDa subunit (Ochem et al., 1997).

C. Regulatory subunit of the DNA-dependent protein kinase

The DNA-dependent protein kinase (DNA-PK), a serine/threonine kinase, is the only modifying enzyme that requires DNA for its kinase activity: it is regulated by DNA, but does not use it as a substrate; rather, its substrate is DNA binding proteins. The DNA-PK holoenzyme is composed of a 470 kDa catalytic subunit, DNA-PKcs, and of a DNA binding subunit, Ku antigen (Ku70/Ku86) of approximately 150 kDa.

DNA-PK phosphorylates several nuclear DNA binding proteins including SV40 T-Ag, p53, Sp-1 transcription factor, Oct-1, DNA topoisomerases, and RP-A (reviewed in Anderson, 1992; 1993; Tuteja and Tuteja, 2000). Also, DNA-PK can autophosphorylate: the DNA-PKcs, Ku70 and Ku86 are phosphorylated in a DNA and ATP-dependent manner, resulting in inhibition of the kinase activity (Lees-Miller et al., 1990; Chan and Lees-Miller, 1996).

DNA-PKcs homologues have been described in human cells, in mouse (Araki et al., 1997), horse (Shin et al., 1997), *Xenopus* (Labhart, 1997), and *Drosophila* . DNA-PKcs has not been described in single cell eukaryotes, such as yeast or molds, neither in plants.

DNA-PKcs possesses a recognizable catalytic kinase domain that belongs to the phosphatidyl/inositol 3-kinase family (Hartley et al., 1995). DNA-PKcs contains a leucine zipper repeat, with six repeats, which likely mediates protein-protein interactions (Hartley et al., 1995).

The kinase activity is activated by both double- and single-stranded DNA (Suwa et al., 1994; Leuther et al., 1999). UV crosslinking (Gottlieb and Jackson, 1993) mobility shift and atomic force microscopy analyses (West et al., 1998) indicated that DNA-PKcs can directly contact DNA and its kinase activity can be activated *in vitro* by DNA, in the absence of Ku (Hammarsten and Chu, 1998; West et al., 1998). However, the kinase activity is stimulated up to 10-fold in the presence of Ku, when compared to only the presence of DNA (Yaneva et al., 1997), but the DNA has to be long enough (above 18-

bp) to allow the independent binding of both Ku and DNA-PKcs (West et al., 1998). The Ku86 subunit has been shown to activate the kinase activity (Singleton et al., 1999). DNA-PKcs is activated by 700-fold by any linear double-stranded DNA (Carter et al., 1990; Lees-Miller et al., 1990) with a minimum length of 12-bp. Closed circular DNA and linear genomic adenovirus DNA with the terminal protein (TP) covalently attached are poor activators of DNA-PK (reviewed in Anderson, 1992). Short single-stranded DNA oligonucleotides are also capable of activating DNA-PKcs (Hammarsten et al., 2000).

iii) Functions of Ku antigen

A. Double-stranded break (DSB) repair and V(D)J recombination

Several reports unequivocally demonstrate that Ku and DNA-PK play important roles in double-strand break (DSB) repair in mammalian cells (reviewed in Featherstone and Jackson, 1999; Tuteja and Tuteja, 2000). The complementation groups of X-ray sensitive mammalian cell lines IR5 and IR7 are deficient in DNA-PK activity and can be complemented by the XRCC5 gene (encoding Ku86) and the XRCC7 gene (encoding DNA-PKcs), respectively (reviewed in Jeggo et al., 1995). IR6 complementation group has also been described and they harbour a mutation in Ku70. These deficient cell lines have defective DNA DSB rejoining, defective DNA-end binding, and are sensitive to ionizing radiation and other agents that produce DSB. In contrast, they are not greatly affected by agents that produce other types of DNA damage, such as ultraviolet light and alkylating agents (Zdzienicka, 1995).

Ku and DNA-PK are also required for efficient V(D)J recombination and to generate the diverse antigen binding functions of the mammalian immune system. The severe combined immunodeficiency (SCID) mouse has a radiosensitive and DSB repair defective phenotype that is due to a mutation in the XRCC7 gene (Blunt et al., 1995; Kirchgessner et al., 1995). The immunodeficiency of the SCID mouse results from defects in the rearrangement of the immunoglobulin and T-cell receptor gene segments which encode the variable (V), diversity (D), and joining (J) regions of the molecules that contribute to antibody and T-cell receptor diversity. V(D)J recombination requires the

formation and rejoining of DNA DSB which are impaired in the SCID mouse. Furthermore, cells of the IR5, IR6, and IR7 complementation groups are also defective in V(D)J recombination and Ku70 and Ku86 knockout mice are also immunodeficient (Nussenzweig et al., 1996; Gu et al., 1997; Li et al., 1998; Grawunder et al., 1998).

Ku may function in DSB repair by facilitating ligation of the broken DNA ends. Ku has been shown to interact with DNA ligases and to bridge two DNA molecules (Ramsden and Gellert, 1998). Recently, Ku has been shown to interact with DNA ligase IV and its accessory protein XRCC4, and stimulate ligase binding, following Ku binding to DNA ends (McElhinny et al., 2000). Ku has also been described as an alignment factor in nonhomologous end-joining (Feldmann et al. 2000). Since some DSB require additional processing prior to rejoining, Ku may recruit and regulate appropriate nucleases to the site of DSB. Candidates for such nucleases are the mammalian homologues of RAD50 and MRE1, which are required for non-homologous end-joining (Paull et al., 1998; Trujillo et al., 1998). Moreover, by interacting and recruiting DNA-PKcs, other repair proteins may be recruited or activated by phosphorylation. Alternatively, the activation of DNA-PK could signal a DNA damage that may subsequently arrest cell cycle progression and lead to apoptosis (reviewed in Featherstone and Jackson, 1999).

B. Transcription

One role proposed for Ku antigen, based on many lines of evidence, has been in transcription. Numerous reports have indicated that Ku binds in a sequence-specific manner to transcriptional regulatory elements (see section X. ii) A. b) Sequence-specific binding). For instance, U1 gene transcription is activated by Ku (Knuth et al., 1990), whereas glucocorticoid-induced MMTV transcription is repressed by Ku (Giffin et al., 1996). Also, Ku, along with the TATA box binding proteins, binds to the CTC box of the human collagen gene and controls its transcription (Generesch et al., 1995). More recently, overexpression of Ku86 in a rat fibroblast cell line has resulted in hypermethylation and silencing of the metallothionein-I-Promoter (Majumdar et al., 1999). Furthermore, Ku70 has been shown to bind to the GATA motif of the glycophorin B promoter in human erythroid cells and negatively regulates the promoter (Camara-

Clayette et al., 1999). Ku and DNA-PK may also affect transcription by phosphorylating the RNA polymerase I transcription machinery (Kuhn et al., 1995), thus inhibiting the high rates of ribosomal genes in the presence of DSB. The RNA polymerase I machinery is responsible for transcription of the large ribosomal RNA precursor.

C. Telomere maintenance

Ku is part of the *S. cerevisiae* and *S. pombe* telomeric complex and is involved in telomere maintenance (Boulton and Jackson, 1998; Gravel et al., 1998; reviewed in Haber, 1999; Baumann and Cech, 2000). Telomeres, which are composed of G-rich tandem repeats, represent the physical ends of chromosomes. Telomeres function to overcome the replication problem associated with linear DNA ends and to protect the chromosomal ends from being treated like DSB. The telomere chromatin structure is in a highly condensed structure that prevents the access of the transcription machinery. When a gene is placed in the telomeric region, transcription does not occur, rendering the gene silent. This is referred to as telomeric silencing or telomere position effect. Telomeric silencing is regulated by a multiprotein complex that includes the silent information regulatory proteins Sir2, Sir3, and Sir4.

When the yeast Ku homologues are inactivated, the telomere ends are shortened by 65% (Boulton and Jackson, 1996; Porter et al., 1996) and telomeric silencing is abolished (Boulton and Jackson, 1998; Laroche et al., 1998; Nugent et al., 1998). However, only silencing at telomere ends is affected since repression of the silent mating-type locus at internal chromosomal sites is normal in Ku mutants (Laroche et al., 1998). Ku has been shown to associate with the Sir proteins involved in transcriptional silencing (Martin et al., 1999). In response to DNA damage, Ku relocalizes from telomeres and binds to DSB (Martin et al., 1999). Consequently, genes near telomere ends that were silenced by Ku and Sir become strongly transcribed. Also, Ku's role at telomeres is likely distinct from DSB repair since yeast cells that lack the DNA ligase IV homologue, an important component of the DSB repair machinery, have normal telomere lengths (Teo et al., 1997). Furthermore, a short carboxyl-terminal region of 30 amino acids of Ku70 is essential for telomere maintenance, but not for end joining (Driller et al., 2000). Other

mutant yeast cells with shortened telomeres have normal silencing (Boulton and Jackson, 1998).

It has been proposed that Ku might protect telomere ends from nucleases and recombinases (Gravel et al., 1998; Polotnianka et al., 1998). Ku can be crosslinked to telomere ends *in vivo* (Gravel et al., 1998). However, Ku dissociates from telomeres at S phase, perhaps allowing nuclease degradation of one strand that can subsequently form a single-stranded DNA binding site for telomerase (reviewed in Featherstone and Jackson, 1999). Recently, Ku has been shown to bind to mammalian telomeres *in vitro* (Bianchi and Lange, 1999).

D. Cell cycle regulation

The functions of Ku in cell cycle regulation are only starting to be investigated. Fibroblast cells from Ku86 knockout mice have a prolonged cell cycle (Nussenzweig et al., 1996). Furthermore, Ku86 deficient Chinese hamster ovary cells were blocked at G2 phase with ICRF-193, a DNA topoisomerase II inhibitor, at a concentration that did not affect wild-type cells (Munoz et al., 1998).

E. Replicative senescence

Senescence describes only the deteriorative changes that occur with time during postmaturational life that underlie an increasing vulnerability to challenges, thereby decreasing the ability of the organism to survive, whereas aging encompasses all the time-related changes that may have positive, negative, or deteriorative effects (Vogel al., 1999). Ku86 knockout mice exhibit a senescent phenotype and show an early onset of age-related changes and shortened life span (Vogel et al., 1999). The mice, when compared to the control littermates, prematurely exhibit osteopenia, epiphysis closure, atrophic skin and hair follicles, hepatocellular degeneration, and age-specific mortality. Cancer and sepsis occur in the control and Ku86-/- mice, although they occur earlier in the knockout mice. Thus, Ku86 influences senescence.

Replicative senescence in both Ku86 and p53 mutant mice is caused by a p53-dependent cell cycle response to DNA damage (Lim et al., 2000). Reducing the levels of

p53, a tumour suppressor protein that is important for cell cycle checkpoints in response to DNA damage and replicative senescence, in Ku86-/- mice results in higher cancer incidence. Furthermore, reducing the levels of p53 rescued Ku86-/- cells from replicative senescence, by enabling spontaneous immortalisation (Lim et al., 2000). Ku86 has also been described as a caretaker gene that maintains the integrity of the genome by suppressing chromosomal rearrangements since Ku86-/- mice display more chromosomal aberrations, such as breakage, translocations, and aneuploidy (Difilippantonio et al., 2000).

F. Aging

Werner's syndrome, WS, is an inherited autosomal recessive premature aging disorder. Cells from WS patients show typical aging characteristics, increased chromosomal abnormalities, and rapid onset of cellular senescence. WS is caused by a mutation in WRN, a protein that is homologous to the REQ family of DNA helicases (Yu et al., 1996). WRN catalyzes DNA unwinding (Gray et al., 1997) and possesses a 3'-5' exonuclease activity (Kamath-Loeb et al., 1998; Shen et al., 1998; Machwe et al., 2000). WRN interacts with RP-A (Brosh et al., 1999) and is also part of the DNA replication complex (Lebel et al., 1999). A physical interaction between Ku and WRN has been reported (Cooper et al., 2000). This functional interaction stimulates the WRN 3'-5' exonuclease activity. It is plausible that WRN and Ku function in a common metabolic pathway, such as DNA replication, and that a defective exonuclease could lead to an aging phenotype.

G. DNA replication

A role for Ku in DNA replication and replication fork movement was proposed a decade ago based on the observation that Ku bound to DNA ends during S phase (Paillard and Strauss, 1991). Since then, evidence for the involvement of Ku in DNA replication has been accumulating. Ku has been shown to bind to several origins of DNA replication, including the adenovirus type 2 origin (de Vries et al., 1989), the B48 human DNA region (Toth et al., 1993), the A3/4 sequence in the minimal origin of *ors*8 and the hamster

DHFR oriβ (Ruiz et al., 1999; [see chapter three of this thesis]), and the human dnmt-1 (DNA methyltransferase) origin (Araujo et al., 1999). A Ku-like protein from *S. cerevisiae*, OBF2 (identified as Ku70), binds to the yeast ARS121 origin and supports the formation of a stable multiprotein complex (Shakibai et al., 1996). The ARS binding factor (ABF-1) and the core binding protein (CBF) are the other two components of the complex. Stable binding of CBF, a protein with similar properties to ORC, occurred only in the presence of OBF2 and ABF-1. Also, a yeast Ku70 mutant strain exhibits a high DNA content during mitotic growth, further implicating Ku in DNA replication (Barnes and Rio, 1997).

Furthermore, Ku has been shown to bind to matrix attachment regions (MAR) with high affinity and sequence-specificity (Galande and Kohwi-Shigematsu, 1999). MAR are believed to represent the sites of initiation of DNA replication. Moreover, binding of Ku to Alu family DNA, suggested to provide cis-elements that affect chromatin structure and origins of DNA replication, has also been reported (Tsuchiya et al., 1998).

Ku is the DNA-dependent ATPase from HeLa cells (Cao et al., 1994) which co-fractionated with a 21S DNA polymerase α multiprotein complex that is able to support SV40 *in vitro* DNA replication (Vishwanatha and Baril, 1990). Recently, Ku was shown to interact with WRN (Cooper et al., 2000), a DNA helicase with 3'-5' exonuclease activity that is part of the replication complex (Lebel et al., 1999) and interacts with RP-A (Brosh et al., 1999).

Ku may also be involved in DNA replication through its association with DNA-PKcs; DNA-PK phosphorylates several DNA biding proteins that are involved in DNA replication, such as RP-A (Brush et al., 1994), DNA topoisomerases I and II (Anderson and Lees-Miller, 1992), SV40 T-Ag (Chen et al., 1991), Oct-1 (Anderson and Lees-Miller, 1992), and Ku antigen (Chan and Lees-Miller, 1996). Moreover, DNA-PKcs has recently been implicated in DNA replication following radiation damage (Guan et al., 2000). The effect of DNA-PKcs on DNA replication was determined by measuring total DNA synthesis or size distribution of nascent DNA, using alkaline sucrose gradient centrifugation. Cells proficient or deficient in DNA-PKcs, and cells deficient in other components required for DSB repair (such as DNA ligase IV and XRCC4) were exposed

to radiation, resulting in inhibition of DNA replication in all cell types. However, the inhibition in the wild-type cells, the DNA ligase IV and XRCC4 deficient cells was subsequently recovered, whereas no recovery was observed in the DNA-PKcs deficient cells. Thus, although a deficiency of DNA-PKcs has no direct measurable effect on DNA synthesis inhibition after irradiation, it causes a pronounced defect in the ability of cells to recover following irradiation (Guan et al., 2000). Other studies have indicated that DNA-PKcs is not required in the mammalian *in vitro* DNA replication in the absence of irradiation (Ruiz et al., 1999; [see chapter three of this thesis]).

Chapters three, four and five of this thesis deal with the role of Ku in mammalian DNA replication. The results presented in these chapters suggest that Ku is an origin specific binding protein that recruits the replication complex at the origin site and may proceed to unwind the DNA with its helicase activity (Ruiz et al., 1999; Matheos et al., submitted; Matheos et al., submitted).

iv) Ku knockout mice and Ku deficient cell lines

Ku70 and Ku86 knockout mice have been generated (Nussenzweig et al., 1996; Gu et al., 1997). The mice are viable but they exhibit many phenotypic defects. The mice are defective in V(D)J recombination, which leads to a decrease of B and T lymphocytes. Ku70 and Ku86 knockout mice differ because the Ku70 knockout mice have low levels of T cells in the thymus, spleen, and periphery, in addition to having thymic lymphomas, whereas Ku86-/- do not have T cells or lymphomas. This suggests that some of the Ku86 remaining in the Ku70-/- may function as a monomer or homodimer (reviewed in Featherstone and Jackson, 1999). The knockout mice are only half the size of their heterozygous littermates and the knockout female fail to nurture their young (Nussenzweig et al., 1996). Furthermore, embryo fibroblast cells from the knockouts have prolonged doubling times. Also, the non-proliferating cells from the mice arrest at G1 phase in early passages with polyploid cells, indicating premature senescence. The cells are also radiosensitive and fail to resume the cell cycle after radiation-induced checkpoint arrest.

The phenotypes of the Ku knockout mice, although supportive of Ku's involvement in DNA repair, do not rule out the possibility that it may also be involved in other processes,

such as DNA replication (reviewed in Featherstone and Jackson, 1999). The immunodeficiency, the radiosensitivity, and the failure of the cells to resume progress through the cell cycle after checkpoint arrest caused by ionizing radiation, are all phenotypes that may easily be explained by a role of Ku in DSB repair. However, the small size of the Ku-deficient mice, the failure of the cells to proliferate in culture, their prolonged doubling time, and the premature senescence suggest a role of Ku in other processes, such as DNA replication.

Xrs mutant cells are derivatives of the Chinese hamster ovary (CHO) K1 cells that were initially described as the first mammalian cell mutants with a defect in DSB rejoining (Kemp et al., 1984). The *xrs* mutant cells were isolated by a simple technique involving the transfer of heavily mutagenized cells from single colonies, following irradiation with a dose determined not to affect survival of wild-type CHO K1 (Jeggo and Kemp, 1983). *Xrs*-5 is a member of the IR5 X-ray complementation group that is defective in V(D)J recombination, DSB repair and DNA-end binding (Kemp et al., 1984; Pergola et al., 1993; Taccioli et al., 1993; Rathmell and Chu, 1994a,b; Getts and Stamato, 1994; Singleton et al., 1997). The defective repair and recombination phenotypes of the *xrs*-5 cells have been traced to the 86 kDa subunit of Ku, since Ku86 cDNA restored DNA end-binding, X-ray sensitivity, and V(D)J recombination (Getts and Stamato, 1994; Rathmell and Chu, 1994a,b; Smider et al., 1994; Taccioli et al., 1994).

The replication profile of the *xrs*-5 cells is the subject of chapter five of this thesis (Matheos et al., submitted). *In vitro* replication assays showed that total and cytoplasmic extracts from *xrs*-5 cells sustained *in vitro* replication as efficiently as the wild-type CHO K1 cells. In contrast, *xrs*-5 nuclear cell extracts lacked replication activity. Addition of Ku to the *xrs*-5 nuclear extracts restored their ability to replicate DNA. A Ku-like or modified Ku protein is suggested to be present in the cytoplasm of the *xrs*-5 cells that allows them to replicate efficiently.

CHAPTER TWO

Oct-1 enhances the *in vitro* replication of a mammalian autonomously replicating DNA sequence

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I. ABSTRACT

A 186 base pair fragment of *ors*8, a mammalian autonomously replicating DNA sequence isolated by extrusion of nascent monkey DNA in early S phase, has previously been identified as the minimal sequence required for replication function *in vitro* and *in vivo*. This 186 base pair fragment contains, among other sequence characteristics, an imperfect consensus-binding site for the ubiquitous transcription factor Oct-1. We have investigated the role of Oct-1 protein in the *in vitro* replication of this mammalian origin. Depletion of the endogenous Oct-1 protein, by inclusion of an oligonucleotide comprising the Oct-1 binding site, inhibited the *in vitro* replication of p186 to approximately 15-20% of the control, whereas a mutated Oct-1 and a nonspecific oligonucleotide had no effect. Furthermore, immunodepletion of the Oct-1 protein from the HeLa cell extracts by addition of an anti-POU antibody to the *in vitro* replication reaction inhibited p186 replication to 25% of control levels. This inhibition of replication could be partially reversed to 50-65% of control levels, a two- to three-fold increase, upon the addition of exogenous Oct-1 POU domain protein. Site-directed mutagenesis of the octamer binding site in p186 resulted in a mutant clone, p186-MutOct, which abolished Oct-1 binding but was still able to replicate as efficiently as the wild type p186. The results suggest that Oct-1 protein is an enhancing component in the *in vitro* replication of p186 but that effect on replication is not caused through direct binding to the octamer motif.

II. INTRODUCTION

The specific initiation of DNA replication is a crucial step in cell growth and reproduction. Eukaryotic origins of DNA replication consist of a core component, which is indispensable for origin function, and cis-acting auxiliary components, which enhance core origin function (DePamphilis, 1993). These auxiliary components include binding sites for specific cellular transcription factors. Understanding how these transcription factors activate eukaryotic origins of DNA replication might aid in understanding the mechanisms that regulate the initiation of DNA replication.

Several mechanisms have been proposed to explain the direct role of transcription factors in activating replication origins of eukaryotic viruses (DePamphilis, 1993). In one model, transcription factors activate origins by initiating RNA primer synthesis at the initiation of replication site (Clayton, 1991). Transcription factors may also stimulate replication by facilitating the assembly and activity of an initiation complex (Chiang et al., 1992; Mul and van der Vliet, 1992). Evidence also exists in support of a model, wherein transcription factor binding may prevent repression of origin activity due to chromatin structure (reviewed in DePamphilis, 1993).

Several mammalian origins of DNA replication also contain putative auxiliary transcription factor binding sites. For example, the Chinese hamster DHFR origin has a consensus binding site for Oct-1 and AP-1 (DePamphilis, 1993), the hamster rhodopsin origin has AP-1 and Oct-1 sites, and CP-1/CAAT box (Gale et al., 1992), while the murine IgH intronic enhancer Eμ contains binding sites for Oct-1, -2, and -3 (Staudt and Lenardo, 1991). Mammalian origin-enriched (*ors*) and autonomous replicating sequences also contain various transcriptional regulatory elements, such as CTF/NF-1 and related sequences, CACCC, the β-globin transcriptional control sequence, as well as Oct-1 sequence (Rao et al., 1990; Landry and Zannis-Hadjopoulos, 1991; Wu et al., 1993). Thus, the octamer consensus motif is present in the auxiliary regions of several viral and eukaryotic origins (Iguchi-Ariga et al., 1988; Staudt and Lenardo, 1991; DePamphilis, 1993). The octamer sequence has also been reported to serve as a putative origin for

cellular DNA replication, since enhanced replication of a transfected plasmid depended on the presence of an intact octamer binding site (Iguchi-Ariga et al., 1993).

The octamer consensus sequence, ATGC(A/T)AAT, is recognized by a group of proteins that have been identified at various developmental stages and in a variety of tissues and organs (reviewed in Schöler, 1991). One of the octamer recognizing proteins is the 93 kDa, ubiquitously expressed transcription factor, Oct-1 (Singh et al., 1986; Staudt et al., 1986; Sturm and Herr, 1988). It is a member of the POU family of transcription factors that have been implicated in tissue-specific gene expression, cell differentiation and DNA replication (Pruijn et al., 1986; Rosenfield, 1991). Oct-1 protein is composed of a transcription activation domain and a DNA binding domain, the POU domain. The POU domain consists of two subdomains: a 60 amino acid carboxyl-terminus domain, called the homeodomain, recognizing the 3' half of the octamer sequence (TAAT), and a 75 amino acid amino-terminus POU-specific domain, binding to the 5' half of the octamer sequence (TATGCA) (Verrijzer et al., 1992). The A/T overlap is recognized by both subdomains.

The Oct-1 POU domain is sufficient to stimulate adenovirus DNA replication, as demonstrated by deletion analyses (Verrijzer et al., 1990). *In vitro*, Oct-1 enhances adenovirus replication three- to seven-fold, depending on the pTP-pol concentration and on a DNA-independent interaction between the pTP-pol complex and the POU homeodomain (Coenjaerts et al., 1994). Furthermore, mutations in the Oct-1 POU specific recognition helix decreased the ability of the POU domain to enhance adenovirus replication (van Leeuwen et al., 1995). Recently, it was shown that the Oct-1 POU domain stimulates replication by increasing the binding of the pre-terminal protein-DNA polymerase complex (pTP-pol) to the core origin and stabilizing the pre-initiation complex *via* a direct interaction between the pTP and the POU homeodomain (van Leeuwen et al., 1997). Thus, the DNA binding domain of Oct-1 not only binds to the octamer motif, but it also directly interacts with a key component of the adenovirus replication machinery, the pTP protein.

In adenovirus, the Oct-1 binding site is important for optimal replication *in vivo*. However, if other compensating DNA sequences, such as the transcription factor binding sites SP1, ATF and EBP-1, are present, deletion of the octamer sequence fails to produce

a replication effect, indicating functional redundancy between different transcription factor binding sites at the left terminus of the adenovirus genome (Hatfield and Hearing, 1993). Furthermore, bending at the octamer site, produced by Oct-1 binding, is not sufficient for replication enhancement (Verrijzer et al., 1991). DNA bending may have an architectural role in the formation of an initiation complex by facilitating interactions between different proteins. Substitution of the Oct-1 binding site by an AP1 site does not stimulate replication in the presence of fos-jun, which are able to bend DNA in a similar fashion to Oct-1 (Coenjaerts et al., 1994; reviewed in van der Vliet, 1996). Thus, protein-protein interactions between the POU DNA binding domain of Oct-1 and pTP are critical for enhancement of adenovirus replication.

Previously, we cloned origin rich sequences (*ors*) isolated by extrusion of nascent DNA from replicating monkey (CV-1) cells at the onset of S phase (Kaufmann et al., 1985; Zannis-Hadjopoulos et al., 1985). The *ors* are capable of transient autonomous replication *in vivo*, upon transfection in monkey (CV-1 and Cos-7) and human (HeLa) cells (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991) and in an *in vitro* replication system that uses HeLa cell extracts (Pearson et al., 1991). Both *in vivo* and *in vitro* replication initiates within the *ors*, is semiconservative, bidirectional and sensitive to the action of aphidicolin (Frappier and Zannis-Hadjopoulos, 1987; Pearson et al., 1991, Pearson et al., 1994a). Among the functional *ors*, *ors*8 has been analyzed in detail both *in vivo* (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991; Zannis-Hadjopoulos et al., 1992) and *in vitro* (Pearson et al., 1991; Zannis-Hadjopoulos et al., 1992). *In vitro* replication of *ors*8 is semiconservative, as shown by the *in vitro* incorporation of bromodeoxyuridine (BrdUTP) and separation of BrdUTP-substituted DNA on both neutral and alkaline CsCl density gradients (Pearson et al., 1991; Zannis-Hadjopoulos et al., 1992). Time-course and electron microscopy mapping of the origin of replication on the *ors*8 plasmid showed that replication starts within the *ors* and proceeds bidirectionally (Pearson et al., 1991; 1994a). Recently, we identified, by deletion analyses, a 186 base pair (bp) fragment of *ors*8 as the minimal sequence required for origin function *in vivo* and *in vitro* (Todd et al., 1995). This minimal replicating DNA sequence contains an imperfect 44-bp IR capable of extruding into a cruciform, several direct repeats, an imperfect consensus binding site

for Oct-1, and several binding sites for the GATA family of transcription factors (Merika and Orkin, 1993; Pevry et al., 1991) (Figure 1). A measurable sequence-directed DNA bent was also detected within the minimal origin of *ors*8 (Todd et al., 1995).

In order to determine the role of transcription factors on the replication activity of a mammalian origin, we investigated the role of Oct-1 in the *in vitro* replication of p186, a pBR322-based plasmid containing the minimal *ors*8 autonomously replicating sequence (Todd et al., 1995).

In this study, we show that Oct-1 enhances the *in vitro* replication of p186. Addition of an Oct-1-specific double-stranded oligonucleotide in the *in vitro* reaction inhibited p186 replication by approximately 80-85%, whereas neither a nonspecific nor a mutated Oct-1 oligonucleotide produced any effect. Furthermore, immunodepletion of the Oct-1 protein by addition of an anti-POU antibody inhibited replication by 75-80%. In both instances, the inhibition was reversed to 50%-65% of control levels when purified Oct-1 POU was added exogenously. Replication levels of a p186 mutant clone, however, which is unable to bind Oct-1, were similar to wild type p186 levels, indicating that the enhancing replication effect of Oct-1 does not result from its direct binding to the octamer motif within the origin, but rather through an indirect mechanism, most likely, involving protein-protein interaction.

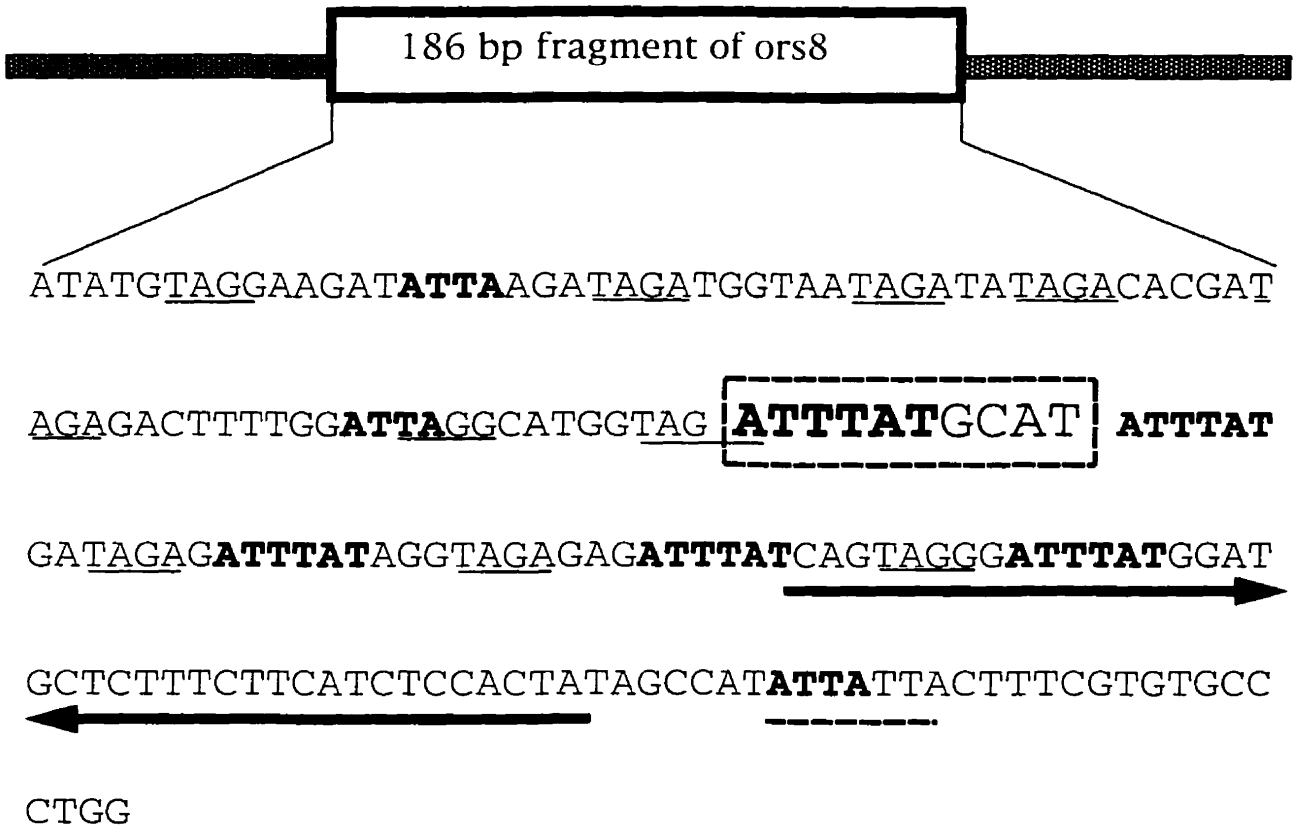


Figure 1. Sequence characteristics of the 186-bp minimal origin of *ors*8. The imperfect Oct-1 binding site (boxed), the imperfect inverted repeat (head-head arrows), the direct repeat (dashed underline), and the repeated sequence motifs TAGA and TAGG (solid underline) and ATTA and ATTTAT (bold) are indicated (Adapted from reference Todd et al., 1995).

III. MATERIALS AND METHODS

Plasmids

Plasmid p186 consists of the *Nde*I-*Rsa*I sub-fragment of *ors*8 sub-cloned in the *Nru*I site of pBR322 (Todd et al., 1995). It is capable of autonomous replication *in vivo* and *in vitro* (Todd et al., 1995). Plasmids p186 and pBluescript II KS- (used as a negative control) were propagated in *Escherichia coli* HB101 as previously described (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991). Plasmid p186-MutOct consists of the p186 plasmid with a mutated Oct-1 binding site (see Site-Directed Mutagenesis below). It was propagated in Epicurian coli XL-1 Blue (Stratagene, LaJolla, CA). Plasmid DNAs were isolated using the QIAGEN-tip 500 columns according to the manufacturer's specifications (QIAGEN, Santa Clarita, CA).

The 186-bp fragment of *ors*8 and the 206-bp fragment of pBR322 (from nucleotide 860-1065) used in the competition bandshift assay were amplified by PCR and purified by isotachophoresis, as previously described (Ruiz et al., 1995), or using the QIAEX II gel extraction kit (QIAGEN).

Oligonucleotides

The Oct-1-specific oligonucleotide, 5'TGTCGAATGCAAATCACTAGAA3', was purchased from Promega Corporation (Madison, WI, OCT1 Consensus Oligonucleotide, Cat. # E3241) as a double-stranded fragment and used in the *in vitro* replication, competition and bandshift assays. It is not homologous to the 186-bp fragment, but contains a perfect Oct-1 binding site that can readily compete for Oct-1 binding in the *in vitro* replication assays. A nonspecific double-stranded DNA fragment was prepared by annealing two oligonucleotides (5'TTCCGAATACCGCAAG3', synthesized by Sheldon Biotechnology, Montréal, Canada), representing the region of pBR322 from nucleotides 838-854. The mutated Oct-1 oligonucleotide was prepared by annealing two single strands of a DNA fragment representing a 31-bp region of the 186-bp fragment of *ors*8 (synthesized by Sheldon Biotechnology, Montréal, Canada), in which the Oct-1 binding site was altered according to Kemler et al. (1989) in order to abolish Oct-1 binding. The

sequence of the mutated Oct-1 oligonucleotide is 5'GGCATGGTAG**CGACCGTG**ATATTTATGATAG 3' (the nucleotides in bold indicate the mutated Oct-1 binding site).

All the annealing reactions, including that of the Oct-1-specific oligonucleotide (Promega), were performed using a 1:1 ratio of the two complementary single-stranded oligonucleotides in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl2, 1 mM DTT, 1 mM EDTA. The oligonucleotides were placed at 90° C for 2 minutes, and allowed to cool to room temperature. The efficiency of annealing was verified by 5' end-labeling an aliquot (typically 10 ng) of the reaction mixture with 1 unit T4 polynucleotide kinase (New England Biolabs, Mississauga, Ontario, Canada) in the presence of 200 μCi [γ^{32}P]-ATP and was found to be approximately 80-90%.

Preparation of cell extracts

HeLa S3 nuclei and cytosol were purchased from Cellex Biosciences, Inc. (Minneapolis, MN). The extracts were made as previously described (Pearson et al., 1991; Todd et al., 1995). The protein concentrations of the nuclear and cytoplasmic extracts were 4.1 mg/ml and 6.4 mg/ml, respectively.

In vitro replication assay

In vitro replication was carried out as previously described (Pearson et al., 1991), with slight modifications. Standard reactions included HeLa cytoplasmic (70 μg) and nuclear (18.5 μg) extracts, 2 mM ATP, 100 mM each CTP, GTP, UTP, dATP, and dGTP, 10 μCi of [α^{32}P]-dTTP and 10 μCi [α^{32}P]-dCTP, 0.2 units of pyruvate kinase (Boehringer Mannheim, Laval, Québec, Canada), and equimolar amounts of either p186, p186-MutOct, or pBluescript (200 ng, 200 ng, and 130 ng, respectively).

The molar excess of the Oct-1-specific, mutated Oct-1, and nonspecific double-stranded oligonucleotides or of the anti-POU antibody (a gift from Dr. Peter C. van der Vliet, Utrecht University, The Netherlands) was in relation to the p186 input plasmid DNA. (That is to say, if 200 ng of p186 plasmid DNA (4549) was used, then 0.97 ng of the Oct-1 specific oligonucleotide (22-bp) would represent equimolar amounts. If a 100-fold molar excess of the oligonucleotide was required, then 97 ng would be used). The

experiments were performed by preincubating the HeLa cell extracts with the oligonucleotides or the anti-Oct-1 POU antibody for 20 minutes on ice.

When the Oct-1 POU protein (a gift from Dr. Peter C. van der Vliet, Utrecht University, The Netherlands) or BSA (New England BioLabs) was used, it was added to the reaction mixture containing the extracts and 250-fold molar excess of Oct-1 oligonucleotide or the extracts and the anti-Oct-1 POU that had been preincubated on ice for 20 minutes. The POU domain and BSA proteins were added to replace the depleted levels of endogenous Oct-1, and preincubated on ice for a further 20 minutes.

When aphidicolin (Boehringer-Mannheim) was used, it was added to a final concentration of 80 μM to the reactions containing the extracts and oligonucleotides that had been preincubated on ice and the reactions were subsequently preincubated at 30°C for 15 minutes.

Following the pretreatment steps, the DNA, nucleotides, pyruvate kinase and PEG solution (125 mM Hepes-KOH pH 7.8, 6 mM EGTA, 32 mM $MgCl_2$, 1.5 M ethylene glycol, 32% wt/vol. polyethylene glycol) were added and the reactions were incubated at 30°C for 1 hour. The reactions were terminated by the addition of 30 mM EDTA/1% SDS. The input DNA and the reaction products were purified as previously described (Pearson et al., 1991; Zannis-Hadjopoulos et al., 1994; Todd et al., 1995). The reaction products were divided into three samples; one third was digested with 1 unit *Dpn*I (New England BioLabs) for 60 minutes at 37°C in the presence of 1X NEB buffer 4 (50 mM KCl, 20 mM Tris acetate, 10 mM magnesium acetate, 1 mM DTT), 200 mM NaCl and 300 ng of pBluescript DNA, used as carrier DNA and indicator of *Dpn*I digestion. The *Dpn*I-digested (1/3) and undigested (1/3) products were resolved on 1% agarose gel in 1X TAE buffer (16-20 hours, 50-55 Volts). The gels were dried and exposed to Kodak BioMax MS film.

Quantification was performed, as previously described (Diaz-Perez et al., 1996), on *Dpn*I-digested products by densitometric measurements using a Phosphoimager analyzer (Fuji BAS 2000) or a Bio Image Densitometer (MillGen/Biosearch); both methods gave similar results. Quantification involved measuring the density of the bands corresponding to the relaxed circular (II) and linear (III) forms of the plasmid DNA. Quantification of the supercoiled (form I) band was not possible because of overlap with

the *Dpn*I digestion products. These results were corrected for the amount of DNA recovered from the *in vitro* replication assay by quantitative analysis of the ethidium bromide picture of the gel. The amount of radioactive precursor incorporated into the DNA was expressed as a percentage of the control p186 *in vitro* reaction that did not contain any oligonucleotide or antibody and was considered to have 100% replication activity.

DNA binding assay

DNA binding assays were performed as previously described (Pearson et al., 1994b; Ruiz et al., 1995). Reactions were performed in 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 1 µg poly(dI-dC) (Pharmacia, Baie d'Urfé, Québec, Canada), with 10 µg total HeLa cell extracts or 2.5 ng of the Oct-1 POU domain protein, and 1 ng ^{32}P-labeled Oct-1-specific or -mutated oligonucleotide (6.8 fmol/reaction) or 0.1 ng of labeled 186-bp DNA (0.78 fmol/reaction), respectively, in final volume of 20 µl. When the anti-Oct-1 antibody (Trans Cruz Gel Supershift reagent, Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, Cat.# sc-232) was used in the binding reaction to interfere with the binding of endogenous Oct-1 to the Oct-1- specific or -mutated oligonucleotides, the extracts and the probe were preincubated at room temperature for 20 minutes, and then 1.5 µg of anti-Oct-1 antibody was added and incubated for a further 25 minutes at room temperature. When competition studies were done, cold DNA, either Oct-1 oligonucleotide and nonspecific 16-bp pBR322 fragment (nucleotide 838-854), or 186-bp fragment DNA and 206-bp pBR322 DNA fragment (nucleotide 860-1065), was included in the reaction in molar excess, as indicated in the Figure legends. After a 30 minute incubation on ice, loading dye (25% Ficoll, 25 mM EDTA, 0.2% BPB, 0.2% xylene cyanol) was added and the samples were subjected to electrophoresis on a 4% polyacrylamide gel in 1X TBE at 180 Volts for 1 hour at room temperature. The gels were then dried and exposed for autoradiography.

Immunodetection assay

Western blots were performed as previously described (Pearson et al., 1994b; Ruiz et al., 1995). Polyacrylamide gel electrophoresis (PAGE) was performed as

previously described (Laemmli, 1970), using 500 ng bacterially expressed His6-tagged Oct-1 POU domain (a gift from Dr. P. C. van der Vliet, Utrecht University, The Netherlands), 70 µg HeLa cell cytoplasmic extracts and 18.5 µg nuclear extracts (these amounts correspond to those used in the *in vitro* replication assays). Proteins were electroblotted to Immobilon-P membranes (Millipore) and probed with anti-POU rabbit polyclonal antibody (a gift from Dr. P.C. van der Vliet). The blots were developed using the ECL Detection system (Amersham, Oakville, Ontario, Canada) with the HRP-labeled anti-rabbit antibody. The membranes were exposed for autoradiography. Determination of the amount of Oct-1 protein in the HeLa nuclear extracts was performed by preparing a Western blot with different dilutions of the Oct-1 POU protein. Then, the films were scanned using a Bio Image Densitometer (Milligen/Biosearch) and a graph of peak area versus protein concentration was plotted. The concentration of the Oct-1 protein present in the HeLa nuclear extracts was determined from the standard curve.

Site directed mutagenesis and sequencing reactions

Mutagenesis of the Oct-1 binding site in p186 was performed using the Chameleon Double-Stranded Site-Directed Mutagenesis kit (Stratagene, Cat.# 200509) with the following modifications. 0.25 pmol of p186 DNA, 25 pmol of *ScaI*/*MluI* unique site elimination selection primer (Pharmacia Biotech, Cat. # 27-1666-01) and 100 pmol of mutagenic primer, carrying the Oct-1 site mutation, were used. The mutagenic primer was synthesized, 5'-phosphorylated and purified by PAGE by Gibco BRL (Burlington, Ontario, Canada). The sequence of the 33-bp primer is 5' -P GGCATGGTAGCGACCGTGATATTTATGATAGAC 3'. This sequence is identical to the mutated oligonucleotide used in the *in vitro* replication assays except for the additional two bases, AC, at the 3'. The primers were extended by incubating the annealed primers/template reaction mixture with a T7 DNA polymerase/T4 DNA ligase mixture at 37°C for 2.0 hours. The reactions were digested with 20 units of *ScaI* (Stratagene) and the subsequent steps were performed as per manufacturer's specifications.

The potential mutant clones were screened by restriction endonuclease digestion with *ScaI* (Stratagene) and *MluI* (Gibco BRL). Clones which were not digested by *ScaI*,

yet linearized by *Mlu*I, were sequenced using the T7 Sequencing kit from Pharmacia Biotech, as per manufacturer's instructions to "read short", with the following modifications. The sequencing reactions contained 1.0 μg miniprep DNA , 2.5 μM of primer H' (from region 994-980 of pBR322), and 12.5 μCi [α^{35}S]-dATP. Then, 3.0 μl of the reaction mixture was denatured and loaded on an 8% sequencing gel. The gel was run at 1700 volts for 1.0 hour. Subsequently, the gel was dried and exposed for autoradiography. The mutant clone, p186-MutOct, was then used in the *in vitro* replication reaction as described above.

IV. RESULTS

The endogenous Oct-1 protein binds the 186-bp fragment of ors8

To verify that our preparation of HeLa cell extracts contained the ubiquitous Oct-1 protein, Western blot analysis of nuclear and cytoplasmic extracts was performed using a rabbit polyclonal antibody against the Oct-1 POU domain protein (Figure 2A). The amount of nuclear and cytoplasmic extracts in these analyses corresponded to the amount used in a single *in vitro* replication assay, 18.5 μg and 70 μg, respectively. The 24 kDa Oct-1 POU domain protein was used as positive control. The HeLa nuclear extracts contained the Oct-1 protein (approximately 93 kDa), in agreement with Oct-1 being a nuclear protein (Figure 2A). By densitometric analyses, we determined that 18.5 μg protein from HeLa nuclear extracts contained approximately 100 ng of Oct-1 protein. Upon longer exposures, very low levels of Oct-1 protein were also detected in the cytoplasmic extracts, suggesting some protein leakage into the cytoplasm during the preparation of the extracts (data not shown).

To determine whether the endogenous Oct-1 protein was able to bind to the imperfect octamer motif within the 186-bp fragment (Figure 1), bandshift and competition bandshift assays were performed in which labeled 186-bp DNA fragment was reacted with protein from total HeLa cell extracts. We have previously shown that, when labeled 186-bp fragment DNA was used in a bandshift with total HeLa cell extracts, three major protein-DNA complexes were formed (Ruiz et al., 1995 and Figure 2B, lane 2). Complexes 1 and 2 were due to specific binding, since they were competed when cold 186-bp fragment DNA was used as a specific competitor, whereas complex 3 was shown to be due to nonspecific binding, as it was abolished in the presence of increasing molar amounts of a 206-bp fragment of pBR322, used as nonspecific competitor (Ruiz et al., 1995). Here, we show that complex 1 is specifically competed by a 10-fold molar excess of the cold Oct-1-specific oligonucleotide (Figure 2B, lane 3). By 50-fold excess of cold competitor, the complex completely disappeared (Figure 2B, lane 4). Furthermore, complex 1 is supershifted when the anti-POU antibody is included in the binding reaction (data not shown). This suggests that this complex represents a specific interaction

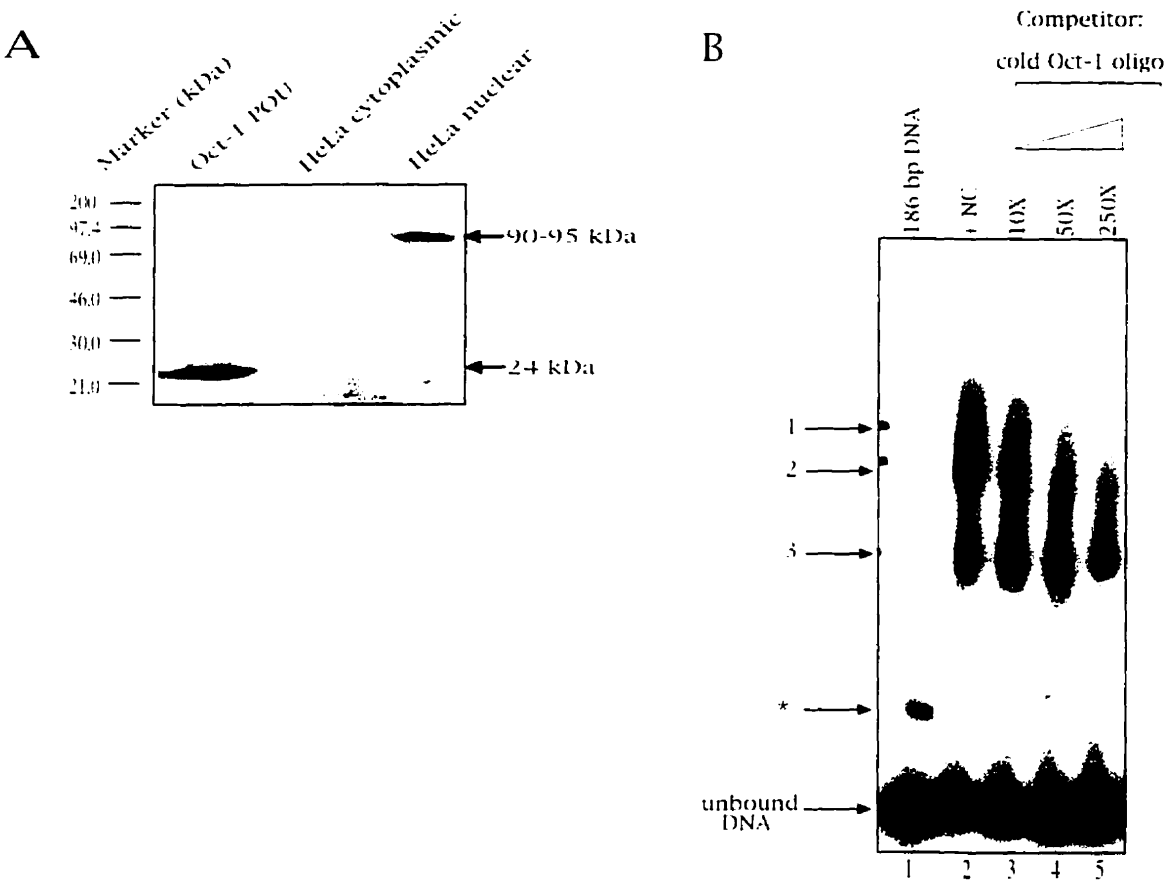


Figure 2. Engogenous HeLa Oct-1 binds to the 186-bp origin. A. Western Blot analysis. 500 ng of purified, bacterially expressed His6-tagged, Oct-1 POU domain protein, 70 µg protein from HeLa cytoplasmic- and 18.5 µg protein from HeLa nuclear- extracts were loaded on a 8% PAGE. The proteins were electrotransferred to Immobilon-P membranes for 1 hour at 100 V in transfer buffer containing 20% methanol. The blot was probed with anti-POU polyclonal antibody and developed as described in Material and Methods. The 24 kDa POU domain protein and the 90-95 kDa Oct-1 protein are indicated. B. Competition bandshift assay showing the binding specificity of the endogenous Oct-1 protein to the 186-bp fragment of *ors*8. Reactions contained 1 ng of labeled 186-bp fragment DNA and 10 µg protein from total HeLa cell extracts (+NC, lane 2) in the presence of increasing molar fold excess amounts of the cold Oct-1 oligonucleotide (lanes 3-5), as indicated. The unbound 186-bp fragment DNA, the three major protein complexes (1, 2, 3), and the 186-bp secondary structure (*) are indicated.

between the 186-bp fragment and the endogenous Oct-1 protein. Complex 2 was also competed in the presence of much higher amounts, 250-fold molar excess, of the Oct-1 specific oligonucleotide. As we have shown previously, complex 2 is formed by the interaction of the 186-bp fragment with the *ors* binding activity protein (OBA) (Ruiz et al., 1995). The competition of complex 2 by the Oct-1 specific oligonucleotide might be due to an interaction of OBA with Oct-1. Finally, complex 3 was not competed even in the presence of 250-fold molar excess of cold competitor, suggesting that Oct-1 binding is not involved in its formation.

Specific binding of endogenous Oct-1 protein found in HeLa extracts to an oligonucleotide containing the Oct-1 binding site

A bandshift assay was used to show the presence of binding activity for the Oct-1-specific double-stranded oligonucleotide in total HeLa cell extracts (Figure 3A). Three major specific protein-DNA complexes were formed (Figure 3A, lane 2, Complex A, B, C). The binding specificity of the oligonucleotide in each the complexes was tested by competition bandshift assays (Figure 3A), with increasing molar excess amounts (5- to 150-fold) of cold Oct-1 oligonucleotide (lanes 3-6), used as specific competitor, or cold pBR322 oligonucleotide (lanes 7-10), used as nonspecific competitor. At 5-fold and 25-fold molar excess of the specific competitor, only the binding of complex A was competed for (lanes 3 and 4), while at 50-fold and 150-fold molar excess, all three complexes were specifically competed by the cold Oct-1 oligonucleotide (lanes 5 and 6). In contrast, none of the complexes were competed by the nonspecific competitor (lanes 7-10). Hence, all three complexes represent a specific interaction of the extracts with the Oct-1-specific oligonucleotide.

To demonstrate which of the complexes were the result of Oct-1 binding, an anti-Oct-1 antibody was included in the binding reaction. Addition of the antibody supershifted complex A (**), but had no major effect on the two faster migrating complexes B and C (Figure 3B, lane 2). To show that complex A was indeed the result of the endogenous Oct-1 protein binding to the Oct-1-specific oligonucleotide, an oligonucleotide with a mutated Oct-1 binding site was also used in a bandshift reaction with total HeLa cell extracts (Figure 3B, lane 3). Complex A, which results from Oct-1

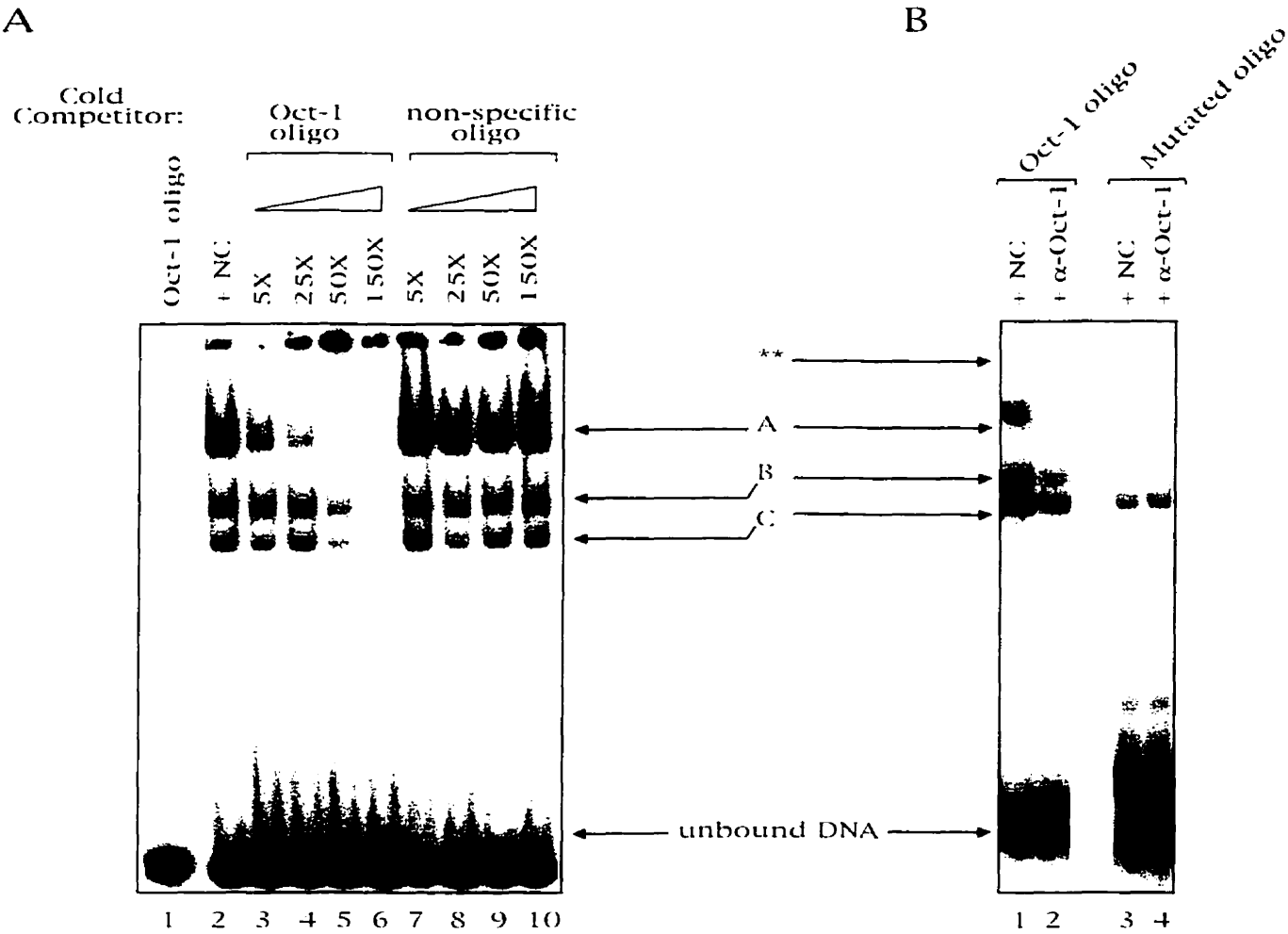


Figure 3. The Oct-1 oligonucleotide binds to the 186-bp origin. A. Competition bandshift assay showing binding specificity of the Oct-1 oligonucleotide to the HeLa cell endogenous Oct-1. Reactions contained 1 ng labeled Oct-1 oligonucleotide and 10 µg protein from total HeLa cell extracts (+NC, lane 2) in the presence of increasing molar excess amounts of cold Oct-1 oligonucleotide (lanes 3-6), added as specific competitor, or cold 16-bp pBR322 DNA fragment (nucleotides 838-854; lanes 7-10), added as nonspecific competitor, as indicated. The free Oct-1 oligonucleotide DNA (unbound DNA) and the three major complexes (A, B, C) are indicated. B. Interference of Oct-1 binding to the 186-bp fragment by addition of the anti-Oct-1 antibody (α-Oct-1). Reactions were performed by preincubating the extracts and the labeled oligonucleotide (Oct-1-specific (lane 1) or mutated (lane 3)) at room temperature for 20 minutes prior to the addition of 1.5 µg of the anti-Oct-1 antibody (lanes 2 and 4, respectively). Upon addition of the antibody, the reaction was incubated for a further 25 minutes at room temperature. The reactions were loaded on a 4% PAGE gel and ran at 180 volts for 1 hour. The three major complexes (A, B, C), the Oct-1 supershifted complex (**), and the unbound Oct-1-specific or mutated Oct-1 oligonucleotides are indicated.

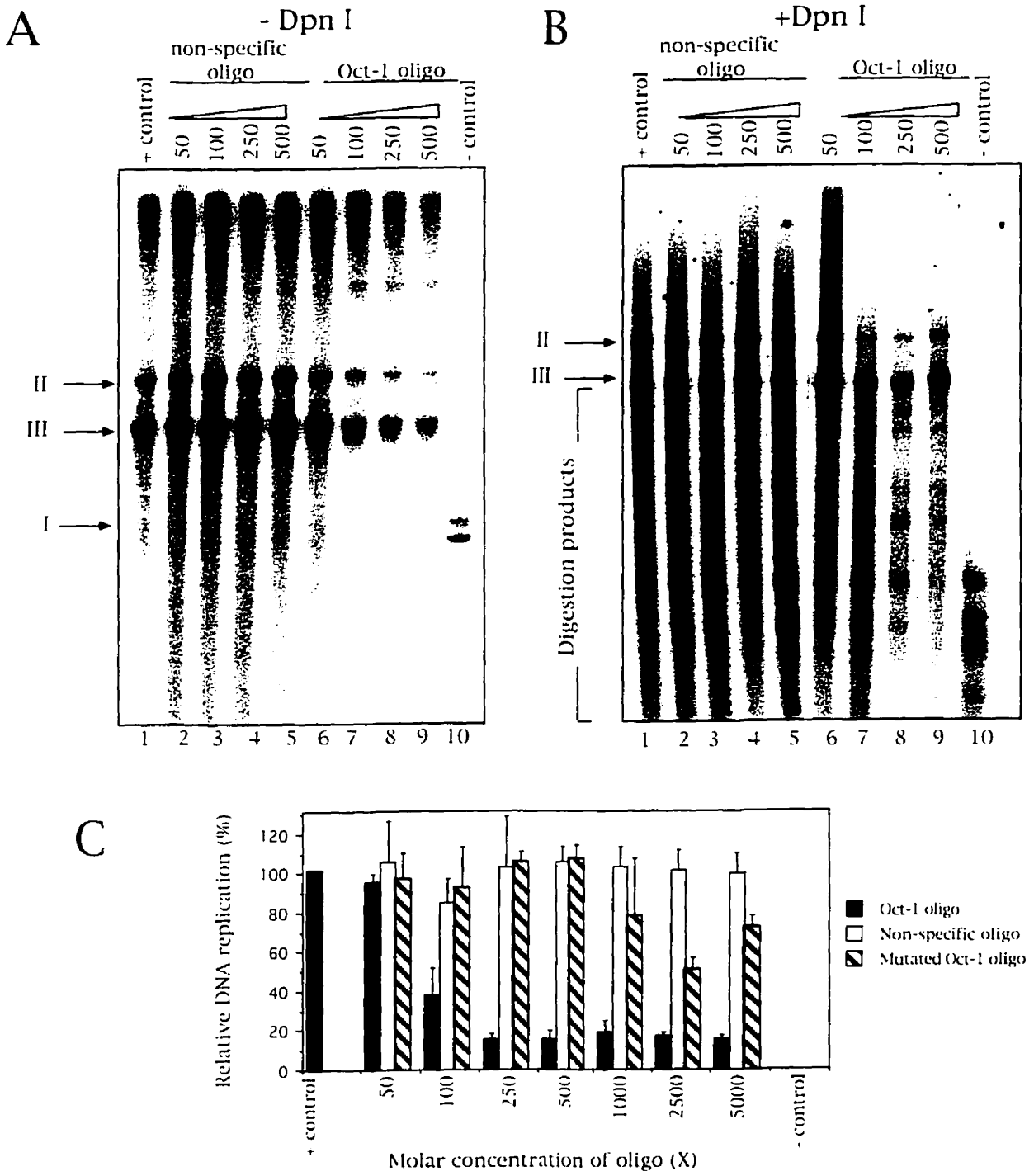
binding, did not form, while the two protein DNA complexes corresponding to complexes B and C did (Figure 3B, lane 3). Addition of the anti-Oct-1 antibody did not supershift either of these complexes (Figure 3B, lane 4). These results show that the Oct-1-specific oligonucleotide specifically binds the endogenous Oct-1 protein, whereas the mutated oligonucleotide does not, and confirms that the retarded complex A is due to the binding of Oct-1. Complexes B and C are the result of the Oct-1 oligonucleotide binding to unidentified proteins whose binding was not competed even in the presence of cold oligonucleotide corresponding to the SP1, AP1, and AP2 binding sites (Promega, personal communication). These two specific complexes are not the result of Oct-1 binding since they also form in the presence of the mutated Oct-1 oligonucleotide and they are not recognized by the Oct-1 antibody.

Addition of the Oct-1-specific oligonucleotide results in inhibition of replication

In vitro replication assays of the p186 plasmid were performed as described previously (Todd et al., 1995), except that increasing molar excess amounts, relative to the amount of the input p186 DNA, of the Oct-1-specific, mutated Oct-1, and nonspecific oligonucleotides were also included in the reaction, in order to study the effect of removal of the endogenous Oct-1 protein on the *in vitro* replication of p186. The extracts and the oligonucleotides were preincubated on ice for 20 minutes, to allow binding of the oligonucleotide to the endogenous Oct-1 protein present in the extracts. The products of the *in vitro* replication reactions included open circular (II), linear (III), and supercoiled (I) forms of the plasmid DNAs (Figure 4A). Lower levels of form I DNA were recovered after the *in vitro* replication reaction, by comparison to those of form II and form III DNA, in agreement with other studies (Decker et al., 1986; Pearson et al., 1991). The ladder of bands between the relaxed circular and supercoiled plasmid DNAs in the gel of undigested samples (Figure 4A, -*Dpn*I) corresponds to topoisomeric molecules (Pearson et al., 1991). DNA forms that migrate slower than the open circular form II represent catenated dimers and replicative intermediates (Pearson et al., 1991; Zannis-Hadjopoulos et al., 1994).

Inclusion of increasing amounts of the Oct-1-specific oligonucleotide decreased the total incorporation of label in p186 (Figure 4A, lanes 6-10), whereas the nonspecific

Figure 4. Oct-1 oligonucleotide inhibits mammalian *in vitro* DNA repliction. HeLa cell extracts were incubated with increasing amounts of Oct-1-specific oligonucleotide or nonspecific oligonucleotide of pBR322 on ice for 20 minutes. Then, p186 DNA and the remaining components of the *in vitro* reaction were added and the reactions were incubated at 30°C for 1 hour. The negative control corresponds to the pBluescript plasmid. The DNA products were purified and concentrated. One third of the reaction was left untreated (-*Dpn*I) and a third was digested with 1 unit of *Dpn*I (+*Dpn*I). The samples were electrophoresed on a 1% agarose gel in 1X TAE buffer at 50-55 V for 16-20 hour. The gels were dried and exposed for autoradiography. A. A typical autoradiograph of undigested *in vitro* products is shown. The open circular (II), linear (III), and supercoiled (I) forms of the replicated DNA are indicated. B. *Dpn*I digestions of the samples in part A. Forms II and III, and the *Dpn*I digestion products are indicated. C. Bar graph showing the relative DNA replication of p186 with increasing amounts of Oct-1-specific (black bars), nonspecific (white bars), or mutated Oct-1 (striped bars) oligonucleotides . The positive control corresponds to the p186 and the negative control to the pBluescript plasmid in the absence of any oligonucleotide, and are indicated with black bars. Quantifications were done on the *Dpn*I-digested samples as described in the Material and Methods. Data is expressed as a percentage of the control p186 *in vitro* reaction lacking any oligonucleotide. Each bar represents two to seven experiments and one standard deviation is indicated.


A
- Dpn I
non-specific oligo
Oct-1 oligo
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50
100
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Molar concentration of oligo (X)
Oct-1 oligo
Non-specific oligo
Mutated Oct-1 oligo

oligonucleotide (Figure 4A, lanes 2-5) and the mutated Oct-1 oligonucleotide (data not shown) had no effect. In addition to incorporation that is due to replication, some is due to repair (Pearson et al., 1991). To differentiate between incorporation due to repair or replication, the *in vitro* replication products were digested with the restriction endonuclease *Dpn*I (Peden et al., 1980). Using the *Dpn*I resistance assay, we have previously demonstrated that the *in vitro* replication of *ors*8 is semiconservative (Pearson et al., 1991; Zannis-Hadjopoulos et al., 1992), as it is *in vivo* (Frappier and Zannis-Hadjopoulos, 1987).

*Dpn*I-digestion of the samples confirmed that the Oct-1-specific oligonucleotide inhibited the replication of p186 (Figure 4B, lanes 6-10), in contrast to the samples in which the nonspecific oligonucleotide (Figure 4B, lanes 2-5) or the mutated Oct-1 oligonucleotide (data not shown) was included. Quantification of the *Dpn*I-resistant bands corresponding to forms II and III was performed (as described in Materials and Methods) and the percent of DNA replication relative to the positive control (Figure 4B, lane 1), p186, was plotted (Figure 4C). A decrease in DNA replication was not observed at the 50-fold molar excess level of Oct-1-specific oligonucleotide, probably because this amount was not sufficient to bind all the endogenous Oct-1 present in the extracts. However, the relative percent of DNA replication decreased to approximately 37% in the presence of competitive 100-fold molar excess of Oct-1-specific oligonucleotide, and down to 12-25% with 250-molar fold of the same oligonucleotide (Figure 4C, black bars). This level of replication remained constant even when the specific oligonucleotide was increased to a molar excess of up to 5000-fold (Figure 4C, black bars). No inhibition of replication was observed when the nonspecific oligonucleotide (white bars) or the mutated Oct-1 oligonucleotide (striped bars) were included in the *in vitro* replication reaction of p186 up to levels of 500-1000-fold molar excess (Figure 4C). Above 1000-fold molar excess, some nonspecific effects were eventually detected for the mutated Oct-1 oligonucleotide (striped bars), although the nonspecific oligonucleotide still exhibited no effect on the level of DNA replication (white bars). Similar results were obtained when the extracts and oligonucleotides were preincubated at 30°C rather than on ice for 20 minutes (data not shown). The data suggest that, depletion/competition of the Oct-1

protein, by the addition of > 250-fold molar excess of Oct-1-specific oligonucleotide, inhibited DNA replication to 18% of the control.

In vitro nucleotide incorporation in the presence of the Oct-1 oligonucleotide is sensitive to aphidicolin

The incorporation of precursor nucleotide into p186 plasmid DNA during its replication *in vitro* in the presence of the Oct-1 oligonucleotide was sensitive to 80 μM aphidicolin (Figure 5). Aphidicolin, a tetracyclic diterpenoid, is an inhibitor of the eukaryotic replicative polymerases α and δ (Lee et al., 1985), while the non-replicative, eukaryotic DNA polymerases β and γ, are resistant to it. Therefore, the results suggest that it is the replicative DNA polymerases that are largely responsible for the *in vitro* incorporation of precursor nucleotide, observed in the presence of both the Oct-1-specific and nonspecific oligonucleotides. Incorporation attributable to repair synthesis in the presence of the oligonucleotides was constant, approximately 15-30%, as determined by quantification of the *in vitro* products following correction for the amount of DNA loaded onto the gel (data not shown). These data are in agreement with previously reported results (Pearson et al., 1991; Zannis-Hadjopoulos et al., 1994).

Addition of purified Oct-1 POU partially reverses the inhibition of replication

To determine whether the inhibition of replication observed in the presence of the Oct-1- specific oligonucleotide was due to the depletion of the Oct-1 protein, purified Oct-1 POU domain protein was added back to the inhibited reaction. First, the binding of the Oct-1 POU domain to the imperfect octamer site present in 186-bp fragment was verified by a bandshift assay (Figure 6A, lane 2; Figure 6B, lane 1). The specificity of the binding was tested in a competition bandshift assay in which either cold 186-bp fragment DNA, used as a specific competitor (Figure 6A, lanes 3-7), or a 206-bp pBR322 fragment, used as nonspecific competitor, was added (Figure 6B, lanes 2-6). The 186-bp fragment-Oct-1 POU complex (**) was specifically competed with 50- to 500-fold molar excess of cold, specific competitor, whereas competition was not observed with increasing amounts (up to 500-fold molar excess amounts) of cold, nonspecific competitor. The slower migrating band seen in all the lanes (indicated by * in Figure 6A

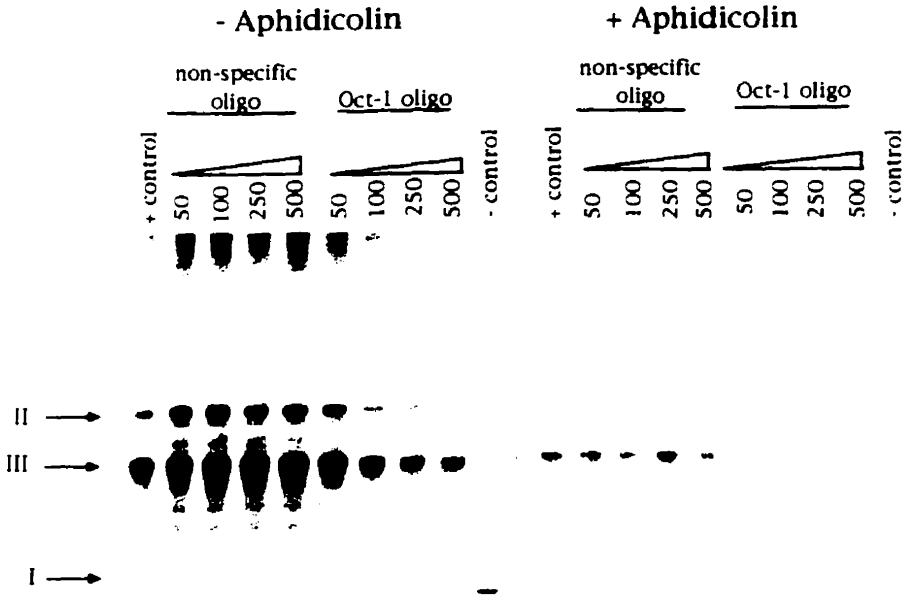

Figure 5. *In vitro* replication assay in the presence of aphidicolin. Reactions were performed by pre- incubating the extracts with the indicated molar excess amounts of the Oct-1-specific or nonspecific as described in Figure 4. Aphidicolin was added to a final concentration of 80 μM and the reactions were incubated at 30°C for 15 minutes. The DNA products were purified and concentrated. The samples were electrophoresed on 1% agarose in 1X TAE buffer at 50-55 V for 16-20 hour. Gels were dried and exposed for autoradiography. Forms I, II, and III are indicated. The + and - control correspond to p186 and pBluescript, respectively, in the absence of any oligonucleotide.

and 6B) results from the formation of a secondary structure induced during the purification of the 186-bp fragment (Ruiz et al., 1995). This complex is not competed for in the presence of increasing amounts of the cold competitor, further confirming that the observed binding/competition with the POU-186-bp fragment is specific.

The purified Oct-1 POU domain was then added to the Oct-1 oligonucleotide-inhibited *in vitro* replication reaction of p186 in order to restore normal replication levels. The *in vitro* replication of p186 was first inhibited by preincubating the extracts with 250-fold molar excess of Oct-1-specific oligonucleotide, resulting in the depletion of the endogenous Oct-1 protein (approximately 100 ng, as deduced from Figure 2A) and inhibition of replication to 18% (Figure 4C). Then, increasing amounts of the Oct-1 POU domain or BSA protein (used as a control) were added to the reaction mixture and incubated for a further 20 minutes on ice before carrying out the *in vitro* reaction. The results indicate that addition of 1.5 ng of POU domain protein increased replication only marginally (increase of 5%), while addition of 80 ng and 160 ng of the POU domain protein restored replication to nearly 50% of control levels (Figure 6C, black bars). Consequently, when recombinant Oct-1 POU domain protein was added at amounts approximately replacing the oligonucleotide-depleted Oct-1 levels, a two-fold restoration in replication was achieved. Addition of the same amounts of BSA did not result in any increase in replication (Figure 6C, striped bars). Also, when purified POU domain protein was added to the *in vitro* replication reaction of p186, in the absence of any oligonucleotide, no significant effect on DNA synthesis was observed (data not shown), suggesting that the mere addition of the POU protein does not result in stimulation of replication and that there exists a threshold level of Oct-1 in the extracts. The results suggest that the increase in replication obtained with the addition of purified Oct-1 POU was due to the restoration of the depleted Oct-1 protein (Figure 6C).

Specific immunodepletion of Oct-1 protein inhibits the in vitro replication of p186

The inability of the purified POU domain protein to fully restore replication to control levels (Figure 6C) may be explained by several possibilities (discussed below). One of them is that the Oct-1-specific oligonucleotide is depleting not only the Oct-1 protein but also other unidentified protein(s)/factors (see Figure 3A, complex B or C),

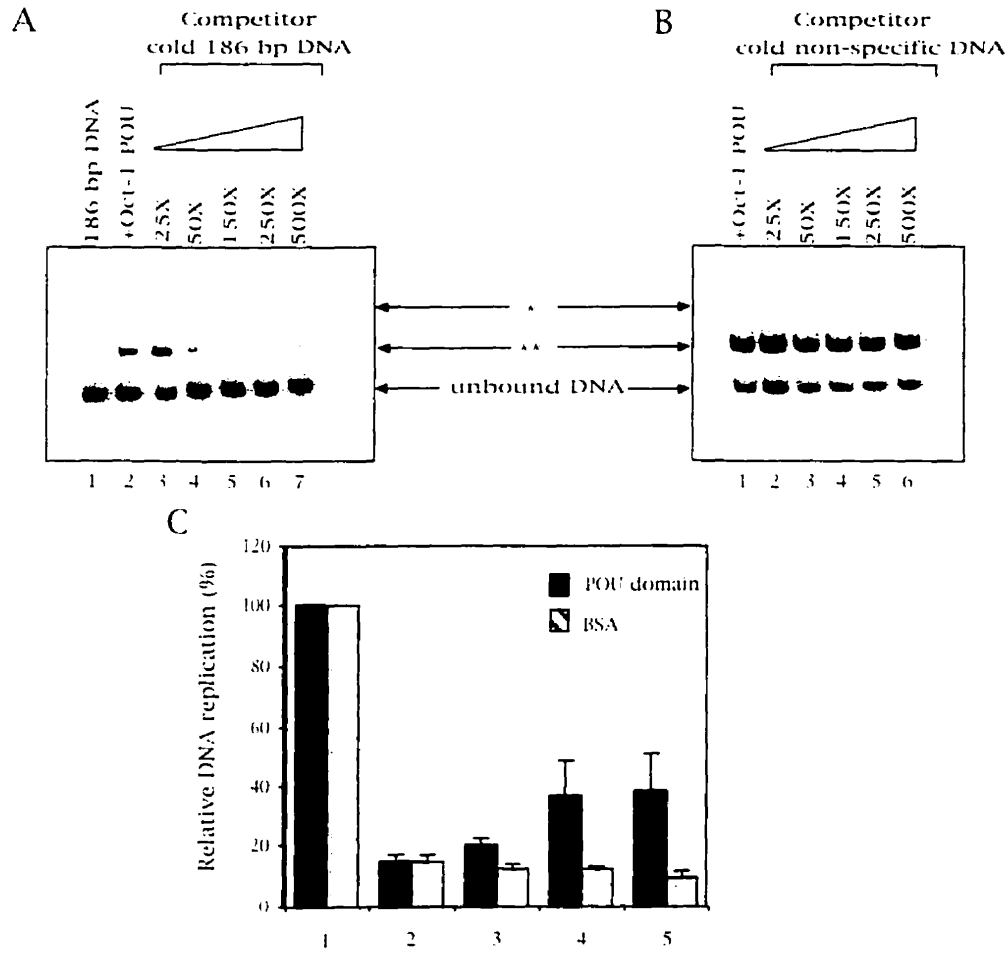

Figure 6. Recombinant POU I inds to the 186-bp origin. A. Competition bandshift assay showing specificity of binding of the Oct-1 POU domain protein to the 186-bp fragment. Reactions contained 0.1 ng of ^{32}P-labeled 186-bp fragment, 2.5 ng Oct-1 POU protein and increasing molar excess amounts of cold 186-bp fragment DNA, as indicated. The free 186-bp DNA (unbound DNA), the protein DNA complex (**), and the 186-bp secondary structure(*) are indicated. B. As in (A) above, except that increasing molar excess amounts of a cold nonspecific competitor from nucleotides 860-1065 of pBR322 was added to the binding reaction. C. *In vitro* replication data showing the reversal of replication inhibition with the addition of exogenous Oct-1 POU. *In vitro* replication was inhibited by preincubating the HeLa extracts with 250-fold molar excess of Oct-1 oligonucleotide (relative to the input p186 DNA) for 20 minutes on ice. Then, different amounts of the Oct-1 POU domain protein or BSA protein were added to replace the amount of Oct-1 specific oligonucleotide depleted Oct-1 and further incubated on ice for 20 minutes. The reactions were performed as described in the Material and Methods. The data is expressed as a percentage of replication relative to a control reaction lacking any oligonucleotide or exogenous Oct-1 POU protein (lane 1). The black bars represent addition of purified POU domain protein (lane 2: 0 ng; lane 3: 1.5 ng; lane 4: 80 ng; lane 5: 160 ng), whereas the striped bars represent addition of equivalent amounts of BSA. The bars represent results obtained from two different experiments and one standard deviation is indicated.

whose depletion may contribute in part to the observed inhibition of replication. To determine the contribution, if any, of these putative Oct-1 oligonucleotide binding protein(s) on the *in vitro* replication of p186, we immunodepleted the HeLa cell extracts of Oct-1 protein using an anti-POU antibody (a gift from Dr. van der Vliet). This antibody recognizes a single protein from total HeLa cell extracts, as determined by Western Blot analysis (Figure 2A) and is specific for the Oct-1 protein (Figure 3B, lane 2).

Addition of a 50-fold molar excess of the antibody relative to the endogenous Oct-1 protein inhibited replication to approximately 28% of the control (Figure 7, anti-POU), while at 150- and 250-fold molar excess the antibody did not inhibit replication further (data not shown). Thus, the maximum level of inhibition of replication observed upon immunodepletion of the extracts corresponds to that which is obtained upon depletion of the Oct-1 protein by inclusion of the Oct-1-specific oligonucleotide (Figure 4A, B, C). Addition of nonspecific polyclonal antibodies, such as anti-goat or anti-mouse IgG, at greater concentrations (70- and 140-fold) than that of the anti-POU antibody did not affect the *in vitro* replication of p186 (data not shown).

To determine whether the inhibition of replication was due to the specific immunodepletion of the Oct-1 protein, purified POU domain was, once again, added exogenously. Addition of a 50-fold molar excess of the POU domain restored replication to approximately 65% of control levels (Figure 7, anti-POU + Oct-1 POU). Addition of higher amounts of the purified POU protein did not result in a greater increase in replication (data not shown).

Mutation of the Oct-1 binding site does not affect the in vitro replication of p186

Since the endogenous Oct-1 is able to bind specifically to the 186-bp fragment and since its depletion, either by addition of the Oct-1-specific oligonucleotide or by addition of the anti-POU antibody, inhibited p186 replication, we investigated whether Oct-1 enhanced p186 replication *via* a mechanism that involved its direct binding to the octamer motif. We, therefore, mutated the Oct-1 binding site in p186 (see Materials and Methods) from TAAATACG to GCTGGCAC. Figure 8a shows the sequencing reactions of one of the resulting mutant clones, p186-MutOct, and wild-type p186, with

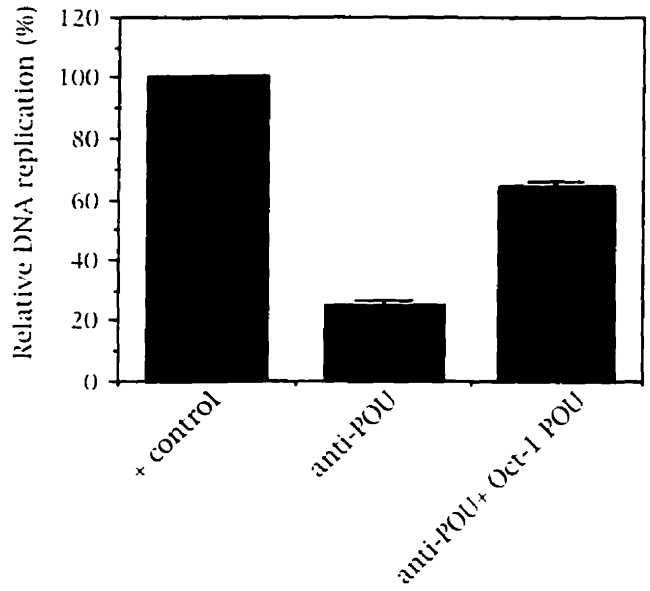

Figure 7. Addition of the anti-POU antibody inhibits DNA replication. *In vitro* replication was inhibited by preincubating a 50-fold molar excess of anti-POU antibody (relative to the endogenous Oct-1) on ice for 20 minutes. Then, a 50-fold molar excess of purified POU domain protein (relative to the amount of anti-POU antibody) was added exogenously and further incubated on ice for 20 minutes. The reactions were performed as described in Materials and Methods. The data are expressed as a percentage of replication relative to a control reaction with no antibody or exogenous Oct-1 POU protein. The bars are results from two different experiments.

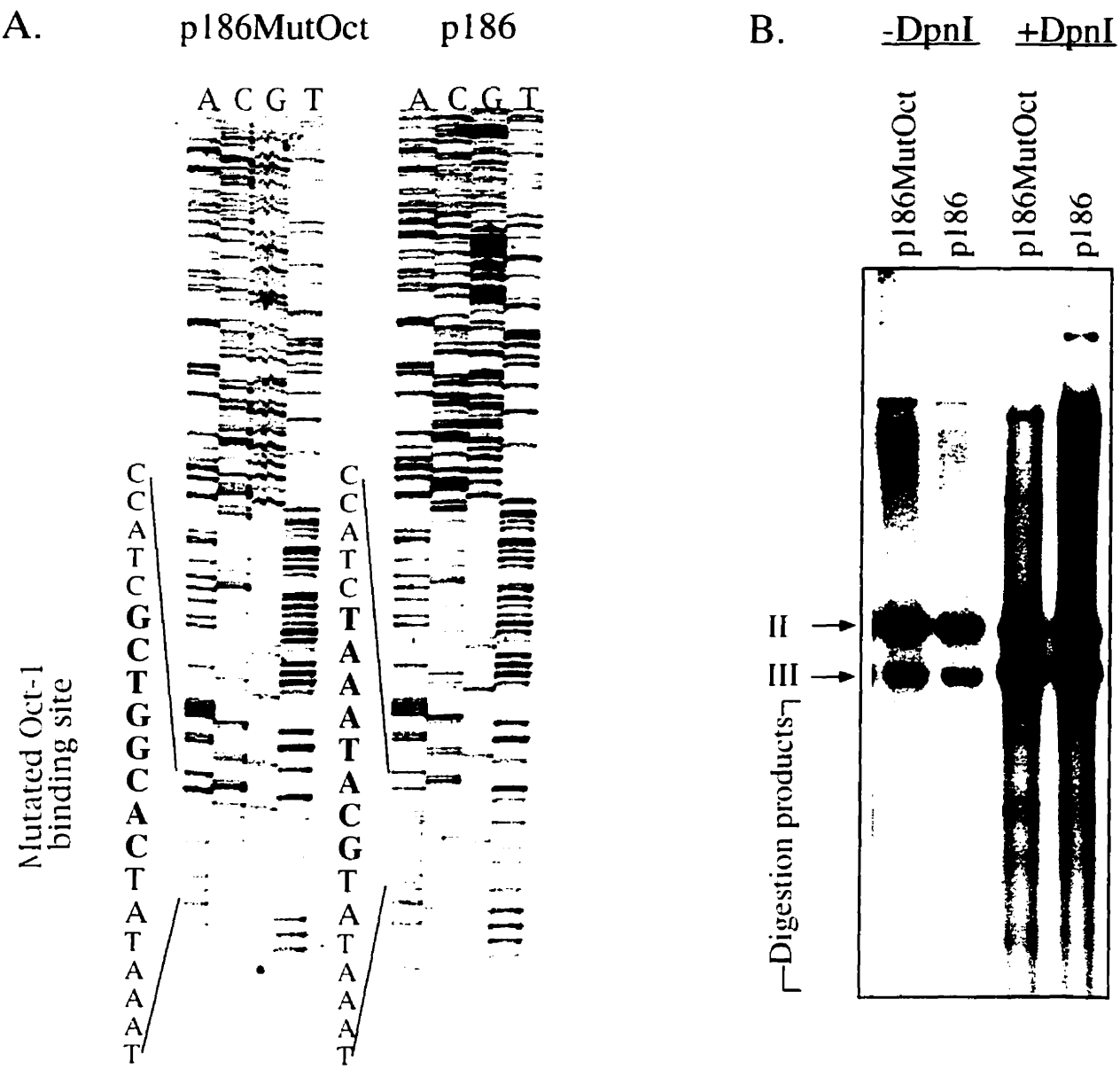

Figure 8. Mutation of the octamer motif in the origin does not abolish DNA replication. Site-directed mutagenesis was employed to alter the Oct-1 binding site in the p186, as described in Materials and Methods. Potential clones were first screened by restriction endonuclease digestion with *Sca*I and *Mlu*I and then sequenced. A. Autoradiograph of an 8% sequencing gel with the mutated Oct-1 binding site in p186-MutOct and the wild type octamer site of p186 indicated in bold. B. Comparison of the replication efficiency of the mutant and wild type plasmids. *In vitro* replication was performed using 200 ng of either p186 or p186-MutOct template DNA. An autoradiograph of the undigested *in vitro* products (lane 1 and 2) and the *Dpn*I-digested products (lane 3 and 4) is shown.

the mutated or wild type Oct-1 binding sites indicated in bold. A comparison of the ability of these two plasmids to undergo *in vitro* replication showed that p186-MutOct replicated *in vitro* with the same efficiency as wild-type p186 (Figure 8b). Quantitation of the *Dpn*I-resistant bands corresponding to forms II and III showed that the mutant plasmid replicated with an average efficiency of 99% compared to 100% for p186. Moreover, competition-depletion (using the Oct-1 oligonucleotide) and reconstitution (adding the POU domain) assays with p186-MutOct as template yielded results similar to those obtained with p186 (data not shown). Finally, to rule out the possibility that Oct-1 was able to bind elsewhere within the mutated 186-bp fragment, a bandshift assay was performed using purified POU domain protein and the mutated 186-bp fragment. No binding was detected by these assays (data not shown).

V. DISCUSSION

We have previously shown that *ors*8 (483-bp) can function as an origin of DNA replication in autonomously replicating plasmids *in vivo* (Frappier and Zannis-Hadjopoulos, 1987) and in a mammalian cell-free replication system (Pearson et al., 1991; Pearson et al., 1994a). We have also shown that a minimal origin lies within an internal fragment of 186-bp, which is also capable of autonomous replication *in vivo* and *in vitro* (Todd et al., 1995). This minimal replicating DNA fragment of *ors*8 contains an imperfect 44-bp IR, several direct repeats, and an imperfect consensus binding site for Oct-1 (Figure 1).

Here, we present evidence that Oct-1 protein, a cellular transcription factor, is an enhancing component in the *in vitro* replication of p186, but it seemingly does not exert its effect through direct binding to the octamer motif in the origin. We confirmed the presence of Oct-1 protein in the HeLa cell extracts that were used for the *in vitro* replication and determined that the HeLa nuclear extracts contained approximately 100 ng of Oct-1 protein (Figure 2A). We also confirmed the potential for specific binding of this endogenous Oct-1 protein to the minimal origin of *ors*8 (Complex 1, Figure 2B). Furthermore, we demonstrated, by competitive bandshift and supershift assays, specific binding of the endogenous Oct-1 protein to a double-stranded oligonucleotide containing the Oct-1 binding site (Complex A, Figure 3A, B). Complex A was the result of Oct-1 binding, since it was: i) competed by increasing amounts of Oct-1 specific oligonucleotide, ii) supershifted by the anti-Oct-1 antibody and, finally, iii) not formed with the mutated Oct-1 oligonucleotide. Complexes B and C were also competed by increasing amounts of the Oct-1 specific oligonucleotide, but not by the nonspecific competitor, suggesting that they are the result of specific binding. However, these complexes were shown to be independent of Oct-1 since they also formed with the mutated Oct-1 oligonucleotide and were not recognized by the antibody against Oct-1. Therefore, these complexes do not result because of Oct-1 binding.

Addition of the Oct-1 specific oligonucleotide did not affect repair-type synthesis during the *in vitro* replication assays, since the background level of aphidicolin-resistant DNA repair (15%-30%) incorporation remained constant regardless of the molar excess

amount of oligonucleotide used and could be attributed to some amount of nicking in the input DNA (Figure 5). Treatment with aphidicolin inhibited incorporation significantly (70%-85%) when compared to reactions without aphidicolin, confirming that the *in vitro* nucleotide incorporation is largely due to the replicative DNA polymerases α/δ.

Addition of > 250-fold molar excess amounts of the Oct-1-specific oligonucleotide to the *in vitro* reaction, resulted in the depletion of the endogenous Oct-1 protein and inhibited replication of p186 by 80-85% compared to the control reaction in which p186 was replicated in the absence of this oligonucleotide (Figure 4B, C); in contrast, the use of a mutated Oct-1 oligonucleotide (up to 1000-fold excess) or a nonspecific oligonucleotide (up to 5000-fold excess) produced no effect (Figure 4C). The partial (20%-40%) inhibition observed at high concentrations (> 1000-fold molar excess) of the mutated oligonucleotide may be caused by the removal of other, unidentified factors that bind to the mutated oligonucleotide (see Figure 3B, lane 3). Although the Oct-1-specific oligonucleotide may also bind to these factors, the inhibition observed upon addition of this oligonucleotide (see Figure 4C) is much higher and occurs at lower oligonucleotide concentrations. Furthermore, depletion of these unidentified factors does not seem to affect replication, since addition of the anti-POU antibody (Figure 7), which only recognizes Oct-1 (see Figure 2A), yielded similar results to those obtained with the Oct-1-specific oligonucleotide. The maximum level of inhibition by the Oct-1 specific oligonucleotide was 85%, even in the presence of 5000-fold excess, suggesting that some basal level of replication does occur in the absence of Oct-1. On the other hand, this low level of replication may, in part, be due to residual activity of the oligonucleotide-bound Oct-1 protein, which is not removed in its bound state during the actual *in vitro* replication reaction. Nevertheless, the Oct-1-specific oligonucleotide inhibits replication, on the average, by 85%, by comparison to the mutated Oct-1 or nonspecific oligonucleotides, and the activity of the oligonucleotide-bound Oct-1, if any, is low.

We demonstrated that the inhibition of replication caused by the addition of the Oct-1 specific oligonucleotide was due, in major part, to depletion of Oct-1, since addition of the Oct-1 POU domain to the inhibited reactions restored replication, up to 50% of control levels (Figure 6C). The amount of recombinant Oct-1 POU domain protein necessary to obtain a two-fold restoration of replication correlates with the levels

of endogenous Oct-1 protein that were depleted (see results). In the adenovirus reconstituted DNA replication system, 5 binding units of POU domain (1 binding unit represents 1.7 ng POU domain) were used to stimulate DNA replication with 50 ng pTP-pol, 1 μg DNA binding-protein (DBP) and 50 ng adenovirus-TP DNA (Coenjaerts et al., 1995). In our system, four times as much input DNA (200 ng) was used, thus requiring 34 ng POU domain. However, since we use cell extracts rather than a reconstituted system, it is not surprising that more protein is required. We also showed that immunodepletion of Oct-1 from the HeLa cell extracts using the anti-POU antibody could maximally inhibit p186 replication to approximately 25% of control levels (Figure 7) and that the inhibition of replication was reversed, to 65% of control levels when recombinant POU domain protein was added exogenously (Figure 7). Finally, although Oct-1 is able to bind to the imperfect wild type octamer motif in p186 (Figure 3A, B and Figure 6A, B), it does not seemingly exert its effect on replication through direct binding, since mutation of this site yielded a clone with wild-type replication efficiency.

The inability of the POU domain to completely reverse the inhibition in the *in vitro* replication of p186 indicates that it is not sufficient by itself to fully enhance p186 replication. This is in contrast to adenovirus replication where, the Oct-1 POU domain and the DNA binding domain of nuclear factor I (NFI) suffice for enhancement of replication of the viral DNA (Gounari et al., 1990; Verrijzer et al., 1990). The POU domain stimulates replication through a direct interaction with the pTP during the pre-initiation step (Coenjaerts et al., 1994; van Leeuwen et al., 1997). Through this protein-protein interaction, the POU domain stabilizes the pre-initiation complex, and together with NFI, positions the complex to the core origin, leading to increased binding of the pTP-pol to its target site. This enhanced binding of the pTP-pol correlates very well with the increased levels of stimulation on replication (van Leeuwen et al., 1997). In adenovirus, the four initiator proteins, pTP-pol, NFI and Oct-1, can form a stable complex even in the absence of DNA (van Leeuwen et al., 1997). Direction of the pTP-pol complex to its target site is also dependent upon the specificity of the intact POU domain; mutation of the POU domain resulted in a decrease in adenovirus DNA replication (van Leeuwen et al., 1995). One explanation for the lack of full restoration of p186 replication *in vitro* might be that the POU domain protein may not function effectively in other

protein interactions that may be required to execute that reaction in higher eukaryotes. In the adenovirus *in vitro* DNA replication system, the POU domain is only interacting with virally encoded proteins and not with any cellular proteins. Furthermore, unlike the adenovirus system, the transcription activation domain, in addition to the binding domain of Oct-1, may also be required for the full activation of replication of p186. For example, the transcription activation domain could be required for enhancement if the mechanism by which stimulation occurs involves distortions to the origin structure by the transcription factor, thus facilitating the assembly of a pre-initiation complex (van der Vliet, 1996). In addition, the flexible nature of the transcription activation domain may increase the potential for different, specific interactions with replication proteins (van der Vliet, 1996).

Significant conformational changes (e.g. bending) in the DNA must occur to promote all the necessary protein-protein interactions and destabilize the origin, thus facilitating the initial unwinding step of replication. Such bending is achieved by several ways in adenovirus. First, the protein-free origin is intrinsically bent (Zorbas et al., 1989). Second, binding of NFI enhances the intrinsic bent (Zorbas et al., 1989). Finally, the POU domain is also capable of bending its recognition sequence to some degree (Klemm et al., 1994). Therefore, although binding of pTP is essential, it has been postulated that it may not be the only role of the adenovirus POU domain. However, the *in vitro* replication efficiency of a virus with a mutated octamer sequence has not been examined thus far. What has been looked at is the ability of other transcription factors to compensate for a deleted octamer sequence *in vivo*. When other DNA sequences, such as SP1, ATF and EBP-1 sites, are present at the left terminus of the adenovirus genome, deletion of the octamer sequence does not inhibit replication, indicating functional redundancy between different transcription factor binding sites (Hatfield and Hearing, 1993). In those studies, depletion of the Oct-1 protein was not investigated. However, when the Oct-1 binding site was substituted by an AP1 site, to which fos-jun can bind and bend DNA similarly to Oct-1, replication was not stimulated (Coenjarts et al., 1994), again indicating the importance of protein-protein interaction between the POU DNA binding domain of Oct-1 and the pTP protein of adenovirus.

The data presented here suggest that the specific depletion of Oct-1 inhibits replication, although mutation of its binding site does not produce any effect (Figure 8b). Consequently, a direct role of Oct-1 in unwinding the minimal origin of *ors*8 at its binding site is unlikely, even though it has the potential to bind to this site (Figure 2B). As discussed earlier, a more plausible function for this transcription factor would be its involvement in protein-protein interactions, as is its main role in adenovirus (van Leeuween et al., 1997). Thus, Oct-1 may be interacting with other replication factor(s), which would also become depleted during the *in vitro* -competition assays.

A growing number of origins in multicellular organisms are being shown to contain binding sites for specific cellular transcription factors, in addition to binding sites for the replication initiator proteins. Several reports suggest that not only are transcriptional regulatory elements a common feature of viral, yeast, and lower eukaryotic origins of DNA replication, but they also modulate initiation of replication by binding to their recognition sites in the origin (reviewed in Boulikas, 1996; DePamphilis, 1993). However, the significance of these sites is still unclear in higher eukaryotic origins since mutational analyses have not been performed and their contribution remains uncertain. It is tempting to assume that the transcription factor binding sites in eukaryotes act as enhancers of replication in a similar mechanism as in yeast and viral systems (DePamphilis, 1988; Heintz, 1992; van der Vliet, 1991). Using p186, which contains the minimal origin of *ors*8, the data presented here indicate that depletion of the Oct-1 transcription factor inhibits the replication of p186, although the actual octamer binding site is not essential for *in vitro* replication. Therefore, the involvement of Oct-1 in p186 DNA replication most likely occurs through protein-protein interaction with the replication machinery. The POU domain proteins have been postulated to be regulators of viral replication through their involvement in interaction with viral proteins (van Leeuwen et al., 1997). For instance, a direct interaction has been reported between Oct-1 and the Herpes simplex transactivator protein (O'Hare et al., 1988), between Oct-6 and the JC papovavirus T antigen (Renner et al., 1994) and between Oct-1 and adenovirus pTP (van Leeuwen et al., 1997).

A better understanding of the relationship between transcription factors, transcription factor binding and the initiation of DNA replication will require the

examination of several different eukaryotic origins in order to eventually lead to an understanding of the regulation of this process.

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CONNECTING TEXT

In the preceding chapter, I have demonstrated that the Oct-1 transcription factor is an enhancing component of mammalian *in vitro* DNA replication. I have shown that, although Oct-1 can bind to the imperfect octamer site in the minimal origin or *ors*8, it does not exert its effect on DNA replication by direct DNA binding. I proposed that Oct-1 affects DNA replication by protein-protein interactions with other origin binding proteins.

The next chapter describes the purification and the identification of an origin binding activity, OBA, from HeLa cells that binds sequence-specifically to the minimal origin of *ors*8 and to the A3/4 origin consensus sequence. The role of OBA in mammalian DNA replication was investigated.

CHAPTER THREE

OBA/Ku86: DNA binding specificity and involvement in mammalian DNA replication

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I. ABSTRACT

OBA (*Ors* Binding Activity) was previously semipurified from HeLa cells, through its ability to interact specifically with the 186-bp minimal replication origin of *ors8* and support *ors8* replication *in vitro*. Here, through competition band-shift analyses, using as competitors various subfragments of the 186-bp minimal ori, we identified an internal region of 59-bp that competed for OBA binding as efficiently as the full 186-bp fragment. The 59-bp fragment has homology to a 36-bp sequence (A3/4) generated by comparing various mammalian replication origins, including the *ors*. A3/4 is, by itself, capable of competing most efficiently for OBA binding to the 186-bp fragment. Band-shift elution of the A3/4-OBA complex, followed by Southwestern analysis using the A3/4 sequence as probe, revealed a major band of approximately 92 kDa involved in the DNA binding activity of OBA. Microsequencing analysis revealed that the 92 kDa polypeptide is identical to the 86 kDa subunit of human Ku antigen. The affinity-purified OBA fraction obtained using an A3/4 affinity column also contained the 70 kDa subunit of Ku and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). *In vitro* DNA replication experiments in the presence of A3/4 oligonucleotide or anti-Ku70 and anti-Ku86 antibodies implicate Ku in mammalian DNA replication.

II. INTRODUCTION

Ku antigen (autoantigen) is a heterodimeric (p70/p86) DNA binding protein recognized by autoantibodies from the sera of certain patients with systemic rheumatic diseases (Mimori et al., 1981; Reeves, 1985; Yaneva et al., 1985; Mimori and Hardin, 1986). It consists of two polypeptides of 86 and 70 kDa (Yaneva et al., 1985). Ku is identical to a DNA-dependent ATPase isolated from HeLa cells (Cao et al., 1994), which had been previously reported to cofractionate with a 21S multiprotein complex competent for DNA synthesis from HeLa cells (Vishwanatha and Baril, 1990). Furthermore, the interaction of Ku antigen with a human DNA region (B48) containing a replication origin was reported (Tóth et al., 1993), while a novel ATP-dependent DNA unwinding enzyme, DNA helicase II (HDH II), was identified as Ku (Tuteja et al., 1994). Recently, Ochem et al., (1997) reported that the Ku70 subunit is the one associated with the helicase activity in the Ku70/Ku86 heterodimer. Moreover, a role for Ku70 as a tumor suppressor for murine T cell lymphoma has been suggested, since Ku70 deficiency facilitates neoplastic growth (Li et al., 1998). Ku has been shown to be the DNA binding subunit of the DNA-dependent protein kinase (DNA-PK) holoenzyme (Gottlieb and Jackson, 1993; Suwa et al., 1994), a nuclear component that phosphorylates a number of DNA binding, regulatory proteins, including transcription factors (Sp1, p53), RNA polymerase II, topoisomerases I and II, Ku antigen, and SV40 large T antigen (Anderson, 1993, and references therein). Although Ku has been characterized as a DNA end binding protein, it was recently shown that it is also a sequence specific DNA-binding protein, binding to negative regulatory element 1 (NRE-1) in the long terminal repeat of MMTV (Giffin et al., 1996). It has also been recently reported that a Ku-like protein from *Saccharomyces cerevisiae* is required for the *in vitro* assembly of a multiprotein complex at the *ARS121* origin of replication (Shakibai et al, 1996).

Our laboratory has previously isolated and cloned early-replicating origin enriched sequences (*ors*) from synchronized African Green monkey kidney (CV-1) cells (Kaufmann, et al, 1985). The *ors* containing plasmids are capable of transient autonomous replication *in vivo*, when transfected into monkey (CV-1 and COS-7) and human (HeLa) cells (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991) and in an *in vitro*

replication system that uses HeLa cell extracts (Pearson et al., 1991). Both *in* vivo and *in vitro*, replication is semi-conservative, bidirectional, depends on the presence of an *ors*-containing template and initiates within the *ors* sequence (Frappier and Zannis-Hadjopoulos, 1987; Pearson et al., 1991; Zannis-Hadjopoulos et al., 1992; Pearson et al., 1994). We have recently shown that one of the functional *ors*, *ors*12 serves as a chromosomal origin of DNA replication in CV-1 cells (Pelletier et al., 1999).

The fractionation of HeLa cell replication proteins with *Ors*-Binding Activity, OBA, was previously reported (Ruiz et al., 1995). OBA sediments at approximately 150 kDa in a glycerol gradient. The OBA-containing fraction is enriched for polymerases α and δ, topoisomerase II and RP-A, and can support the *in vitro* replication of *ors*8 plasmid (Ruiz et al., 1995). Partial purification of OBA was achieved through its sequence-specific binding to a 186-bp subfragment of *ors*8, which was previously identified as the minimal sequence required for *ors*8 function as a replication origin *in vivo* and *in vitro* (Todd et al., 1995).

In this study, we have identified the DNA binding activity of OBA as the 86 kDa subunit of Ku (Ku86) antigen. We have also affinity-purified OBA (apOBA) based on its ability to specifically bind to A3/4, a sequence derived by comparison of mammalian DNA replication origins. Sequence-specific binding of OBA/Ku was also supported by band-shift competition analysis using a supercoiled A3/4-containing plasmid. Furthermore, inhibition of *in vitro* DNA replication was observed in the presence of either increasing amounts of the A3/4 oligonucleotide, or anti-Ku70 and anti-Ku86 antibodies. The results indicate an involvement of OBA/Ku antigen in mammalian DNA replication.

III. MATERIAL AND METHODS

Cells and Plasmids

pBR322, p186, p*ors*8, and p*ors*12 plasmids were propagated in *Escherichia coli* HB101, as previously described (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991). *Ors8* (GenBank Accession No: M26221) plasmid has been previously described (Kaufmann et al., 1985; Zannis-Hadjopoulos et al. 1985; Rao et al., 1990). p186 consists of the *Nde*I-*Rsa*I fragment (186-bp) of *ors8* inserted into the *Nru*I site of pBR322 (Todd et al., 1995). Linearized A3/4/pBR322 and pBR322 plasmids were obtained by digestion with *Nru*I enzyme.

Preparation of DNA Fragments, Oligonucleotides and End labeling

In order to obtain the 186-bp fragment for bandshift experiments, p *ors*8 plasmid was used as template in PCR reactions for amplification of the *ors*8 insert, which was then digested with *Nde*I and *Rsa*I, as described previously (Ruiz et al., 1995). A nonspecific competitor fragment, pBRfg (206-bp), was prepared by PCR amplification of pBR322 DNA, as previously described (Ruiz et al., 1995). Oligonucleotides containing the A3/4 sequence (36 nucleotides in length; 5'-CCTCAAATGGTCTCCAATTTTCCTTTGGCAAATTCC-3') and a nonspecific competitor derived from pBR322 (16 nucleotides in length; 5'-TTCCGAATACCGCAAG-3') were synthesized (Sheldon Biotechnology Centre, McGill University, Montreal), further purified by denaturing polyacrylamide gel electrophoresis (PAGE) and annealed as described in Wall, 1988. 5' end-labeling of the 186-bp fragment and A3/4 double-stranded oligonucleotide were performed as previously described (Ruiz et al., 1995).

Fractionation of HeLa cell extracts

HeLa S3 nuclei and cytosol (Cellex Biosciences Inc., Minneapolis) were used to prepare nuclear and cytosolic extracts as described previously (Pearson et al., 1991). Nuclear and cytosolic extracts were mixed (total cell extracts) and dialyzed against buffer A (26 mM

Hepes, pH 7.8, 82 mM potassium acetate, 5.0 mM $MgCl_2$, 2.5 mM EDTA, 1.0 mM DTT, 1.0 mM PMSF, 1.0 μM pepstatin A, 1.0 μM leupeptin, 10% glycerol). The chromatographic steps for the purification of OBA were performed essentially as described before (Ruiz et al., 1995), except that a Sephacryl S-300 step replaced the 10-40% glycerol gradient and an affinity purification step was added at the end. Briefly, total cell extracts were applied to a DEAE Sephadex A-50 column previously equilibrated in buffer A. The flowthrough (FT) was collected and the bound protein was eluted with a linear salt gradient of potassium acetate (0.082-1.0 M) in buffer A. The fractions collected from the elution gradient were pooled into four different pools (A, B, C and D), based on their salt concentration. Pool B was then dialyzed against buffer B (0.01 M $KHPO_4$, pH 7.4, 0.15 M NaCl, 2.5 mM EDTA, 1.0 mM DTT, 1.0 mM PMSF, 1.0 μM pepstatin A, 1.0 μM leupeptin, 10% glycerol) and loaded onto an Affi-Gel Heparin column. The FT was collected and the proteins bound to the matrix were eluted with a linear salt gradient of NaCl (0.15-1.0 M) in buffer B. The fractions were monitored for OBA activity by band-shift analyses and those that were positive (300 mM NaCl) were concentrated, dialyzed and labeled as pool E. Pool E was subsequently loaded onto a Sephacryl S-300 column in buffer B and fractions were collected and monitored for OBA activity as above. The OBA-positive fractions were pooled (pool F), concentrated and loaded onto an A3/4 DNA affinity column (see below), equilibrated with buffer B. The FT was recovered and the protein bound to the matrix eluted with a linear salt gradient of NaCl (0.15-1 M) in buffer B. OBA-positive fractions were pooled (apOBA), concentrated, dialyzed against buffer B and frozen in aliquots at -70 °C.

DNA affinity column

Oligonucleotides complementary (antisense) to the A3/4 sequence oligonucleotide (sense; see above) were synthesized and 5' directional overhangs (5'-GATC) were added, yielding a 40-mer oligonucleotide (Sheldon Biotechnology Centre, McGill University, Montreal). The oligonucleotides were further purified by denaturing PAGE. Equal amounts (220 μg) of antisense and sense oligonucleotides were mixed, annealed, phosphorylated, and ligated, as previously described (Kadonaga and Tjian, 1986). The multimers were then coupled to 10 ml of Sepharose CL-2B (Pharmacia LKB Biotechnology Inc.) freshly activated by cyanogen bromide (Kadonaga and Tjian, 1986).

Gel mobility shift assays and competition experiments

Gel mobility shift (bandshift) assays were typically performed as previously described (Ruiz et al., 1995) in 20 μl volume, by incubating 0.1 ng (0.81 fmoles) of end-labeled 186-bp fragment or 0.5 ng (21 fmoles), or other indicated amount, of double-stranded, end-labeled oligonucleotide containing the A3/4 sequence, with 200 ng of protein from Pool F (Sephacryl S-300). Reactions were carried out in binding buffer (10 mM Tris-HCL, pH 7.5, 80 mM NaCl, 1 mM EDTA, 10 mM 2-Mercaptoethanol, 0.1% Triton X-100, 4% glycerol), in the presence of 1 μg of double-stranded (ds) poly (dI-dC) (Pharmacia), used as nonspecific competitor. After incubation on ice for 30 min, the reaction mixture was analyzed by 4% PAGE; the gel was then dried and exposed for autoradiography. For band-shift competition experiments, a constant amount (0.1 ng) of radioactively labeled 186-bp fragment was mixed with increasing molar excess amounts of either the 186-bp fragment or various subfragments (75-, 69-, 59-, and 42-bp, respectively) generated from it, as well as the A3/4 oligonucleotide, used as cold competitors. pBRfg (see above) was also used as nonspecific competitor. A constant amount of protein (200 ng) from Pool F (Sephacryl S-300), was then added to the mixture, and the reaction was left to proceed as described above. The shifted complexes were quantitated by densitometry performed using a Phosphoimager (Fuji BAS 2000) and the results were expressed as % reduction in complex formation with increasing amounts of competitor. In competition reactions using the A3/4 oligonucleotide as probe, increasing molar amounts of either the double- or single-stranded A3/4 oligonucleotides were used as cold competitors. Competition reactions were also performed using either supercoiled or linearized plasmid containing the A3/4 sequence (A3/4/pBR322), or the vector (pBR322) alone; 260 ng of affinity-purified (ap)OBA were incubated for 1h with 50X and 500X molar excess amounts of either the supercoiled or the linearized plasmid, relative to the radioactive A3/4 sequence (0.25 ng; 10.5 fmoles), which was added last, and the reaction was left to proceed as described above.

When the mobility-shift assays were followed by Western blotting analyses, the reactions were performed using 0.25 ng/reaction of the probe, and increasing amounts of apOBA (100, 250 and 350 ng, respectively), using the conditions described above. Half of the reactions were performed using radioactively labeled DNA, and the other half were performed

using cold A3/4 as probe. A control reaction was also carried out in the absence of DNA. The reactions were analyzed using the mini-protean II slab cell electrophoresis system (Bio-Rad, Richmond, CA). After electrophoresis (free probe was run out of the gel), the radioactive part of the gel was dried and exposed for autoradiography, while the equivalent "cold" part of the gel was prepared for Western blotting as described below.

In vitro DNA replication

In vitro replication reactions were performed as previously described (Pearson et al., 1991) with modifications according to Matheos et al. (1998). In the experiments involving the addition of the A3/4 oligonucleotide competitor, increasing molar excess amounts (relative to the input 200 ng p186 or p*ors*12 template DNA) of either the A3/4 or the nonspecific oligonucleotide (see above) were preincubated with the HeLa cell extracts on ice for 20 minutes.

Experiments involving the addition of the anti-Ku antibodies were carried out in a similar manner as previously described (Lin et al., 1997). Anti-Ku70 (Santa Cruz, CA, sc-1486) and anti-Ku86 (Santa Cruz, CA, sc-1484) antibodies, 3.5, 7.0, and 14.0 μg of a 1.5 mg/ml antibody stock, concentrated using Microcon-10 microconcentrators (Amicon), were preincubated, respectively, with the HeLa cell extracts, on ice for 20 minutes. A goat IgG antibody (Sigma) was used as a control.

The antibodies were neutralized as recommended by the manufacturer by reacting 14.0 μg of either the anti-Ku70 or anti-Ku86 antibody with a 7-fold (by weight) excess of the Ku70 (Santa Cruz, CA, sc-1486P) or the Ku86 (Santa Cruz, CA, sc-1484P) blocking peptides or with a mixture of GATA-1 (Santa Cruz, CA, sc-1233P), GATA-2 (Santa Cruz, CA, sc-1235P) and DNA-PK (Santa Cruz, CA, sc-1552P) blocking peptides, the latter three serving as nonspecific blocking peptides. The incubations were carried out overnight at 4°C. Subsequently, the neutralized antibodies were preincubated with the extracts and finally added to the *in vitro* replication reaction, carried out as described above.

The *in vitro* replication products were divided into three aliquots: one third was digested with 1 unit of *Dpn*I (New England BioLabs) for 60 minutes at 37°C, as previously described (Matheos et al., 1998). The *Dpn*I-digested and one third of the undigested products were subjected to electrophoresis on 1% agarose gel in 1X TAE buffer (16-20 h, 50-55 Volts).

The gels were dried and exposed to DuPont reflection NEF-autoradiographic film. Quantification was performed by densitometric measurements of the *Dpn*I-digested products, as previously described (Diaz-Perez et al., 1996; Matheos et al., 1998), using a phosphoimager analyzer (Fuji BAS 2000). The amount of radioactive precursor incorporated into the DNA was expressed as a percentage of the control p186 reaction (100%).

Bandshift elution of OBA-A3/4 complexes followed by Southwestern analysis

Ten bandshift reactions were performed with radioactively labeled A3/4 DNA (10 ng/reaction) and 3.75 μg/reaction of protein from Pool F (from the Sephacryl-S300 column, see Materials and Methods), using the conditions described above. As a control, similar reactions were carried out in the absence of DNA. The band-shifts were analyzed by electrophoresis in a native 4% polyacrylamide gel, and the wet gel was exposed for 5h at 4°C for autoradiography. The OBA-DNA complexes were then excised from the gel, the proteins and the DNA were eluted from the gel by isotachophoresis (Ofverstedt et at., 1984) and then subjected to electrophoresis on 8% SDS-polyacrylamide gel under reducing conditions. The proteins were then transferred electrically to an Immobilon-P membrane (Millipore, Bedford, MA) and subjected to Southwestern analysis, following the protocol described in Philippe (1994), with some modifications. Briefly, the membrane was incubated overnight (14-16 h) in blocking solution (buffer S: 25 mM Hepes-KOH, pH 7.7, 25 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT containing 5% skim milk and 0.05% NP-40). The next day the membrane was subjected to a process of denaturation-renaturation, as follows: it was incubated for 10 min in a denaturing solution of 6 M guanidine hydrochloride in buffer S, followed by 10 min incubations in 3 M, 1.5 M, 0.75 M, 0.375 M and 0.187 M guanidine hydrochloride, respectively, diluted in buffer S; it was then washed twice for 10 min with buffer S, and incubated for 2h in blocking buffer, followed by 1h incubation in buffer S + 1% skim milk. The membrane was then incubated overnight in hybridization solution (20 mM Hepes, pH 7.7, 75 mM KCL, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 1% skim milk, 0.05 % NP-40) containing radioactively labeled A3/4 oligonucleotide (5.2 ng/ml, 2.6 x 10^6 cpm/ml) in the presence of poly dI-dC (50 μg/ml) and pBRfg DNA (454 ng/ml) as nonspecific competitors. Finally, the membrane was washed three times with hybridization solution and subsequently exposed for

autoradiography. The entire procedure was carried out at 4°C and the incubations were done on a rocking platform.

Western Blotting experiments and SDS-PAGE

Denaturing PAGE was performed as previously described (Laemmli, 1970), using the mini-protean II slab cell electrophoresis system (Bio-Rad, Richmond, CA). Western blot analysis was performed essentially as previously described (Burnette, 1981), using the ECL detection kit (Amersham). All the antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). For immunodetection of Ku autoantigen subunits (Ku86 and Ku70), 10 and 20 μg of total cell extracts (NC), and 1 and 3 μg of affinity purified OBA were subjected to electrophoresis on 8% SDS-PAGE and electrically transferred to Immobilon-P (Millipore, Bedford, MA). The membrane was first probed with anti-Ku86 (C-20) antibody (1 μg/ml); it was then stripped and reprobed with anti-Ku70 (C-19) antibody (2 μg/ml) using the ECL detection kit protocol. For DNA-PKcs detection, 20 and 40 μg of total cell extracts (NC), and 3 and 6 μg of affinity purified OBA were run on a 6% SDS-PAGE and transferred onto Immobilon-P membrane, as described above. The membrane was then probed with anti-DNA-PKcs (C-19) antibody (4 μg/ml). An anti-goat IgG-horseradish peroxidase-conjugated secondary antibody (1/2000) was used in the immunoblots. A similar procedure was carried out for the Western analysis done on the membranes that had been previously used for Southwestern or band-shift analyses. Visualization of the proteins in the gels was done using the Rapid Silver Staining Kit (Sigma, St. Louis, MO).

Microsequencing analysis of OBA

Protein concentration was determined by the method of Bradford (Bradford, 1976) and the Nucleic Acid Soft-Pack module from a DU-65 Spectrophotometer (Beckman, Mississauga, Ontario, Canada). 29 μg of affinity-purified OBA fraction were subjected to electrophoresis on a 8% SDS-PAGE under reducing conditions. Proteins were blotted onto a Problott membrane (Applied Biosystems) and visualized by staining with Ponceau S (Sigma) (0.2% w/v in 1% v/v acetic acid). The excess of dye was washed off with 1% acetic acid and a protein band estimated as approximately 92 kDa was subsequently excised from the membrane and sent for internal sequencing analysis (Harvard Microchemistry Facility, Cambridge, MA).

IV. RESULTS

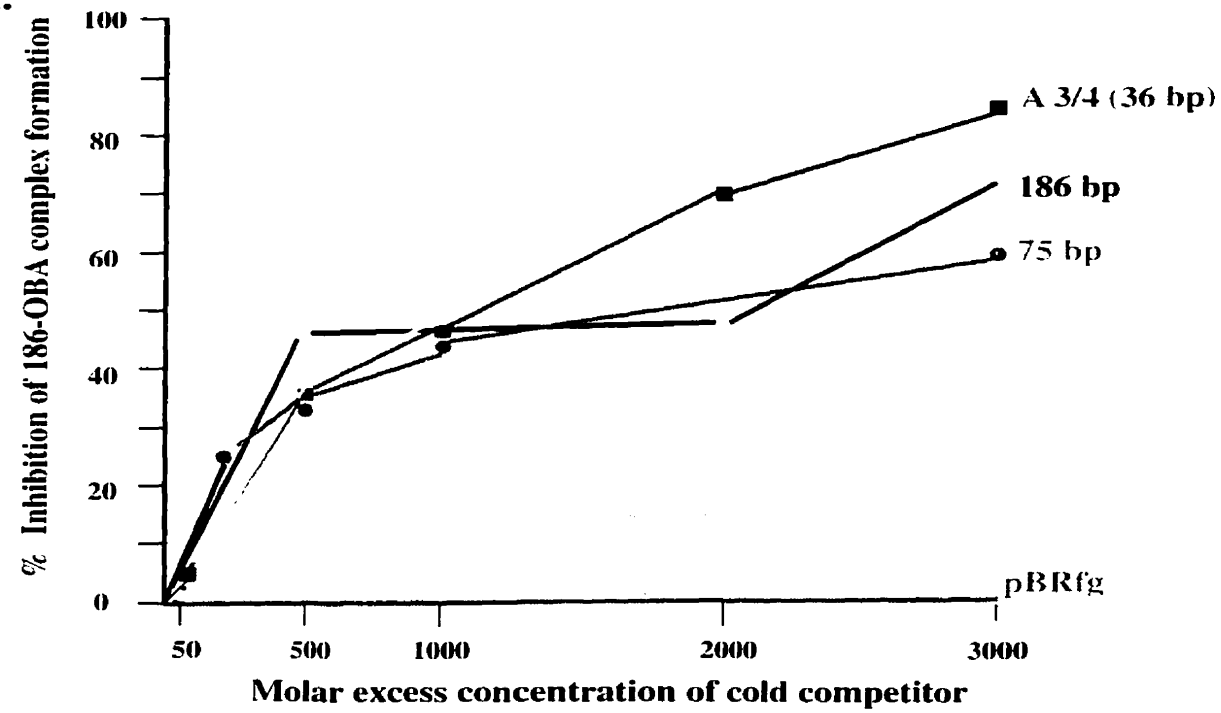
Specific interaction between OBA and the A3/4 DNA consensus sequence

The map and sequence characteristics of the 186-bp fragment of *ors*8 have previously been reported (Ruiz et al., 1995; Todd et al., 1995). In the present study, band-shift competition experiments were performed in order to localize the OBA binding site within the 186-bp minimal origin of *ors*8 (Figure 1A). In contrast to the nonspecific competitor, pBRfg, which did not compete, the different subfragments of the 186-bp competed to different extents for OBA binding. The most efficient competitor was the internal 59-bp fragment (Figure 1A), generated by from the digestion of the 186-bp with *MslI* and *FokI* (Figure 1B), which competed as efficiently as, or better than the 186-bp fragment itself. The 59-bp fragment (Figure 1C) contains two 7-bp stretches with 85% identity to a 36-bp DNA sequence, A3/4, (Figure 1C) deduced from different *ors* (Kaufmann, 1985; Rao et al., 1990) and human replication origins isolated in our laboratory (Bell et al., 1991; Wu et al, 1993; Nielsen et al., 1994). When the A3/4 sequence was tested as competitor for OBA binding to the 186-bp fragment in the bandshift assay, it was able to compete just as well as the 59-bp fragment for OBA binding (Figure 1A). Furthermore, the specificity of OBA binding to the A3/4 sequence was tested in a series of competition bandshift assays, using the A3/4 oligonucleotide as specific competitor, in increasing (50-2000X) molar-fold amounts (Figure 2). Formation of the OBA-A3/4 complex decreased in the presence of 50-fold molar excess of cold A3/4 oligonucleotide competitor and it was 95% abolished at 500-fold molar excess of A3/4 (Figure 2, A3/4ds). In contrast, when similar competition reactions were carried out using the two single-stranded oligonucleotides of the A3/4 sequence as competitors, neither was able to compete the OBA-A3/4 complex (Figure 2; leading and complementary).

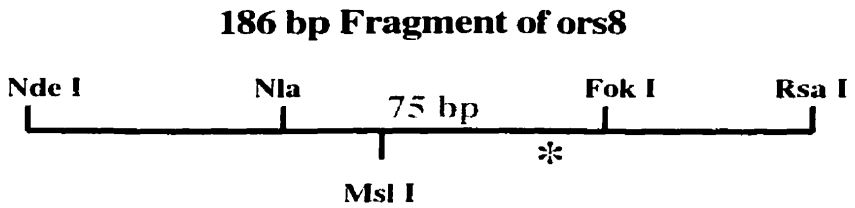
A3/4 oligonucleotide inhibits the in vitro DNA replication of p186

To investigate the effect of the A3/4 sequence on the replication of p186, we performed *in vitro* DNA replication assays (Pearson et al., 1991; 1994; Matheos et al., 1998), in which the HeLa cell extracts were preincubated with increasing molar excess amounts of either the A3/4 oligonucleotide or a nonspecific oligonucleotide used as competitors (Figure

A.



B.



C.

TTATGATAGAGATTTATAGGTAGAAGATTTATCAGTAGGGATTTATGGATGCTCTTTCTT

Figure 1. Competition bandshift analysis of OBA binding. A. Band-shift reactions were performed by incubating constant amounts of both, protein (Pool F; 200 ng) and radioactively labeled DNA (186-bp fragment; 0.1 ng). The various subfragments of the 186 bp and the A3/4 (36 bp) sequence were used as cold competitors at the molar-fold excess level indicated. The shifted complexes were quantitated by densitometry and the results expressed as percent reduction in complex formation. B. Restriction map of the 186-bp sequence of *ors*8 indicating the fragments used as competitors. The *Fok*I site (arrow point) bisects the inverted repeat (><). The position of the A3/4 homologous sequence is indicated (*). C. Sequence of the 59 bp fragment of *ors*8. The nucleotides that are identical to the A3/4 36-bp sequence are indicated (*).

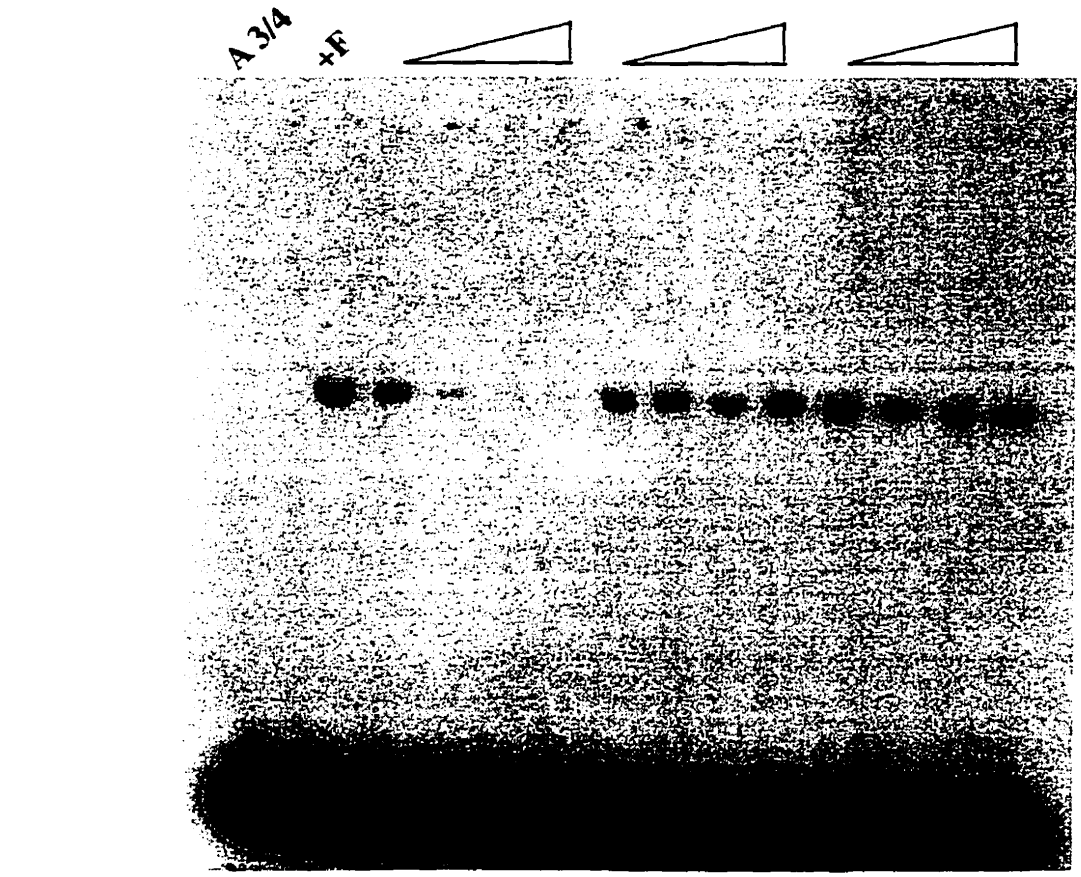


Figure 2. Specificity of OBA binding to the A3/4 sequence. Band-shift reactions were carried out incubating constant amounts of radioactively labeled A3/4 DNA (0.5 ng) and Pool F (200 ng). Increasing molar excess (50x, 500x, 1000x and 2000x) amounts of the A3/4 sequence, either double-stranded (ds), or leading and complementary A3/4 single strands, were used as cold competitors.

3). Addition of increasing amounts of the A3/4 oligonucleotide strongly inhibited p186 replication *in vitro*, decreasing it by approximately 4-fold at 10X molar excess and by approximately 10-fold at 250X molar excess amounts relative to the control (Figure 3). In contrast, addition of the nonspecific oligonucleotide did not affect the replication of p186 (Figure 3), nor did that of a 29-bp random oligonucleotide (data not shown). Addition of the A3/4 oligonucleotide similarly inhibited the *in* vitro replication reaction of p*ors*12, a plasmid containing *ors*12, a functional *ors* that serves as a chromosomal origin of DNA replication, whereas the nonspecific oligonucleotide did not have an inhibitory effect (data not shown). The products of the *in vitro* replication reaction included open circular (form II), linear (III) and supercoiled (I) forms of the plasmid DNA. In addition, replicative intermediates and topoisomeric forms of the plasmid DNA were also obtained, in agreement with previous observations (Pearson et al., 1991; Zannis-Hadjopoulos, 1994; and Matheos et al., 1998). As previously shown, the *in vitro* replication system mimics *in vivo* replication, in that replication initiates specifically within the *ors*, is bidirectional, semiconservative and sensitive to aphidicolin (Pearson et al., 1991, 1994).

Identification of the polypeptide involved in the DNA binding activity of OBA

To analyze the peptide(s) involved in the DNA binding activity of OBA, band-shift elution (Ruiz et al., 1995) of the OBA-A3/4 complex, followed by Southwestern blot analysis were performed (Figure 4). Several band-shift reactions were carried out simultaneously, and the OBA-A3/4 complex was excised and eluted from the gel by isotachophoresis. The OBA proteins were then subjected to Southwestern analysis (see Materials and Methods), using the A3/4 oligonucleotide as radioactive probe. A major band of approximately 92 kDa was revealed as the one primarily involved in the DNA binding activity of OBA (Figure 4, arrow).

OBA purification on an A3/4 affinity column

An A3/4 DNA sequence-specific affinity column was prepared in order to enrich for the DNA binding activity of OBA, as determined by Southwestern analysis. Pool F (from Sephacryl-S300 column) was loaded onto the column, the flowthrough was recovered and the proteins bound to the column were eluted with a linear salt (NaCl) gradient (see Materials and Methods). The apOBA, localized by band-shift analyses using the A3/4 oligonucleotide as

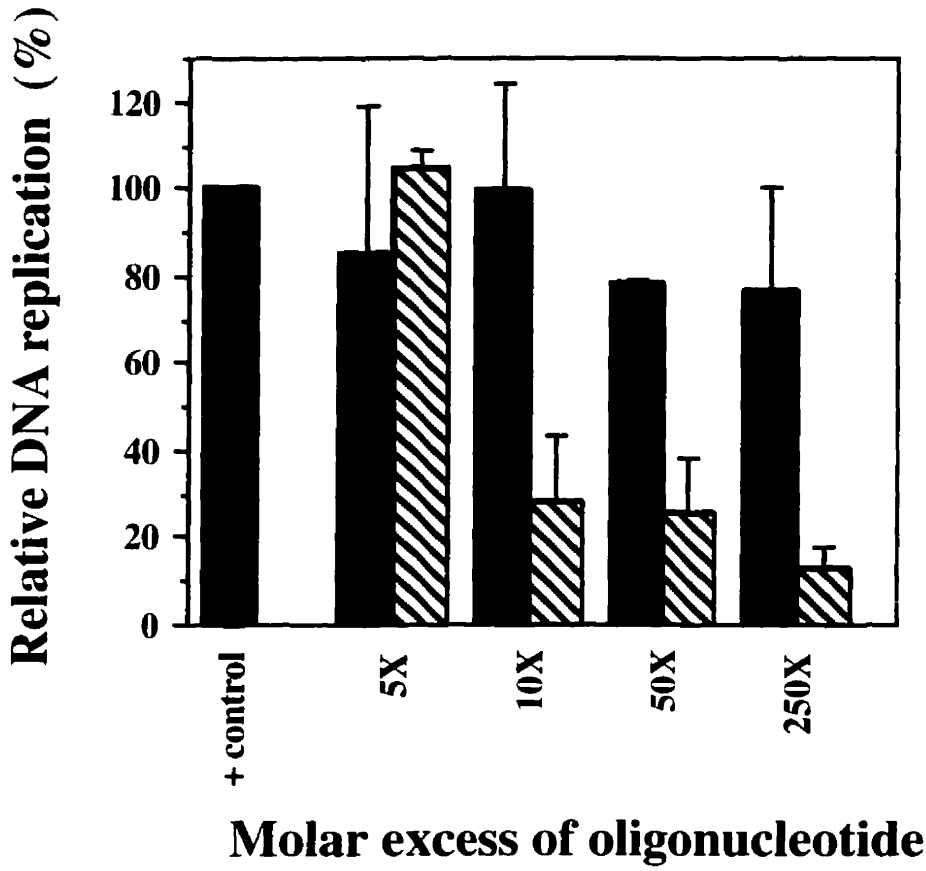


Figure 3. Effect of the A3/4 oligonucleotide sequence on the *in vitro* replication of p186. A p186 plasmid (200 ng), containing the 186-bp fragment of *ors*8, was used as template for *in vitro* replication. Increasing molar excess amounts (indicated) of the specific A3/4 (dashed bars) or nonspecific pBR322 (black bars) oligonucleotides, relative to the p186 template, were used.

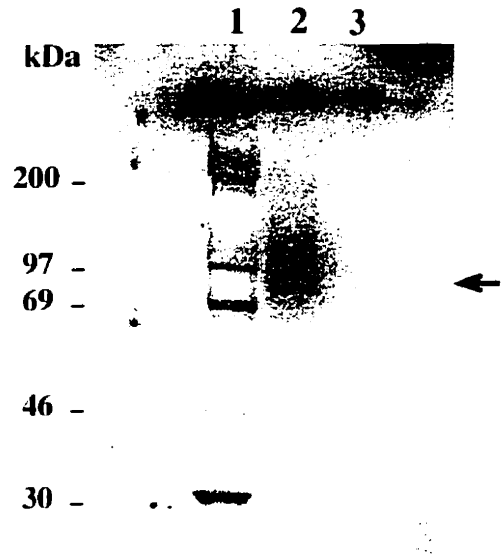


Figure 4. Southwestern analysis of OBA-A3/4 complex. OBA-A3/4 band-shifted complex (see Figure 2) was eluted from the gel by isotachophoresis and subjected to 8% SDS-PAGE under reducing conditions. A control area from the same gel containing protein, but not DNA, was excised and similarly treated. The gel was blotted onto an immobilon membrane and probed with radioactively labeled A3/4 DNA, in the presence of ds poly (dI-dC) and pBRfg as nonspecific competitors. Lanes: 1, molecular size markers (molecular sizes are indicated); 2, OBA from OBA-A3/4 eluted complex; 3, protein control lane.

probe, was eluted at 0.45 M NaCl as a single peak around fraction 72 (Figure 5) and generated the same characteristic complexes as those that had been obtained with OBA in the previous chromatographic steps (Ruiz et al., 1995). PAGE analysis of apOBA (Figure 6A) revealed three predominant bands with estimated relative molecular mass of 78,000 (78 kDa), 92,000 (92 kDa) and >200,000 (>200 kDa). In addition, a band with molecular mass of 130,000 (130 kDa; Figure 6A, lane apOBA, *), as well as a doublet of lesser abundance with estimated molecular mass of 104,000 (104 kDa) and 110,000 (110 kDa), respectively, were observed.

DNA binding polypeptide of OBA is Ku86

ApOBA was electroblotted onto Problott™ membrane (Perkin-Elmer) and stained with Ponceau-S. The 92 kDa, exhibiting DNA-binding (see Figure 4) was excised from the membrane and sequenced at the Harvard Microchemistry Facility by collisionally activated dissociation (CAD) mass spectroscopy on a Finnigan TSQ 700 triple quadrupole mass spectrometer. The three peptide sequences obtained (Table I) were subjected to homology searches using the BLAST program (Altschul et al., 1990) and all three were found to have 100% identity to the Ku86 subunit of human Ku autoantigen (Table I).

The enrichment of OBA throughout the purification scheme was visualized by 8% denaturing PAGE of the different pools (Figure 6A, lanes NC to apOBA). In order to verify the presence of Ku antigen in the affinity-purified OBA fractions, Western blotting analysis were performed, using antibodies raised against the Ku86 and Ku70 subunits (Santa Cruz, CA). The results (Figure 6, B and C) show that the two major OBA bands, estimated as migrating as 92 kDa and 78 kDa, correspond to the Ku86 and Ku70 subunits of Ku autoantigen, respectively. Similar Western blotting analyses using anti-DNA-PKcs antibodies revealed that the high molecular weight predominant band (>200 kDa) present in the affinity-purified OBA (Figure 6A, arrow) corresponded to DNA-PKcs (p465) (Figure 6D, arrow). The 130-kDa band present in apOBA (Figure 6A, *) and in the Western blot is a degradation product of DNA-PKcs (Figure 6D, *). Western analysis performed on the same membrane that had been used for Southwestern analysis (see Figure 4) confirmed that the OBA band with DNA binding activity, which was also sequenced, is the Ku86 subunit of Ku autoantigen (Figure 7A). Although the Ku70 subunit could also be detected (Figure 7B), no DNA binding

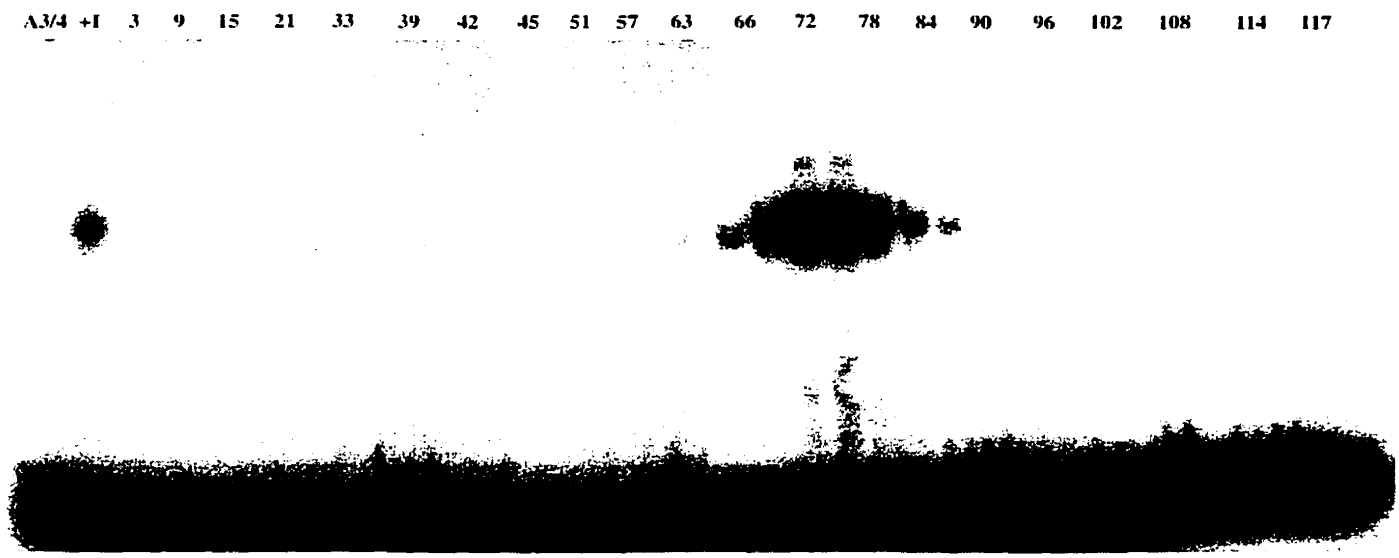


Figure 5. Elution profile of OBA from the A3/4 affinity column. A DNA sequence-specific affinity column was prepared by binding of oligomers of the A3/4 oligonucleotide sequence to Sepharose CL-2B, freshly activated with cyanogen bromide. Pool F (see Materials and Methods) was loaded onto the column, the flowthrough was recovered, and the proteins bound to the matrix were eluted with a linear salt gradient (150 mM-1.0 M) of NaCl in buffer B. The elution of OBA (0.45 M NaCl) was localized by band-shift reactions, incubating 16 µl of every second fraction with the A3/4 DNA as probe.

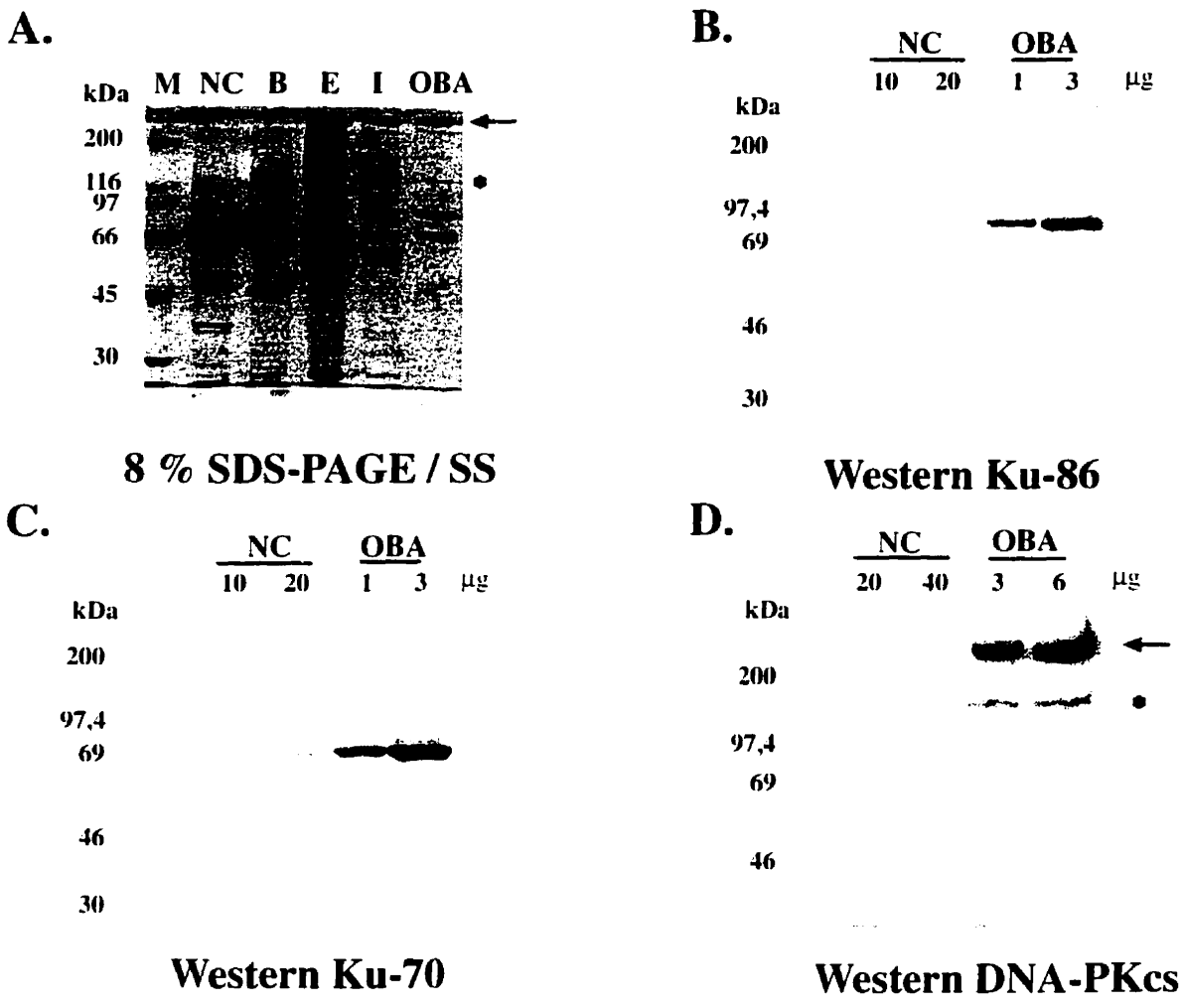


Figure 6. Ku antigen is present in the affinity-purified OBA fraction. A. OBA enrichment profile in the nuclear and cytoplasmic (NC) HeLa cell extracts and in pools b, e, i and affinity-purified OBA (apOBA) through the purification steps. 2.5 µg of pools NC, B, E, I as well as 1.0 µg of affinity-purified OBA were subjected to 8% SDS-PAGE and silver stained. The position of the DNA-PKcs band is indicated (arrow). western blot analyses of the affinity-purified OBA fraction were performed using: (B), anti-Ku86 antibody (1 µg/ml); (C), anti-Ku70 antibody (2 µg/ml); (D), anti-DNA-PKcs antibody (4 µg/ml). The DNA-PKcs band is indicated (D, arrow); the faster migrating band is a degradation product of DNA-PKcs (*). Western analyses were done on 8% SDS-PAGE for Ku86 and Ku70, and on 6% SDS-PAGE for DNA-PKcs.

Table I. Microsequencing analysis of tryptic peptides of the OBA band.

Peptides	Amino acid sequence	Homology	Position
Aff92-PK160	SQIPLSK	Ku86 (100%)	526-532
Aff92-PK312	TLFPLIEAK	Ku86 (100%)	535-543
Aff92-PK296	TDTLEDLFPTTK	Ku86 (100%)	470-481

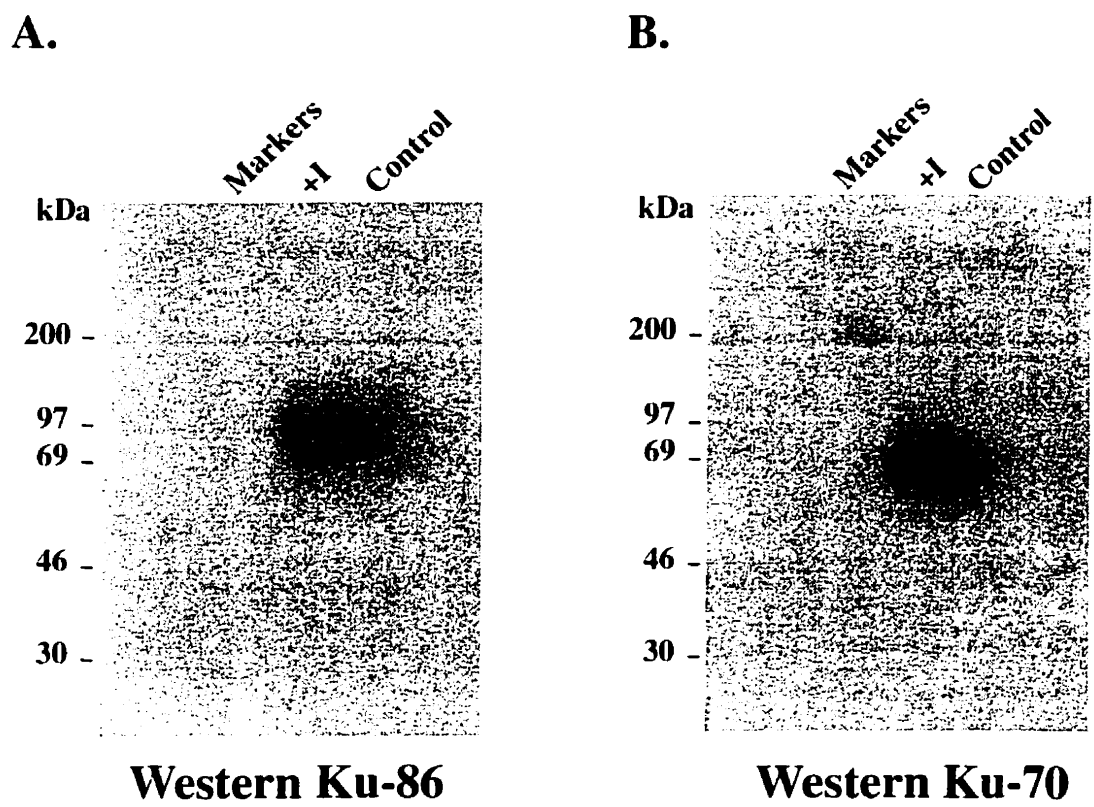


Figure 7. OBA corresponds to the Ku86 subunit of Ku antigen. The membrane used for Southwestern analysis (Fig. 4) was probed with, A. anti-Ku86 (1 μg/ml) and, B. anti-Ku70 (2 μg/ml) antibodies, respectively.

activity was associated with it, in contrast to previous reports (Zhang and Yaneva, 1992; Griffith et al., 1992; Wu and Lieber, 1996).

Bandshift analysis of OBA

When apOBA is used in bandshift assays with A3/4 DNA (Figure 8), a faster migrating band (Figure 8, arrow) is detected below the regular OBA complex (Figure 8, asterisk). To investigate the nature of this band, band-shift reactions were performed in duplicate, using radioactively labeled and unlabeled A3/4 oligonucleotide as probe. The radioactive part of the gel was used for bandshift analysis, where the expected band-shift pattern was obtained with increasing amounts of apOBA (Figure 8.I). The nonradioactive part of the gel was transferred after electrophoresis to a membrane and subjected to Western blotting analysis, as described above. The results (Figure 8.II and III) showed that Ku86 is present only in the upper OBA complex (Figure 8.II), together with Ku70 (Figure 8.III), while in the faster migrating band only the p70 subunit of Ku was detected (Figure 8.III).

OBA/Ku86 binds the DNA in a sequence-specific manner

To investigate whether OBA/Ku interacts with the A3/4 DNA in a sequence-specific manner or whether it binds to the ends of double-stranded linear DNA without any sequence preference, competition bandshift assays were carried out using as competitor a supercoiled double-stranded plasmid containing the A3/4 sequence, A3/4/pBR322. Similar control reactions were carried out using the supercoiled vector plasmid, pBR322, lacking the A3/4 sequence. The results (Figure 9.I) show that at 50X molar excess, the supercoiled A3/4/pBR322 plasmid competed to a small extend (2.9%) for the OBA binding to the A3/4 oligonucleotide, while at 500X molar excess it competed very efficiently, reducing the formation of the OBA-A3/4 complex by 97% (Figure 9.I, A3/4/pBR322); the nonspecific supercoiled competitor (pBR322), on the other hand, was not able to affect at all the OBA-A3/4 complex formation (Figure 9.I). When the same plasmids (A3/4/pBR322 and pBR322) were linearized and used as competitors (A3/4/pBR322/*Nru* I and pBR322/*Nru* I, respectively, Figure 9.II), at 50X molar excess only the A3/4/pBR322 plasmid competed to a small extend (4.4%), while at 500X molar excess both linear plasmids competed for OBA binding to A3/4 with equal efficiencies (98 %) (Figure 9.II).

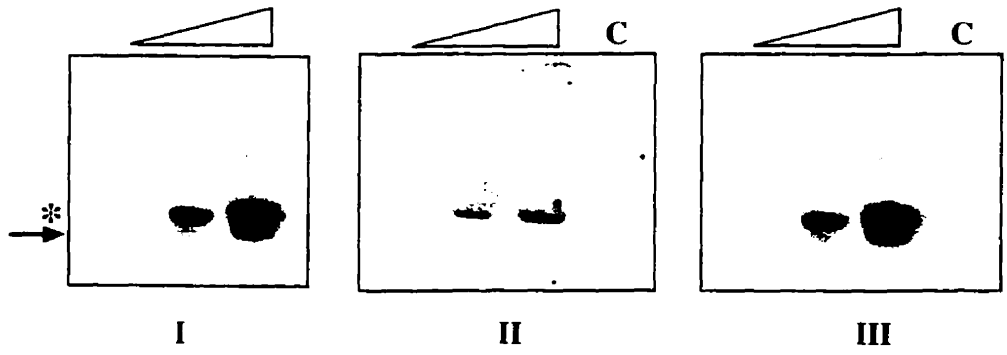


Figure 8. Western blot analysis of the band-shifted complexes generated with apOBA. (I). Band-shift reactions were performed under regular conditions, using 0.25 ng/reaction of A3/4 radioactive probe and increasing amounts of apOBA (100, 250 and 350 ng, respectively). A control reaction was done in which the DNA was absent. A duplicate set of reactions was also performed using non-radioactive A3/4 as probe. All reactions were subjected to electrophoresis in a minigel apparatus so that the DNA probe ran out of the gel. The non-radioactive half of the gel was transferred to a membrane and subjected to Western Blotting analysis with (II) anti-Ku86 antibody, and (III) anti-Ku70 antibody. The position of the main OBA-A3/4 (asterisk) and of the faster migrating (arrow) complexes is indicated.

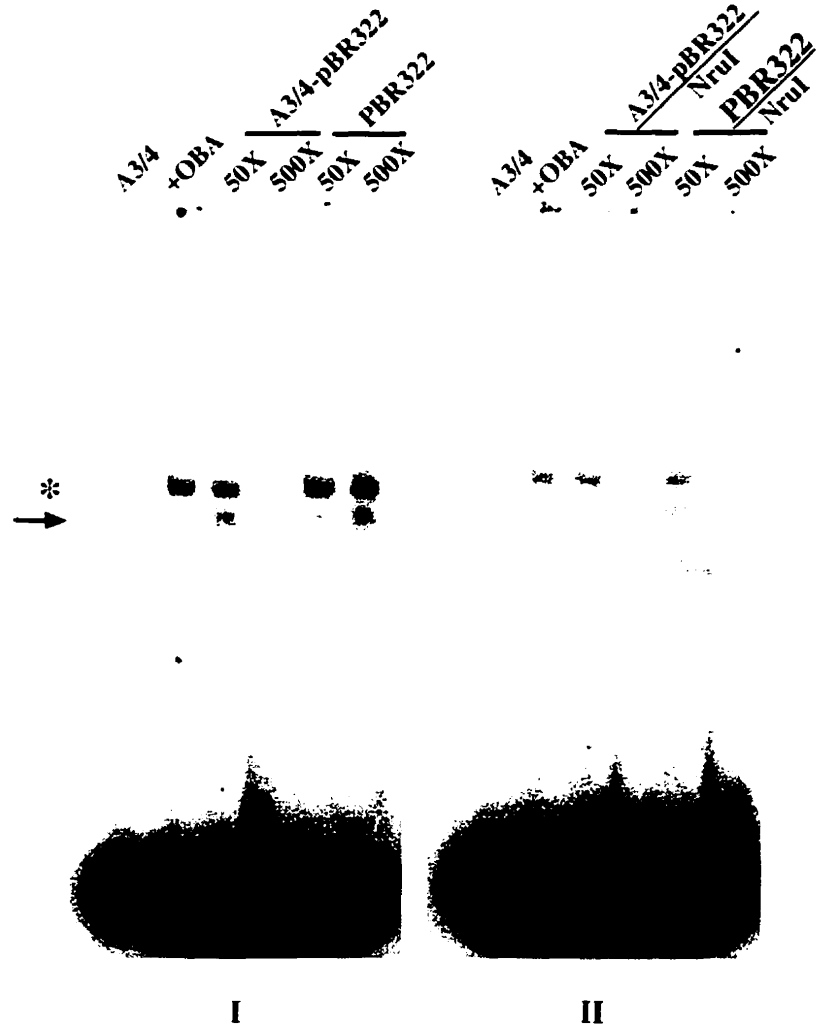


Figure 9. Sequence-specific binding of apOBA to A3/4. Bandshift reactions were performed by incubating radioactively labeled A3/4 DNA (0.25 ng) and apOBA (260 ng). I. The supercoiled plasmids A3/4/pBR322 and pBR322 were used as competitors at 50X and 500X molar excess, respectively. II. Similar experiments were carried out in the presence of linearized A3/4/pBR322 (A3/4/pBR322/NruI) and pBR322 (pBR322/NruI) plasmids. The position of the main OBA/A3/4 (*) and of the fater migrating complex (arrow) in indicated.

Ku70 and Ku86 antibodies inhibit the in vitro DNA replication of p186

To investigate whether Ku is involved in the replication of p186, we performed *in vitro* replication reactions preincubating the HeLa cell extracts with increasing (3.5 μg, 7 μg and 14 μg) amounts of the anti-Ku70 and anti-Ku86 antibodies, as well as a control, goat IgG antibody (Figure 10A). Addition of 14 μg of either the anti-Ku70 or anti-Ku86 antibodies inhibited the relative *in vitro* replication of p186 by 62% and 56%, respectively. In contrast, addition of the nonspecific goat IgG antibody had no effect over the entire range of concentrations assayed, indicating that the observed inhibition is specific to the Ku antibodies.

To confirm that the inhibition of p186 *in vitro* replication was in fact due to specific interaction of the antibodies with Ku antigen in the reaction, the respective antibodies were neutralized with either the Ku-specific or nonspecific blocking peptides prior to their addition to the reaction (Figure 10B). Addition of anti-Ku70 and anti-Ku86 antibodies that had been neutralized with the Ku70 and Ku86 specific peptides, respectively, reversed the inhibition of p186 replication *in vitro* (Figure 10B, lane 3), when compared to the reaction with the untreated antibodies (Figure 10B, lane 2). Neutralization of the anti-Ku70 antibody (black bars) restored replication by 47% and of the anti-Ku86 antibody (white bars) by 30%, respectively, bringing replication levels close to the control reaction (Figure 10B, lane 1). In contrast, when the Ku antibodies were blocked with a nonspecific peptide mixture, and then added to the reaction, they failed to restore replication (Figure 10B, lane 4).

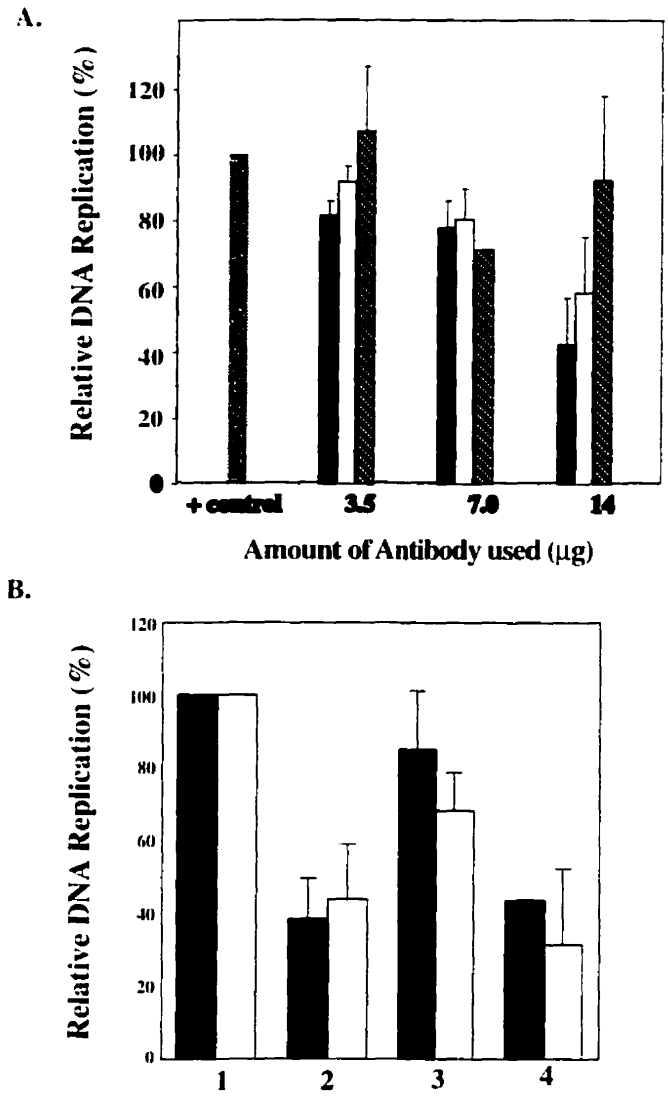


Figure 10. A. Effect of anti-Ku70 and anti-Ku86 antibodies on the *in vitro* replication of p186. Increasing amounts (3.5, 7.0 and 14 µg) of anti-Ku70 (black bars), anti-Ku86 (white bars), and goat IgG (dashed bars) antibodies were preincubated with HeLa cell extracts, prior to the addition of the plasmid DNA template (p186, 200 ng). B. Neutralization of the anti-Ku70 and anti-Ku86 antibodies. A constant amount (14 µg) of the anti-Ku70 (black bars) and anti-Ku86 (white bars) antibodies was neutralized using specific (lane 3) or nonspecific (lane 4) peptides before being added to the reaction. Lane 1 represents the p186 control reaction and lane 2 represents the reaction inhibited with 14 µg of antibodies. *In vitro* reactions were carried out and the data were processed as described in materials and methods.

V. DISCUSSION

Ku is a nuclear protein originally identified as an autoantigen associated with human lupus erythematosus and related overlap syndromes (Mimori et al. 1981). Ku has been reported to be part of a family of related proteins (Griffith et al., 1992), conserved in organisms that range from yeast (Feldmann and Winnacker, 1993; Jacoby and Wensink, 1996) to humans (Mimori et al., 1981). Ku (p70/p86) serves as the DNA binding subunit for the DNA-PK holoenzyme (Gottlieb and Jackson, 1993; Dvir et al., 1992). It mediates the recruitment of DNA-PKcs to DNA and, although it binds to the ends of naked DNA and is translocated along the DNA in an ATP-independent manner (de Vries et al., 1989), it was recently reported as a transcription factor that recruits DNA-PK directly to specific DNA sequences (Giffin et al., 1996; 1997). DNA-PK holoenzyme, composed of the DNA-PKcs (465 kDa) and Ku antigen, has been implicated in multiple nuclear processes including transcription (Kuhn et al., 1995; Finnie et al., 1993; Genersch et al., 1995), double-stranded DNA break repair (Getts and Stamato, 1994; Jeggo et al., 1995; Weaver, 1995), V(D)J recombination (Taccioli et al., 1994; Smider et al., 1994; Blunt et al., 1995; Kirchgessner et al., 1995; McConnell and Dynan, 1996, and references therein, Han et al., 1997), and DNA replication (Anderson and Lees-Miller, 1992; Anderson, 1993).

In this study, we identified a 59-bp region within the 186-bp fragment of *ors*8, to which OBA is specifically binding. OBA, a DNA binding activity from HeLa cells, was previously identified as a protein activity that specifically interacts with the 186-bp fragment of *ors*8 (Ruiz et al., 1995), the minimal sequence required for the replication function of *ors*8 (Todd et al., 1995). The 59-bp subfragment of *ors*8 contains two stretches of 7-bp each with 85% identity to a 36-bp sequence (A3/4) deduced from mammalian replication origins, among them the *ors*. Bandshift competition analyses confirmed that OBA binds the double-stranded A3/4 sequence specifically. Furthermore, addition of increasing amounts of an oligonucleotide derivative of the A3/4 sequence inhibited the *in vitro* replication of p186, a plasmid containing the 186-bp minimal origin, as well as that of p*ors*12, a plasmid containing a chromosomal origin of DNA replication. Southwestern analyses of the OBA-A3/4 bandshifted complex revealed a major peptide of approximately 92 kDa responsible for the DNA binding activity of OBA. Microsequencing analyses of this band revealed identity to the

86 kDa subunit of Ku autoantigen (Mimori et al., 1981; Reeves, 1985; Yaneva et al., 1985). OBA was enriched by affinity purification (apOBA) on a column (Kadonaga and Tjian, 1986) constructed using multimers of the A3/4 sequence.

A supercoiled circular plasmid containing the A3/4 sequence was able to compete for the binding of apOBA to A3/4, as opposed to the vector alone (Figure 9.I), indicating that the interaction of OBA with A3/4 is sequence specific. However, OBA is also capable of binding to DNA termini, since the same plasmids (A3/4-pBR322 and pBR322), when linearized, competed for OBA binding as efficiently as the supercoiled specific competitor (Figure 9.II), in agreement with previous reports regarding Ku binding (Paillard and Strauss, 1991; Gottlieb and Jackson, 1993; Rathmell and Chu, 1994). Thus, the data indicate that, in addition to binding to DNA ends, as expected, OBA/Ku also exhibits efficient sequence-specific binding to the internal A3/4 sequence. These data support recent reports describing sequence-specific binding of Ku to an internal DNA sequence, NRE-1 (Giffin et al., 1996, 1997); the direct binding of Ku/DNA-PK to NRE-1 represses glucocorticoid-induced MMTV transcription (Giffin et al., 1996, 1997). The 26-bp NRE-1 sequence contains a 7-nucleotide stretch match to the 59-bp fragment of *ors*8, overlapping A3/4. Interestingly, a 207-bp fragment from the origin of bidirectional replication (OBR), *oriβ*, associated with the dihydrofolate reductase (DHFR) gene, which contains a region of homology (approximately 70%) to the A3/4 sequence, is also able to form a complex with apOBA, which is efficiently competed by increasing amounts of the A3/4 oligonucleotide (data not shown).

A Ku-like protein from *Saccharomyces cerevisiae* was demonstrated to have affinity for ssDNA (Shakibai et al., 1996), while previous reports identified Ku70 as the DNA binding activity of the Ku(p70/p86) heterodimer, with affinity for DNA ends (Zhang and Yaneva, 1992; Wu and Lieber, 1996). Recently, the sequence-specific binding of Ku autoantigen to the single, upper strand of NRE-1 was shown to be mediated by both Ku subunits (Torrance et al., 1998). The binding of Ku to double stranded NRE-1, however, although it also requires the two subunits, occurs in a two-step fashion. The first step involves binding of the Ku70 subunit to the DNA and is followed by a Mg^{+2}-dependent step which leads to the contact of the Ku86 subunit to DNA. In our study, band-shift competition analyses demonstrated that OBA binding cannot be competed by single-stranded DNA (Figure 2). Microsequencing analyses revealed that apOBA is identical to the p86 subunit of Ku antigen, while Western blotting

showed that both Ku subunits (70 kDa and 86 kDa) were present in the affinity-purified OBA fraction (Figure 6). Furthermore, Western analysis performed on the same membrane that was used in the Southwestern analysis, confirmed that, although both Ku antigen subunits (Ku86 and Ku70) were present, the DNA binding activity was only associated with the Ku86 subunit (Figure 4 and Figure 7). Feldmann et al., (1996) reported the cloning of *HDF2* (high affinity DNA binding factor), the *S. cerevisiae* gene encoding the second subunit of the HDF heterodimer, the yeast Ku homologue. HDF2 is homologous to the Ku86 subunit of Ku antigen and can bind DNA on its own. Since the DNA binding activity of HDF2 is much weaker than the binding activity of the HDF heterodimer, the authors argued that HDF2 is the one involved in DNA binding, while HDF1 (p70 subunit) increases the affinity of the heterodimer for the DNA. On the other hand, other reports argued that both subunits are directly involved in DNA end binding (Milne et al., 1996), and that only the heterodimeric form of recombinant Ku antigen is able to bind DNA ends (Ochem et al., 1997). Interestingly, the identification of Ku antigen isolated from HeLa cells in a 2D-gel database of transformed human amnion cell (AMA) proteins revealed that Ku86 consists of at least three charge variants, whose relative abundance is a function of cell proliferation (Stuiver et al., 1991). Different charge variants of this subunit could explain the discrepancy in the results regarding the DNA binding activity of the polypeptide.

It was recently reported (Klug, 1997; Bliss and Lane, 1997) that in band-shift analyses involving Ku antigen, the order of addition of reagents to the bandshift reaction is crucial in avoiding artifact bands. In light of this, we performed bandshift reactions both under conditions in which all the components of the band-shift assay were present at the same time in the reaction, and by step incubation, in which the competitor DNA (A3/4) and the protein (apOBA) were preincubated together and the radioactive probe was added last (data not shown). These studies further confirmed that, regardless of the order of addition, the interaction between A3/4 and OBA/Ku is sequence-specific, and revealed that preincubation of the A3/4 competitor with apOBA resulted in a higher level of inhibition of formation of the apOBA complex than was obtained otherwise. Interestingly, in band-shift reactions in which the nonspecific competitor (pBRfg), the 186-bp radioactive probe, and the protein fraction were simultaneously incubated, no complex competition was observed (Figure 1, pBRfg). These results indicate that, when the reagents are present simultaneously, OBA/Ku

preferentially binds to its specific internal binding site (contained in the 186 bp fragment of *ors* 8), and not to the DNA termini presented by the linear nonspecific pBRfg (Figure 1). In contrast, in band-shift reactions in which the linear A3/4pBR322 (specific) or pBR322 (nonspecific) plasmids were initially incubated with the protein fraction and the probe was subsequently added, both plasmids were able to compete for OBA/Ku binding, indicating that under these conditions the protein interacted with DNA termini (Figure 9.II).

Although both subunits of Ku antigen (p70 and p86) were detected in the main (slower migrating) OBA-shifted complex (Figure 8, asterisk) as expected of a heterodimeric (p70/p86) DNA binding protein, (Figure 8, arrow) only the p70 subunit was detected in the faster migrating complex (Figure 8, compare II and III). This is due to the fact that the Ku86 antibody used in these analyses was raised against the C-terminal end of the protein (Ku86 (C-20), Santa Cruz, CA), and thus is unable to recognize the Ku86 subunit in the faster migrating complex, which arises by the specific *in vitro* endoproteolysis of Ku86 at the carboxyl-terminus region (Paillard and Strauss, 1993). This proteolytic degradation of the Ku86 subunit gives rise to a 69 kDa peptide that is able to associate with Ku70 to form a lower molecular weight Ku heterodimer, which is still capable of binding DNA (Paillard and Strauss, 1993).

The apOBA preparation is also enriched for a high molecular weight protein, which was shown by Western analysis to correspond to DNA-PKcs (Figure 6D). While DNA-PKcs is present in the preparation, it is absent from the OBA-A3/4 complex (Figure 8 and data not shown). The role of OBA/Ku in p186 *in vitro* replication may be independent of the DNA-PKcs activity, since addition of increasing amounts of anti-DNA-PKcs antibodies to the *in vitro* reaction did not affect p186 replication (data not shown). Interestingly, it was recently reported that DNA-PKcs is able to bind DNA by itself, independently of Ku antigen (Yaneva et al., 1997).

A role of Ku in DNA replication has been suggested before, since it was reported as part of a multiprotein complex competent for T-antigen-dependent SV40 *in vitro* replication (Vishwanatha and Baril., 1990; Cao et al., 1994). More recent studies, also suggest the involvement of Ku in replication, either as an independent protein or through its association with DNA-PKcs. Henricksen et al., (1996) reported that the phosphorylation of replication protein A (RP-A) by DNA-PK is involved indirectly in the modulation of DNA replication. Shakibai et al., (1996) reported the purification of OBF2 (origin binding factor 2) from

Saccharomyces cerevisiae, which is identical to HDF, the yeast homologue of the mammalian Ku antigen. OBF2 binds to the *ARS121* replication origin and supports the formation of a protein complex at the origin. In our study, the inhibition of p186 replication, observed in the presence of either the A3/4 oligonucleotide or the anti-Ku antibodies directed against either subunit of Ku, also suggests a role of OBA/Ku in mammalian DNA replication. The specificity of the inhibition of p186 replication, due to the sequestering of p70 and p86 proteins, was demonstrated by the neutralization of these antibodies with their specific peptides. Since *in vitro* DNA replication is not fully inhibited by the presence of the Ku antibodies (Figure 10), there may also be alternative pathways to carry out OBA(Ku) activity in the absence of this protein.

Recently, Ku86 and Ku70 knockouts were obtained (Nussenzweig et al., 1996; Gu et al., 1997) and both of them are viable. Although *Ku86*$^{-/-}$ mice have a growth defect, neither, the *Ku86*$^{-/-}$ mice nor the murine embryonic stem cell line which lacks Ku70 expression have been investigated for defects in DNA replication. The hypersensitivity of Ku86-deficient cell lines and mice to DNA damage support the role of Ku86 in growth regulation (Nussenzweig et al., 1997). It has been demonstrated that multiple genes encode a family of Ku70-related polypeptides (Griffith et al., 1992a). This could explain in part the diversity of functions and contradictory data that have been reported for Ku antigen with regard to its DNA binding activity and the phenotype obtained in the knockout experiments.

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CONNECTING TEXT

In the preceding chapter, OBA was identified as Ku antigen. Sequence-specific binding of OBA to the double-stranded A3/4 origin sequence was shown. *In vitro* replication experiments demonstrated that OBA/Ku was involved in mammalian DNA replication.

The next chapter further describes the role of OBA/Ku in mammalian and viral DNA replication. The helicase activity of OBA/Ku and its ability to interact with the replication machinery, including with Oct-1 (see chapter two), was investigated. A model of the role of OBA/Ku in mammalian DNA replication is presented.

CHAPTER FOUR

OBA/Ku: an origin-specific binding protein that associates with replication proteins is required for mammalian DNA replication

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I. ABSTRACT

OBA represents a HeLa cell protein activity that binds in a sequence-specific manner to A3/4, a 36-bp mammalian replication origin sequence. OBA's DNA binding domain is identical to the 86 kDa subunit of Ku antigen. Addition of an A3/4 double-stranded oligonucleotide inhibited *in vitro* DNA replication of p186, p*ors*12, and pX24, plasmids containing the monkey replication origins of *ors*8, *ors*12, and the Chinese hamster DHFR oriβ, respectively. In contrast, *in vitro* SV40 DNA replication remained unaffected. The inhibitory effect of A3/4 oligonucleotide was fully reversed upon addition of OBA. Furthermore, depletion of OBA/Ku by inclusion of an antibody recognizing the Ku heterodimer, Ku70/Ku86, decreased mammalian *in vitro* replication to basal levels. By co-immunoprecipitation analyses, OBA/Ku was found to interact with DNA polymerases δ and ε, PCNA, topoisomerase II, RF-C, DNA-PKcs, and Oct-1, a transcription factor that enhances p186 and pX24 replication by a mechanism other than direct DNA binding. The data suggest an involvement of OBA/Ku in mammalian DNA replication as an origin-specific-binding protein with DNA helicase activity. OBA/Ku acts at the initiation step of replication and requires an A3/4-homologous sequence for origin binding. The physical association of OBA/Ku with Oct-1 and the replication proteins reveals a possible mechanism by which these proteins are recruited to mammalian origins.

II. INTRODUCTION

The heterodimeric Ku antigen (reviewed in Tuteja and Tuteja, 2000) consists of a 70 kDa and 86 kDa subunit and appears to be present in all eukaryotes, suggesting that it has a conserved function. Discovered originally as an autoimmune antigen (Mimori et al., 1981), it is now known that Ku is both the DNA binding subunit and the allosteric activator of the DNA-dependent protein kinase, DNA-PK (Gottlieb and Jackson, 1993). DNA-PK, which consists of Ku and the DNA-PK catalytic subunit (DNA-PKcs), is critical for non-homologous DNA double-strand break repair and site-specific V(D)J recombination (reviewed in Anderson, 1993; Jin et al., 1997; Featherstone and Jackson, 1999; Pastink and Lohman, 1999; Kanaar et al., 1999). Recent studies, using Ku knockout mice and yeast, support additional functions for Ku in transcription (Finnie et al., 1993; Kuhn et al., 1995; Genersch et al., 1995; Giffin et al., 1996), telomeric maintenance (Boulton et al., 1996; Porter et al., 1996; Gravel et al., 1998; Polotnianka et al., 1998), replicative senescence (Woo et al., 1998) and DNA replication (Ruiz et al., 1999). Ku86 has also been shown to suppress chromosomal aberrations and malignant transformation (Difilippantonio et al., 2000).

Evidence for the involvement of Ku in DNA replication is accumulating. Ku is identical to the DNA-dependent ATPase purified from HeLa cells (Cao et al., 1994), which cofractionated with a 21S multiprotein complex that is able to support SV40 *in vitro* DNA replication (Vishwanatha and Baril, 1990). Ku has been shown to bind to several origins of DNA replication, including the adenovirus type 2 origin (de Vries et al., 1989), the B48 human DNA region (Toth et al., 1993), the A3/4 sequence comprised in the minimal origin of the monkey *ors*8 (Ruiz et al., 1999), the dihydrofolate reductase (DHFR) Chinese hamster replication origin, oriβ (Ruiz et al., 1999), and the human *dnmt* 1 (DNA-methyltransferase) origin (Araujo et al., 1999). Also, a Ku-like protein from *Saccharomyces cerevisiae*, OBF2, binds to the yeast ARS121 origin of replication and supports the formation of a stable multiprotein complex at essential replication sequences (Shakibai et al., 1996). Furthermore, Ku may be involved in DNA replication through its association with DNA-PK; DNA-PK phosphorylates several DNA-binding proteins (reviewed in Anderson and Lees-Miller, 1992; Lees-Miller, 1996), some of which are

involved in DNA replication. These include RPA (Brush et al., 1994), topoisomerase I and II (Anderson and Lees-Miller, 1992), SV40 large T antigen (LTAg) (Chen et al., 1991), Oct-1 (Anderson and Lees-Miller, 1992), and Ku antigen (Chan and Lees-Miller, 1996). Finally, the phenotypes of the Ku knockout mice, although supportive of Ku's involvement in DNA repair, do not rule out the possibility that it may also be involved in DNA replication. The immunodeficiency, the radiosensitivity and the failure of cells to resume progress through the cell cycle after checkpoint arrest caused by ionizing radiation, are all phenotypes of the Ku knockout mice that may easily be explained by a role of Ku in double-strand break repair (Nussenzweig et al., 1996, 1997; Gu et al., 1997). However, the small size of the Ku-deficient mice, the failure of the cells to proliferate in culture, their prolonged doubling time, and their premature senescence suggest a role of Ku in other processes, such as DNA replication (Featherstone and Jackson, 1999).

Our laboratory's focus has been on isolating mammalian origins of DNA replication and identifying origin-binding proteins. The origin-enriched-sequences, *ors*, previously isolated from early-replicating CV-1 monkey cells (Kaufmann et al., 1985; reviewed in Zannis-Hadjopoulos and Price, 1998, 1999), are capable of conferring autonomous replication to plasmids *in vivo* (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991), and *in vitro* (Pearson et al., 1991). Initiation of autonomous replication is site-specific for the *ors*, both *in vivo* and *in vitro*, as mapped by electron microscopy (Frappier and Zannis-Hadjopoulos, 1987; Pearson et al., 1994) and by earliest-labeled fragment analysis (Pearson et al., 1991). Furthermore, using non-competitive and competitive PCR-based mapping technologies, we demonstrated that, *in vivo*, DNA replication initiates preferentially in an *ors*-containing region on the chromosome (Pelletier et al., 1999), as well as in other human autonomously replicating sequences such as 343, NOA3, S14, S3 and F15 (Wu et al., 1993; Tao et al., 1997, 2000). Among the functional *ors*, *ors*8 has been studied extensively both *in vivo* (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991) and *in vitro* (Zannis-Hadjopoulos et al., 1992; Pearson et al., 1991; Pearson et al., 1994). By deletion analyses, a region of 186-bp was identified as the minimal origin of *ors*8 (Todd et al., 1995). This 186-bp fragment contains an imperfect inverted repeat sequence capable of extruding into a cruciform, several direct repeats, binding sites for the GATA family of transcription

factors, and two 7-bp stretches identical to portions of the A3/4 sequence (Ruiz et al., 1999), a 36-bp mammalian origin sequence that is capable of supporting replication *in vivo* and *in vitro* (Price et al., in preparation). The 186-bp fragment of *ors*8 also contains an imperfect binding site for the transcription factor, Oct-1 (Todd et al., 1995). Oct-1 is an enhancing component of the *in vitro* replication of p186, a pBR322-based plasmid that contains the minimal origin of *ors*8 (Matheos et al., 1998).

With the goal of identifying proteins that interact with mammalian origins of DNA replication, we purified an *Ors* Binding Activity (OBA) (Ruiz et al., 1995) and identified its DNA binding activity as the 86 kDa subunit of Ku antigen (Ruiz et al., 1999). In addition to its ability to bind to DNA ends, sequence-specific binding of OBA/Ku to A3/4 was demonstrated and preliminary experiments suggested OBA/Ku's involvement in mammalian DNA replication (Ruiz et al., 1999).

In the present study, we further examined the role of OBA/Ku on mammalian and viral DNA replication. Addition of the A3/4 double-stranded (ds) oligonucleotide inhibited the *in vitro* replication reaction of plasmids p186, p*ors*12 (Pelletier et al., 1997) and pX24. pX24 contains the DHFR origin of replication oriβ (Burhans et al., 1986). It replicates autonomously *in vivo* and *in vitro* (Zannis-Hadjopoulos et al., 1994) and DNA replication initiates within the origin sequence (Diaz-Perez et al., 1996). Addition of the A3/4 ds oligonucleotide did not, however, inhibit replication of pSV40 (Li and Kelly, 1984), containing the SV40 replication origin. Addition of exogenous, affinity-purified OBA (apOBA) to the A3/4-inhibited reactions fully restored replication levels. Furthermore, addition of clone 162, a Ku-specific antibody that recognizes the Ku70/Ku86 heterodimer, also inhibited *in vitro* replication, as did an anti-DNA polymerase δantibody. By co-immunoprecipitation assays, an interaction was found between OBA/Ku and Oct-1; POU, the DNA binding domain of Oct-1, was able to partially restore replication in reactions where the A3/4 ds oligonucleotide was competing for OBA/Ku. An association of OBA/Ku with PCNA, DNA polymerases δ and ε, topoisomerase II, RF-C and DNA-PKcs, but not with DNA primase or ORC-1, was also found. The data suggest that OBA/Ku is involved in mammalian DNA replication, at the level of initiation rather than elongation, requires an A3/4 homologous region for origin binding, and directs a replication complex that includes Oct-1 at the origin site.

III. MATERIALS AND METHODS

Plasmids

Plasmid p186 consists of the *NdeI/RsaI* (186-bp) fragment of *ors8* (GenBank accession number M26221) cloned in the *NruI* site of pBR322 (Todd et al., 1995). Plasmid p*ors*12 consists of the *ScaI/StyI* (215-bp) fragment of *ors*12 (GenBank accession number M26225), subcloned in the *NruI* site of pML2 (Pelletier et al., 1997). The pX24 plasmid contains the 4.8-kbp *XbaI* fragment of the DHFR origin of replication inserted into the *XbaI* site of pUC13 (Burhans et al., 1986; Zannis-Hadjopoulos et al., 1994; Diaz-Perez et al., 1996). The pSV40 (pUC-HSO) plasmid contains the *HindIII/SphI* SV40 fragment cloned into pUC19 (Li and Kelly, 1984).

Plasmid DNAs were isolated using the QIAGEN-tip 500 columns, according to the manufacturer's specifications (QIAGEN, Mississauga, Ontario, Canada). M13mp19 single-stranded DNA (Yanisch-Peron et al., 1985) was prepared as described in Sambrook et al. (1989).

Oligonucleotides

The oligonucleotides of the 36-nt A3/4 sequence (5' CCTCAAATGGTCTCC AATTTTCCTTTGGCAAATTCC 3'), the 16-nt nonspecific oligonucleotide, derived from nucleotides 838-854 of pBR322 (5' TTCCGAATACCGCAAG 3'), and the 29-nt random oligonucleotide (5' AGACCTGCAGTCTGCTGTCGTCTAGAGGA 3') were synthesized by Sheldon Biotechnology (Montréal, Québec, Canada). The oligonucleotides were purified by PAGE and annealed as described in Matheos et al. (1998). The 22-bp Oct-1 oligonucleotide (5' TGTCGAATGCAAATCACTAGAA 3') was purchased as a double-stranded oligonucleotide from Promega Corporation (Madison, WI) (Matheos et al., 1998). The 27-bp GATA oligonucleotide (5' CACTTGATAACAGAAAGT GATAACTCT 3') was purchased as a double-stranded oligonucleotide from Santa Cruz Biotechnology, Inc (Santa Cruz, CA).

Antibodies

Clone 162 mouse monoclonal antibody (Neomarkers, Union City, CA) recognizes a conformational epitope of the Ku70/Ku86 dimer and was used in the *in vitro* replication and the supershift assays. The anti-Ku86 (C-20) goat polyclonal, anti-Oct-1 (C-21) rabbit polyclonal, anti-PCNA (PC10) mouse monoclonal, anti-DNA-PKcs (C-19) goat polyclonal, anti-topoisomerase IIβ (C-19) goat polyclonal, and the anti-actin (H-196) rabbit polyclonal antibodies, used in Western blot analyses, were purchased from Santa Cruz Biotechnologies, Inc. The anti-DNA polymerase ε (clone 93GIA) mouse monoclonal and the anti-DNA primase (p58) rabbit polyclonal antibodies, also used in Western blot assays, were purchased from Neomarkers. The anti-DNA polymerase δ (clone 22) mouse monoclonal antibody was purchased from Transduction Laboratories/BD PharMingen (Mississauga, Ontario, Canada). The anti-RF-C mouse monoclonal antibody was kindly provided by Dr. B. Stillman (Cold Spring Harbour Laboratory, Cold Spring Harbour, New York, NY). The anti-ORC-1 rabbit polyclonal antibody was kindly provided by Dr. D.G. Quintana (Brigham and Women's Hospital and Harvard Medical School, Boston, MA). The control antibody used in the *in vitro* replication and the supershift assays is a goat polyclonal IgG (Sigma, St-Louis, MO).

Preparation of cell extracts

HeLa S3 nuclei and cytosol were purchased from Cellex Biosciences (Minneapolis, MN). The extracts were made as previously described (Pearson et al., 1991; Matheos et al., 1998). The protein concentrations of the nuclear and cytoplasmic extracts were 5.3 mg/ml and 7.0 mg/ml, respectively.

Affinity Purification of OBA (apOBA)

Total HeLa cell extracts were fractionated and OBA was purified on an A3/4 DNA affinity column, as previously described (Ruiz et al., 1999).

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSA) were performed as described previously (Matheos et al., 1998; Ruiz et al., 1999). Reactions were carried out in 20 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, 1 μg poly (dI-dC) (Amersham Pharmacia Biotech, Baie d'Urfé, Québec, Canada), with 10 μg total HeLa cell extracts or 50 ng affinity-purified OBA (apOBA), and 10 ng of ^{32}P-labeled 186-bp DNA or 0.5 ng ^{32}P-labeled A3/4, respectively, in a final volume of 20 μl. The reaction mixtures were incubated for 30 minutes on ice. In the supershift assays, 1.5 μg of antibody was added to the EMSA reaction, following the 30 minute incubation, and further incubated for 3 hours at 4°C. The samples were subjected to electrophoresis on a 6% PAGE gel in 1X TBE. The gels were dried and exposed for autoradiography.

In vitro DNA Replication Assays

I. Mammalian *in vitro* DNA replication

In vitro replication was carried out as previously described (Matheos et al., 1998), with slight modifications. Standard reactions included HeLa cytoplasmic (79.5 μg) and nuclear (28.8 μg) extracts, 2 mM ATP, 100 mM each CTP, GTP, UTP, dATP, and dGTP, 10 μCi each of [α^{32}P]-dTTP and [α^{32}P]-dCTP, 2 units of pyruvate kinase, and 200 ng of input plasmid DNA (or equimolar amounts, relative to p186, if different origin-containing plasmids were to be compared).

The molar excess of the various ds oligonucleotides was in relation to the input plasmid DNA. For example, if 200 ng of p186 plasmid DNA (4549-bp) was used, then 1.58 ng of A3/4 ds oligonucleotide (36-bp) would represent equimolar amounts and 15.8 ng would represent 100-fold molar excess.

The experiments were performed by pre-incubating the HeLa cell extracts with the ds oligonucleotide or the antibody on ice for 20 minutes. When apOBA or BSA were used to replace the OBA/Ku that was competed for by the A3/4 ds oligonucleotide, they were added to the reaction mixture containing the extracts and 10-fold molar excess of A3/4 ds oligonucleotide.

Following the pre-treatment steps, the reactions were performed as described in Matheos et al. (1998), at 30°C for 1 hour. The reaction products were purified using the QIAquick PCR Purification kit (QIAGEN).

Samples were digested with 1 unit of *Dpn*I (New England Biolabs, Mississauga, Ontario, Canada) for 60 minutes at 37°C in the presence of 1X NEB 4 buffer (50mM KCl, 20 mM Tris acetate, 10 mM magnesium acetate, 1 mM DTT) and 200 mM NaCl. The samples were separated on 1% agarose gel in 1X TAE buffer (16-20 hours, 50-55 Volts).

Quantitation was performed as previously described (Matheos et al., 1998), on *Dpn*I-digested products by densitometric measurements, using a phosphoimager analyzer (Fuji BAS2000, Stamford, CT). Quantitation of the supercoiled (form I) band was not possible because of overlap with the *Dpn*I digestion products. These results were corrected for the amount of DNA recovered from the *in vitro* replication assay by quantitative analysis of the ethidium bromide picture of the gel (not shown). The amount of radioactive precursor incorporated into the DNA was expressed as a percentage of the control reaction.

II. SV40 *in vitro* DNA replication

SV40 *in vitro* replication assays were performed using the CHIMERx (Madison, WI) SV40 large T Antigen DNA replication assay kit, according to the manufacturer's specifications.

Immunoprecipitation and Immunodetection Assays

Total HeLa cell extracts (700 μg) were incubated overnight with 4 μg of clone 162 antibody, anti-Oct-1 antibody or a pre-immune rabbit serum (Santa Cruz) at 4°C, on a rocker platform. The antibody-bound complex was recovered by adding 30 μl of Protein-A agarose beads (Santa Cruz). After a 3 hour incubation at 4°C, the immunoprecipitates were collected by centrifugation at 2500 rpm for 5 minutes at 4°C. The pellet was washed four times with RIPA (1X PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) and resuspended in a final volume of 40 μl electrophoresis sample buffer (50 mM Tris-HCl (pH 6.8) 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol).

Western blots were performed as previously described (Matheos et al., 1998). In brief, 20 µl of the immunoprecipitates were resolved by 6% or 8% SDS-PAGE, proteins were electroblotted to Immobilon-P membranes (Millipore, Mississauga, Ontario, Canada) and probed with various antibodies, as stated in the figure legends. The blots were developed using the Western Blot Chemiluminescence Reagent (NEN Life Sciences, Guelph, Ontario) with the appropriate horse-radish-peroxidase (HRP)-labeled conjugated antibodies (Santa Cruz). The membranes were exposed for autoradiography.

Helicase Assays

a. Preparation of the helicase substrate

The helicase substrate, consisting of an γ^{32}P-labeled oligonucleotide annealed to single-stranded M13mp19 phage DNA to create a partial duplex, was prepared as described in Tuteja et al. (1990), with the following modifications. A 17-mer, 5' GTTTTCCCAGTCACGAC 3', was synthesized by GIBCO BRL. 100 ng of the 17-mer was end-labeled with T4 polynucleotide kinase (New England Biolabs) and [γ^{32}P]-ATP as described in Ruiz et al. (1999) and purified by NICK column (Amersham Pharmacia Biotech). The labeled oligonucleotide was annealed to 2.5 µg of single-stranded circular M13mp19 DNA in 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl and 1 mM DTT, by heating at 95°C for 1 minute, 65°C for 2 minutes, and then cooling slowly to room temperature. The annealed helicase substrate was purified by passage through a microcon-50 column (Amicon, Beverly, MA).

b. Helicase assay

The ability of OBA to unwind the helicase substrate was determined as described in Tuteja et al. (1990). The reaction mixture contained 20mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 4 mM ATP, 60 mM KCl, 8 mM DTT, 4% w/v sucrose, 80 µg/ml BSA, 4000 cpm ^{32}P-labeled helicase substrate and 500 ng of apOBA (unless otherwise indicated). The reactions were resolved by 12% non-denaturing PAGE followed by autoradiography. DNA unwinding activity was expressed as percent unwinding and was determined by

quantitation using a FUJI BAS 2000 phosphoimager. The percent unwinding represents the amount of unwound 17-mer/amount of annealed substrate.

IV. RESULTS

The anti-Ku antibody inhibits in vitro DNA replication

A specific interaction between OBA and the A3/4 DNA sequence has been previously demonstrated (Ruiz et al., 1999). To confirm that both subunits of the Ku protein, Ku70 and Ku86, were involved in the binding of the apOBA to A3/4, a supershift assay was performed using the clone 162 antibody, which recognizes the Ku heterodimer. When ^{32}P-labeled A3/4 DNA was reacted with apOBA, two complexes were detected (Figure 1, lane 2). The slower migrating complex results from the interaction between Ku70/Ku86 with A3/4, while the faster migrating complex arises from the association with Ku70/Ku69; Ku69 is a truncated form of Ku86 which results from site-specific cleavage by a leupeptin-sensitive protease (Knuth et al., 1990; Paillard and Strauss, 1993; Quinn et al., 1993, Han et al., 1996; Jeng et al., 1999). The levels of the truncated complex may vary depending on the leupeptin concentration (Jeng et al., 1999). The same two complexes, in addition to some faster-migrating degradation or nonspecific complexes, were obtained when A3/4 was reacted with HeLa cell extracts (Figure 1, lane 5), but in this case, the level of the truncated complex was reduced due to a higher leupeptin concentration used in the preparation of the HeLa cell extracts (5 μM) compared to that of OBA (1 μM). Both of these complexes, the Ku70/Ku86-A3/4 and the Ku70/Ku69-A3/4, were supershifted by clone 162 antibody (Figure 1A, lanes 3 and 6), but not by the control antibody, a goat polyclonal IgG (Figure 1, lanes 4 and 7).

To investigate the role of Ku antigen in replication, clone 162 antibody was added to the *in vitro* DNA replication reaction of p186 (Todd et al., 1995). The HeLa cell extracts were preincubated with the antibody for 20 minutes, then the origin-containing plasmid was added and the reaction was carried out (see Materials and Methods). The replication reaction products typically included open circular (form II), linear (form III) and supercoiled (form I) forms of the plasmid DNA (Figure 2A). Topoisomeric forms (T), and replicative intermediates (RI) were also obtained, in agreement with previous studies (Decker et al., 1986; Pearson et al., 1991; Zannis-Hadjopoulos et al., 1994;

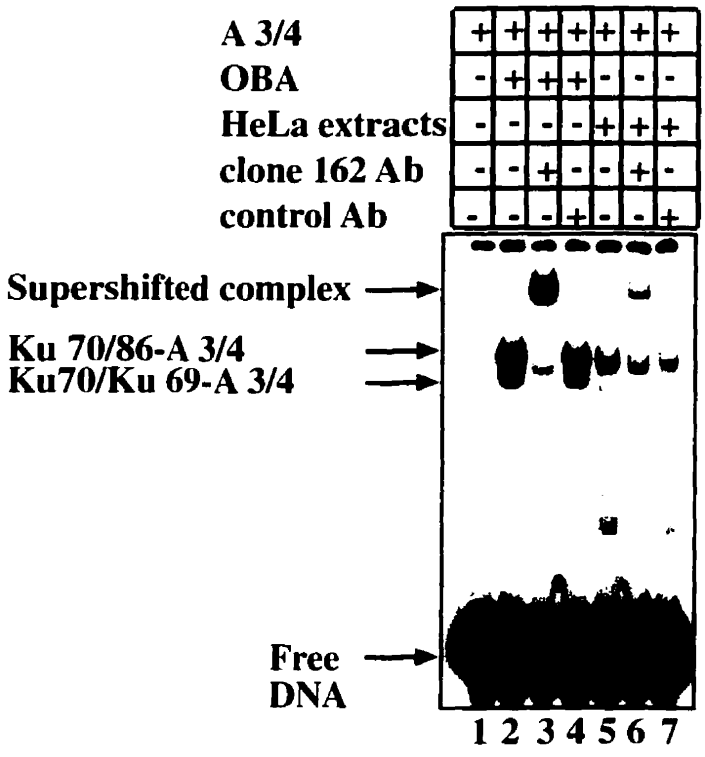
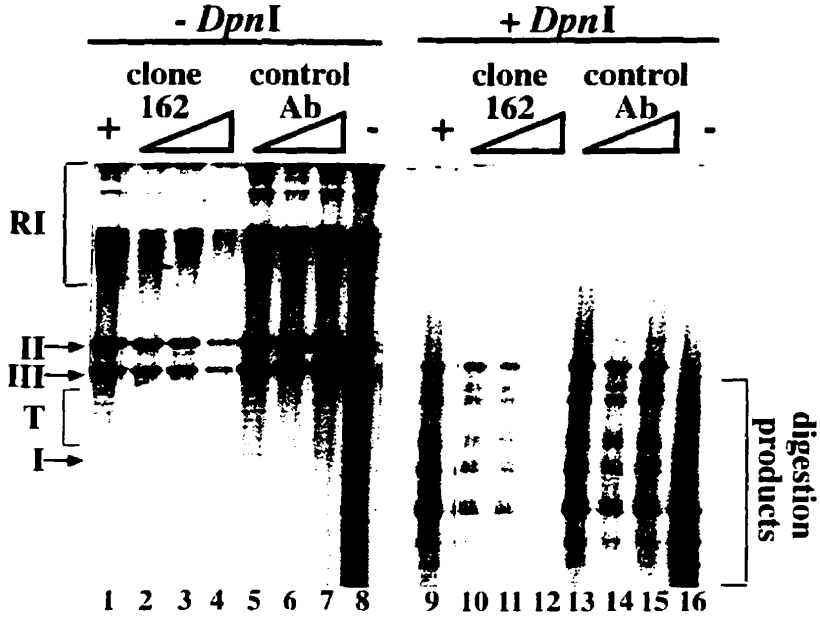


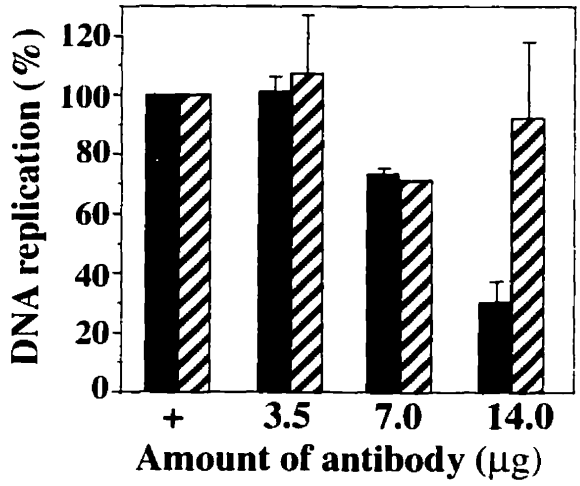
Figure 1. Bandshift/supershift assay illustrating the binding specificity of OBA/Ku to the A3/4 DNA sequence. Reactions contained 0.5 ng of labeled A3/4 and 50 ng apOBA or 10 µg of total HeLa cell extracts, as indicated. The bandshift reaction was incubated on ice for 30 minutes. Then, 1.5 µg of clone 162 or control goat IgG antibody was added and further incubated at 4°C for 3 hours. The reactions were loaded on a 6% PAGE gel and subjected to electrophoresis at 180 Volts. The Ku70/Ku86-A3/4, the Ku70/Ku69-A3/4, and the supershifted complexes are indicated.

Figure 2. A. Clone 162 antibody inhibits p186 DNA replication. Typical autoradiograph of the *in vitro* replication products upon addition of the clone 162 (lanes 2-4, 10-12) or control antibodies (lanes 5-8, 13-15). HeLa cell extracts were incubated with increasing concentrations of clone 162 or control antibody (lanes 2, 5, 10, 13: 3.5 μg; lanes 3, 6, 11, 14: 7.0 μg; lanes 4, 7, 12, 15: 14.0 μg) on ice for 20 minutes. Subsequently, the p186 template DNA and the remaining *in vitro* replication reaction components were added and the samples were incubated at 30°C for 1 hour. The products were purified as described in Materials and Methods. A fraction of the reaction was undigested (-*Dpn*I; lanes 1-8), while the rest was digested (+ *Dpn*I; lanes 9-16) with 1 unit of *Dpn*I for 1 hour. The samples were electrophoresed on a 1% agarose gel in 1X TAE buffer at 50 Volts for 16-20 hours. The gels were dried and exposed to autoradiography. Lane 1 contains the + control (p186 reaction without any antibody) while lane 8 contains the negative control (pBR322 plasmid without a mammalian replication origin). The supercoiled (I), the relaxed circular (II), the linear (III), the topoisomeric (T), and the replicative intermediate (RI) forms of the DNA are indicated. B. Quantitation data performed on the *Dpn*I-digested samples. Data are expressed as a percentage of the control p186 *in vitro* reaction lacking any antibody. Each error bar represents 6 experiments. Black bars represent addition of clone 162 antibody and striped bars represent addition of the control goat polyclonal antibody. C. As in A. except that anti-DNA polymerase δ antibody was added to the replication reaction of p186 at the amounts indicated. Blacks bars represent addition of anti-DNA polymerase δ antibody and striped bars represent addition of the control antibody,

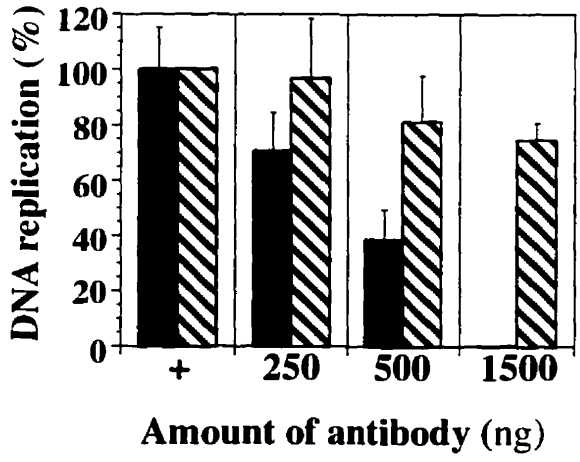
A.


- DpnI
+ DpnI
clone 162
control Ab
+
-
RI
II
III
T
I
digestion products
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

B.


DNA replication (%)
120
100
80
60
40
20
0
+
3.5
7.0
14.0
Amount of antibody (μg)

C.


DNA replication (%)
120
100
80
60
40
20
0
+
250
500
1500
Amount of antibody (ng)

Matheos et al., 1998). Quantitation of the *Dpn*I-resistant DNA, performed as described previously (Matheos et al., 1998), showed that addition of the Ku-specific antibody inhibited replication by as much as seventy percent at the highest concentration (14 μg) used (Figure 2B). In contrast, the control antibody did not affect the replication levels. The level of inhibition obtained with the clone 162 antibody, which targets both Ku subunits, is stronger by comparison to the inhibition previously seen with either the Ku70 or the Ku86 antibodies separately (Ruiz et al., 1999). The specificity of the molar amount of antibody used has previously been demonstrated by the neutralization of the Ku-specific antibodies with their specific blocking peptides (Ruiz et al., 1999).

As shown previously, *in vitro* replication is dependent on DNA polymerases α and/or δ (Pearson et al., 1991). To further validate this finding and our strategy for the *in vitro* Ku competition-replication assay, anti-DNA polymerase δ antibody was added to the *in vitro* replication reaction of p186 (Figure 2C). 60% inhibition of replication was obtained with only 500 ng anti-DNA polymerase δ antibody, and no replication was observed with 1500 ng of antibody. These results confirm the previous ones indicating that DNA polymerase δ is required in the mammalian *in vitro* replication system to mediate DNA synthesis. The amount of anti-DNA polymerase δ antibody required to completely abolish DNA synthesis was significantly lower than the amount of clone 162 antibody. This might be due either to different affinities or concentration of the two specific antibodies or to the essential role of DNA polymerase δ on replication. Similar results with anti-DNA polymerase δ have also been obtained using the SV40 DNA replication system (Lin et al., 1997).

A3/4 inhibits the in vitro DNA replication of mammalian origins

It was previously shown that the A3/4 ds oligonucleotide inhibits the *in vitro* DNA replication of p186 (Ruiz et al., 1999). To investigate the effect of the A3/4 sequence on the replication of other origin-containing plasmids, we performed similar *in vitro* DNA replication assays in which the HeLa cell extracts were preincubated with increasing amounts of the A3/4 ds oligonucleotide and then different origin-containing plasmids were used as template in the *in vitro* replication reaction (Figure 3A). The plasmids used included: a) p*ors*12, a plasmid containing the 215-bp minimal origin of

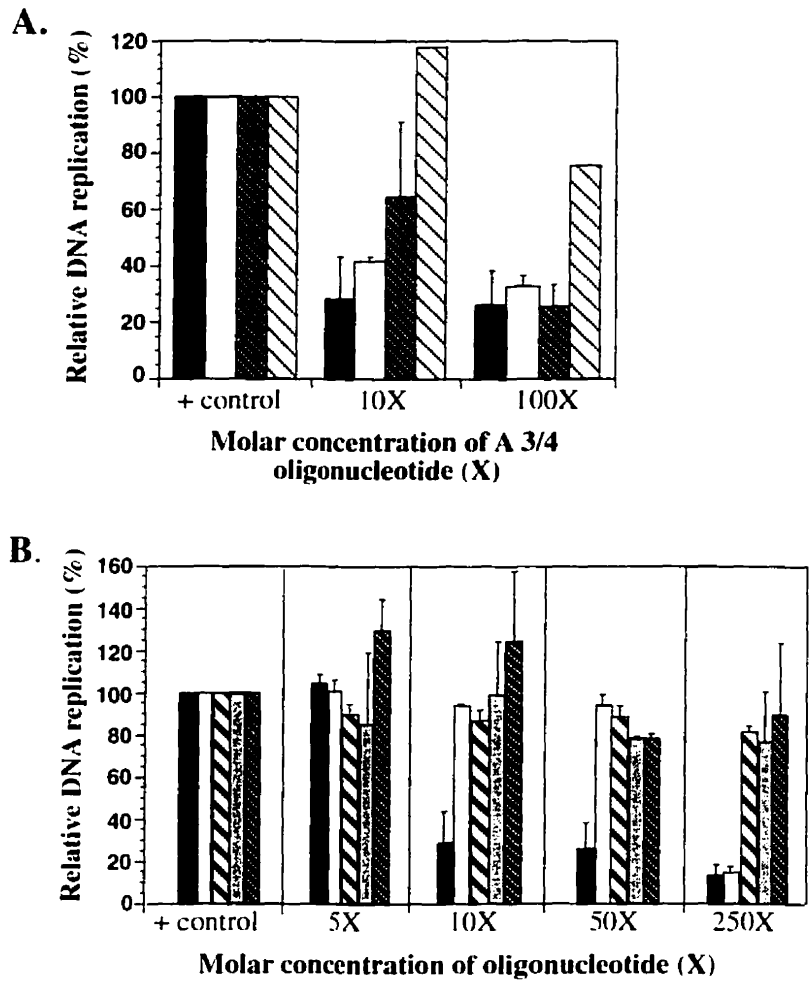


Figure 3. A. Effect of the addition of the A3/4 ds oligonucleotide on the *in vitro* replication of different origin-containing plasmids. Plasmids p186 (black bars), p*ors*12 (white bars), pX24 (black bars with stripes) or pSV40 (white bars with stripes) were used as template for *in vitro* replication. Increasing molar excess amounts (10 or 100X- molar excess) of the A3/4 ds oligonucleotide, relative to the input template DNA, were added and incubated with HeLa cell extracts, on ice for 20 minutes, prior to the replication reaction. The *in vitro* replication products were purified, digested with *Dpn*I, and the *Dpn*I-resistant bands were quantitated using a phosphoimager. The amount of radioactive precursor incorporated into the DNA is expressed as a percentage of the control (no oligonucleotide added) reaction. Error bars indicate at least 4 experiments. B. Effect of different ds oligonucleotides on the *in vitro* replication reaction of p186. 5X, 10X, 50X and 250X molar excess of five different oligonucleotides (A3/4: black bars; Oct-1: white bars, GATA: white bars with stripes; nonspecific: gray bars; random: black bars with stripes) were pre-incubated with the HeLa cell extracts, as above. The reactions and the quantitations were performed as indicated in part A. Error bars represent at least 4 experiments.

*ors*12 (Pelletier et al., 1997), a monkey autonomously replicating sequence (Frappier and Zannis-Hadjopoulos, 1987), which has been shown to function as an origin at its chromosomal location (Pelletier et al., 1999); b) pX24, which contains the Chinese hamster DHFR origin of DNA replication, oriβ (Burhans et al., 1986) and which can replicate autonomously *in vivo* and *in vitro* (Zannis-Hadjopoulos et al., 1994; Diaz-Perez et al., 1996), and finally, c) pSV40, which consists of a recombinant pUC plasmid containing the viral SV40 origin (Li and Kelly, 1984). The *in vitro* replication reaction of p186 was also included for comparison (Figure 3A). Addition of increasing amounts (10X and 100X molar excess) of the A3/4 ds oligonucleotide to the *in vitro* reaction inhibited p186 replication by approximately 70-75% by comparison to the control reaction (Figure 3A, black bars). It also strongly inhibited p*ors*12 (55% at 10X and 65% at 100X molar excess) (Figure 3A, white bars) and pX24 (30% at 10X and 75% at 100X molar excess) (Figure 3A, black bars with stripes) replication levels. pX24 has been shown to bind apOBA (Ruiz et al., 1999) and p*ors*12 contains an A3/4 homologous region (Price et al., in preparation). In contrast, no significant effect on pSV40 *in vitro* replication resulted by the addition of the A3/4 ds oligonucleotide (Figure 3A, white bars with stripes). A 20% decrease in replication was observed at the 100X molar excess of the A3/4 ds oligonucleotide, which is comparable to the effect observed with nonspecific oligonucleotides (Figure 3B). The pSV40 plasmid contains no significant homologies to A3/4. The inhibition of replication induced by the A3/4 ds oligonucleotide is apparently produced at the level of initiation of replication, rather than elongation, since only origins with A3/4 homology were affected, while the replication of the SV40 origin-containing plasmid was not.

The specificity of the A3/4 effect was examined through the addition of several different oligonucleotides to the p186 replication reaction (Figure 3B). The Oct-1 ds oligonucleotide (white bars), comprising the binding site for the Oct-1 transcription factor, has previously been shown to inhibit replication of p186 (Matheos et al., 1998), although to a lesser extent than A3/4 (black bars). Here, a higher concentration (250X-molar excess) of Oct-1 ds oligonucleotide, compared to A3/4 (10X-molar excess), was required to decrease replication to basal levels. No inhibition was observed upon addition of the 27-bp GATA ds oligonucleotide (white bars with stripes). GATA-1 (Pevny et al.,

1991; Merika and Orkin, 1993) is a transcription factor that has been shown to inhibit bacterial initiation of DNA replication (Trudel et al., 1996). Several binding sites for the GATA family of transcription factors are present in the 186-bp fragment of *ors*8 (unpublished data). Finally, a 16-bp nonspecific (grey bars) and a 29-bp random (black bars with stripes) ds oligonucleotide did not affect *in vitro* DNA replication at the concentrations used. Thus, the inhibitory effect of both the A3/4 and Oct-1 ds oligonucleotides on p186 replication *in vitro* is specific.

Addition of apOBA to the A3/4-inhibited reaction restores replication levels

To test whether the inhibitory replication effect of the A3/4 ds oligonucleotide was caused by the competition for OBA/Ku, increasing amounts of apOBA were added to the p186 *in vitro* replication reaction that had been inhibited by the addition of 10X-molar excess of A3/4 ds oligonucleotide (Figure 4A, B). The products of the replication reaction were digested with *Dpn*I and subjected to electrophoresis (Figure 4A). The amount of DNA loaded in each lane was comparable, as verified by agarose gel-ethidium bromide staining (data not shown). Quantitation of the *Dpn*I-resistant DNA showed that addition of apOBA fully restored replication levels, whereas addition of the same amount of BSA did not result in any change (Figure 4B). The results indicate that the increase in replication obtained with the addition of apOBA is due to the restoration of the essential levels of OBA/Ku.

Interaction between OBA/Ku and Oct-1

Both Oct-1 and OBA can specifically bind to the minimal origin of *ors*8 and are required for *in vitro* DNA replication (Matheos et al., 1998; Ruiz et al., 1999). However, the enhancing replication effect of Oct-1 is not due to its direct binding to the origin, but rather through a mechanism involving protein-protein interactions (Matheos et al., 1998). Co-immunoprecipitation assays were carried out to determine whether an interaction between Oct-1 and OBA is taking place (Figure 5A). When OBA/Ku was immunoprecipitated from HeLa cell extracts using clone 162 antibody, Oct-1 was also detectable (Figure 5A, lane 2). In a complementary experiment, when Oct-1 was immunoprecipitated using an anti-Oct-1 antibody, a high amount of Ku was also brought

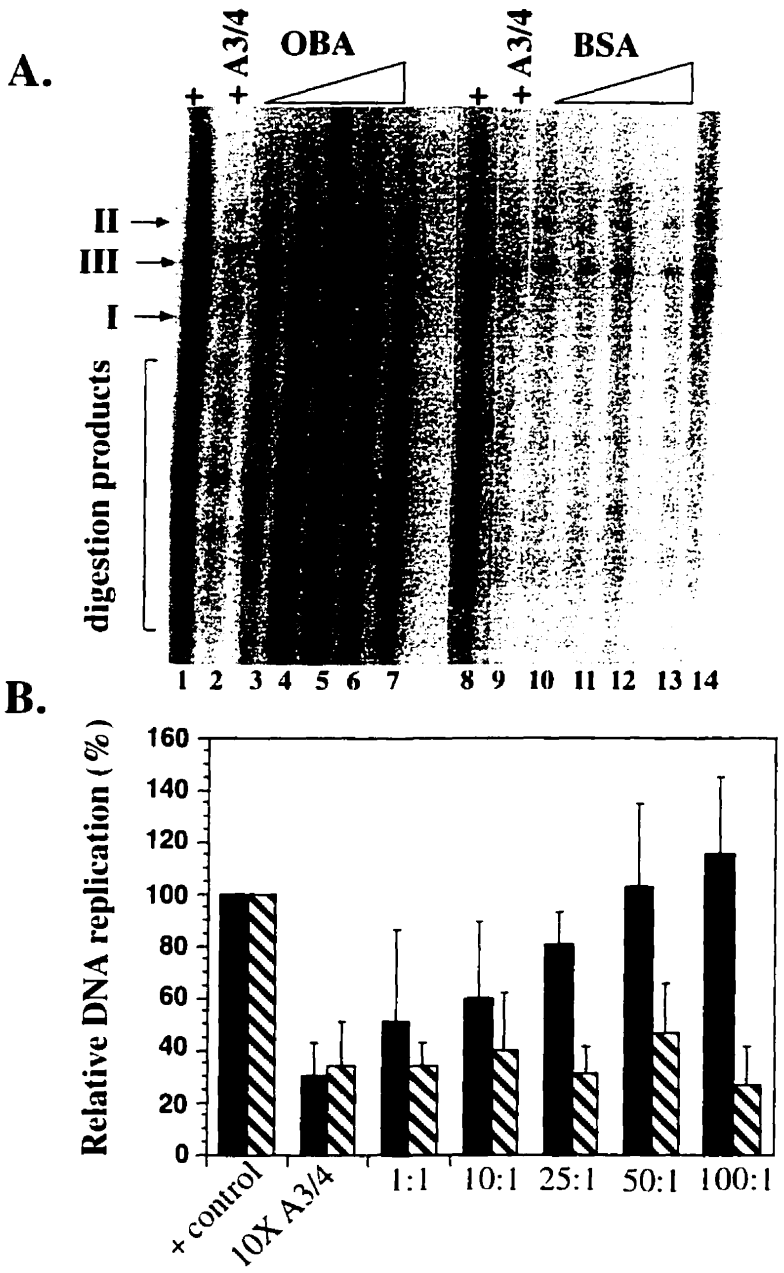


Figure 4. Addition of OBA to the A3/4-inhibited p186 reaction restores replication levels. Increasing amounts of apOBA or BSA were added to the p186 *in vitro* replication reaction that had previously been inhibited by the addition of 10X molar excess of A3/4 ds oligonucleotide. The estimated molar amount of protein added was in excess of the A3/4 ds oligonucleotide. A. Autoradiograph of *Dpn*I-digested products (lanes 1-7, apOBA added; lanes 8-14, BSA added). The open circular, linear, and supercoiled forms of the plasmid, in addition to the *Dpn*I-digestion products are indicated. B. Bar graph showing the relative DNA replication of the A3/4-inhibited p186 reaction in the presence of increasing amounts of OBA (black bars) or BSA (striped bars). The ratio indicated represents the amount of protein added in molar excess of the A3/4 ds oligonucleotide. Error bars represent 4 experiments performed in duplicate.

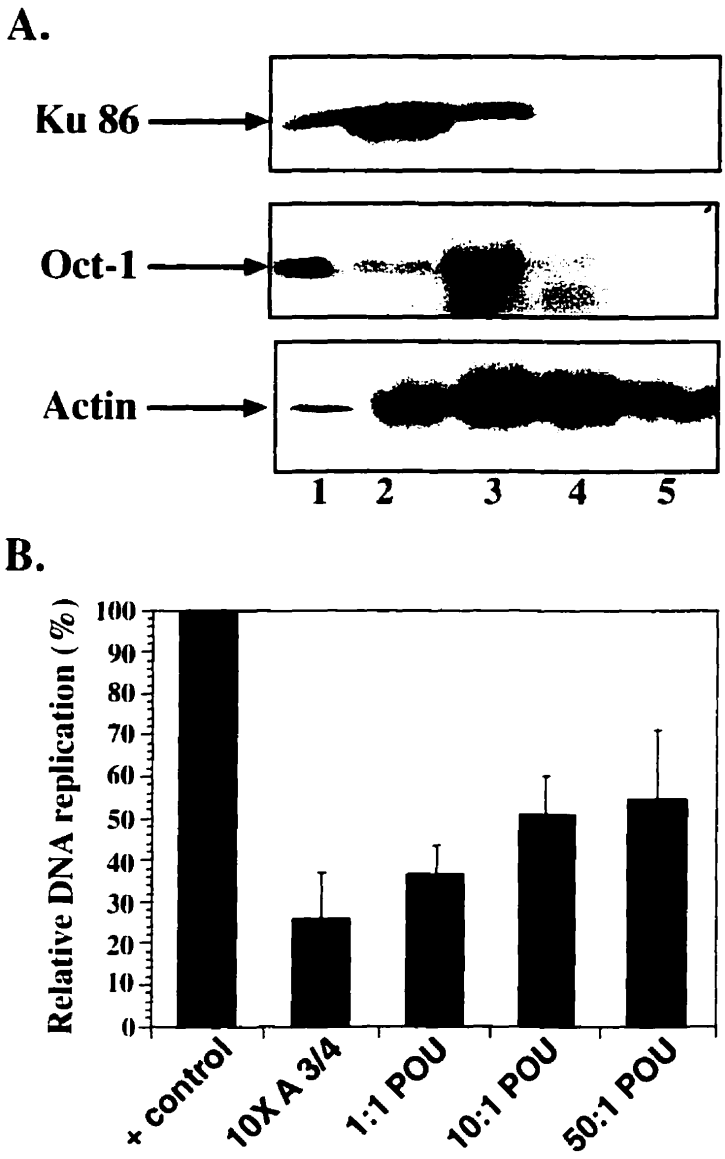


Figure 5. A. Immunoprecipitation assay showing the interaction of OBA/Ku and Oct-1. HeLa cell extracts (lane 1) were incubated with an anti-Ku (clone 162) (lane 2) antibody, anti-Oct-1 (lane 3) antibody or pre-immune rabbit serum (NRS) (lane 4). Immunodepletion was performed by adding Protein-A agarose beads. The beads were washed and recovered by centrifugation. Then, they were loaded on 8% SDS-PAGE gel, transferred and probed with 1/500 anti-Ku86, 1/500 anti-Oct-1 or 1/1000 anti-actin. Lane 1: 50µg HeLa cell extracts, lane 2: clone 162 precipitate, lane 3: anti-Oct-1 antibody precipitate, lane 4: NRS precipitate, lane 5: Protein-A agarose beads alone. B. Effect of the addition of Oct-1 POU domain protein to the A3/4-inhibited reaction. The indicated molar amounts of Oct-1 POU protein (relative to the amount of A3/4 ds oligonucleotide) were added to the p186 *in vitro* reaction that had previously been inhibited by the addition of a 10X molar excess of A3/4 ds oligonucleotide.

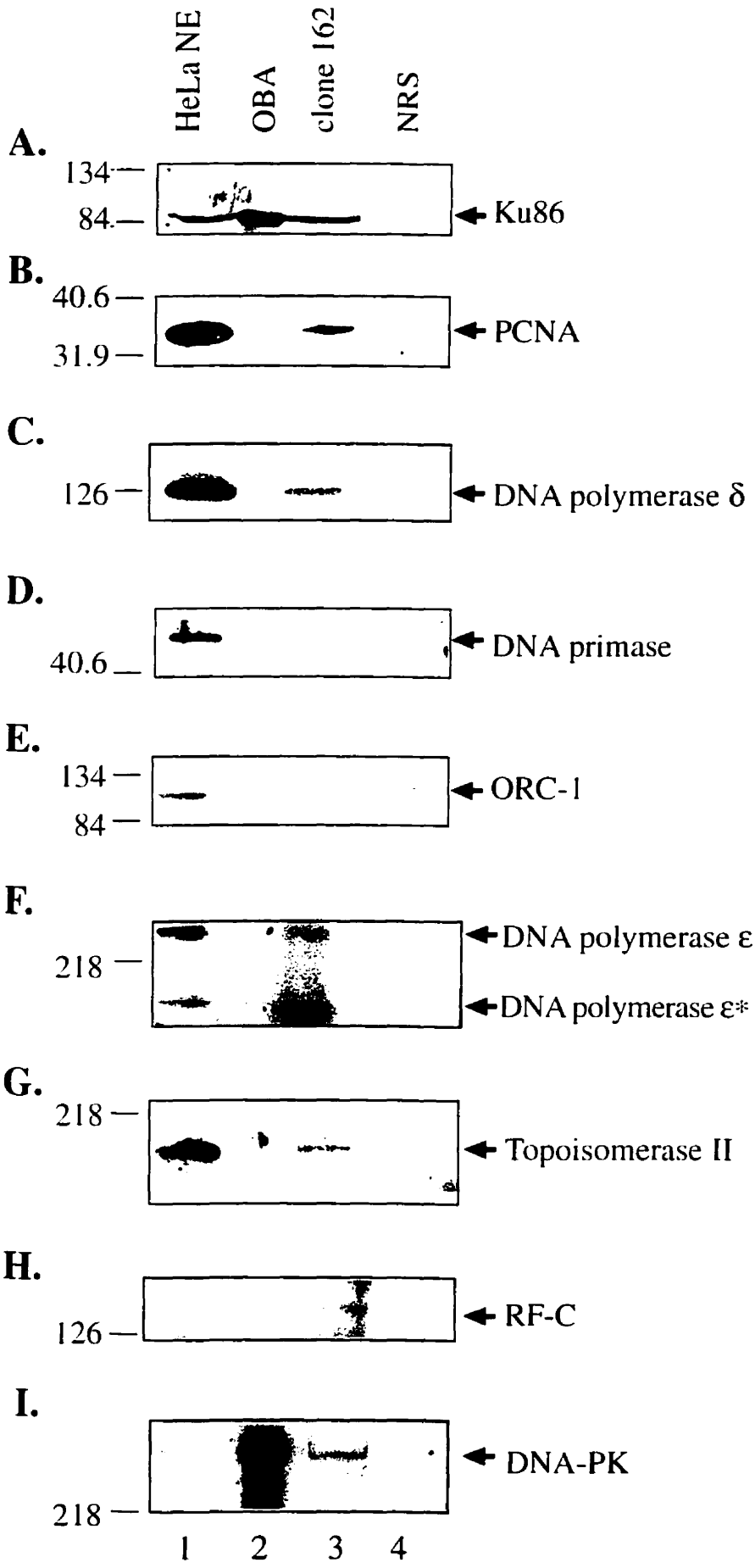
down (Figure 5A, lane 3). The level of Oct-1 precipitated with the Ku antibody is lower than the level of Ku precipitated with the Oct-1 antibody (Figure 5A, lanes 2). A possible reason for this result might be that most of the Oct-1 present in the cell extracts is associated with Ku, but a substantial amount of Ku is not associated with Oct-1. In contrast, no precipitate was obtained either when normal rabbit serum (NRS) (Figure 5A, lane 4) or when the beads alone (Figure 5A, lane 5) were used, indicating the specificity of the Ku/Oct-1 interaction.

In light of the interaction found between OBA/Ku and Oct-1 and since both these proteins are involved in p186 *in vitro* replication, we tested whether addition of Oct-1 alone, could restore the p186 *in vitro* replication levels, in reactions that had been previously inhibited by the addition of 10X molar excess of A3/4 ds oligonucleotide. As shown in Figure 5B, addition of increasing amounts of the POU domain of Oct-1 to these reactions, steadily increased the replication levels of p186, partially restoring the inhibition caused by the A3/4 ds oligonucleotide. At the highest ratio (50:1) of POU added relative to the amount of A3/4 ds oligonucleotide used to inhibit replication, the level of replication increased by approximately a factor of 2, to 50-60% of control (Figure 5B). These results are in agreement with those obtained previously in reconstitution experiments of Oct-1-depleted extracts, in which the POU domain did not fully restore replication (Matheos et al., 1998).

Association of OBA/Ku with replication proteins

To test whether Ku associated with other proteins involved in DNA replication, further immunoprecipitation assays were performed (Figure 6). When OBA/Ku was immunoprecipitated from HeLa nuclear extracts using clone 162 antibody, PCNA (36 kDa, Figure 6B) DNA polymerase δ (125 kDa, Figure 6C), DNA polymerase ε (>200 kDa Figure 6F), topoisomerase II (180 kDa, Figure 6G), and RF-C (140 kDa, Figure 6H) were also precipitated. The anti-DNA polymerase εantibody also recognized the truncated DNA polymerase ε form, DNA polymerase ε* (140 kDa, Figure 6F), that has lost its C-terminus (Prapurna and Rao, 1997). The anti-RF-C antibody recognized extremely low levels of RF-C in the nuclear extracts (Figure 6H, lane 1), visible only upon long autoradiographic exposures (data not shown), in contrast to the clone 162

Figure 6. Immunoprecipitation assay showing OBA/Ku interactions with replication proteins. Ku was precipitated from HeLa cells using clone 162 antibody or normal rabbit serum (NRS) and protein-A agarose beads. Following electrophoresis on 8% SDS-PAGE (or 6% SDS-PAGE for DNA polymerase ε, Topoisomerase II, and DNA-PK_{cs}) and transfer to Immobilon-P membrane, blots were probed with: A. 1/400 anti-Ku86, B. 1/200 anti-PCNA, C. 1/1000 DNA polymerase δ, D. 1/1000 anti-DNA primase, E. 1/2000 anti-ORC-1, F. 1/100 anti-DNA polymerase ε, G. 1/100 anti-topoisomerase II, H. 1/100 anti-RF-C, and I. 1/25 anti-DNA-PK_{cs}. Lane 1: 60-80 μg HeLa nuclear extract, lane 2: 300 ng apOBA, lane 3: clone 162 precipitate, lane 4: NRS precipitate.


HeLa NE
OBA
clone 162
NRS
A.
134
84
Ku86
B.
40.6
31.9
PCNA
C.
126
DNA polymerase δ
D.
40.6
DNA primase
E.
134
84
ORC-1
F.
218
DNA polymerase ε
DNA polymerase ε*
G.
218
Topoisomerase II
H.
126
RF-C
I.
218
DNA-PK
1
2
3
4

precipitate (Figure 6H, lane 3), where a significant amount of RF-C was detectable. None of the replication proteins precipitated when the normal rabbit serum (NRS) was used (Figure 6, lane 4), demonstrating their specific association with OBA/Ku. The replication proteins tested are part of a mammalian DNA replication complex, the DNA synthesome, that is capable of supporting SV40 *in vitro* DNA replication (reviewed in Malkas, 1998). Not surprisingly, the catalytic subunit of DNA-PK, DNA-PKcs, was also found associated with Ku (Figure 6I, 475 kDa), since together they form the DNA-PK holoenzyme. The apOBA preparation (Figure 6I, lane 2) also contained DNA-PKcs, as previously reported (Ruiz et al., 1999). In contrast, OBA/Ku was not found to be associated with either DNA primase (Figure 6D, 58 kDa) or ORC-1 (Figure 6E, 110 kDa). In these reactions, low background levels of the respective proteins were found in both the Ku and NRS immunoprecipitates. DNA primase synthesizes short RNA primers to initiate DNA replication (Wang, 1991). No direct proof of ORC's involvement in the initiation of mammalian DNA replication has yet been demonstrated (Quintana and Dutta, 1999).

OBA has DNA helicase activity

Human DNA helicase II (HDH II), a novel ATP-dependent DNA unwinding enzyme from HeLa cells that exclusively unwinds DNA duplexes in the 3' to 5' direction, has been identified as Ku antigen (Tuteja et al., 1994). Several variants of the Ku dimer exist (Griffith et al., 1992). Because the DNA binding subunit of OBA is identical to Ku86, we tested the helicase activity of the apOBA by assaying the unwinding of radiolabeled oligonucleotide annealed to circular M13mp19 ss DNA (Figure 7). Titration of the apOBA amount required for helicase activity showed that maximal unwinding activity was obtained with 1000 ng of protein (Figures 7A, B). At higher protein concentrations, a drastic drop in helicase activity was observed, which may be due to the excess protein aggregating and no longer being functionally available to unwind the DNA (Figure 7B). The overall unwinding reaction using 1000 ng of apOBA was linear for 20 minutes and reached 70%-75% completion after 30 minutes (Figure 7C, D). The kinetics of the OBA helicase activity are similar to those previously reported to HDH II, in which a maximum value of ~90% was obtained in 30 minutes (Tuteja et al., 1994).

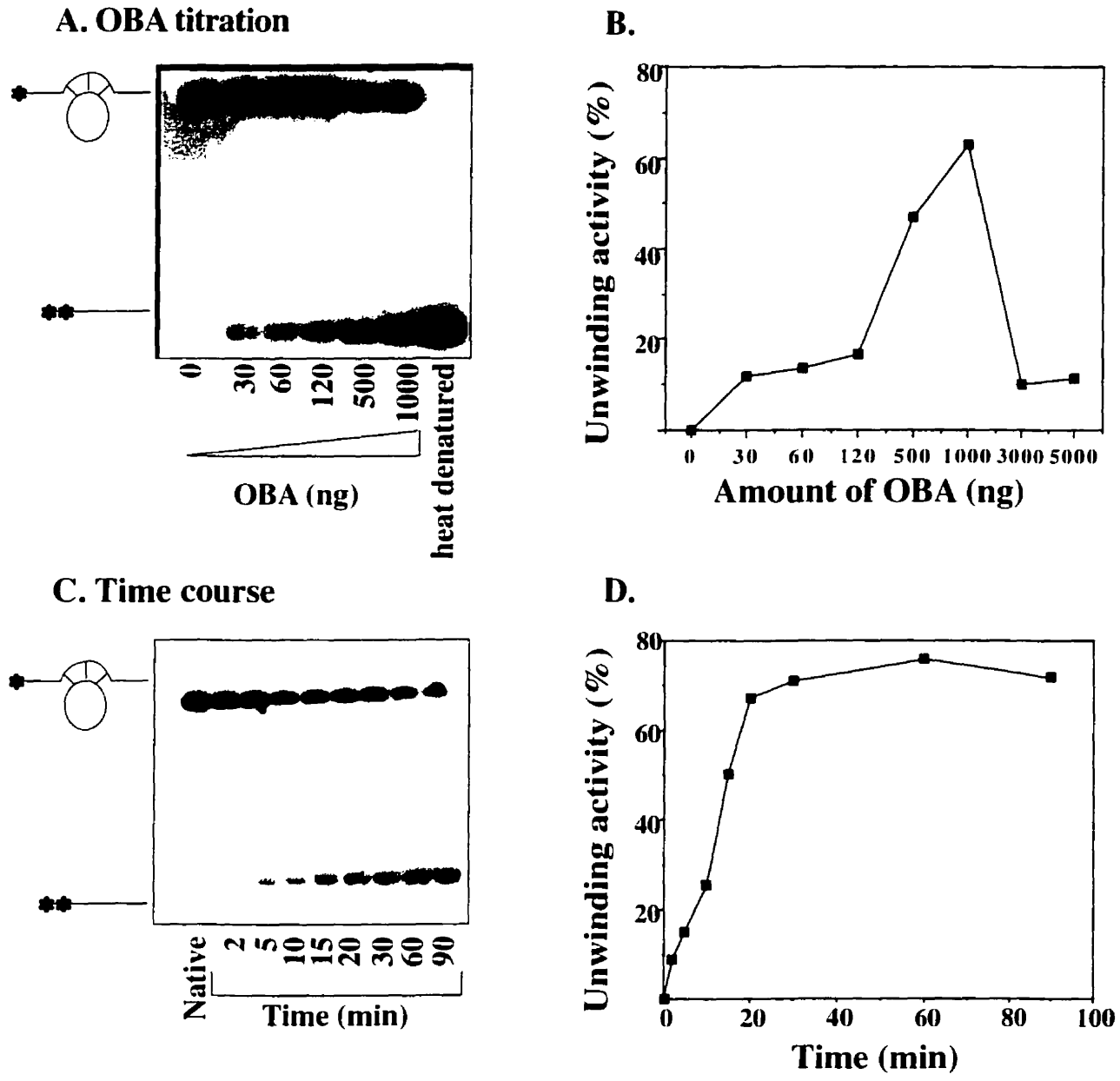


Figure 7. OBA can function as a DNA helicase. A. Titration of amount of apOBA required to unwind the helicase substrate (end-labeled 17-mer annealed to M13). Autoradiograph showing the substrate (*) and the unwound product (**) with increasing amounts of apOBA. B. Quantitation of apOBA's unwinding activity by the use of a phosphoimager. C. Kinetics of the OBA unwinding reaction: 1000 ng of apOBA were reacted with 4000 cpm (1.0 ng) of helicase substrate for the indicated periods of time. D. Quantitation of the time-course reaction depicted in panel C.

V. DISCUSSION

We have previously isolated mammalian (monkey and human) origin-enriched-sequences, *ors*, which are active replication origins in early S-phase. Their functional characterization relied on various replication assays, such as transient autonomous replication *in vivo* (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991), long-term persistence as episomes in mammalian cells (Nielsen et al., 2000), *in vitro* replication (Pearson et al., 1991; Zannis-Hadjopoulos et al., 1992; 1994), electron microscopic analysis of replicating DNA (Frappier and Zannis-Hadjopoulos, 1987; Pearson et al., 1994) and PCR-based mapping *in vivo* (Pelletier et al., 1999). In all the cases examined, the initiation site has been mapped to the *ors*. For all origins examined thus far, the initiation site mapped *in vivo* by PCR methods co-localizes with the sites mapped in transient *in vivo* or *in vitro* replication assays. The *in vitro* replication assay is a faster acceptable alternative to the other *in vivo* replication assays (Pearson et al., 1991, 1994; Zannis-Hadjopoulos et al., 1994).

Using the 186-bp minimal origin of *ors*8 (Todd et al., 1995), we recently identified a specific *Ors*-Binding Activity, OBA, from HeLa cells (Ruiz et al., 1995, 1999). By competition band-shift analyses, the OBA binding site was found to lie within a region of the 186-bp fragment that shares homology with A3/4, a 36-bp sequence generated by comparing various mammalian origins, including the *ors*. OBA was subsequently affinity-purified using an A3/4 affinity column. Microsequencing analysis revealed that the DNA binding polypeptide of apOBA is identical to the 86 kDa subunit of the heterodimeric Ku antigen. The apOBA also contained the 70 kDa subunit and the DNA-PKcs (Ruiz et al., 1999). Preliminary studies suggested a role for Ku in mammalian DNA replication, since its depletion, either by addition of antibodies raised against each of the subunits, or by addition of the A3/4 ds oligonucleotide inhibited *in vitro* DNA replication of p186 (Ruiz et al., 1999).

In the present study, we further examined the role of OBA/Ku in *in vitro* mammalian and viral DNA replication and investigated its interactions with proteins of the replication machinery.

Addition of the A3/4 ds oligonucleotide inhibited the *in vitro* replication of p186 and p*ors*12, plasmids containing the minimal replication origins of *ors*8 and *ors*12 (Todd et al., 1995; Pelletier et al., 1997), respectively, and of pX24, a plasmid containing the Chinese hamster DHFR origin, oriβ, (Zannis-Hadjopoulos et al., 1994; Diaz-Perez et al., 1996) (Figure 3A). All three plasmids were similarly inhibited by the A3/4 ds oligonucleotide; namely, approximately a 70% decrease in replication was observed at the 100-fold molar excess of the oligonucleotide (Figure 3A). p186 contains a region of 85% homology (Ruiz et al., 1999), while p*ors*12 and pX24 contain a region of approximately 70% homology to A3/4 (Ruiz et al., 1999; Price et al., in preparation). Specific binding of OBA to p186 and pX24 has been previously shown (Ruiz et al., 1999). In contrast, SV40 *in vitro* DNA replication was not significantly affected by the addition of the A3/4 ds oligonucleotide (Figure 3A), even at a concentration as high as 500-fold molar excess (data not shown). SV40 has a well-defined replication origin, where the viral large T antigen binds and initiates DNA replication (reviewed in Bullock, 1997). All the other components required for SV40 replication are provided by the mammalian host. Since the SV40 origin does not have a strong homology to the A3/4 sequence, and since the A3/4 ds oligonucleotide does not inhibit SV40 replication, the results suggest that OBA/Ku is involved in the initiation, rather than elongation, step of replication and requires an A3/4-homologous sequence at the origin for binding. Although Ku is the DNA-dependent ATPase purified from HeLa cells which cofractionated with a 21S complex that is able to support SV40 *in vitro* DNA replication (Vishwanathe and Baril, 1990; Cao et al., 1994), our data indicate that Ku is not required for SV40 replication. This is not surprising since origin recognition and DNA unwinding, two of Ku's apparent replication functions, are being carried out by the viral large T antigen.

The specificity of the A3/4 ds oligonucleotide effect was examined through the addition of several different, double-stranded oligonucleotides to the p186 replication reaction (Figure 3B). The oligonucleotides used can be divided into three groups: 1) oligonucleotides that recognize proteins that bind to the minimal origin of *ors*8, such as

the Oct-1 and GATA-1 oligonucleotide; 2) oligonucleotides that represent Ku binding sites, such as the A3/4 and the Negative Regulatory Element 1 (NRE-1) (Schild-Poulter et al., in preparation) oligonucleotide; and 3) oligonucleotides comprised of any DNA sequence, such as the nonspecific and the random oligonucleotide. The 22-bp Oct-1 ds oligonucleotide, which binds to the Oct-1 transcription factor, inhibited p186 replication to basal levels at the 250-fold molar excess concentration (Figure 3B). Oct-1 has been implicated in adenovirus DNA replication (Verrijzer et al., 1990; Coenjaerts et al., 1994; van Leeuwen et al., 1995), where it increases the binding of the pre-terminal protein-polymerase complex to the adenovirus core origin and stabilizes the pre-initiation complex through a direct interaction with the pre-terminal protein (van Leeuwen et al., 1997). Moreover, Oct-1 was found to be an enhancing component of mammalian DNA replication (Matheos et al., 1998). Although depletion of Oct-1, either by addition of the Oct-1 ds oligonucleotide or an anti-Oct-1 antibody, inhibited p186 DNA replication, site-directed mutagenesis showed that the Oct-1 binding site in the origin was not required for efficient replication, suggesting that the Oct-1 protein was not exerting its effect through direct binding to *ors*8, but rather through protein-protein interactions (Matheos et al., 1998). In contrast to Oct-1, the 27-bp GATA ds oligonucleotide did not affect p186 replication (Figure 3B). This oligonucleotide binds the GATA-1 transcription factor for which there exist several potential binding sites within the minimal origin of *ors*8 (unpublished data). GATA-1 is a zinc finger DNA-binding protein that is essential for the development of red blood cells (Pevny et al., 1991). GATA-1 also binds three of the four DnaA protein-binding sites in the *Escherichia coli* origin of replication, oriC, and inhibits initiation of DNA replication (Trudel et al., 1996). A 26-bp NRE-1 ds oligonucleotide, which represents another sequence-specific binding site for Ku antigen (Giffin et al., 1996), has also been tested in the mammalian *in vitro* DNA replication system (Schild-Poulter et al., in preparation). The direct binding of Ku/DNA-PK to NRE-1 represses glucocorticoid-induced MMTV transcription (Giffin et al., 1996, 1997). Surprisingly, the NRE-1 ds oligonucleotide did not affect p186 *in vitro* replication. The most likely explanation for this is a different type of mechanism of Ku sequence-specific binding to NRE-1 versus A3/4. Torrance et al. (1998) reported that, although binding of Ku to double-stranded NRE-1 requires both subunits, it occurs in a two-step fashion, where the

Ku70 subunit binds first to the DNA and then, in a Mg^{+2} dependent step, Ku86 makes contact. Also, Ku has preference for the single, upper strand of NRE-1 (Torrance et al., 1998). This mechanism of Ku binding to NRE-1 is different from that to A3/4, where it is the Ku86 subunit that binds to the double-stranded form of A3/4 DNA; neither of the A3/4 single-strands is bound by Ku, and the presence of Mg^{+2} does not affect the binding (Ruiz et al., 1999). Furthermore, Ku bound to covalently closed microcircles containing the NRE-1 but not the A3/4 sequence (Schild-Poulter et al., submitted), perhaps suggesting that additional factors and/or interactions may aid Ku's binding to A3/4 and contribute to its role in DNA replication (see below). Finally, neither of the two nonspecific oligonucleotides of different size, the 16-bp nonspecific ds oligonucleotide derived from pBR322 and the 29-bp randomly generated ds oligonucleotide, inhibited DNA replication, demonstrating that the *in vitro* replication reaction is not sensitive to the exogenous addition of nonspecific oligonucleotides. Hence, the inhibitory effect of the A3/4 and Oct-1 ds oligonucleotides is specific.

We recently reported that antibodies directed against each of the Ku subunits inhibit replication by approximately 55-65%, whereas nonspecific (control) antibodies do not have any effect (Ruiz et al., 1999). When the antibodies were pre-neutralized with Ku-specific blocking peptides, replication remained at control levels. Here, we showed that the specific inhibition of replication caused by the addition of the A3/4 ds oligonucleotide is due to competition for OBA/Ku and addition of increasing amounts of apOBA fully restored replication levels, while addition of BSA did not have any effect (Figure 3A, B). Taken together, these results suggest that Ku is required for mammalian DNA replication.

Oct-1 and OBA/Ku can specifically bind to the 186-bp fragment of *ors*8 and can enhance DNA replication (Matheos et al., 1998; Ruiz et al., 1999). Oct-1 exerts its effect through a mechanism other than direct binding to the octamer motif in the origin (Matheos et al., 1998). Co-immunoprecipitation studies demonstrated an interaction between OBA/Ku and Oct-1 (Figure 5). Furthermore, the DNA binding POU domain of Oct-1 was partially able to restore replication in an A3/4-inhibited reaction (Figure 5B), suggesting that the interaction between Oct-1 and OBA/Ku is required for optimal *in vitro* replication. In viral systems, the POU domain proteins act as regulators of replication by

interacting with viral initiator proteins. For example, an interaction has been shown between Oct-1 and the adenovirus pre-terminal protein (van Leeuwen et al., 1997), between Oct-1 and the Herpes simplex transactivator protein (O'Hare et al., 1988) and between Oct-6 and the JC papovavirus T-Ag (Renner et al., 1994). Through its interaction with viral initiator proteins, the POU domain stabilizes the pre-initiation complex and positions the replication complex to its target site at the origin (van Leeuwen et al., 1997).

Ku antigen is identical to human DNA helicase II (HDH II), an ATP-dependent DNA unwinding enzyme from HeLa cells (Tuteja et al., 1994). HDH II/Ku exclusively unwinds DNA duplexes in the 3' to 5' direction. Many human DNA helicases have been described (Tuteja et al., 1990, 1993; Seo et al., 1991; Seo and Hurwitz, 1993). Helicases are required during replication, repair, recombination and transcription to unwind the parental DNA strands. Ku antigen is probably not a homogenous protein, since the 70 kDa subunit is produced by a gene family, and thus different variants of the dimer could be produced by the cell for different purposes (Griffith et al., 1992). Upon testing the DNA unwinding activity of OBA, we found that apOBA possessed DNA helicase activity (Figure 7). The maximum unwinding activity obtained was 70%-75% at 30-60 minutes and then reached saturation. This is comparable to the kinetics of HDH II, which produces ~90% unwinding in 30 minutes (Tuteja et al., 1994). Other helicases that have been implicated in DNA replication also have similar unwinding kinetics. The HDH I (Tuteja et al., 1990) and HDH IV (Tuteja et al., 1991) unwinding reactions reach 80%-85% completion in 90 minutes.

Since OBA/Ku binds specifically to mammalian origin sequences, possesses DNA helicase activity, and is involved in DNA replication, we examined its interaction with other proteins involved in mammalian DNA replication. By co-immunoprecipitation analyses, OBA/Ku was found to interact with PCNA, DNA polymerase δ, DNA polymerase ε, topoisomerase II, and RF-C, but not with DNA primase and ORC-1 (Figure 6). PCNA, DNA polymerases δ and ε, topoisomerase II, DNA primase and RF-C are all part of the DNA synthesome, a mammalian cell multiprotein DNA replication complex that is stable and fully functional to support *in vitro* DNA replication (Malkas et al., 1990; Wu et al., 1994; Applegren et al., 1995; Coll et al., 1996; Tom et al., 1996; Lin et al., 1997; Jiang et al., 1998). This complex contains other replication proteins,

including DNA polymerase α, DNA ligase I, RPA, and topoisomerase I. DNA helicase activity has also been associated with this replication complex (Sekowski et al., 1998). The helicases previously associated with this complex are helicases I and IV (Sekowski et al., 1998). PCNA functions as an accessory actor for DNA polymerase δ and may participate in the coordination of leading and lagging strand synthesis (Tan et al., 1986; Malkas, 1998). DNA polymerase δ conducts both leading and lagging strand DNA synthesis during the elongation phase of DNA replication (Tsirimoto et al., 1990). DNA polymerase ε has been implicated in the conversion of DNA primers into Okazaki pieces and in acting as a molecular sensor of DNA damage in eukaryotic cells (Nethanel and Kaufmann, 1990; Zlotkin et al., 1996). Topoisomerase II unwinds the DNA by making a double-strand break and allows for the progression of the replication forks (reviewed in Roca, 1995). RF-C mediates polymerase switching between leading and lagging strand synthesis (Waga and Stillman, 1994). DNA primase synthesizes short RNA primers required to initiate synthesis and it is tightly associated with DNA polymerase α (Wang, 1991). Ku has been shown to stimulate DNA polymerase α-primase (Vishwanatha and Baril, 1990). Surprisingly, no interaction was found between Ku and DNA primase (Figure 6D), possibly indicating that these two proteins may interact weakly, in a fashion that is not detectable by coimmunoprecipitation. Alternatively, it might be possible that Ku is interacting with DNA polymerase α and not the DNA primase. ORC has not been described in the DNA synthesome. ORC is a six subunit origin recognition complex that binds to the *S. cerevisiae* ARS sequence and is required for initiation of DNA replication in yeast (Bell and Stillman, 1992; Dutta and Bell, 1997). Although mammalian homologues of ORC have been found, the role of ORC in the initiation of mammalian DNA replication is not clear (reviewed in Quintana and Dutta, 1999).

Based on the results of the present study, a preliminary model for the involvement of OBA/Ku in mammalian DNA replication can be presented (Figure 8). First, OBA/Ku binds to the A3/4 in mammalian origins of replication. OBA/Ku can either bind by itself or as part of the replication complex. Oct-1 will then interact with OBA/Ku and further aid in positioning the replication complex, as it does in viral systems. Ku, if not already associated with the replication complex, will interact with other replication proteins, and aid in recruiting the other required proteins to the initiation site. Following the initial

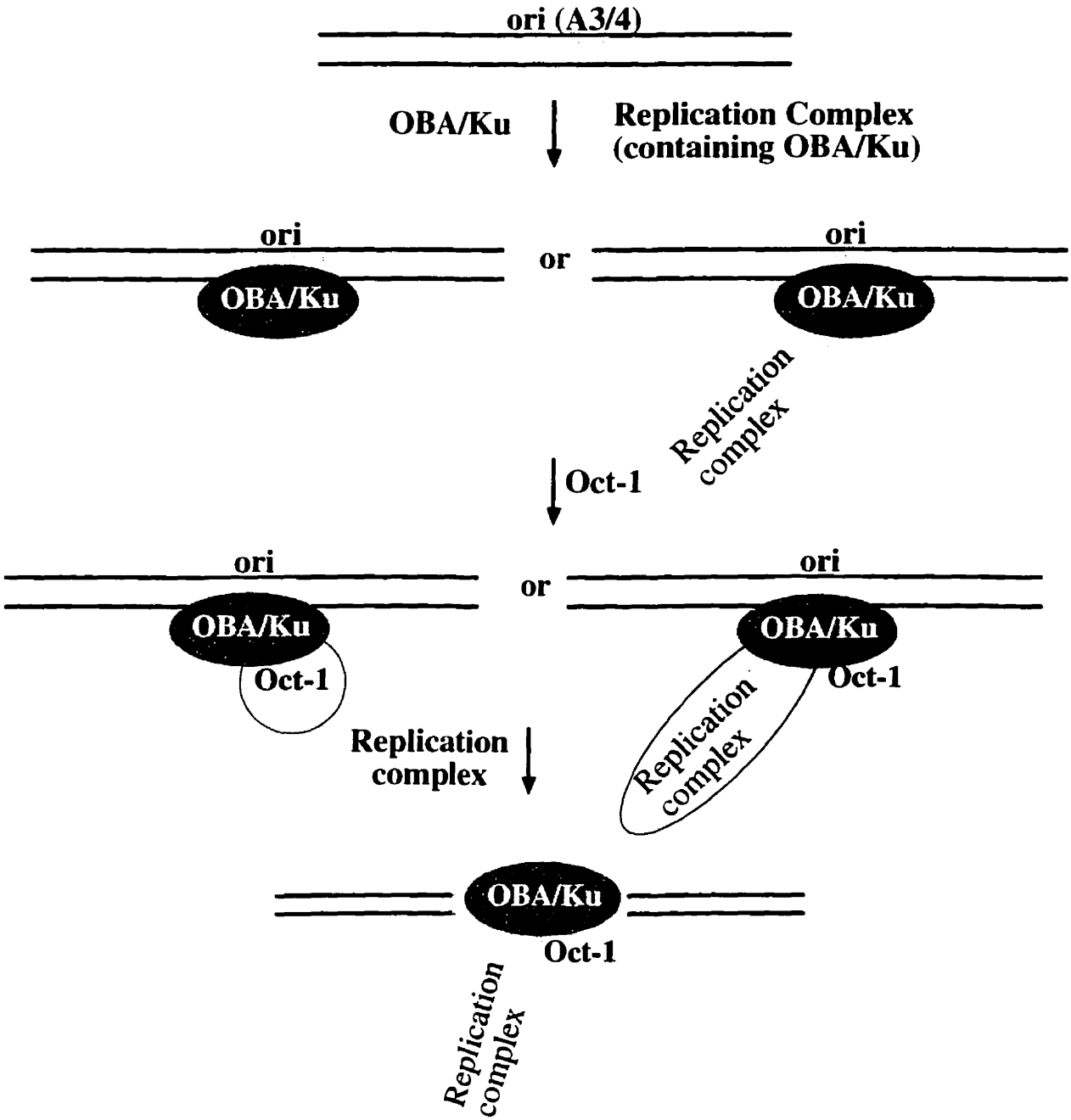


Figure 8. Proposed model for the involvement of OBA/Ku in mammalian DNA replication. Hatched box represent the origin of DNA replication with the A3/4 sequence.

steps of origin binding and loading of the replication complex, Ku, either alone or with other helicases, will proceed to unwind the DNA.

Various roles of Ku have been proposed for many important cellular metabolic processes, such as DNA double-strand break repair, V(D)J recombination, DNA replication, transcription regulation, regulation of heat shock-induced responses, cell signaling, and telomere maintenance (reviewed in Tuteja and Tuteja, 2000). The involvement of Ku in DNA replication is now becoming more evident. Because of its ability to bind to DNA ends during S-phase, it has been proposed that Ku is required for replication fork movement (Paillard and Strauss, 1991). Ku has been found bound to viral (de Vries et al., 1989), yeast (Shakibai et al., 1996) and mammalian (Toth et al., 1993; Ruiz et al., 1999; Araujo et al., 1999) origins of DNA replication and can support the formation of a stable multiprotein complex at yeast origins (Shakibai et al., 1996). It is also part of a mammalian multiprotein replication complex (Vishwanatha and Baril, 1990). Ku is the DNA-binding subunit of DNA-PK which phosphorylates several DNA replication proteins (reviewed in Anderson and Lees-Miller, 1992; Brush et al., 1994). Our findings support a role of OBA/Ku in the initiation of mammalian DNA replication, as an origin-specific binding protein with helicase activity that interacts with replication proteins. Further *in vivo* studies will be required to verify the *in vitro* replication results and to ascertain its role in DNA replication.

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CONNECTING TEXT

In the preceding chapter, OBA/Ku was shown to be involved in mammalian DNA replication as an origin-specific binding protein with DNA helicase activity. OBA/Ku acts at the initiation step of replication and requires an A3/4 homologous sequence for origin binding.

The next chapter describes the replication activity of the *xrs*-5 cell line, a radiosensitive mutant CHO K1 derivative that is defective in Ku86.

CHAPTER FIVE

Analysis of the DNA replication competence of the *xrs*-5 mutant cells defective in Ku86

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I. ABSTRACT

The radiosensitive mutant *xrs*-5, a derivative of the Chinese hamster ovary (CHO) K1 cell line, is defective in DNA double-strand break repair and V(D)J recombination. The defective phenotypes of *xrs*-5 cells are complemented by the 86 kDa subunit of Ku antigen. Ku is an abundant, heterodimeric, nuclear protein that consists of a 70 kDa and 86 kDa subunit. Ku has been implicated in many cellular metabolic processes, including DNA replication. OBA is a protein, previously purified from HeLa cells that binds in a sequence-specific manner to mammalian origins of DNA replication. The DNA binding subunit of OBA has been identified as Ku86. We tested the *xrs*-5 cell line for its ability to replicate a mammalian origin-containing plasmid, p186, in an *in vitro* replication system. Total and cytoplasmic cell extracts from *xrs*-5 cells replicated the p186 DNA with the same efficiency as the parental CHO K1 cell extracts. In contrast, *xrs*-5 nuclear cell extracts did not possess any replication activity. Addition of OBA restored replication in the *xrs*-5 nuclear extract reaction in a similar way that Ku86 complements the *xrs*-5 defective repair and recombination phenotypes. The data implicate Ku antigen in a direct role in DNA replication and suggest that the lack of replication defect in the *xrs*-5 cells results from the presence of a Ku-like origin-specific binding protein present in the cytoplasm which compensates for the lack of Ku86 in the nucleus.

II. INTRODUCTION

The radiosensitive mutant cell line, *xrs*-5, a derivative of the Chinese hamster ovary (CHO) K1, is defective in V(D)J recombination and DNA double-strand break (DSB) rejoining (Kemp et al., 1984; Pergola et al., 1993; Taccioli et al., 1993; Rathmell and Chu, 1994). The *xrs* mutants were initially described as the first mammalian cell mutants with a defect in DSB rejoining (Kemp et al., 1984). They were isolated by a simple technique involving transfer of heavily mutagenized cells from single colonies, followed by irradiation with a dose determined not to affect survival of wild-type CHO K1 cells (Jeggo and Kemp, 1983). The *xrs*-5 cells, along with other members of the x-ray complementation group 5, also lack DNA end-binding activity (Rathmell and Chu, 1994; Getts and Stamato, 1994; Singleton et al., 1997). The defective repair and recombination phenotypes of the *xrs*-5 cells have been traced to the 86 kDa subunit of the Ku protein, since Ku86 cDNA restored DNA end binding, x-ray sensitivity and V(D)J recombination (Getts and Stamato, 1994; Rathmell and Chu, 1994; Smider et al., 1994; Taccioli et al., 1994).

Ku (reviewed in Tuteja and Tuteja, 2000) is a heterodimeric DNA-binding protein of 70 kDa and 86 kDa subunits (Mimori et al., 1986). Ku binds avidly to DNA ends, whether blunt or with 5' or 3' overhangs as well as to other discontinuities in the DNA structure (Mimori and Hardin, 1986). Sequence-specific binding of Ku has also been demonstrated, including binding to mammalian origins of DNA replication (Giffin et al., 1996; Ruiz et al., 1999; Araujo et al., 1999; Schild-Poulter et al., submitted). Ku is necessary for the processing of DNA DSBs that are induced by damaging agents such as ionizing radiation or oxidative reactions, by endogenous recombination processes and by certain chemotherapeutic drugs (reviewed in Featherstone and Jackson, 1999; Tuteja and Tuteja, 2000). An involvement of Ku has also been suggested in transcription (Giffin et al., 1996; Finnie et al., 1993; Kuhn et al., 1995; Generesch et al., 1995), telomeric maintenance (Boulton and Jackson, 1996; Porter et al., 1996; Polotnianka et al., 1998), replicative senescence (Woo et al., 1998), suppression of chromosomal aberrations and malignant transformation (Difilippantonio et al., 2000), aging (Vogel et al., 1999; Cooper

et al., 2000) and DNA replication (Ruiz et al., 1999; Vishwanatha and Baril, 1990; Cao et al., 1994; Shakibai et al., 1996; Matheos et al., submitted).

We have previously shown that OBA (Ruiz et al., 1995), a HeLa cell activity whose DNA binding subunit has been identified as Ku86, is involved in mammalian DNA replication (Ruiz et al., 1999). OBA/Ku binds to a mammalian replication origin sequence termed A3/4, a 36-bp mammalian origin sequence that is capable of supporting replication *in vivo* and *in vitro* (Price, G.B and Zannis-Hadjopoulos, M., in preparation). Depletion of OBA/Ku, either by inclusion of an A3/4 oligonucleotide or by antibodies directed against the Ku protein, inhibited DNA replication in a mammalian *in vitro* replication system (Ruiz et al., 1999; Matheos et al., submitted). OBA/Ku also possesses DNA helicase activity and can interact with proteins of the replication machinery, including DNA polymerases α and δ, PCNA, topoisomerase II, and replication factor C (RF-C). The *in vitro* replication cell-free system mimics eukaryotic nuclear DNA replication *in vivo* (Pearson et al., 1991; Pearson et al., 1994; Zannis-Hadjopoulos et al., 1994; Todd et al., 1995; Pelletier et al., 1997). It uses a plasmid containing a mammalian origin of DNA replication and extracts from HeLa cells (Pearson et al., 1991). Moreover, we have used this system to study the effects on DNA replication of cancer chemotherapeutic drugs (Diaz-Perez et al., 1996; Diaz-Perez et al., 1998) and of various proteins including the Oct-1 transcription factor (Matheos et al., 1998), the GATA-1 factor, Ku antigen and DNA polymerase δ (Ruiz et al., 1999; Matheos et al., submitted) and a mammalian polynucleotide kinase (Jilani et al., 1999).

Since the *xrs*-5 mutant cells have severely reduced levels of Ku86 and Ku70 proteins, in this study we tested their potential for replication in the *in vitro* replication system. We found that total and cytoplasmic extracts from *xrs*-5 cells replicated p186, a mammalian origin-containing plasmid, with the same efficiency as CHO K1 cell extracts. In contrast, *xrs*-5 nuclear cell extracts did not possess any replication activity. Addition of OBA/Ku to the *xrs*-5 nuclear cell extracts restored replication activity, in a similar way that Ku86 cDNA complements the defective repair and recombination phenotypes (Smider et al., 1994). Moreover, we identified a factor in the *xrs*-5 cytoplasmic cell extracts that bound in a sequence-specific manner to the A3/4 sequence and likely allowed for efficient replication of the *xrs*-5 total and cytoplasmic cell extracts. The data suggest that OBA/Ku

participates in mammalian DNA replication and that the cytoplasmic factor, potentially a modified Ku protein can compensate for the lack of Ku in the nucleus.

III. MATERIALS AND METHODS

Cell culture

The *xrs*-5 cell line was derived from the CHO K1 cell line on the basis of its sensitivity to ionizing radiation (Jeggo and Kemp, 1983). CHO K1 and *xrs*-5 cells were cultured in RPMI medium (Gibco-BRL, Burlington, Ontario, Canada) supplemented with penicillin (100 u/ml), streptomycin (0.1 mg/ml), 1 mM glutamine and 10% fetal bovine serum (Gibco-BRL). Cells were maintained at 37°C in 5% CO_2.

Extract Preparation

Cell extracts from log phase CHO K1 and *xrs*-5 cell monolayers were prepared as described by Pearson et al. (Pearson et al., 1991). Briefly, monolayers were washed twice with isotonic buffer (20 mM Tris-HCl pH 7.4, 137 mM NaCl, 5mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 250 mM glucose). The cells were collected and lysed in a Type B Dounce homogenizer. Nuclei were removed by 5 minutes centrifugation at 1200 X g and were subsequently used to prepare the nuclear extract. The supernatant was centrifuged for 1 hour at 100 000 X g in a Beckman type 50 Ti rotor and the supernatant was used as the cytoplasmic extract. The nuclear pellet was suspended in hypertonic buffer (hypotonic and 500 mM potassium acetate) in 1/2 the volume used for the cytoplasmic extract (for instance, if the cytoplasmic pellet was resuspended in 4 ml, then the nuclear pellet was resuspended in 2 ml). The mixture was incubated for 90 minutes on ice, spun in a Beckman SW50.1 rotor at 300 000 X g for 1 hour and used as the nuclear extract.

Preparation of template DNA for replication assay

Plasmid p186 consists of the minimal origin of *ors*8 (GenBank accession number M26221). It contains the *NdeI*/*Rsa*I subfragment of *ors*8 cloned in the *Nru*I site of pBR322 (Todd et al., 1995). Plasmid DNA was isolated using the QIAGEN-tip 500 column (QIAGEN, Mississauga, Ontario, Canada).

Mammalian in vitro DNA replication

Replication assays were performed as previously described (Pearson et al., 1991; Matheos et al., 1998), with slight modifications. CHO K1 or *xrs*-5 nuclear cell extracts (16 μg), cytoplasmic cell extracts (45 μg), or nuclear and cytoplasmic cell extracts together (8:15 ratio) were added to the replication mixture with 100 ng of input p186 plasmid DNA. Experiments involving the addition of A3/4-affinity-purified OBA (Ruiz et al., 1999) were performed by pre-incubating the nuclear cell extracts with 160 ng or 600 ng of OBA on ice for 20 minutes, prior to the replication reaction. Replication reactions were performed at 30°C for 1 hour. The *in vitro* replication reaction products were purified using the QIAquick PCR purification kit (QIAGEN). Reaction samples were digested with *Dpn*I (New England Biolabs, Mississauga, Ontario, Canada), as previously described (Matheos et al., 1998), and resolved by electrophoresis on 1% agarose gel. Quantitation was performed on *Dpn*I-digested products by densitometric measurements using a phoshorimager analyzer (Fuji BAS2000, Stamford, CT), as described in Diaz-Perez et al. (1996) and Matheos et al. (1998). The total amount of DNA recovered from the *in vitro* replication reaction was determined by quantitative analysis of the picture of the ethidium bromide-stained gel.

Immunoblotting Assay

Nuclear or cytoplasmic extract proteins were resolved by 8% SDS-PAGE, transferred to Immobilon-P (Millipore, Mississauga, Ontario, Canada), probed with human anti-Ku 70 (C-19) or anti-Ku 86 (C-20) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by treatment with horseradish peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology, Inc). The blots were developed using the ECL Western blotting detection kit (Amersham-Pharmacia, Baie d'Urfé, Québec, Canada).

Electrophoretic Mobility-Shift Assay (EMSA)

Nuclear or cytoplasmic cell extracts (10 μg) were incubated with 0.5 ng of ^{32}P-end-labeled A3/4 probe (5' CCTCAAATGGTCTCCAATTTTCCTTTGGCAAATTCC 3') for 30 minutes on ice in the presence of 1 μg poly dI-dC (Amersham-Pharmacia), used as nonspecific competitor, in a final volume of 20 μl including binding buffer (10 mM Tris-

HCl pH 7.5, 80 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.1% Triton X-100, 4% glycerol). The mixtures were electrophoresed on a 6% PAGE gel at 180 Volts in TBE (45 mM Tris-HCl pH 8.0, 45 mM Boric Acid, 1 mM EDTA) and the gel was dried and subjected to autoradiography.

For electrophoretic mobility-shift competition assays, 0.5 ng of ^{32}P-labeled A3/4 was mixed with increasing molar excess amounts of A3/4 or nonspecific (5' TTCCGAATACCGCAAG 3') cold competitor oligonucleotides. 10 μg extract protein was then added, and the reaction was left to proceed as described above.

Electrophoretic Mobility-Supershift Assay

Electrophoretic mobility shift mixtures were prepared as described above, and after the standard 30 minutes incubation, 1.5 μg of clone 162 antibody (Neomarkers, Union City, CA) or control goat IgG (Sigma, St-Louis, MO) was added and further incubated for an additional 3 hours on ice. The samples were then applied to native 6% PAGE and electrophoresed, as described above.

IV. RESULTS

Replication activity of CHO K1 and xrs-5 cell extracts

Previous studies have shown that *xrs*-5 mutants are defective in Ku86, a subunit of the Ku70/Ku86 heterodimeric protein (Taccioli et al., 1993; Getts and Stamato, 1994; Rathmell and Chu, 1994; Smider et al., 1994). This renders the mutants severely defective in the repair and recombination processes. Since Ku86 binds to origins of DNA replication (Ruiz et al., 1999) and it has been shown to be required for mammalian DNA replication (Ruiz et al., 1999; Matheos et al., submitted), we tested the ability of *xrs*-5 cell extracts to support the *in vitro* replication of a mammalian origin-containing plasmid. p186 is a pBR322-based plasmid that contains the minimal monkey cell origin of *ors*8 (Todd et al., 1995). It replicates autonomously when transfected in mammalian cells and in a cell-free *in vitro* system that uses HeLa cell extracts. The replication of this plasmid initiates within the *ors* sequence, is semiconservative, bi-directional, and dependent on the replicative DNA polymerases α and δ (Pearson et al., 1991; Pearson et al., 1994; Todd et al., 1995).

The CHO cell extracts, both wild-type (K1) and mutant (*xrs*-5), yielded similar *in vitro* replication products (Figure 1A) as those routinely obtained with the HeLa cell extracts (Pearson et al., 1991; Zannis-Hadjopoulos et al., 1994; Matheos et al., 1998), namely, relaxed circular (form II), linear (form III), supercoiled (form I), topoisomeric molecules (T) with supercoils (ladder of bands between forms II and I), and replicative intermediates (RI) (migrating slower than form II). However, *in vitro* replication of p186 DNA using the CHO cell extracts was consistently less efficient (approximately nine times) than using HeLa cell extracts (data not shown). This is consistent with our previous findings suggesting that HeLa cell extracts may be producing higher concentrations of initiator proteins, resulting in more efficient replication than that observed with CV-1 or COS-7 cell extracts (Pearson et al., 1999). Other laboratories have also reported differences in *in vitro* replication activities of cell extracts, depending on their source (Stillman and Gluzman, 1985; Wobbe et al., 1985; Li et al., 1986; Guo et al., 1989).

Both the parental CHO (K1) and the mutant (*xrs*-5) total cell extracts were equally efficient in supporting the *in vitro* replication of p186 (Figure 1A, B). The profiles

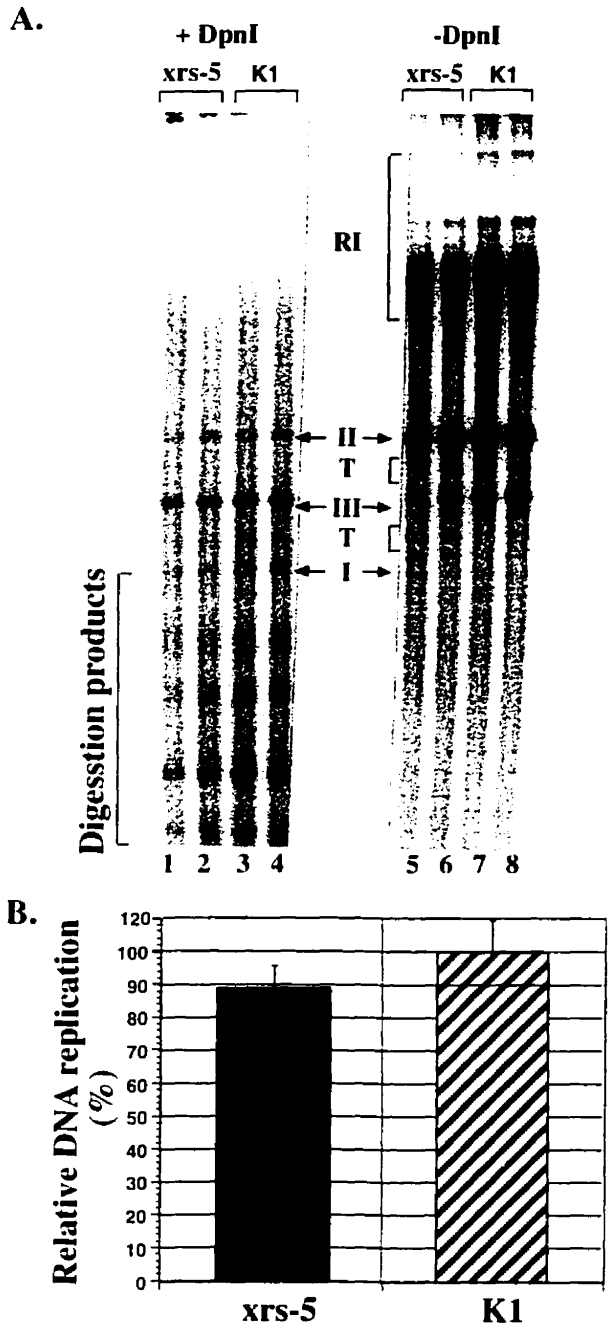


Figure 1. Replication activity of the CHO K1 and *xrs*-5 cell extracts. A. Typical autoradiograph of DNA replication products. p186 was incubated in reaction mixtures containing CHO K1 or *xrs*-5 cell extracts. The DNA was purified, concentrated and a sample was digested with 1 unit of *Dpn*I for 1 hour at 37°C. The undigested (lanes 5-8) and the *Dpn*I-digested (lanes 1-4) samples were subjected to electrophoresis on 1% agarose gel. The supercoiled (I), relaxed circular (II), linear (III), topoisomeric (T) and replicative intermediate (RI) forms of the plasmid and the *Dpn*I-digestion products are indicated. Duplicate samples are shown. B. Bar graph showing the relative DNA replication of p186 with the CHO K1 and *xrs*-5 cell extracts. Quantitations were performed on *Dpn*I-digested samples (see text). Data are expressed as a percentage of the control CHO K1 reaction. Each bar represents the average of four experiments. Standard deviations are shown.

obtained for both, the total incorporation of radioactive precursor nucleotide (Figure 1A, -*Dpn*I), indicating total incorporation due to both replication and repair synthesis, and *Dpn*I-resistance (Figure 1A, +*Dpn*I), indicating incorporation due to replication, were similar for the two types of cell extracts. Furthermore, the quantitation profiles of the *Dpn*I-resistant bands, generated by the two reactions and corresponding to DNA forms II and III, were virtually the same (Figure 1B).

Examination of the levels of Ku protein in xrs-5 mutants

To examine whether any residual Ku protein levels could be detected in *xrs*-5 cell extracts, which might account for their observed replication activity, Western immunoblotting analyses were performed using anti-Ku antibodies (Figure 2). An anti-human Ku86 antibody (C-20) was used that was able to recognize the hamster Ku86 protein, reacting with both the CHO K1 nuclear (K1 N) and cytoplasmic (K1 C) cell extracts (Figure 2A, lanes 2 and 3). No material cross-reacting with this antibody was detectable either in the cytoplasmic (*xrs*-5 C) or nuclear (*xrs*-5 N) cell extracts derived from the *xrs*-5 cells (Figure 2A, lanes 4 and 5), in agreement with previous reports stating that *xrs*-5 cells lack Ku86 protein, as detected by Western blotting, and Ku86 RNA transcript, as detected by Northern blotting (Rathmell and Chu, 1994; Singleton et al., 1997). However, Ku86 transcript has been detected in the *xrs*-5 mutants when the more sensitive technique of RT-PCR was used (Singleton et al., 1997). Each subunit of the Ku heterodimer is required to stabilize the other, and the absence of Ku86 in *xrs*-5 has been shown to result in the loss of the Ku70 subunit (Taccioli et al., 1994; Smider et al., 1994; Singleton et al., 1997). To examine the levels of Ku70 in the *xrs*-5 cell extracts, the same membrane was probed with an anti-human Ku70 antibody (C-19) recognizing the Ku70 protein in the CHO K1 nuclear and cytoplasmic cell extracts (Figure 2B, lanes 2 and 3). In contrast, *xrs*-5 showed dramatically decreased levels of Ku70 (Figure 2B, lanes 4 and 5). Upon longer exposures, a faint band cross- reacting with the anti-Ku70 antibody was detected in the *xrs*-5 cytoplasmic cell extracts.

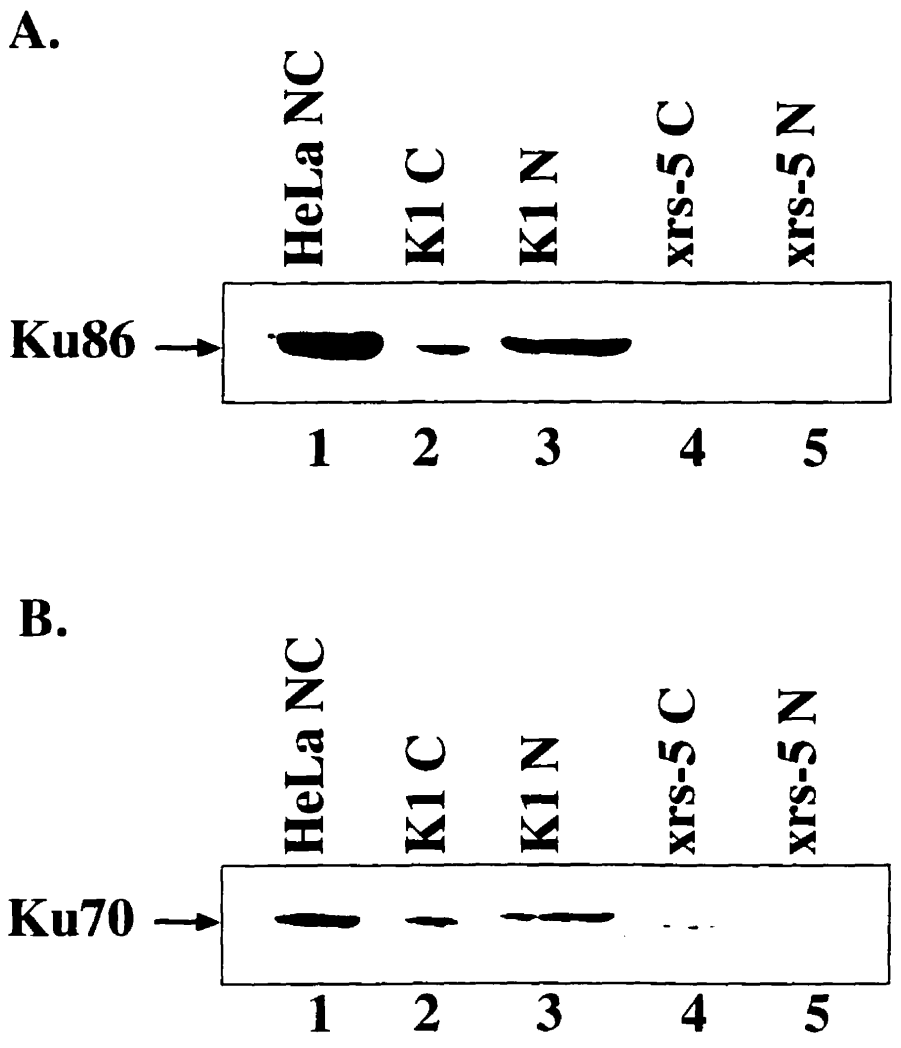


Figure 2. Immunoblot analysis of CHO K1 and *xrs*-5 cell extracts. Nuclear (N; panels A and B, lanes 3 and 5) or cytoplasmic (C; panels (A) and (B), lanes 2 and 4) cell extracts from CHO K1 or *xrs*-5 cells were examined by Western blotting using a Ku86 (A) or Ku70 (B) antibody. HeLa whole-cell extracts (lane 1) were used as controls.

A3/4 binding activity in CHO K1 and xrs-5 cell extracts

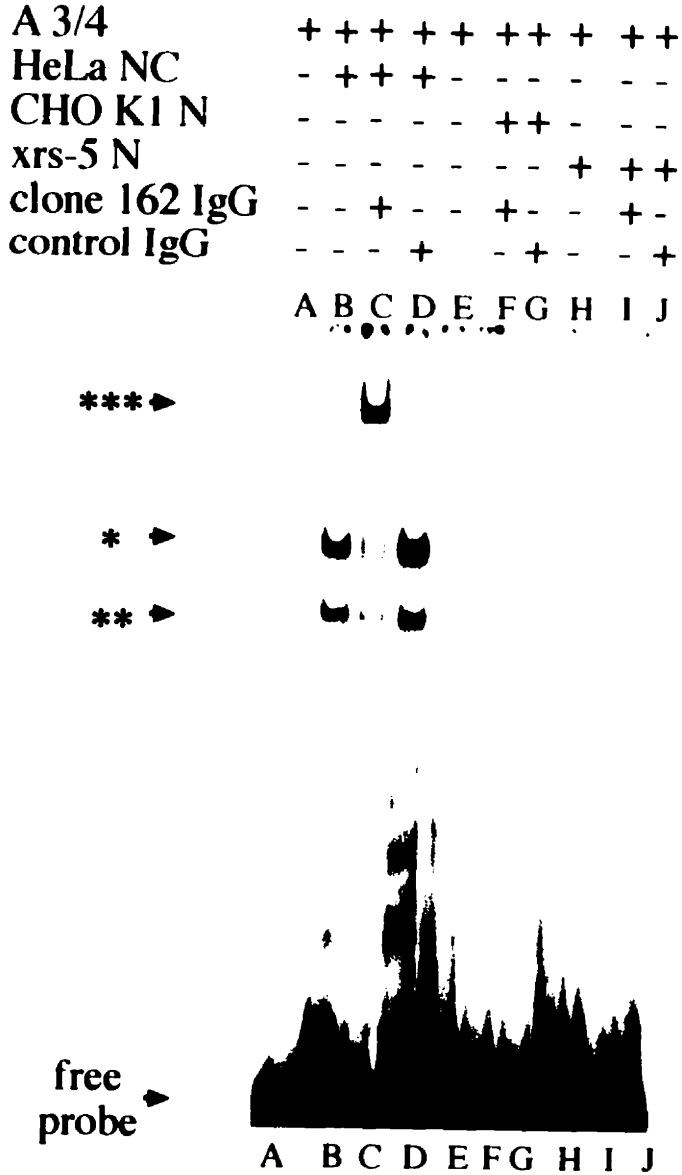
Electrophoretic mobility shift and super-shift assays were performed to examine whether there was A3/4-specific binding present in the wild type and mutant CHO cell extracts (Figure 3A, B). When HeLa cell extracts were reacted with radiolabeled A3/4, two main complexes arose (Figure 3A, lane B). The slower migrating complex (*) results from the interaction of A3/4 with the Ku heterodimer, Ku70/Ku86, while the faster migrating complex (**) arises from its interaction with the truncated Ku, Ku70/Ku69 (Quinn et al., 1993; Han et al., 1996; Jeng et al., 1999). The migration of these complexes is further retarded by clone 162 antibody (Figure 3A, lane C, ***), which recognizes the Ku70/Ku86 heterodimer, but not by the control antibody (Figure 3A, lane D). The reaction of radiolabeled A3/4 oligonucleotide with the CHO K1 nuclear cell extracts yielded a complex of similar migration (Figure 3A, lane E) as that obtained from its reaction with the HeLa cell extracts (compare with Figure 3A, lane B). This complex, was also supershifted by the clone 162 antibody (Figure 3A, lane F), but not by the control antibody (Figure 3A, lane G). The EMSA reaction of radiolabeled A3/4 oligonucleotide with the *xrs*-5 nuclear cell extracts did not result in a Ku-A3/4 complex (Figure 3A, lanes H, I, J) consistent with the results obtained by the Western blot analysis, in which neither Ku70 nor Ku86 were detected (see Figure 2). The EMSA reaction with *xrs*-5 cytoplasmic cell extracts also resulted in a complex with similar migration as the HeLa (Figure 3A, lane B) and CHO K1 nuclear (Figure 3A, lane E) and cytoplasmic (Figure 3B, lane A) cell extracts. This complex, however, was not recognized by the clone 162 antibody (Figure 3B, lane E), unlike the complex generated with the CHO K1 cytoplasmic cell extracts (Fig 3B, lane B). The fact that the *xrs*-5 cytoplasmic cell extracts-A3/4 complex was not recognized by the clone 162 antibody suggests that the epitope that is normally recognized by the antibody is either not present or not accessible. Faster migrating complexes, probably due to degradation, were also detected.

An A3/4-specific binding protein is present in CHO K1 and xrs-5 cytoplasmic cell extracts

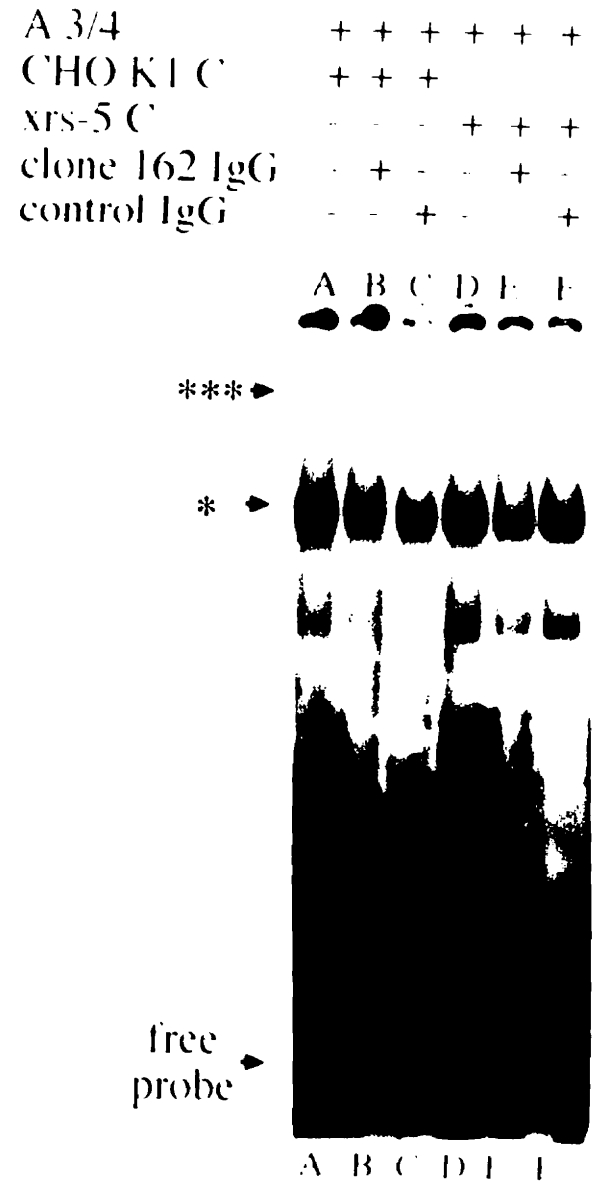
The binding specificity for the A3/4 oligonucleotide in the complexes formed with the *xrs*-5 cytoplasmic cell extracts was tested by competition bandshift assays, with

Figure 3. A3/4 binding activity in CHO K1 and *xrs*-5 cell extracts. (A) Nuclear (N) cell extracts from CHO K1 or *xrs*-5 cells were mixed with radiolabeled double-stranded A3/4 DNA probe. Following the binding reaction, clone 162 or control antibody was added to the mixture, as indicated. The DNA-protein complexes were separated by 6% PAGE. The Ku70/Ku86-A3/4 (*), Ku70/Ku69-A3/4 (**) and the supershifted complexes (***) are indicated. (B) As in panel (A) except that cytoplasmic (C) cell extracts from CHO K1 and *xrs*-5 cells were used.

A.


A 3/4 + + + + + + + + + +
HeLa NC - + + + - - - - - -
CHO K1 N - - - - - + + - - -
xrs-5 N - - - - - - - + + +
clone 162 IgG - - + - - + - - + -
control IgG - - - + - + - - + +
A B C D E F G H I J
***
*
**
free probe
A B C D E F G H I J

B.


A 3/4 + + + + + +
CHO K1 C + + +
xrs-5 C + + +
clone 162 IgG + + -
control IgG + -
***
*
free probe

increasing molar excess of cold A3/4 (Figure 4, lanes 3, 4, 8, 9), used as specific competitor, and cold pBR322-derived oligonucleotide (Figure 4, lanes 5, 6, 10, 11), used as nonspecific competitor. The A3/4-Ku complex formed with both the CHO K1 (Figure 4, lane 7) and *xrs*-5 (Figure 4, lane 2) cytoplasmic cell extracts was specifically competed with increasing concentrations of cold A3/4 but not with cold nonspecific competitor. Hence, the complex formed with these cytoplasmic cell extracts represents a specific protein interaction with A3/4. Some faster migrating complexes were also detected, likely attributed to degradation products.

Replication activity of nuclear, cytoplasmic or total CHO K1 and xrs-5 cell extracts

To test whether the A3/4-specific binding protein present in the cytoplasm *xrs*-5 cells was responsible for the efficient replication from these cell extracts (see Figure 1), we performed *in vitro* DNA replication assays, using either cytoplasmic or nuclear extracts separately, or cytoplasmic and nuclear extracts together, from either CHO K1 or *xrs*-5 cells (Figure 5A, B). The CHO K1 cytoplasmic cell extracts (Figure 5A, lanes 3 and 4) replicated the p186 DNA as efficiently as the CHO K1 total cell extracts (Figure 5A, lanes 1 and 2), whereas the nuclear cell extracts alone (Figure 5A, lanes 5 and 6) were approximately 6-fold less efficient (Figure 5B, CHO K1 N). HeLa cell cytoplasmic extracts have also previously been shown to have the same replication activity as total cell extracts (Diaz-Perez et al., 1996; Diaz-Perez et al., 1998), presumably due to leakage of the replication proteins into the cytoplasm during extract preparation. Nuclear cell extracts, which contain a high salt concentration (500 mM potassium acetate), have previously been shown to inhibit SV40 (Stillman and Gluzman, 1985; Decker et al., 1986) and mammalian (Pearson et al., 1991) *in vitro* DNA replication, but are required for the formation of supercoiled (form I) DNA. The *xrs*-5 cytoplasmic cell extracts (Figure 5A, lanes 9 and 10) also showed comparable replication activity to the *xrs*-5 total (Figure 5A, lanes 7 and 8) and the CHO K1 total (Figure 5A, lanes 1 and 2) and cytoplasmic (Figure 5A, lanes 3 and 4) cell extracts. In contrast, the *xrs*-5 nuclear cell extracts did not support the *in vitro* replication of the p186 DNA, as indicated by the failure to incorporate any radioactive precursor (Figure 5A, lanes 11 and 12; Figure 5B).

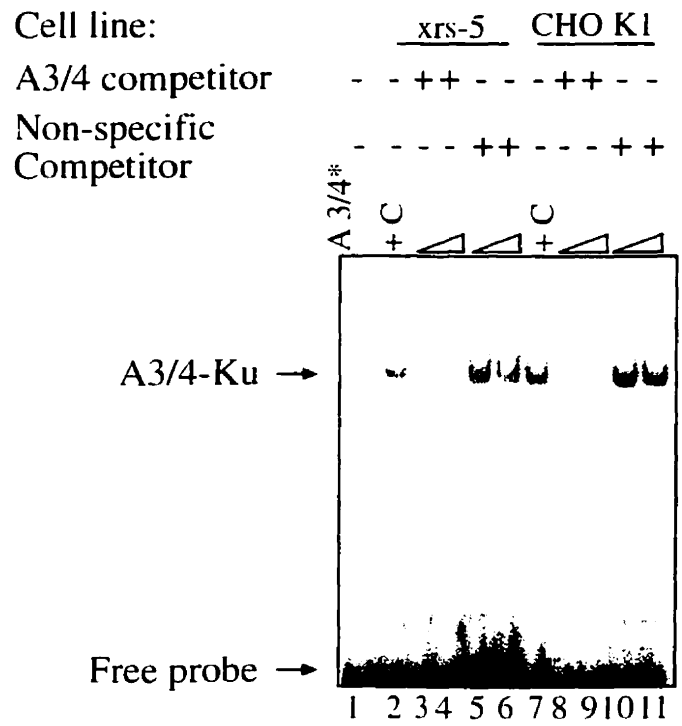


Figure 4. Competition/bandshift assay using CKO K1 and *xrs*-5 cytoplasmic cell extracts. Electrophoretic mobility shift assay was performed by adding cytoplasmic (C) cell extracts from *xrs*-5 (lanes 2-6) or CHO K1 (lanes 7-11) to a mixture containing radiolabeled A3/4 oligonucleotide (lane 1). Some reactions contained no additional competitor DNA (lanes 2 and 7). Lanes 3 and 8 contained 50X molar excess of cold A3/4 competitor, lanes 4 and 9 contained 500X of cold A3/4 competitor, lanes 5 and 10 contained 50X nonspecific cold competitor and lanes 6 and 11 contained 500X nonspecific competitor. The positions of the protein-DNA complexes and of the free probe are indicated.

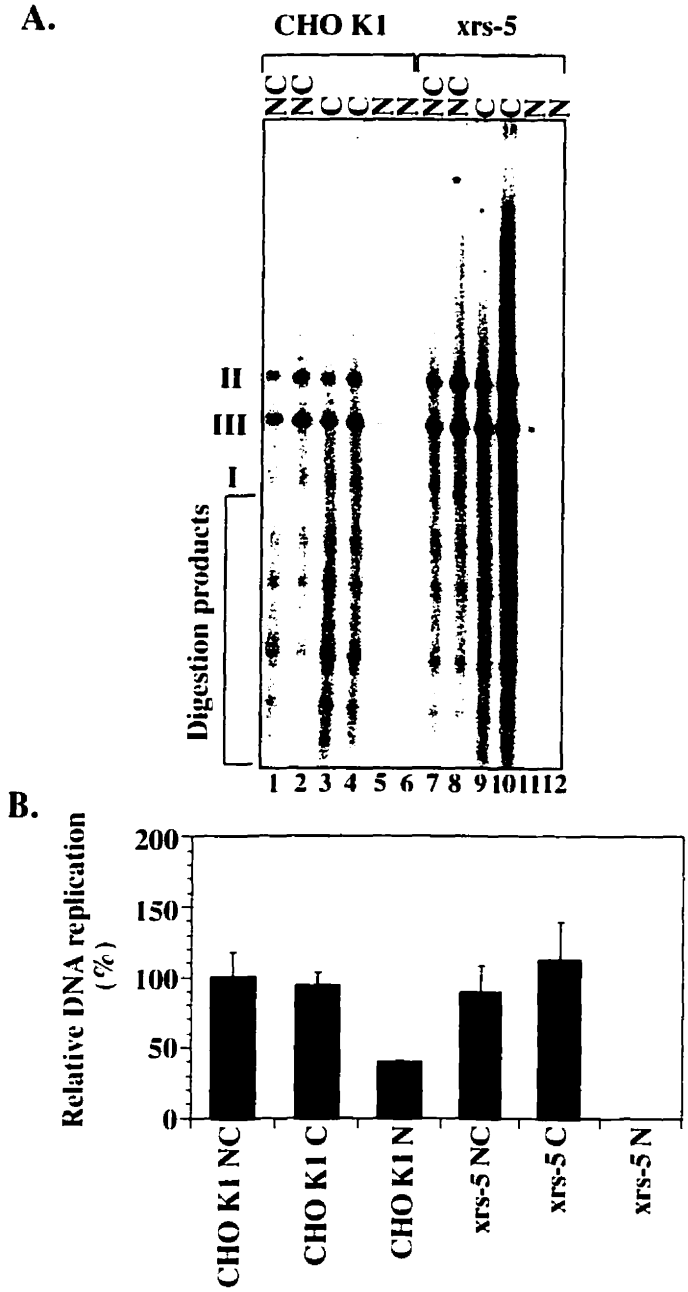


Figure 5. *xrs*-5 nuclear cell extracts do not replicate p186. *In vitro* DNA replication assays were performed with CHO K1 or *xrs*-5 nuclear and cytoplasmic (NC), cytoplasmic (C) or nuclear (N) cell extracts. A. Autoradiograph of *Dpn*I-digested replication products. Duplicate samples are shown. Form I, II and III of the DNA and the *Dpn*I digestion products are indicated. B. Quantitation of DNA replication activities of the cell extracts, related to the CHO K1 NC reaction. Each bar represents the average of four experiments.

OBA restores replication activity of xrs-5 nuclear cell extracts

In order to determine whether Ku could restore replication activity in the *xrs*-5 nuclear cell extracts, additional replication assays were performed where OBA/Ku was added to the *in vitro* replication reaction (Figure 6A, B). The exogenous addition of OBA/Ku increased replication of the *xrs*-5 nuclear cell extracts from 0% to approximately 60%, relative to the replication activity of the K1 nuclear extract (Figure 6B). Hence, OBA/Ku was able to complement the lack of replication activity in the *xrs*-5 nuclear extract, in a similar way that the Ku86 cDNA complements the defective repair and recombination phenotypes (Smider et al., 1994). No significant effect was observed when OBA/Ku was added to the CHO K1 nuclear cell extracts *in vitro* replication reaction (Figure 6A, lanes 2 and 3 and Figure 6B). Addition of OBA/Ku has previously been shown to restore replication in *in vitro* replication reaction that had been inhibited by the addition of the A3/4 oligonucleotide (Matheos et al., submitted).

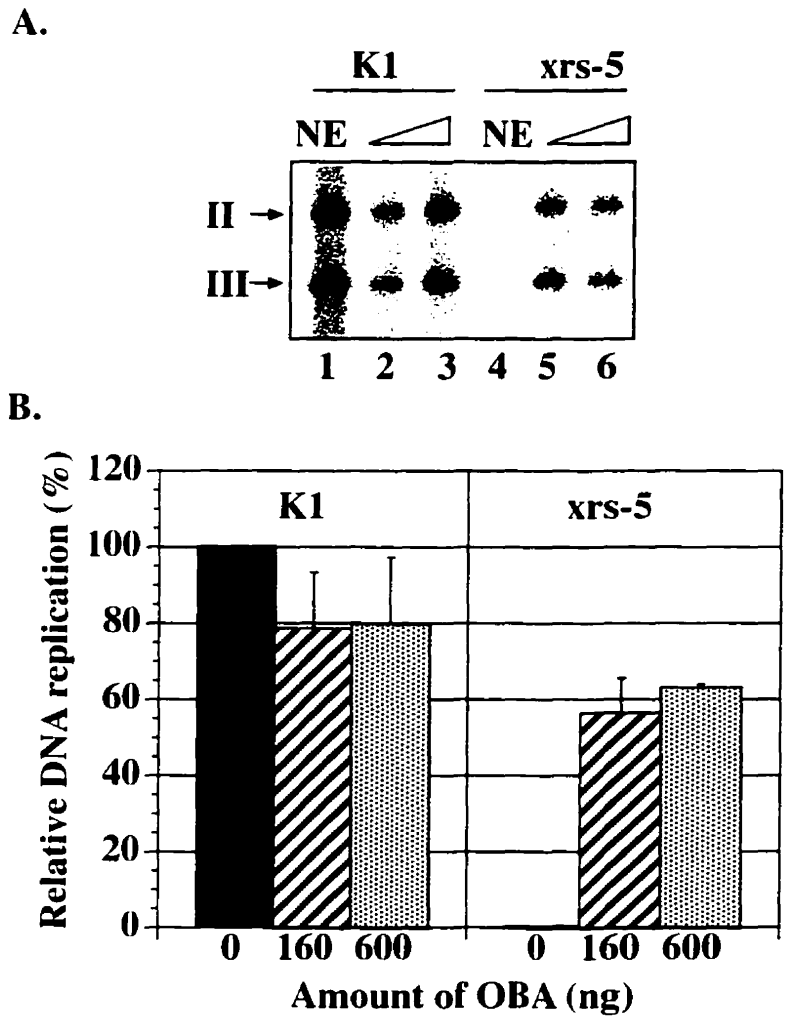


Figure 6. OBA restores replication activity to the *xrs*-5 nuclear cell extracts. *In vitro* DNA replication assays were performed with either K1 or *xrs*-5 nuclear cell extracts in the presence of A3/4-affinity-purified OBA. A. Autoradiograph of the replication products. DNA forms II and III are indicated. Lane 1 and 4: 0 ng OBA; lane 2 and 5: 160 ng OBA; lane 3 and 6: 600 ng OBA. B. DNA replication activities of the OBA-reconstituted nuclear cell extracts, relative to the K1 nuclear extract reaction. Each bar represents the average of three experiments.

V. DISCUSSION

In the present study, we have examined the replication activity of the *xrs*-5 cells, defective in the 86 kDa subunit of Ku antigen, a protein known to be involved in numerous cellular metabolic processes, including DNA replication. We have shown that *xrs*-5 nuclear cell extracts do not possess any replication activity (Figure 5), in contrast to *xrs*-5 cytoplasmic cell extracts (Figure 5) and to *xrs*-5 and CHO K1 total cell extracts (Figure 1). Furthermore, the mutant nuclear cell extracts did not incorporate any radioactive precursor attributed to repair seeing that these cells are also deficient in repair mechanisms. Upon examination of the Ku protein levels in the *xrs*-5 cell extracts by Western blot analyses, it was determined that *xrs*-5 cytoplasmic or nuclear cell extracts did not have any detectable levels of Ku70 or Ku86 proteins (Figure 2). This is in agreement with previous studies, in which neither Ku86 protein or Ku86 transcript were detected in *xrs*-5 cells or other cell lines of the XRCC5 group, by Western or Northern analyses, respectively (Rathmell and Chu, 1994; Singleton et al., 1997; Errami et al., 1998). Since both Ku subunits are required for protein stability (Errami et al., 1996; Singleton et al., 1997) and since Ku86 likely regulates Ku70 levels posttranscriptionally (Chen et al., 1996), it is not surprising to find reduced Ku70 levels as a result of reduced Ku86 levels. Low levels of wild type Ku86 transcripts however have been detected by the more sensitive technique of RT-PCR (Singleton et al., 1997). The low level of wild-type transcript could either represent the presence of some revertants in the *xrs*-5 cell population or a low transcript level in each cell. In contrast, Yasui et al. (1999) have recently reported the presence of both Ku70 and Ku86 proteins in the *xrs*-5 cells, albeit at low concentrations, using Western blot and 2D gel electrophoresis analyses. Furthermore, by indirect immunofluorescence, Ku70 and Ku86 were found distributed over the cytoplasm and nuclei of CHO K1 and *xrs*-5 cells, with enhanced perinuclear localization of Ku86 in the *xrs*-5 cells (Yasui et al., 1999). The subcellular localization of Ku has been quite controversial, as discussed in Koike et al (1999). Reports of purely nuclear (Koike et al., 1999; Bakalkin et al., 1998), membrane (Dalziel et al., 1992), cytoplasmic (Bakalkin et al., 1998), and both nuclear and cytoplasmic (Fewell and Kuff, 1996) localization have been published (reviewed in Koike et al., 1999)

probably due to differences in detection methods or to a change in Ku's subcellular localization during the cell cycle. Ku has been shown to be present in the cytoplasm of mitotic cells and at the periphery of condensed chromosomes (Koike et al., 1999). The same investigators found Ku70 and Ku86 associated as a heterodimer throughout the cell cycle. Although an enhanced perinuclear localization of only Ku86 in *xrs*-5 cells (Yasui et al., 1999) is surprising, it is likely that this is a truncated form of Ku86 that is stable when not complexed to Ku70 (Singleton et al., 1997). Unlike the wild-type protein, truncated stable forms of Ku86 have been detected without Ku70, perhaps due to protein conformational changes or to loss of degradation signal sequences (Singleton et al., 1997). Yasui et al. (1999) postulate that the concentration of Ku86 at the nuclear periphery of *xrs*-5 cells keeps the Ku complex sequestered, thereby preventing it from accessing the DNA. In terms of DNA repair, this could prevent the Ku protein complex from accessing DNA DSBs. The inability of the *xrs*-5 nuclear cell extracts to replicate DNA might be due to either a lack or the presence of very low levels of Ku86. Alternatively, Ku86 might be localized in the nuclear periphery and thus be unavailable for interaction with the DNA and the replication complex.

Replication activity of the *xrs*-5 nuclear cell extracts was restored upon addition of OBA (Figure 6). OBA represents a protein activity purified from HeLa cells (Ruiz et al., 1995) whose DNA binding subunit was identified as Ku86 (Ruiz et al., 1999). OBA also contained the 70 kDa subunit and DNA-PK. OBA's identity as Ku antigen was confirmed my microsequencing, Western blot and supershift analyses (Ruiz et al., 1999). OBA/Ku binds to mammalian origins of DNA replication (Ruiz et al., 1999; Araujo et al., 1999; Schild-Poulter et al., submitted). More specifically, OBA binds to a 36-bp sequence, termed A3/4 (Price, G.B and Zannis-Hadjopoulos, M., *in* preparation), that is common to mammalian origins including the *ors*, the Chinese hamster dihydrofolate reductase oriβ origin, the human *dnmt* 1 (DNA methyltransferase) origin, and the human lamin B2 origin. Other laboratories have also reported Ku binding to other mammalian (Toth et al., 1993) viral de Vries et al., 1989) and yeast (Shakibai et al., 1996) origins of DNA replication. The Ku86 subunit of OBA/Ku was responsible for the binding to A3/4, as assessed by Southwestern (Ruiz et al., 1999) and UV crosslinking analyses (Schild-Poulter et al., submitted). OBA/Ku has also been shown to interact with many replication proteins

including polymerases δ and ε, RF-C, and topoisomerase II, in addition to having DNA helicase activity (Matheos et al., submitted). OBA has been shown to act at the initiation step of replication and require an A3/4-homologous sequence for origin binding (Matheos et al., submitted). The ability of OBA/Ku to restore replication activity in the *xrs*-5 nuclear cell extracts further demonstrates that OBA/Ku plays an important role in mammalian DNA replication. Addition of OBA to the CHO K1 nuclear cell extracts did not increase replication (Figure 6), suggesting that the specific level of OBA/Ku in CHO K1 extracts was sufficient for optimum replication.

xrs-5 cytoplasmic cell extracts were as efficient in replicating p186 DNA as the wild-type CHO K1 cell extracts (Figure 5). Their replication competence may be due to either low levels of the Ku protein, as described in Yasui et al. (1999) which, however, we and others have not detected (see above), or to another, Ku-like, factor. Reaction of the *xrs*-5 cytoplasmic cell extracts with radiolabeled A3/4 produced a protein-DNA complex with similar migration as the Ku-A3/4 complex produced by the HeLa and CHO K1 cell extract (Figure 3). Furthermore, this complex was specific, as determined by competition with cold A3/4 DNA (Figure 4). However, the *xrs*-5 cytoplasmic cell extracts-A3/4 complex was not recognized by the anti-Ku (clone 162) antibody (Figure 3), which recognizes a conformational epitope of the Ku70/Ku86 heterodimer, suggesting that the Ku epitope is either not present or not available. This implies that the cytoplasmic protein that recognizes A3/4 is either a modified Ku protein or another origin-specific binding protein with similar affinities for A3/4. This protein could be a modified form of Ku86 that is able to complex with Ku70, albeit in a manner that is not recognized by the antibody. Further studies will need to be performed to address the nature of this protein.

Absence of the Ku86 protein, either in mice or in *ku86* $^{-/-}$ cells, results in hypersensitivity to ionizing radiation, defective DSB-repair pathways and lymphocyte development, and early onset of an age-related phenotype including osteopenia, hepatocellular degeneration and shortened life span (Vogel et al., 1999; Nussenzweig et al., 1996; Gu et al., 1997). Furthermore, Ku86 knockout mice are half the size of their heterozygous littermates, their cells have prolonged doubling times in culture, due to rapid loss of proliferating cells, and they exhibit replicative senescence (Nussenzweig et al., 1996; Gu et al., 1997). Absence of Ku also results in inhibition of DNA replication to a

basal level of 10-20% of normal *in vitro* replication (Ruiz et al., 1999; Matheos et al., submitted). The fact that the knockout mice are viable and in vitro DNA replication is not completely abolished suggest that other mechanisms may take over in the absence of Ku antigen. In vitro replication experiments performed using cell extracts derived of cell lines from knockout Ku86 mice will ultimately allow analysis of the consequences of complete loss of Ku86 on DNA replication. Our present results would suggest that these extracts would be severely defective in their ability to replicate DNA.

Acknowledgement

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CHAPTER SIX

General Discussion

We have previously isolated mammalian (monkey and human) origin enriched sequences, *ors*, which are active replication origins in early S phase (described in Chapter one, section IV.ii)E.e) Origin enriched sequences). Their functional characterization relied on various replication assays such as transient autonomous replication *in vivo* (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991), long-term persistence as episomes in mammalian cells (Nielsen et al., 2000), *in vitro* replication (Pearson et al., 1991; Zannis-Hadjopoulos et al., 1992; Zannis-Hadjopoulos et al., 1994), electron microscopic analysis of replicating DNA (Frappier and Zannis-Hadjopoulos, 1987; Pearson et al., 1994) and PCR-based mapping *in vivo* (Pelletier et al., 1999). In all cases examined, the initiation site has been mapped to the *ors*. For all origins examined thus far, the initiation site mapped *in vivo* by PCR-mapping methods colocalizes with the sites mapped in transient *in vivo* or *in vitro* replication assays. The *in vitro* replication assay is a faster alternative to the other *in vivo* replication assays (Pearson et al., 1991; Pearson et al., 1994; Zannis-Hadjopoulos et al., 1994).

A 186-bp minimal origin of *ors*8, one of the better-characterized *ors* origins, has also been previously identified (Todd et al., 1995). The minimal origin contains a 44-bp IR, several direct repeats, MAR sites, an imperfect binding site for the Oct-1 transcription factor and an A3/4-homologous region. Using this minimal origin of *ors*8, we previously purified an origin binding protein, OBA (Ruiz et al., 1995), which has subsequently been identified as Ku antigen and has been shown to bind in a sequence-specific manner to A3/4 (Ruiz et al., 1999; [see chapter three of this thesis]).

The objective of this thesis was to investigate the role of the transcription factor Oct-1 and the OBA protein, which bind to the octamer motif and A3/4 site in the origin, respectively, in mammalian *in vitro* DNA replication.

Oct-1 is a member of the POU family of transcription factors that have been implicated in tissue-specific gene expression, cell differentiation and DNA replication. Oct-1 is composed of a transcription activation domain and a DNA binding domain, the POU domain. The POU domain has been shown to stimulate DNA replication of adenovirus by directly interacting with the pTP protein and positioning the pTP-pol complex to the core origin. The Oct-1 binding site has also been shown to be important for optimal DNA replication *in vivo*. However, if other compensating DNA sequences,

such as transcription factor binding sites SP-1, ATF, or EBP-1, are present, deletion of the octamer motif fails to produce a replication effect, indicating functional redundancy between different transcription factor binding sites (Hatfield and Hearing, 1993). Furthermore, DNA bending at the octamer motif, produced by Oct-1 binding, is not crucial for DNA replication (Verrijzer et al., 1991), since substitution of the Oct-1 binding site by an AP-1 site does not stimulate replication in the presence of fos-jun, which are able to bend DNA in a similar fashion to Oct-1 (Coenjaerts et al., 1994). Thus, protein-protein interactions between the POU domain and the initiator protein/complex are critical for enhancement of adenovirus DNA replication.

In this thesis, I have shown that the Oct-1 protein is required for efficient mammalian *in vitro* DNA replication (Matheos et al., 1998; [see chapter two of this thesis]). Depletion of Oct-1 from HeLa cell extracts, either by addition of an Oct-1 oligonucleotide or an anti-POU antibody, inhibited DNA replication to basal levels (Chapter two, Figures 4 and 7). In contrast, addition of nonspecific oligonucleotides or antibodies did not produce any inhibitory effect. This inhibition was due to the specific depletion of Oct-1, since addition of exogenous Oct-1 POU protein to the inhibited reaction restored replication levels by two- to three-fold (Chapter two, Figures 6 and 7). Finally, although Oct-1 is able to bind to the imperfect octamer motif in the origin (Chapter two, Figure 3 and 6), it does not seemingly exert its effect through direct DNA binding, since mutation of this site yielded a clone with wild-type replication efficiency (Chapter two, Figure 8). Also, when the transcription factor GATA-1, for which several binding sites exist in the minimal origin of *ors*8, was depleted from the *in vitro* DNA replication reaction, no inhibition was observed (Chapter four, Figure 3B). GATA-1 is a transcription factor that has been implicated in DNA replication of *E. coli*; binding of GATA-1 to the DnaA boxes in the *E. coli* origin represses initiation of DNA replication. The results show that binding of another transcription factor cannot replace the effect observed with Oct-1, suggesting that Oct-1 most likely paricipates in protein-protein interactions, rather than inducing an architectural change of the origin structure.

The data presented in Chapter two suggest that the specific depletion of Oct-1 inhibits DNA replication, although mutation of its binding site does not produce any replication effect. Consequently, a direct role of Oct-1 in origin unwinding through origin

binding is unlikely. A more plausible function for Oct-1 would be its involvement in protein-protein interactions. Thus, the lack of full restoration of DNA replication upon addition of the POU domain protein may be because the transcription activation domain is required for additional protein-protein interactions. Therefore, Oct-1 is likely interacting with replication factors or initiator proteins in mammalian systems. Such a candidate is OBA/Ku (see below).

OBA was initially purified from HeLa cells based on its ability to interact sequence-specifically with the minimal origin of *ors*8 (Ruiz et al., 1995). In this thesis, we have identified OBA as Ku antigen (Ruiz et al., 1999), by microsequencing (Chapter three, Table I), western blot (Chapter three, Figure 6), and supershift (Chapter four, Figure 1) analyses. Ku antigen (described in Chapter one, section X) is an abundant heterodimeric protein that is composed of a 70 kDa and 86 kDa subunit. Various roles for Ku have been proposed in DSB repair, transcriptional regulation, telomeric maintenance, replicative senescence and DNA replication (reviewed in Featherstone and Jackson, 1999; Tuteja and Tuteja, 2000). Ku is known to bind to double-stranded DNA ends, nicks/gaps, single to double-strand transitions, and hairpin structures such as hairpin loops. Binding to DNA ends is mediated by the Ku70 subunit (Zhang and Yaneva, 1992; Chou et al., 1992; Giffin et al., 1999).

OBA/Ku was shown to bind to A3/4, the 36-bp mammalian origin consensus sequence (described in Chapter one, section IV.ii)E.f)) in a sequence-specific manner, since binding was competed in the presence of supercoiled A3/4-containing plasmid but not with supercoiled plasmid alone or with linerized plasmids (Chapter three, Figure 9). Furthermore, binding to A3/4 is preferred to DNA ends (Schild-Poulter et al., submitted) and is mediated by the Ku86 subunit, as determined by microsequencing (Chapter three, Table I) and Southwestern blot (Chapter three, Figure 4) analyses. Therefore, A3/4 represents a distinct class of a Ku binding site based on the fact that the double-stranded form of A3/4 is recognized in a sequence-specific manner by the Ku86 subunit.

In Chapters three and four, I present evidence that OBA/Ku is involved in mammalian *in vitro* DNA replication. When OBA/Ku was depleted, using anti-Ku70, anti-Ku86 or clone 162 (an antibody recognizing the heterodimer), DNA replication was inhibited to 30%-40% of control levels (Chapter three, Figure 10; Chapter four, Figure 2).

Furthermore, when the Ku specific antibodies were preneutralized with their specific Ku blocking peptides, no inhibition of DNA replication was observed, implying that the effect obtained with the Ku antibodies was due to the specific depletion of OBA/Ku. Likewise, when the A3/4 oligonucleotide was added to the *in vitro* replication of p186, p*ors*12 and pX24 (plasmids containing mammalian DNA replication origins with A3/4 homology), DNA replication was inhibited to 10-15% of control levels (Chapter three, Figure 3; Chapter four, Figure 3A). In contrast, several other oligonucleotides, including a nonspecific, and a random oligonucleotide did not produce any effect (Chapter four, Figure 3B). Moreover, addition of an oligonucleotide comprising the NRE-1 sequence, another sequence-specific binding site for Ku (described in Chapter one, section X.ii)A.b)), did not inhibit DNA replication (Schild-Poulter et al., submitted), further implying that the mechanism of Ku binding to the mammalian origin consensus sequence (A3/4) is distinct from binding to other sequences, as described above. Also, depletion of the catalytic subunit of DNA-PK did not inhibit DNA replication (Ruiz et al., 1999; [see Chapter three of this thesis]), suggesting that the DNA binding subunit (Ku) and the catalytic subunit (DNA-PKcs) can function independently in DNA replication.

Interestingly, addition of the A3/4 oligonucleotide did not inhibit SV40 *in vitro* DNA replication (Chapter four, Figure 3A). SV40 has a well-defined replication origin, where viral T-Ag binds and initiates DNA replication. Since, the SV40 origin does not have strong homology to the A3/4 sequence, unlike the mammalian origins, and since the A3/4 oligonucleotide does not inhibit SV40 DNA replication, the results suggest that OBA/Ku is involved in initiation, rather than elongation, step of mammalian DNA replication and requires an A3/4-homologous sequence for origin binding.

In this thesis, I also show that OBA/Ku can interact with proteins of the replication machinery (Chapter four, Figure 6), including with DNA polymerases δ and ε, DNA topoisomerase II, RF-C, RP-A, PCNA. This suggests that OBA/Ku can directly interact with the replication proteins and recruit them to the site of initiation. This is consistent with other examples of initiator proteins, such as SV40 T-Ag and *S. cerevisiae* ORC, which interact and/or recruit proteins required for DNA replication at the origin. In this way, a functional replication complex is formed following binding of the initiator protein to the origin.

OBA/Ku was also shown to interact with Oct-1 (Chapter four, Figure 5). In viral systems, the POU domain of Oct-1 acts as a regulator of replication by interacting with viral initiator proteins. For example, an interaction has been sown between Oct-1 and the adenovirus pTP (van Leeuwen et al., 1997), between Oct-1 and the Herpes simplex transactivator protein (O'Hare et al., 1988), and between Oct-6 and the JC papovavirus T-Ag (Renner et al., 1994). It is likely that Oct-1 acts in a similar manner in mammalian systems; by interacting with OBA/Ku, which may be serving as an initiator protein, Oct-1 may be stabilizing the OBA/Ku-A3/4 interactions and positioning the initiator protein or the replication complex of which OBA/Ku is part.

Furthermore, since Ku is not a homogenous protein due to the fact that the Ku70 subunit is produced by a gene family, OBA's ability to unwind DNA was examined. I found that OBA possesses DNA helicase activity (Chapter 4, Figure 7) with similar kinetics to Ku and to other helicases reported to be involved in DNA replication (Tuteja et al., 1991; Tuteja et al., 1994). The DNA helicase used at the eukaryotic replication fork has not been identified. It has been postulated that several distinct helicases could function in eukaryotic chromosomal replication and that the role of an individual helicase may be required at a specific step in the replication process (Waga and Stillman, 1998). OBA/Ku is a very plausible candidate for being the eukaryotic DNA helicase, since in addition to its DNA unwinding ability, Ku can also bind to the mammalian origin consensus sequence (Ruiz et al., 1999; [see chapter three of this thesis]), translocate along the genome (de Vries et al., 1989; Yoo and Dynan, 1999), and interact with proteins of the replication machinery (Matheos et al., submitted; [see chapter four of this thesis]).

The data presented in Chapters two, three and four suggest that OBA/Ku can function as a mammalian initiator protein; it can bind in a sequence-specific manner to the origin consensus sequence, unwind DNA, interact/recruit the replication machinery, and interact with a transcription factor that can enhance basal replication levels. However, depletion of OBA/Ku did not fully abolish *in vitro* DNA replication, whereas depletion of another important DNA replication protein, DNA polymerase δ, did (Chapter four, Figure 2C). This suggested that an alternative replication mechanism exists in the absence of OBA/Ku. I, therefore, examined the replication activity of *xrs*-5, a derivative of the CHO

K1 cell line that is defective in Ku86 (Matheos et al., submitted; see Chapter five of this thesis).

The results presented in Chapter five show that total and cytoplasmic extracts from *xrs*-5 cells can support replication of the p186 DNA with the same efficiency as wild-type CHO K1 cell extracts (Chapter five, Figure 1 and 5), whereas the *xrs*-5 nuclear cell extracts cannot (Chapter five, Figure 5). The replication activity of the *xrs*-5 nuclear cell extracts was restored upon addition of OBA (Chapter five, Figure 6). By western blot analysis, I showed that the *xrs*-5 cells lack any detectable Ku86 (Chapter five, Figure 2). Furthermore, although no binding to A3/4 is observed with the *xrs*-5 nuclear extracts, binding is obtained with the cytoplasmic *xrs*-5 extracts (Chapter five, Figure 3). Competition/bandshift analyses confirmed that there exists a cytoplasmic protein in the *xrs*-5 cells that binds in a sequence-specific manner to A3/4. This cytoplasmic protein is either a modified Ku or another origin binding protein with similar affinities for A3/4. *In vivo* replication assays, looking at the entire cell rather than either the nuclear or cytoplasmic cell extracts, will ultimately determine the importance of this cytoplasmic A3/4-binding protein, and any defects in its transport to the nucleus.

Based on all the results presented in this thesis, a preliminary model for the involvement of OBA/Ku in mammalian DNA replication can be presented (Chapter four, Figure 8). First, OBA/Ku binds to the A3/4 sequence present in mammalian origins, either by itself or as part of the replication complex. Oct-1 will then interact with OBA/Ku and further aid in positioning the replication complex, as it does in viral systems. OBA/Ku, if not already associated with the replication complex, will interact with other replication proteins and recruit them to the origin site. Following the initial steps of origin recognition, binding, and loading of the replication complex, OBA/Ku will proceed to unwind the DNA. In the absence of OBA/Ku an alternative mechanism likely predominates, which will involve a cytoplasmic protein binding to the A3/4 sequence.

These studies provide a better understanding of the proteins that bind to mammalian origins, thereby aiding in understanding the mechanisms that regulate the initiation of DNA replication.

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