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The presence of cruciforms in functional mammalian origins of DNA replication and purification of a human cruciform binding protein

by

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November, 1998

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

For my parents Judy and Malcolm

SUMMARY

The proteins and sequence elements necessary to initiate mammalian DNA replication have been, to date, poorly characterized. Here, we have further defined the minimal sequence elements required to support autonomous DNA replication of a mammalian early replicating DNA sequence, *ors8*. In addition, we have identified a protein, CBP, which binds specifically to DNA cruciforms, a DNA secondary structure that can form at inverted repeats which are commonly found within origins of DNA replication.

Cloned fragments from a known mammalian origin from the DHFR locus, *oriβ*, have been shown to replicate autonomously following transient transfection into mammalian cells and when used as templates in an *in vitro* DNA replication system. These *in vivo* and *in vitro* DNA replication assays have been used to define the sequence elements present within the minimal origin of *ors8*, a monkey origin enriched sequence, activated in early S phase. The minimal origin, consisting of an internal sequence of 186 bp, was shown to contain bent DNA, an imperfect Oct-1 binding site, and an inverted repeat which has been shown previously to extrude into a cruciform structure *in vivo*. Cruciform structures have been hypothesized to form at or near origins of DNA replication to serve as sites of recognition for initiator proteins. To further investigate this possibility, a previously described cruciform binding protein, CBP, was identified as a member of the 14-3-3 family of proteins. 14-3-3 proteins are involved in regulating a wide array of cellular processes by acting as adaptor or stabilizing molecules. We have shown that 14-3-3 binding to cruciform structures is required for the *in vitro* DNA replication of the 186-bp minimal origin of *ors8*, further suggesting a crucial role for cruciform structures in DNA replication.

RÉSUMÉ

La caractérisation des éléments de séquence et des protéines nécessaires pour l'initiation de la réplication de l'ADN chez les mammifères est à ce jour incomplète. Nous avons donc caractérisé de façon plus précise les éléments de séquences requis pour la réplication de l'origine de mammifère *ors8*. De plus, nous avons identifié la CPB, une protéine capable de se lier aux structures cruciformes présentes dans les régions d'origine de la réplication de l'ADN.

Des fragments provenant du locus *DHFR*, qui contient l'origine de réplication *oriβ*, ont pu se reproduire de façon autonome dans des cellules mammifères et servir comme *template* dans un système de replication *in vitro*. Nous avons utilisé ces essais de la réplication d'ADN *in vivo* et *in vitro* pour définir les éléments de séquence que l'on retrouve dans l'origine minimale de réplication *ors8* qui représente une séquence enrichie d'origines de réplication de singe activée dans la portion initiale de la phase S. Nous avons démontré que cette origine minimale, qui comporte une séquence interne de 186 pb, contient également de l'ADN courbée, un site de liaison imparfait pour Oct-1, ainsi qu'une séquence inverse répétée capable de former une structure cruciforme *in vivo*. Les structures cruciformes pourraient donc se former aux origines de réplication de l'ADN et induire le recrutement de protéines impliquées dans l'initiation de ce processus. Pour évaluer cette hypothèse de façon plus approfondie, nous avons identifié la protéine CPB qui est capable de se lier aux structures cruciformes comme étant un membre de la famille des protéines 14-3-3. Les protéines 14-3-3 sont impliquées dans la régulation de diverses réponses cellulaires en agissant comme molécules adaptatrices ou stabilisatrices. De plus, nous avons démontré que l'intéraction de 14-3-3 avec les structures cruciformes est requise pour la réplication *in vivo* et *in vitro* de l'origine minimale *ors8*.

FOREWORD

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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 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.

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As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided 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.

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.

CONTRIBUTIONS OF AUTHORS

Chapter 2: Zannis-Hadjopoulos, M., Nielsen, T.O., Todd, A., and Price, G.B. "Autonomous replication *in vivo* and *in vitro* of clones spanning the region of the *DHFR* origin of bidirectional replication (*oriβ*)." 1994. *Gene* **151**:273-277.
I carried out the *in vivo* replication assays together with Maria Zannis-Hadjopoulos, and the *in vitro* DNA replication assays were performed by Torsten Nielsen.

Chapter 3: Todd, A., Landry, S., Pearson, C.E., Khoury, V., Zannis-Hadjopoulos, M. "Deletion analysis of minimal sequence requirements for autonomous replication of *ors8*, a monkey early-replicating DNA sequence." 1995. *J. Cell. Biochem.* **57**:280-289.
Deletion mutants of *ors8* were generated by Suzanne Landry and Viviane Khoury. I carried out all of the *in vivo* DNA replication assays, and the bent DNA assays. Christopher Pearson performed the *in vitro* DNA replication assays.

Chapter 4: Todd, A., Cossons, N., Aitken, A., Price, G.B., and Zannis-Hadjopoulos, M. "Human cruciform binding protein belongs to the 14-3-3 family." 1998. *Biochemistry* **37**:14317-14325.
All of the experiments in this publication are my own with the exception of parts of the immunofluorescence experiments depicted in figure 6, which were performed by Nandini Cossons. Alastair Aitken provided the 14-3-3 antisera and purified proteins.

Chapter 5: Todd, A., Aitken, A., Price, G.B. and Zanni-Hadjopoulos, M. "14-3-3 proteins required for *in vitro* DNA replication. " To be submitted for publication as a short communication in 1999. I performed the band-shift assays, and Maria Zannis-Hadjopoulos performed the *in vitro* DNA replicaton assays. The 14-3-3 antibodies were supplied by Alastair Aitken.

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ABBREVIATIONS

2D	two-dimentional
Ad	adenovirus
ARS	autonomously replicating sequence
βME	β-mercaptoethanol
BLAST	basic alignment search tool
Blotto	TBS-T, 5% skim milk
bp	base pairs
BrdUrd	bromodeoxyuridine
CADMS	collisionally activated dissociation mass spectrometry
CBP	cruciform binding protein
CDK	cyclin dependent kinase
C-terminus	carboxy-terminus
DHFR	dihydrofolate reductase
DHFR	gene encoding DHFR
DMEM	Dulbecco's minimal essential medium
DNase	deoxyribonuclease
ds	double stranded
DTT	dithiothreitol
DUE	DNA Unwinding Element
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EM	electron microscope

ESMS	electrospray mass spectroscopy
FCS	fetal calf serum
FISH	fluorescence *in situ* hybridization
G1	gap 1 phase of the cell cycle
G2	gap 2 phase of the cell cycle
Hepes	N-(2-hydroxyethyl)piperazine-Ní-2-ethane-sulfonic acid
HPLC	high-performance liquid chromatography
HSV	herpes simplex virus
IR	inverted repeat
kb	kilo bp, or 1000 bp
LCR	locus control region of the human β-globin locus
M	mitotic phase of the cell cycle
MAR	matrix attachment region
MCM	minichromosome maintenance protein
MEM	minimal essential medium
NP40	nonylphenoxy polyethoxy ethanol
NRS	normal rabbit serum
nt	nucleotide
N-terminus	amino-terminus
ori	origin(s) of replication
oriβ	bidirectional *ori* of hamster *DHFR* region
ors	ori enriched sequence

PBS	phosphate buffered saline (137mM NaCl, 8mM Na_2HPO_4, 2.7mM KCl, 1.5mM KH_2PO_4)
PCR	polymerase chain reaction
pI	isoelectric point
PKC	Protein kinase C
Post-RC	postreplicative complex
Pre-RC	prereplicative complex
pTP	preterminal protein (Adenovirus)
PVDF	polyvinylidene fluoride
RLF	replication licensing factor
S phase	DNA synthetic phase of the cell cycle
SAR	scaffold attachment region
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPF	S-phase promoting factor
ss	singe stranded
SSB	single stranded binding protein (*E.coli*)
SV40	simian Virus 40
TAg	SV40 Large Tumor Antigen
TBS	10mM Tris-HCl, pH 8.0, 150 mM NaCl
TBS-T	TBS, 0.05% Tween-20
UV	ultra violet

ACKNOWLEDGEMENTS

I would like to thank my thesis supervisor, Dr. Maria Zannis-Hadjopoulos, for providing me with the opportunity to work in her laboratory when I was an uncertain undergraduate student, and her wisdom in deciding to keep me as a graduate student. I am grateful for her never failing support, optimism, and for providing me with the freedom to pursue my ideas. I would also like to thank Dr. G.B. Price for many stimulating discussions, critical advice, and for teaching me to believe. I would like to thank Dr. Alastair Aitken for a successful collaboration and for helpful discussions. I would like to thank JF and Fiona for critical reading and help with this thesis. Thanks to Mike for soft-ball, and JF for hockey. I am indebted to Claude, Ariadni, and Fito for being there and helping me to be who I am today. I thank all the past and present members of Maria's and Gerry's labs, as well as members of the Cancer Centre and Biochemistry department for technical help, advice and encouragement. My thanks are extended to Sarita Benchimol for her constant support of the students, for never letting us go hungry, and to both Sarita and Dr. Stanners for always working hard to make the Cancer Centre a great place to work.

I would like to thank the McGill Faculty of Medicine, Le Défi Corporatif Canderel, the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR), the Henri St-Amour educational fund of the Lachine General Hospital, and the St Andrew's Society of Montréal for financial support.

I am especially grateful to JF for introducing me to la fée des étoiles, for his understanding, endless patience, and love. I must also thank my family for their understanding and support over the last 6 years. I thank most importantly my mother and father for their unconditional love, for encouraging me to pursue whatever I wanted to and for providing me with the opportunities to do so.

CHAPTER ONE

GENERAL INTRODUCTION

The most basic definition of what constitutes 'life' is the ability of an entity to reproduce itself. Arguably, the cell's ability to faithfully duplicate its genome prior to dividing is, therefore, what defines it as a living entity. The initiation step of DNA replication, which is a very important and highly regulated step in the process, is still poorly understood in mammalian cells. Furthering our understanding of the initiation of DNA replication in higher eukaryotes should help us to better understand processes such as malignant transformation, where the strict controls regulating initiation of replication may be disrupted.

I. DNA replication origins in model systems

The study of DNA replication in simple organisms such as bacteria, viruses, and yeast has revealed common features and mechanisms. The small genomes of simple organisms are easy to study in comparison to those of metazoans, and they also share feature and mechanistic similarities with complex genomes. Simple organisms serve therefore as model systems for the study of the more complex genomes of higher eukaryotes. Outlined below are descriptions of some of the more intensely studied model systems for DNA replication (reviewed recently, Hickey and Malkas, 1997; Zannis-Hadjopoulos and Price, 1998).

(i) Prokaryotes

(a) *Escherichia coli* chromosomal origin, *oriC*

The replication of the 4.7 X 10^6 bp genome of the *Escherichia coli* bacterium initiates from a unique site in the circular chromosome, *oriC* (Kornberg and Baker, 1992; Marians, 1992). This minimal origin region of 245 bp (Oka *et al.*, 1980) contains several regions conserved across bacteria, which do not tolerate substitutions, interspersed

between random sequence. There are four 9-mer sequences (5'-TTATC/ACAC/AA-3') which are organised into two inverted repeats (IRs). These repeated sequences, also known as the dnaA boxes, have a highly conserved position (Zyskind *et al.*, 1983), and serve as binding sites for *E. coli*'s initiator protein, dnaA (Fuller and Kornberg, 1983). Three AT rich 13-mer sequences (5'-GATCTNTTNTTTT-3') are located at the left edge of oriC, and serve as the site of helix unwinding after initiation (Kowalski and Eddy, 1989; Figure 1.1). Also found within *oriC* are 11 copies of the recognition sequence for the Dam methylase, (5'-GATC-3'). Methylation of the adenine within these sites regulates the association of *oriC* with the cell membrane, and contributes to the refractory period between initiation events. Initiation at *oriC* is prevented when the origin is hemimethylated (Russell and Zinder, 1987), which occurs following the passage of the replication fork and prior to methylation of the newly synthesised strand by Dam methylase.

Initiation requires a supercoiled template, and begins at *oriC* with the highly co-operative binding of dnaA monomers to the dnaA boxes within *oriC* (Fuller *et al.*, 1984). The 52 kDa dnaA protein binds tightly to ATP, with a Kd of 0.03μM (Sekimizu *et al.*, 1987), and possesses weak DNA dependent ATPase activity. Consequently, dnaA can be found complexed with ATP, ADP or free of nucleotide. The initial binding of dnaA protein to the dnaA boxes occurs in the presence of low concentrations (30nM) of ATP (Sekimizu *et al.*, 1987). In conjunction with the dsDNA-binding protein HU, 10-20 monomers of dnaA become complexed with 200-250 bp of *oriC*, with the DNA wrapped around the multimers of dnaA protein. In the presence of high concentrations of ATP (5 mM) the *oriC*-dnaA-HU complex is converted into an open complex with the unwinding of the three AT rich 13-mer sequences (Bramhill and Kornberg, 1988).

Figure 1.1 ***Echerichia coli*** **chromosomal origin,** ***oriC***

The 245 bp *oriC* contains four 9-mer sequences, or dnaA boxes, which serve as binding sites for the initiator protein, dnaA. Three AT rich 13-mer sequences serve as the site of unwinding, and are located at the left edge of *oriC*

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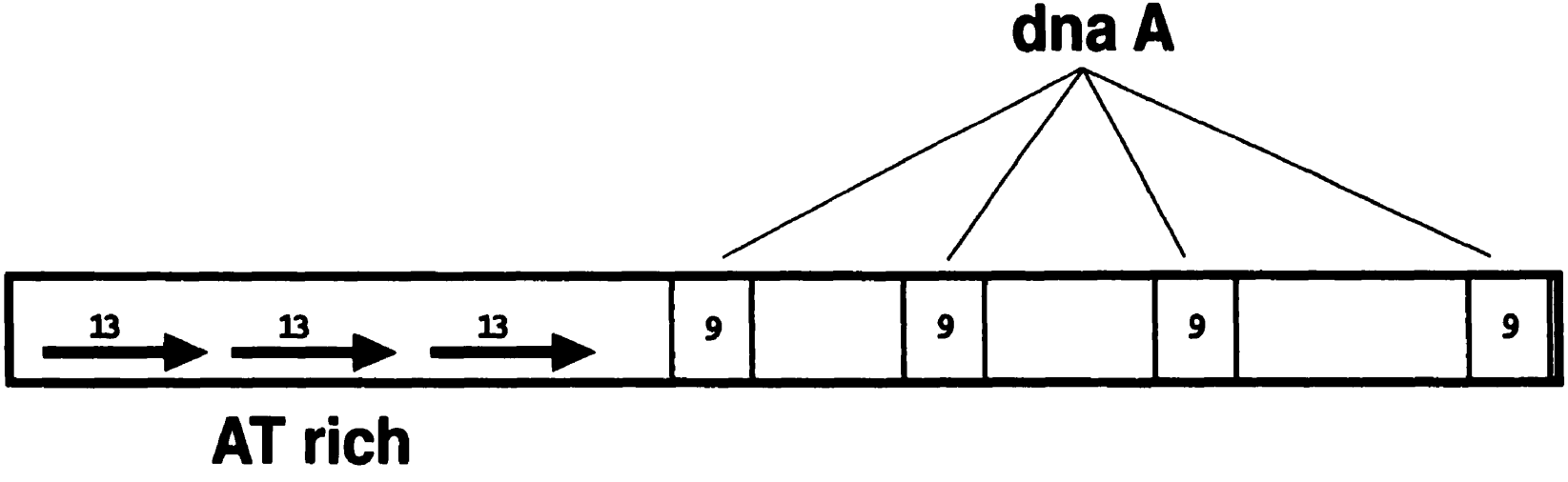
E. coli oriC



The presence of dnaA then guides the *E. coli* helicase, dnaB, from a dnaB-dnaC complex in solution to the opened duplex DNA, thus forming a prepriming complex. In the presence of the single-stranded DNA binding protein (SSB) and DNA gyrase, dnaB unwinds the template bidirectionally from *oriC*. The *E. coli* primase, dnaG, and DNA polymerase III holoenzyme complete the enzymes necessary to form the replication forks, and thus synthesize the daughter molecules.

There is convincing evidence implicating the activity of RNA polymerase in the initiation of replication at *oriC;* the RNA polymerase inhibitor rifampicin can cause inhibition of initiation at *oriC in vivo* (Baker and Kornberg, 1988; Ogawa *et al.*, 1985). The effect of RNA polymerase on the initiation of replication at *oriC* is independent of mRNA synthesis, and does not involve the priming of DNA synthesis (Baker and Kornberg, 1988). Rather, the effect of RNA polymerase is more likely to favour the open complex required for the assembly of the replication complex. Active transcription at even substantial distances from *oriC* can provide a large enough change in the template topology to allow for opening of the duplex and initiation to occur (Kornberg and Baker, 1992).

(b) Bacteriophage lambda origin, oriλ

Bacteriophage λ is a temperate phage with a genome of 48 kb, which can either grow lytically, or lysogenically. The lytic life cycle produces many progeny phage, and lysis of its host, *E.coli*. During the lysogenic state, most phage functions are repressed, with the genome remaining quiescent as a prophage, and integrated into the host *E. coli* chromosome. As such, it is replicated regularly as part of the host DNA. DNA damage or other agents that cause inhibition of host DNA replication induces the lytic life cycle,

resulting in excision of the prophage as a covalently closed circle. As is the case for the *E. coli* chromosome, a supercoiled template is required for replication to proceed. Replication of the excised lambda genome thus allows the phage to escape the fate of its host cell (Calendar, 1988).

Replication of the 48 kb phage λ genome requires all of the host cell replication machinery except the initiator protein, dnaA. Two virally encoded proteins, the products of the O and P genes (λO and λP) are the only factors required for replication that are not supplied by the host. Although the overall structure of the λ phage origin of replication, *oriλ,* is similar to that of *oriC*, the *oriλ* sequence is not related to *oriC*. *oriλ* contains four repeats of a 19 bp palindromic sequence, which are analogous to the four 9-mer repeats of *oriC*, since they serve as binding sites for the λ phage initiator protein, λO (Dodson *et al.*, 1985). Flanking the 19-bp elements is a 40 bp AT rich region, composed of three 11 bp repeated sequences (5'-TTNTCTTTTGT-3'), which is the site of helix unwinding (Zylicz *et al.*, 1984; Figure 1.2). To the right of the AT rich region lies a 28 bp IR. To initiate replication, λO binds tightly to the four 19-bp repeats within oriλ via its N-terminus (Zylicz *et al.*, 1984). Electron microscope (EM) analysis has shown that, analogous to the case for dnaA protein in *E. coli*, the DNA is wrapped around the λO protein aggregate, forming an 'O-some' (Dodson *et al.*, 1985). An intrinsic bend in the oriλ sequence aids in the formation of the O-some, and induces structural instabilities in the flanking AT rich region. Once bound, λO unwinds the flanking AT rich region (Schnos *et al.*, 1988), and interacts with the λP protein via its C-terminus (Zylicz *et al.*, 1984). The λP protein is an analogue of dnaC, and serves to recruit the host dnaB helicase to the origin (Dodson *et*

al., 1986) by virtue of the fact that it has a higher affinity for dnaB than does dnaC (Mallory *et al.*, 1990). The λP protein is brought to the origin in a complex with dnaB. Before replication can proceed, the host heat-shock proteins dnaK, dnaJ and grpE are required to remove λP from the origin, thus releasing dnaB to carry out its helicase function (Dodson *et al.*, 1986). The structural instabilities resulting from the intrinsically bent sequence at *oriλ* probably facilitate the insertion of the dnaB protein between the template strands as it is released from λP. dnaK is a prototypical molecular chaperone which possesses a weak DNA-independent ATPase activity which is stimulated by the presence of both dnaJ and grpE (Liberek *et al.*, 1991). The removal of λP from the origin requires ATP hydrolysis by dnaK (Alfano and McMacken, 1989; Liberek *et al.*, 1988).The assembled replication forks which follow are identical to those of *E. coli*.

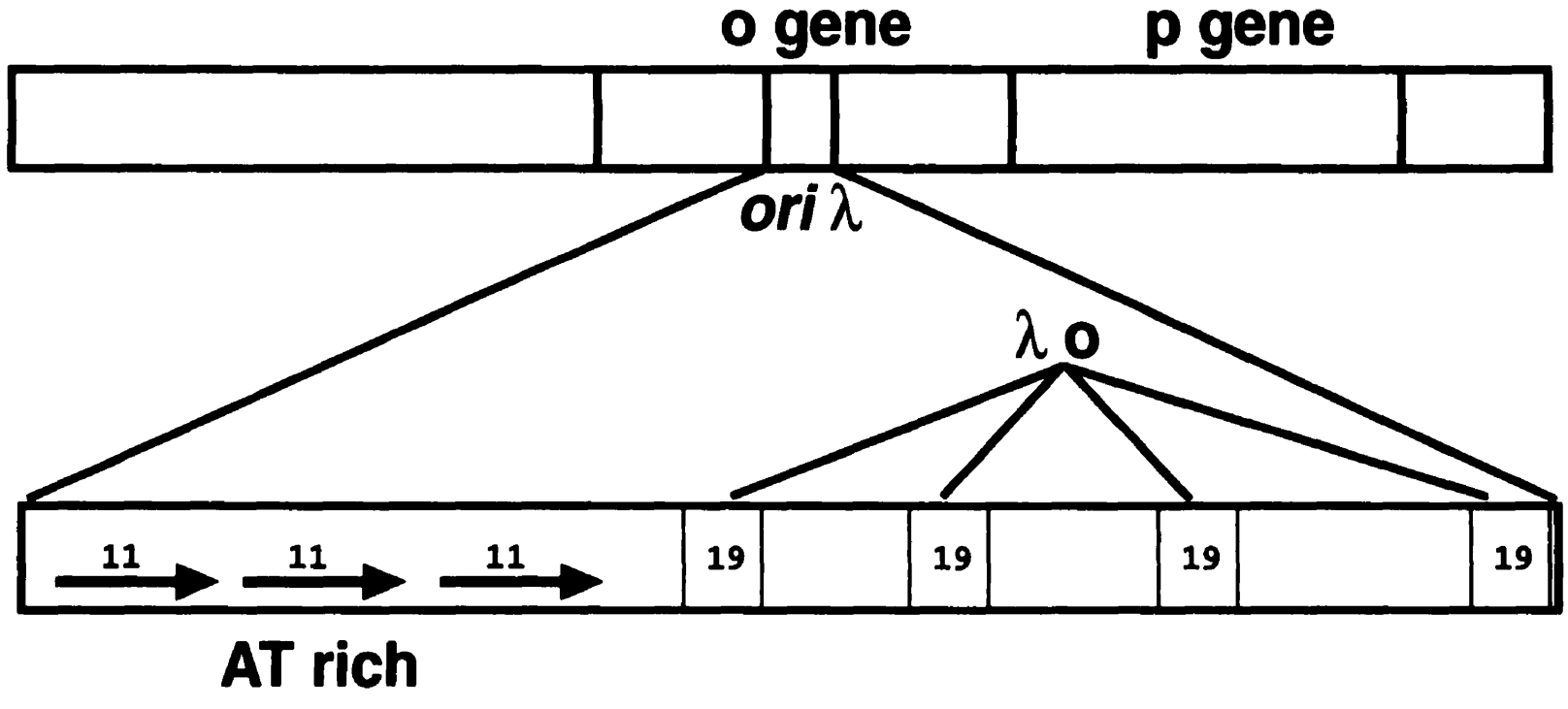
RNA polymerase activity is also required for initiation at *oriλ* (Dove *et al.*, 1971). Transcription through the P gene, which is downstream from *oriλ*, is usually required for initiation. Because the transcription does not necessarily need to cross the origin, it is thought that transcription 'activates' initiation rather than providing the necessary RNA primers (Furth *et al.*, 1982).

In summary, replication at the phage λ origin, *oriλ*, is very similar to that of it's host origin, *oriC*, with only two phage gene products, λO and λP, needed in addition to the host cell proteins. The involvement of the three host molecular chaperones dnaK, dnaJ and grpE in *oriλ* initiation has led to questions regarding their role in initiation at *oriC*. To date their involvement in *E. coli* DNA replication cannot be ruled out.

Figure 1.2 Bacteriophage lambda origin, *oriλ*

In a structure similar to that of *oriC*, *oriλ* contains four repeats of a 19 bp sequence which serve as binding sites for the initiator protein, λO. Flanking this region is an AT rich region consisting of three 11 bp repeated sequences. To the right of the AT rich region lies a 28 bp inverted repeat (not shown).

Bacteriophage lambda *ori* λ



(c) ColE1 origin

Several common plasmids, including the 4.6 kb pBR322, replicate via the ColE1 origin, derived originally from the ColE1 *E. coli* plasmid. This origin uses a strategy to initiate DNA replication which is different to that used by the *oriC* and *oriλ* origins. The ColE1 origin is not recognized by an initiator protein. Rather, origin recognition and origin unwinding are effected by transcription carried out by RNA polymerase. Transcription from a promoter 555 nt upstream of the ColE1 origin opens the double stranded template, and forms a stable RNA-DNA hybrid. In contrast with *oriC* and *oriλ* initiation, the 3' end of this initiating transcript serves to prime replication of the continuous strand (Itoh and Tomizawa, 1980). Processing of the RNA-DNA hybrid by RNaseH results in the formation of a stable D-loop with a 3'-OH that can be extended by a DNA polymerase. Two dnaA boxes, in the form of an IR, lie downstream from the origin and can serve as loading sites for discontinuous strand synthesis.

(ii) Eukaryotes

(A) Viruses

(a) SV40

Simian virus 40 (SV40), a papovavirus family member, replicates in simian cells, and to a lesser extent in human cells. The double stranded circular genome of about 5300 bp is assembled into chromatin which is, other than by the absence of histone H1, indistinguishable from the chromatin of the host cell (Hassell and Brinton, 1996). SV40 replicates its genome strictly during S phase of the cell cycle with the aid of only one virally encoded protein, large tumour antigen (TAg), with the balance of necessary

proteins provided by the host. The simplicity of the SV40 genome, coupled with its use of a mechanism similar to that used by its host to duplicate its genome, has led to its intense study since the 1950s with the goal of furthering the understanding of eukaryotic DNA replication.

The SV40 genetically defined origin is located in a non-coding region of the genome along with early and late promoter elements (reviewed in DePamphilis, 1988). It comprises two functional elements: a core region, known as *ori*-core, and auxiliary regions. The *ori*-core is where replication initiates, and is absolutely required. The auxiliary regions increase the efficiency (5-to 25-fold) and cell-type specificity of replication (Hassell *et al.*, 1986).

Ori-core consists of three essential domains. At the centre of the core is a 27 bp IR which contains four copies of a pentanucleotide motif (5'-GAGGC-3'), to the right of the core lies an AT rich domain of 17 bp, and to the left of the central region is a 15 bp imperfect IR, also known as the early palindrome (Kornberg and Baker, 1992). The central 27 bp IR contains the four pentanucleotide TAg binding sites arranged as two inverted pairs. The spacing, sequence and arrangement of these sites are critical for *ori* function (Dean *et al.*, 1987; Deb and Tegtmeyer, 1987). The AT rich region is a site of DNA bending (Deb *et al.*, 1986b) which is further distorted when TAg binds to the central region of the *ori*-core. This AT rich region is also a likely site of helix unwinding during initiation. It has been suggested that the early palindrome may be functionally similar to the DNA unwinding element (DUE) described in yeast origins (Lin and Kowalski, 1994).

The auxiliary elements fall into two classes: TAg binding sites and transcriptional activator binding sites. Additional TAg binding sites flank both sides of *ori*-core, and the

affinity of TAg for these sites is of similar magnitude as for the sites within *ori*-core. These TAg binding sites may stimulate replication by recruiting TAg to the vicinity of *ori*-core, thus facilitating TAg-mediated unwinding (Gutierrez *et al.*, 1990). The transcriptional activator binding sites are located at the late border (left) of *ori*-core, and are capable of stimulating replication to a much greater extent than the TAg binding sites (Figure 1.3, reviewed in DePamphilis, 1988; DePamphilis and Bradley, 1986; DePamphilis, 1993b). The possible mechanisms of stimulation of replication by transcriptional activators are outlined below.

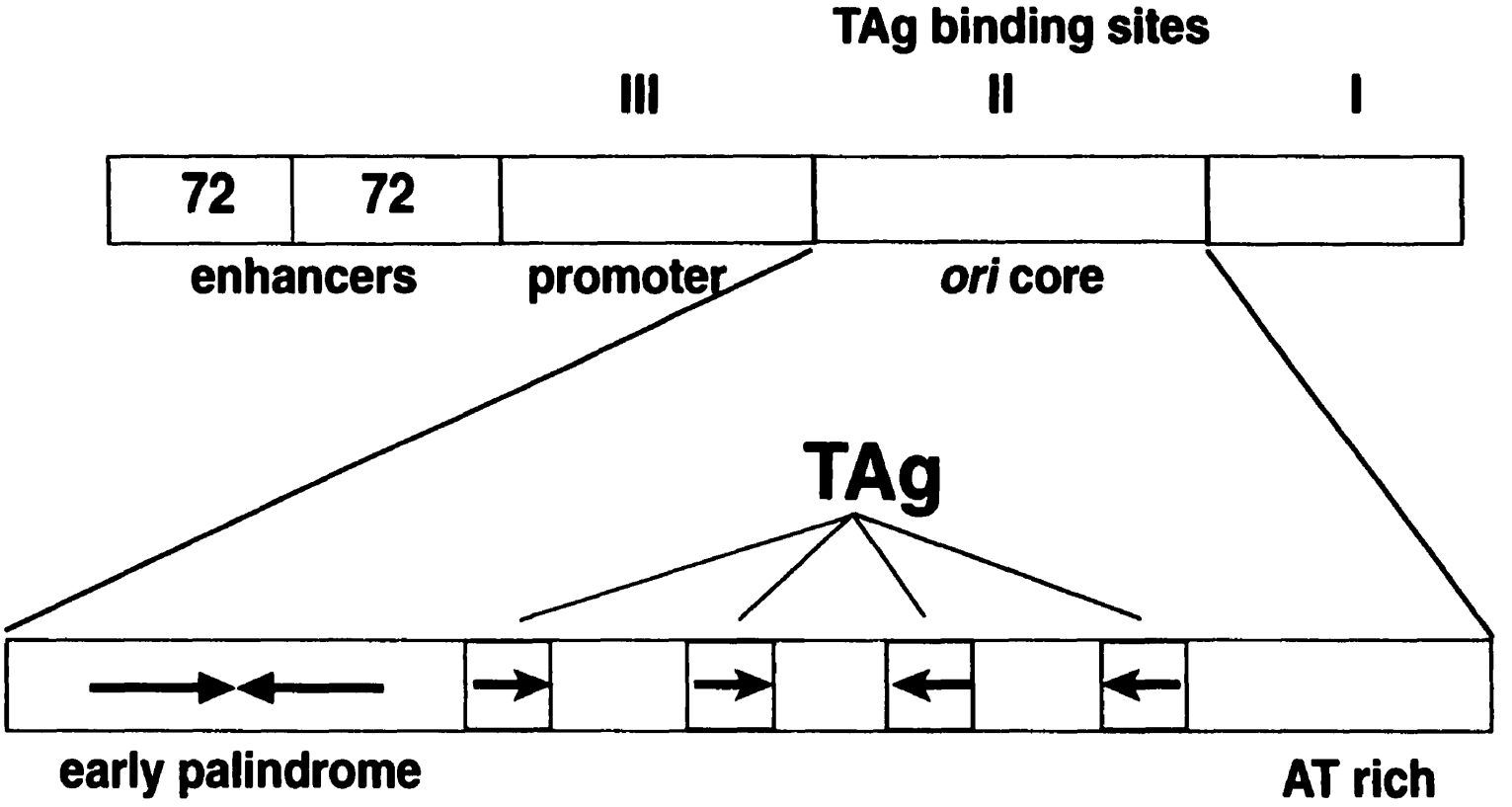
The SV40 initiator protein, large T antigen is a multifunctional nuclear phosphoprotein (reviewed in Fanning, 1992; Fanning and Knippers, 1992; Manfredi and Prives, 1994; Pipas, 1992). TAg's activities include sequence-specific binding to double and single strand DNA, ATPase, and a 3' to 5' helicase, necessary for the advancement of the replication fork. TAg is also known to interact with a number of cellular proteins involved in DNA replication and transcription. These include the replication proteins DNA polymerase-αprimase, the single-stranded binding protein RP-A, and the transcriptional activators p^{105}Rb and p53.

Initiation of SV40 DNA replication begins with the binding of TAg monomers to the four pentanucleotide repeats within *ori*-core (Borowiec *et al.*, 1990; SenGupta and Borowiec, 1994). After the initial TAg binding, additional monomers add to the complex, assembling into two hexamers that flank the *ori*-core. ATP binding by TAg stimulates the process, and is required for the hexameric complex formation. TAg then untwists the AT rich region, and melts the early palindrome.

Figure 1.3 SV40 origin of DNA replication

The core region, *ori*-core, and auxiliary regions of the SV40 origin are coincident with promoter elements of the genome. *Ori*-core consists of: 1) a 27 bp inverted repeat, containing four copies of the 5'-GAGGC-3' sequence (denoted by short arrows) which serve as TAg binding sites, 2) an AT rich domain, and 3) an imperfect inverted repeat also referred to as the early palindrome. The auxiliary elements consist of additional TAg binding sites which flank *ori*-core, and an enhancer region. The enhancer region contains transcriptional activator binding sites.

SV40


TAg binding sites
III
II
I
72
72
enhancers
promoter
ori core
TAg
early palindrome
AT rich

The single-stranded DNA binding protein RP-A is then recruited to the TAg-*ori* complex, facilitating further unwinding of the *ori* by TAg's 3'-5' helicase activity. The TAg/RP-A complex then recruits DNA polymerase-αprimase thus forming the preinitiation complex, which is primed for bidirectional semiconservative DNA replication.

In summary, the initiator protein TAg carries out three functions during the initiation of replication: it alters the structure of the *ori* DNA, it unwinds the DNA ahead of the replication fork, and it recruits replication proteins to the *ori* to form the preinitiation complex.

Despite the considerable amounts of information obtained from the study of SV40, there are limitations to what can be learned about cellular DNA replication from this system. SV40 encodes its own initiator protein, therefore providing no information with respect to the cellular initiation process. In addition, SV40 initiates many times from the same origin within the same S phase unlike the cellular case, where each origin initiates only once per S phase, leaving questions about how this cellular regulation takes place.

(b) Adenovirus

Adenoviruses, of which there are many distinct strains, have been isolated from human, simian and other animal cells, and are often associated with acute respiratory disease. Some strains are oncogenic, while others are only transforming. Adenovirus possesses a linear double stranded genome of 36 kb, which includes two replication origins of 51 bp positioned at either end of the genome within inverted terminal repeats. The inverted terminal repeats allow for a single stranded circular structure to form via a pan-handle structure. The identical origins at each end of the viral DNA consist of three domains (Kornberg and Baker, 1992). Domain A serves as the minimal origin, and

contains a 10 bp consensus sequence conserved throughout all serotypes (5'-ATAATATACC-3'). Domain B contains the consensus sequence 5'-TTGGCN$_5$GCCAA-3' and stimulates replication 30-fold or more. Domain C contains the consensus sequence 5'-TATGATAAT-3' and stimulates replication three-fold. Domain B serves as the binding site for the cellular transcription factor NFI, and Domain C binds the cellular transcription factor NFIII/Oct-1 (Figure 1.4). Adenovirus replication requires three virally encoded proteins: the terminal protein precursor (pTP), the DNA polymerase (Ad pol), and the SSB, (AdDBP). Three cellular proteins (nuclear factors) are also required: NFI, NFIII/Oct-1 and a type I DNA topoisomerase (NFII). *In vitro* replication systems have been developed which have been useful in the elucidation of the mechanism of replication of the adenovirus genome (reviewed in Stillman, 1989). Replication takes place in two stages. In the first stage, initiation and unidirectional elongation from either end produces a duplex daughter strand and a displaced single strand. In the second stage, the displaced daughter strand is replicated to produce a second daughter duplex. The viral terminal protein (TP; processed pTP) is covalently bound to each 5' end of the genome. Initiation involves displacement of the TP by the pTP at the 3' end of the complementary strand, and linkage by the Ad pol of a deoxycytidylate to a serine residue of the pTP. Replication by the Ad pol then follows. Initiation is stimulated by the two cellular transcription factors, NFI (Mul and Van der Vliet, 1992) and NFIII/Oct-1, which act synergistically (van Leeuwen *et al.*, 1997).

Figure 1.4 Adenovirus origin

The 36 kb adenoviral genome contains two origins of replication, each consisting of three domains. Domain A is the minimal origin, and binding site for the pTP/pol. Domains B and C stimulate replication, and serve as binding sites for the cellular factors NFI and NFIII/Oct-1 respectively

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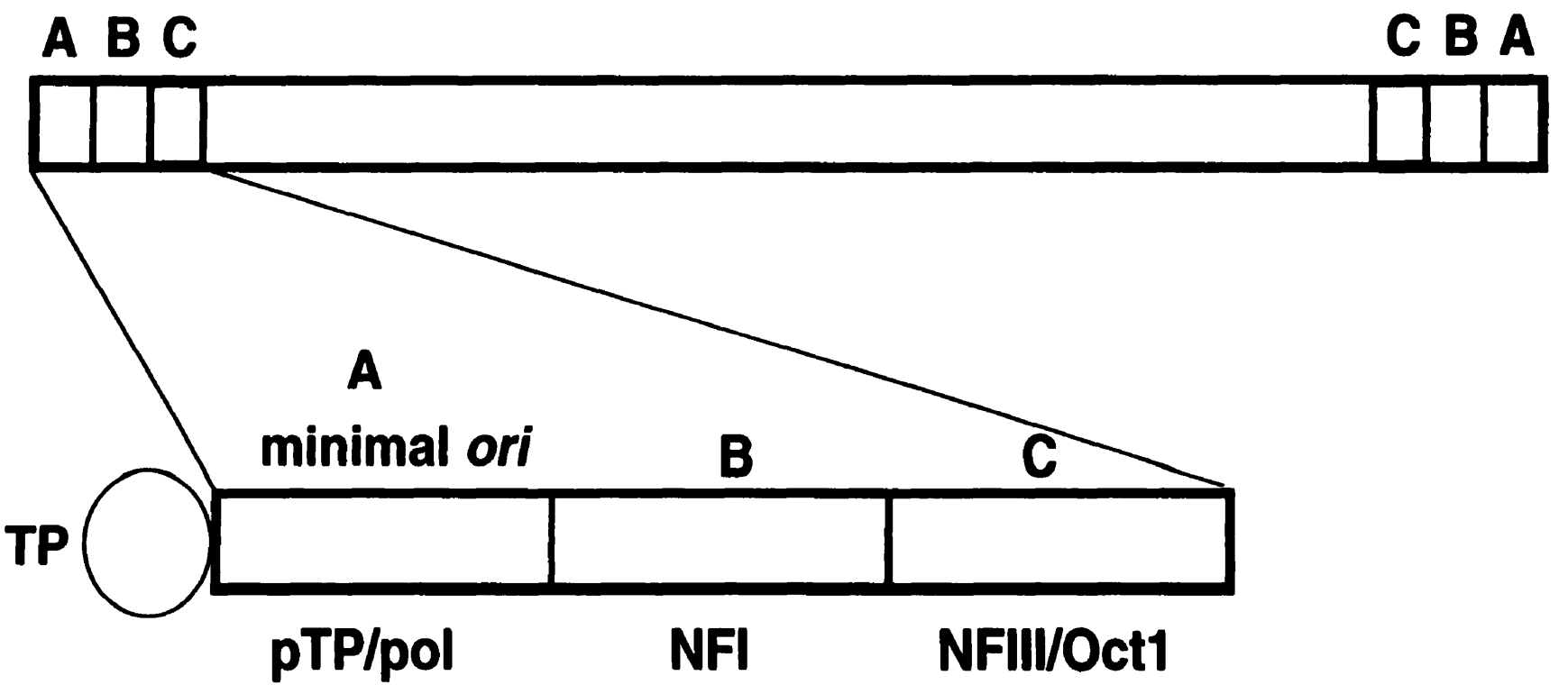
Adenovirus



(B) Yeast

The budding yeast *Saccharomyces cerevisiae* replicates its genome from an estimated 250-400 origins of replication distributed on 16 chromosomes, thus serving as a more relevant model for the replication of the genomes of higher eukaryotes, which are estimated to have hundreds of thousands (10^4-10^6) of origins of replication distributed on 23 pairs of chromosomes (Hand, 1978).

Autonomously Replicating Sequences (ARS elements), were first discovered in *S. cerevisiae* as DNA sequences that allow plasmids carrying selectable markers to transform yeast at high frequency (Hsiao and Carbon, 1979; Stinchcomb *et al.*, 1980). In these transformed cells, the extrachromosomal plasmids were maintained autonomously, suggesting that ARSs may be origins of replication (reviewed in Campbell and Newlon, 1991; Toone *et al.*, 1997). Indeed, ARS elements were later shown by 2D gel electrophoresis techniques which examine replicative intermediates to direct the initiation of DNA replication in yeast (Brewer and Fangman, 1987; Huberman *et al.*, 1987). While all chromosomal replication origins identified to date can act as ARSs on plasmids, only a subset of ARS elements actually function as origins in the chromosomal context (reviewed in Newlon and Theis, 1993).

Mutation and linker substitution analyses of ARS1 have revealed a modular structure, consisting of a highly conserved 11-bp ARS consensus sequence, (ACS; 5'-(A/T)TTTAT(A/G)TTT(A/T)-3'), and immediate flanking sequences (domain A), which are essential but not sufficient for ARS function (Van Houten and Newlon, 1990), as well as domains B1, B2 and B3 (Marahrens and Stillman, 1992) and domain C1. The spacing

between the A and B elements as well as their orientation is critical for ARS function (Diffley and Stillman, 1988). Functionally, the modular elements A and B3 serve as binding sites for the initiator complex, ORC, and the transcription factor, Abf1, respectively, while it is thought that the B1 or B2 elements of ARS1 may be providing a region of thermal instability, or a DNA unwinding element (Figure 1.5; DUE; Rowley *et al.*, 1994).

The Origin Recognition Complex (ORC) consists of six polypeptides ranging in size from 104-50 kDa; each subunit is named according to size: the largest subunit is referred to as ORC1 and the smallest as ORC6. Multiple ORC subunits bind to the ACS and B1 regions in a sequence-specific, ATP dependent fashion (Bell and Stillman, 1992; Rao and Stillman, 1995; Rowley *et al.*, 1995). Protein-DNA crosslinking studies have not definitively identified which subunits are responsible for the DNA recognition. However, no individual ORC subunit is capable of specific DNA binding, and the ORC6 subunit is dispensable for DNA recognition (Dutta and Bell, 1997). ORC seems to be a good candidate for the initiator protein in *S. cerevisiae*, since mutations within the ACS that reduce ORC binding *in vitro* also reduce ARS function *in vivo*, as measured by plasmid stability assays (Bell and Stillman, 1992). Once the genes encoding the six ORC subunits had been cloned, further genetic evidence followed corroborating ORC's role as the initiator protein. Strains with viable mutations in ORC2 and ORC5 show defects in initiation from chromosomal origins and in plasmid maintenance (Bell *et al.*, 1993; Foss *et al.*, 1993; Fox *et al.*, 1995; Loo and Rine, 1994; Micklem *et al.*, 1993). In addition, there are multiple genetic interactions between the ORC2 and ORC5 mutants and mutations in a number of other genes involved in regulating DNA replication (Loo *et al.*, 1995).

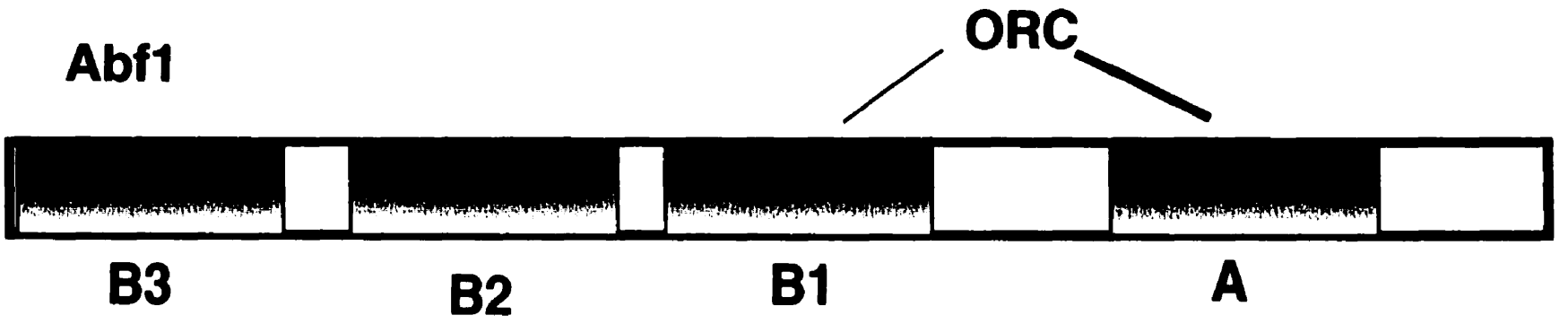
Although ORC appeared to be an ideal candidate for the yeast initiator protein, it was found associated with the ACS throughout the cell cycle, suggesting that ORC cannot be acting alone as the signal for the initiation of DNA replication (Diffley and Cocker, 1992; Diffley *et al.*, 1994).

It is interesting to note that ORC homologues have been cloned from a number of other species, including *Drosophila melanogaster*, *Xenopus laevis*, *Mus musculus*, and *Homo sapiens* (reviewed in Dutta and Bell, 1997). Since DNA sequences that define replication origins in higher eukaryotes have yet to be defined, the role that ORC plays in these species is as yet unclear. The yeast ARS sequences do not direct specific initiation of DNA replication in human cells (Tran *et al.*, 1993), nor do yeast ARS elements that occur naturally in the DNA of other eukaryotes mediate replication in these species, despite the fact that these ARS elements are functional in yeast (Newlon, 1988). The yeast ACS is also not found in the minimal sequence required to support autonomous replication of the mammalian origin enriched sequence (*ors*) 8 (Chapter 2, Todd *et al.*, 1995). This evidence suggests that in higher eukaryotes, the mechanism of initiation of replication differs from that described in yeast.

Figure 1.5 ***S. cerevisiae*** **Autonomously Replicating Sequence, ARS**

Shown are the yeast ARS A, B1,B2, and B3domains. Domain A contains the 11 bp consensus sequence, ACS, and serves together with domain B1 as binding sites for the initiator protein ORC. Domain B3 serves as the binding site for the Abf1 transcription factor. Domains B1 or B2 may serve as a region of unwinding or DUE.

S. cerevisiae **ARS**



(C) Mammalian origins

In contrast to the simple origins of prokaryotes, eukaryotic viruses and yeast, origins of replication found within the chromosomes of metazoan animals are considerably more complex. Although it is well established that DNA replication begins at defined sites in simple genomes, evidence supporting initiation of replication at specific metazoan chromosomal sites is just now beginning to come to light through the use of origin mapping techniques. The initiation sites for DNA replication at about 22 different genomic locations from flies, frogs, and mammals have been mapped to date (DePamphilis, 1996; Kobayashi *et al.*, 1998 and references therein). Mapping methods allow the *in vivo* detection of initiation sites to within a few hundred bp (reviewed in DePamphilis, 1997). From the analysis of these metazoan origins, there have been no reports of specific DNA consensus sequences required for initiation of DNA replication. A degenerate consensus sequence has been described (Dobbs *et al.*, 1994), but it has yet to be functionally tested.

(a) Chinese Hamster Dihydrofolate Reductase locus

By far the most intensely studied mammalian locus of DNA replication is that of the Chinese Hamster Dihydrofolate Reductase (*DHFR*) gene (Burhans *et al.*, 1990). This region was first studied in the methotrexate resistant cell line CHOC 400, because it contains 1000 copies of a 250 kbp region encompassing the *DHFR* gene. Each *DHFR* amplicon has been shown to contain at least three transcription units, and a replication origin activated in early S phase (Milbrandt *et al.*, 1981). Cloning and early mapping studies showed that the earliest labelled restriction fragments (ELFs) were located between the *DHFR* and *2BE2121* transcription units (Heintz *et al.*, 1983). Many techniques have been used to map the initiation site at this locus, including identifying the

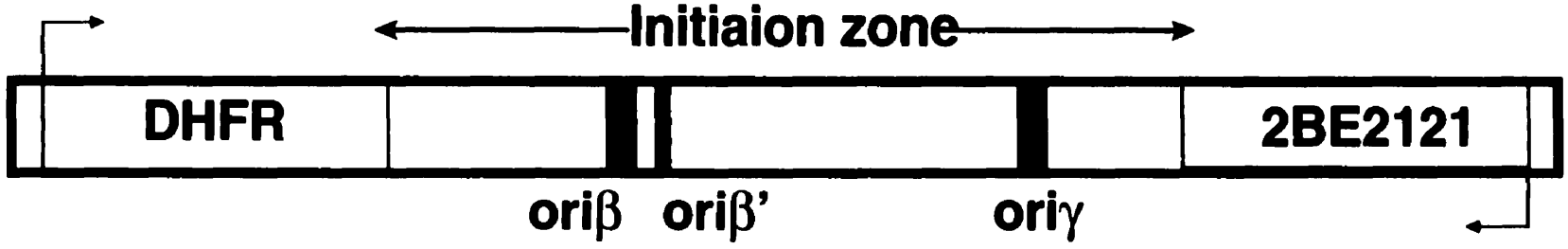
earliest labelled nascent fragment, determining the replication fork polarity (by measuring the Okazaki fragment and leading-strand biases) and by measuring the growth and relative abundance of nascent strands (Kobayashi *et al.*, 1998 and references therein; reviewed in DePamphilis, 1993a). These genetic studies reveal a strong origin, *oriβ*, located approximately 17 kb downstream from the *DHFR* gene, and a weaker origin, *oriγ* located approximately 40 kb downstream. A third, previsouly unidentified origin, *oriβ'*, was recently located 5 kb further downstream from *oriβ* (Kobayashi *et al.*, 1998; Figure 1.6). In conflict with this data, the analysis of replication intermediates by 2D gel electrophoresis techniques points to random initiation events distributed throughout the 55 kb region between the *DHFR* and *2BE2121* genes (Dijkwel and Hamlin, 1995 and references therein). The two conflicting results can be reconciled by a consideration of the sensitivities of the two techniques. The 2D gel electrophoretic methods are sensitive to even a small number of initiations, whereas the methods that measure the nascent strand length/abundance or hybridization biases of leading and lagging nascent strands detect only the most active initiation sites (Kalejta *et al.*, 1996).

oriβ contains several stretches of AT rich DNA, and multiple near matches to the yeast ARS consensus sequence (Heintz, 1996). Downstream from *oriβ* is a region of stably bent DNA that binds multiple proteins, including AP1 and Oct-1 (Held *et al.*, 1992) as well as the DNA-binding protein RIP60, which binds to an ATT-repeat motif located 3' of the AP1 site (Dailey *et al.*, 1990). Also included in the *oriβ* region are other elements commonly associated with origins of DNA replication, such as DUEs, (Caddle *et al.*, 1990b) and matrix attachment regions (MARs, Dijkwel and Hamlin, 1988).

Figure 1.6 Chinese Hamster Dihydrofolate Reductase (DHFR) locus

Three initiation sites have been located within the 55 kb region between the *DHFR* and *2BE2121* genes. These are, in order of relative frequency of activation, *oriβ*, *oriγ*, and *oriβ'*, which are located 17, 40 and 22 kb downstream from the *DHFR* gene, respectively.

DHFR amplicon



(b) Human β-globin locus

The human β-globin locus consists of five linked genes, ε, γ_A, γ_G, δ and β, which exhibit erythroid-specific, developmentally regulated expression. This region has been very well characterized in humans because it contains a large number of genetic defects that cause β-thalassaemias. Replication initiates from the intergenic region between the δ and β genes, regardless of expression (Kitsberg *et al.*, 1993; Figure 1.7). The locus control region (LCR), an upstream element, regulates gene expression and replication timing; absence of the LCR prevents β-globin expression and shifts replication timing from early to late in S-phase. Sequences encompassing the LCR, 40-60 kb 5' to the mapped initiation site appear to be necessary for origin activation, as the origin is not active in somatic cell hybrids containing a 35 kb deletion within the LCR (Aladjem *et al.*, 1995). The β–globin region has recently been shown to be capable of initiating replication *in vivo* after having been transferred to a new chromosomal location by site-specific recombination. This work provides evidence supporting initiation of replication at specific DNA sequences in higher eukaryotes (Aladjem *et al.*, 1998).

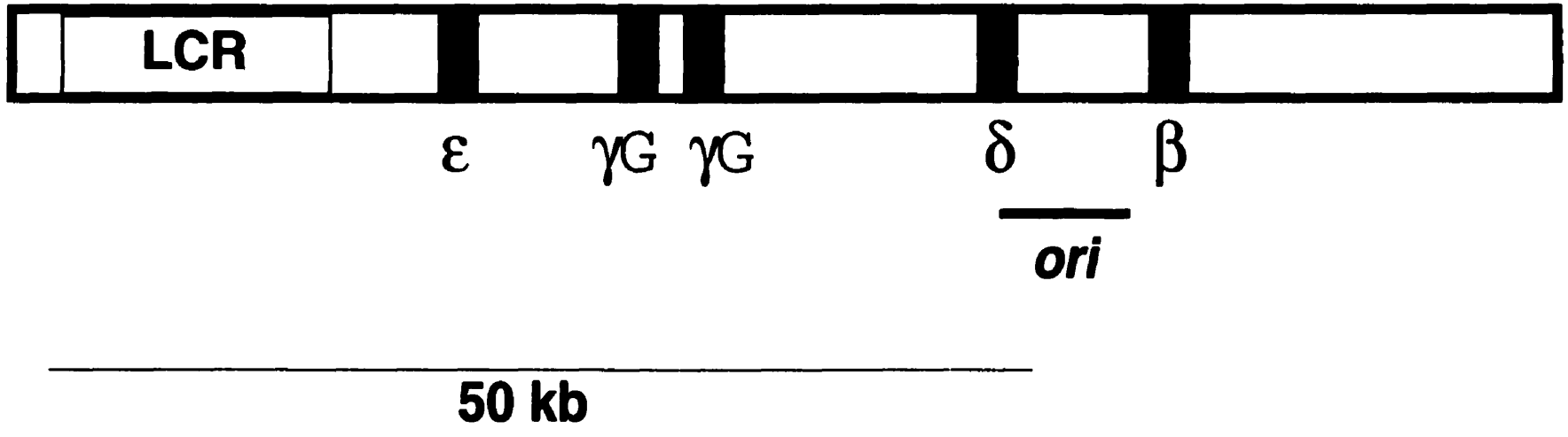
(c) Mammalian early replicating origin enriched sequences (*ors*)

An elegant method, developed by Zannis-Hadjopoulos *et al.* (Zannis-Hadjopoulos *et al.*, 1983; Zannis-Hadjopoulos *et al.*, 1981), for the study of mammalian origin structure involves isolating short nascent DNA sequences that have initiated DNA replication early in S phase, rather than carrying out physical mapping studies of origins in their chromosomal context (Figure 1.8).

Figure 1.7 Human β-globin locus

The β-globin locus consists of five linked genes, ε, γ_A, γ_G, δ and β whose expression is controlled by the locus control region (LCR). The LCR also regulates replication which initiates from the intergenic region between the δ and β genes (denoted as *ori*).

Human β-globin locus



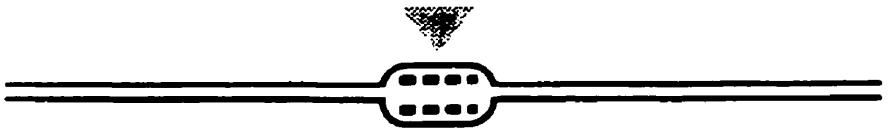
The rationale behind this nascent strand extrusion method is that, if short nascent strands are isolated, there should be an origin of bidirectional replication at or near its center and, once these sequences are isolated, they can be cloned into vectors for further study. Early replicating sequences have been isolated by this method from both human and monkey cells, creating a library of origin enriched sequences, or *ors*, that are activated early in S phase (Kaufmann *et al.*, 1985; Zannis-Hadjopoulos *et al.*, 1985). Functional analyses of these sequences by autonomous plasmid replication assays *in vivo* (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991), *in vitro* replication assays using a cell-free system (Pearson *et al.*, 1991), examination of replicative intermediates by electron microscopy (Pearson *et al.*, 1994b), and by *in vivo* chromosomal mapping techniques (Wu *et al.*, 1993b; Pelletier *et al.*, submitted) revealed that the *ors* are capable of initiating semiconservative bidirectional DNA replication. Nucleotide sequence analyses of more than fifty *ors* revealed sequence and structural elements that are associated with mammalian origins of bidirectional replication. These included AT rich sequences, inverted repeats with the capacity to form cruciform structures, nuclear scaffold and matrix attachment regions (SARs and MARs), and eukaryotic transcriptional regulatory elements (see below; Rao *et al.*, 1990).

Figure 1.8 Nascent strand extrusion of early replicating DNA sequences

Method used to isolate origin enriched sequences (*ors*) from early replicating monkey (CV-1) DNA. Briefly, cells were synchronized at the G1/S boundary and then released into S phase *in vivo* in the presence of ^{3}H- thymidine for one minute. Nuclei were prepared, and DNA replication was allowed to continue *in vitro* for a further 10 minutes in the presence of Hg-dCTP. Nascent strands were extruded from replication bubbles and affinity purified followed by cloning into pBR322 (Zannis-Hadjopoulos *et al.*, 1981); (Kaufmann *et al.*, 1985) Figure reproduced from Vassilev and DePamphilis, 1992.

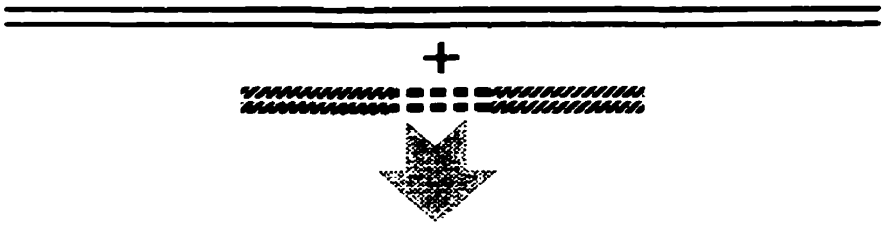
Origin of Bidirectional Replication

- Synchronize Cells at G1/S Interphase
- Release Cells and Label DNA *in vivo* [1 min, ^{3}H-TdR (▬▬)]



- Continue DNA Replication *in vitro* (10 min, Hg-dCTP (▨))

- Extrusion of Nascent DNA (50 °C)



- Isolate 0.5 - 2 kb ^{3}H-DNA by Velocity Sedimentation
- Purify Hg-DNA by Affinity Chromatography
- Clone Isolated DNA

II. Eukaryotic origin structure

There has yet to be described a consensus sequence shared by all origins of DNA replication, rather, from the study of the origins of model systems, several DNA sequence and structure elements have emerged as being common elements of all known origins of replication described to date (reviewed in DePamphilis, 1996). These elements are described below:

(i) Origin recognition element (ORE)

An ORE is the DNA-binding site for an origin recognition protein, which is required to initiate DNA replication. In the simple genomes of prokaryotes, eukaryotic viruses and yeast, the ORE is a defined consensus sequence that serves as the binding site for one or more initiator proteins. The initiator protein initiates DNA replication by carrying out at least two tasks. The first is the initiation of DNA unwinding via their helicase activity (e.g. SV40 TAg) or by recruiting a helicase to the origin (e.g. *E. coli* dnaA protein recruits the dnaB helicase to the origin). The second task is the recruitment of other replication proteins to the origin. For example, SV40 TAg recruits the DNA polymerase-αprimase to the replication fork, which is responsible for laying down the first RNA-primed DNA strand. Similarly, the Adenovirus preterminal protein (Ad pTP) which initiates Ad replication, is complexed with the Ad DNA polymerase, which is responsible for DNA synthesis of the Ad genome.

(ii) AT rich DNA

Most origins contain AT rich DNA consisting of an A rich strand and a T rich strand. The length of the AT rich DNA can be important for origin function, which is observed in SV40 (Gerard and Gluzman, 1986) and Herpes Simplex Virus (HSV, Koff *et al.*, 1991).

(iii) Bent DNA

Sequence-directed curvature of the helix axis, or bent DNA, arises from oligo (dA) tracts of 3-6 bp in length which deflect the axis of the helix from a straight line. When the spacing of the tracts is in phase with the helical repeat of the DNA (10-11 bp), macroscopic bending results (reviewed in Hagerman, 1990). Bent DNA has been observed within regulatory regions for transcription, recombination, and origins of DNA replication. Bent DNA is found within the ARS elements of yeast, several prokaryotic origins, viral origins, the human mitochrondrial L-strand origin as well as several mammalian chromosomal origins characterized to date including the Chinese hamster *DHFR* locus, and the human *c-myc* associated origins (Eckdahl and Anderson, 1990; Caddle *et al.*, 1990b; Kumar and Leffak, 1989; Hagerman, 1990 and references therein). Bent DNA has been found to be of functional importance in several cases. The SV40 origin of replication has a region of bent DNA that, if altered, significantly reduces the ability of SV40 to replicate (Deb *et al.*, 1986b). The minimal DNA sequence of the monkey early replicating sequence *ors8*, that is required to support autonomous replication both *in vivo* and *in vitro* , also contains bent DNA (Todd *et al.*, 1995). Bent DNA is thought to facilitate protein binding (Eckdahl and Anderson, 1990); in fact a number of proteins have been documented to bind to regions of bent DNA within origins

of replication, including the λO initiator protein (Zahn and Blattner, 1987). The binding of proteins to bent DNA has also been found to further distort the helix, as in the case of the RIP 60 protein which binds to a region of bent DNA within the *oriβ* origin of the *DHFR* locus (Caddle *et al.*, 1990a). Such protein-induced distortion may serve to facilitate helix unwinding, or further protein interactions (DePamphilis, 1996).

(iv) DNA unwinding element, DUE

DNA unwinding following origin activation appears to begin at easily unwound sequences termed DNA unwinding elements (DUE). A DUE is defined as a DNA sequence that, when mutated, renders the DNA less easily unwound, and reduces replication (Umek and Kowalski, 1990). DUEs are usually AT rich, and sequence-specific, with the base stacking interactions determining the energy required for unwinding. There is, however, no consensus sequence for a DUE. Easily unwound sequences can substitute for DUEs in yeast and *E. coli* (Kowalski and Eddy, 1989; Umek and Kowalski, 1988). The spacing between the DUE and ORE is critical, implying that a protein interacting with the ORE may also interact with the DUE (DePamphilis, 1996). In fact, binding of proteins to the ORE induces unwinding at the DUE in *E. coli* (Kowalski and Eddy, 1989) and SV40 (DePamphilis, 1996). The DUE is believed to be the site of entry of the replication machinery to begin DNA synthesis.

(v) Nuclear matrix attachment sites

Human somatic cell chromatin is organized into loops of ~50-100 kb (Vogelstein *et al.*, 1980). The base of the DNA loops are attached to the nuclear scaffold (metaphase) or matrix (interphase), a proteinaceous scaffold that remains in the nucleus following nuclease digestion of the chromatin (Georgiev *et al.*, 1991). The DNA sequences that

attach to the scaffold or matrix (Scaffold or Matrix Attachment Region, SAR or MAR) have been described for several gene loci, including the chicken lysozyme gene, the human interferon-β gene, the human β-globin gene, the chicken α-globin gene, p53, and the human protamine gene cluster (Singh *et al.*, 1997 and references therein). MARs are relatively short DNA sequences, commonly containing ATTTA and ATTA sequences (Boulikas, 1992). IR sequences are also associated with matrix-bound DNA (Boulikas, 1993a). Approximately 100,000 matrix attachment sites are estimated to exist in the mammalian cell nucleus, nearly half of which include origins of replication (Bode *et al.*, 1996). MARs have been found within several characterized replication origins, including the origin associated with the *DHFR* gene (Dijkwel and Hamlin, 1988), the mouse IgH enhancer origin (Ariizumi *et al.*, 1993), the human 343 (Wu *et al.*, 1993a) and β-globin origins (Boulikas, 1993b) as well as the mammalian autonomously replicating *ors* (Mah *et al.*, 1993; Rao *et al.*, 1990).

(vi) Inverted Repeats and Cruciforms

IRs are commonly found at prokaryotic and eukaryotic control regions, including origins of DNA replication. Under conditions of negative supercoiling, IRs can form DNA cruciforms, which have been implicated in the regulation of DNA replication. These topics will be addressed further in section IV.

III. Regulation of origin usage: cell cycle control

(i) Why regulate DNA replication?

In order to replicate their large volumes of DNA which are distributed over many chromosomes, eukaryotic cells use multiple replication origins to initiate bidirectional

DNA replication (Cairns, 1966; Huberman and Riggs, 1966; Huberman and Riggs, 1968). A typical human fibroblast has an estimated genome size of 10^9 bp, containing $10^4 - 10^6$ replication origins (Hand, 1978). Each of these active replication origins must initiate DNA replication once per S phase of the cell cycle in order to ensure the faithful replication of the entire genome. To further complicate the process, with the exception of the early embryo (Blumenthal *et al.*, 1974), not all of the replication origins initiate DNA synthesis at the same time in S phase (Taylor, 1960). This presents an organizational challenge for the cell, since as S phase progresses, origins that have not fired must be identified and activated in a growing pool of replicated DNA. Furthermore, only a subset of potential origins are used in a normal adult S phase (Harland and Laskey, 1980).

There is, therefore, a sophisticated regulatory process in place ensuring the firing of each and every active origin of replication once and only once per S phase, as well as the repression of the non-active origins. This process has been reviewed recently (Diffley, 1996; Stillman, 1996; Toone *et al.*, 1997)

(ii) The replicon model

The replicon model, proposed by Jacob *et al.* (1964), was the first description of how DNA replication was proposed to be regulated. This model, based originally on the regulation of *E. coli* chromosomal replication, predicted that an initiator protein would activate DNA replication through a controlling DNA sequence called a replicator. The model was confirmed experimentally in *E. coli*, where the initiator protein was identified as dnaA, which acted upon the replicator, *oriC* (Kornberg and Baker, 1992). The replicon model has far reaching applicability, correctly describing the mechanism of initiation in bacteria, viruses and yeast. The model may also be relevant for mammalian genomes,

where it has already been established that initiation occurs at specific sites. A *bona fide* mammalian initiator protein has yet to be identified, and this subsquently remains an area of intense research.

(iii) Evidence for cell cycle regulation

Cell fusion studies, in which cells were synchronized at various stages of the cell cycle prior to being fused together (Rao and Johnson, 1970; also reviewed in Stillman, 1996), provided the first observations that DNA replication was indeed cell cycle regulated. These experiments revealed three important phenomena: first, only G1 nuclei are capable of initiating DNA replication; second, S-phase cells, but not those in G1 or G2 contain an activator of initiation of DNA replication that can prematurely initiate replication in competent G1 nuclei; and third, G2 nuclei cannot re-replicate their DNA until the cell has passed through mitosis. Cells exist, therefore, in two distinct states of competence for the initiation of DNA replication: a prereplicative state, which is competent for replication, and a postreplicative state which is no longer competent until the cell has passed through mitosis.

(iv) Licensing DNA replication

The discovery that nuclei could be induced to re-replicate their DNA during the same S phase if their nuclear membranes were transiently permeabilized (Blow and Laskey, 1988) led to a refinement of the previously proposed licensing factor model (Harland and Laskey, 1980; Harland, 1981)). This model proposes a licensing factor, or essential initiation factor, which is chromatin bound, able to support a single initiation event, and then "cancelled" by the passage of the replication fork. Active licensing factor,

then, would be able to gain access to the chromatin only after the breakdown of the nuclear envelope during mitosis, (due to a lack of a nuclear localization sequence for example) in agreement with the early cell fusion experiments. Based on observations in *Xenopus laevis*, the licensing factor has been separated into two discreet activities. The first, replication licensing factor (RLF), licenses replication origins by rendering them 'replication-competent'. The second activity, S-phase promoting factor (SPF), induces licensed origins to initiate, and in doing so removes the license (reviewed in Chong *et al.*, 1996). RLF has been further separated into two activities, RLF-M and RLF-B. RLF-M has been identified as a complex of MCM proteins (Chong *et al.*, 1995; see below) while RLF-B has yet to be identified. SPF has been identified as a cyclin-dependent kinase.

(v) Initiator

The search for "the" *S. cerevisiae* initiator protein led to the discovery of the ORC complex which binds to the A and B1 elements of *S. cerevisiae* ARS sequences (see the Yeast section above). ORC genomic footprinting studies showed ORC bound to DNA throughout the cell cycle, clearly disqualifying ORC as the initiator protein (Diffley and Cocker, 1992). However, the ORC genomic footprint does change with the cell cycle. Starting at late M until the end of S phase, there are extended regions of nuclease protection adjacent to the ACS (Diffley *et al.*, 1994), suggesting that additional proteins are recruited to this region. This lead to the idea that there are two states of the chromatin: a prereplicative state, which is competent for DNA replication, and a postreplicative state which is not. ORC is present in both the prereplicative (pre-RC) and postreplicative (post-RC) complexes, and is therefore thought to serve as a landing pad for other proteins necessary for initiation (Figure 1.9).

(vi) CDC6

The CDC6 protein of *S. cerevisiae* (the *Schizosaccharomyces pombe* homolog is encoded by the cdc18 gene) is essential for DNA replication, required for the formation and maintenance of the pre-RC (Cocker *et al.*, 1996), and interacts with ORC *in vitro* (Liang *et al.*, 1995). If cells enter the cell cycle without CDC6, they proceed to an abortive pseudo-mitotic state without having replicated their DNA (Piatti *et al.*, 1995). Both CDC6 and cdc18 are unstable proteins, with half lives of the order of 5 minutes (Diffley, 1996 and references therein), and *de novo* CDC6 and cdc18 protein synthesis is required in each cell cycle for DNA replication to occur (Kelly *et al.*, 1993; Piatti *et al.*, 1996). CDC6 is not only required for the formation of the pre-RC and hence DNA replication, but it also ensures that replication occurs before mitosis. A probable scenario is that CDC6 loads onto ORC, which is bound at the yeast origin of replication, forming the pre-RC (Figure 1.9).

(vii) MCM Proteins

MCM (minichromosome maintenance) proteins were first identified by genetic studies in yeast (reviewed in Chong *et al.*, 1996). They form a gene family with six members identified to date in several eukaryotes. MCM proteins form complexes with themselves, are required for the initiation of DNA replication in *S. cerevisiae*, and have been identified as a component of the licensing system. The RLF-M has been identified in *Xenopus* as a complex of the MCM2, MCM3 and MCM5 proteins (Chong *et al.*, 1995). MCM proteins associate with chromatin late in M phase and remain there until they are removed as S phase progresses (Kubota *et al.*, 1995; Romanowski *et al.*, 1996; Stillman, 1996 and references therein). MCMs have also been found to physically interact with

ORC1 in *S. Pombe* (Grallert and Nurse, 1996). Furthermore, the interaction of the MCM proteins with chromatin is dependent on CDC6 (Stillman, 1996 and references therein). In yeast, the MCM proteins are found in the nucleus during late mitosis and G1, but disappear from the nucleus during early S phase (Dalton and Whitbread, 1995; Hennessy *et al.*, 1990; Yan *et al.*, 1993). MCM proteins are not localized to sites of DNA synthesis during S phase (Kimura *et al.*, 1994; Kubota *et al.*, 1995; Madine *et al.*, 1995; Todorov *et al.*, 1995), further supporting the hypothesis that MCM proteins are involved in initiation of replication and are displaced from the chromatin after initiation occurs. However, there has been a report of weak helicase activity associated with the MCM proteins (Ishimi, 1997), which lends support to the theory that MCM proteins are redistributed on the chromatin following initiation, leading to their association with replication forks (Aparicio *et al.*, 1997). In mammalian cells the MCM proteins are constitutively nuclear (Kimura *et al.*, 1994; Thommes *et al.*, 1992; Todorov *et al.*, 1994), but they appear to display cell cycle regulated chromatin association, in keeping with the licensing model (Chong *et al.*, 1996). This regulation may be mediated by phosphorylation, since mammalian MCM2 and MCM3 are both chromatin bound and hypophosphorylated in G1 phase, but during S phase become hyperphosphorylated and no longer associated with chromatin (Figure 1.9; Romanowski and Madine, 1996) until mitosis.

(viii) CDC45

The product of the *S. cerevisiae* CDC45 gene is essential for the initiation of DNA replication (Hardy *et al.*, 1997; Hopwood and Dalton, 1996; Zou *et al.*, 1997). CDC45 protein levels are constant throughout the cell cycle (Owens *et al.*, 1997), despite a periodic mRNA expression pattern with levels peaking at the G1/S interface (Hardy *et al.*,

1997). Yeast cells with defective CDC45 protein are not capable of activating pre-RCs to initiate DNA replication (Owens *et al.*, 1997). In addition, it appears that the activity of the Cdc7/dbf4 kinase is dependent on CDC45 protein (Owens *et al.*, 1997). Recently, it has been reported that CDC45 is loaded onto chromatin in a CDK-cyclin dependent manner, but that this chromatin binding is temporally distinct from the pre-RC formation (Figure 1.9; Zou and Stillman, 1998).

(ix) Cyclin-CDKs

In G1, the competent state of *S. cerevisiae* chromosomes requires, during a narrow window of opportunity within the cell cycle, the establishment of the pre-RC, which to date has been found to consist of at least ORC, CDC6, CDC45, and MCM proteins. There are other proteins involved in the regulation of the formation of this complex, including the cyclin-CDK complexes. Cyclin-dependent kinases (CDK) are a family of serine/threonine kinases that require an associated cell cycle regulated cyclin for kinase activity. In addition, the cyclin-CDK kinase activity is itself regulated by phosphorylation. Cyclin-CDK complexes have cell cycle-specific roles in a number of pivotal functions, including, among others, control of entry into S phase and mitosis, co-ordination of mitosis, and regulation of replication to once per cell cycle. In each cell cycle-specific role, the regulatory cyclin of the kinase differs (reviewed in Nasmyth, 1996). The *S. cerevisiae* cyclin B-CDKs function to both activate DNA replication and to block the formation of the pre-RC following initiation (Dahmann *et al.*, 1995; Figure 1.9). The cyclin B-CDK complexes may be interacting with CDC6, ORC or both (Stillman, 1996 and references therein). The mammalian cyclin-dependent kinase CDK2 complexes with both cyclin A

and cyclin E to promote entry into S phase. Both cyclins A and E have been found to possess SPF activity in *Xenopus* extracts (Strausfeld *et al.*, 1996).

(x) CDC7-Dbf4

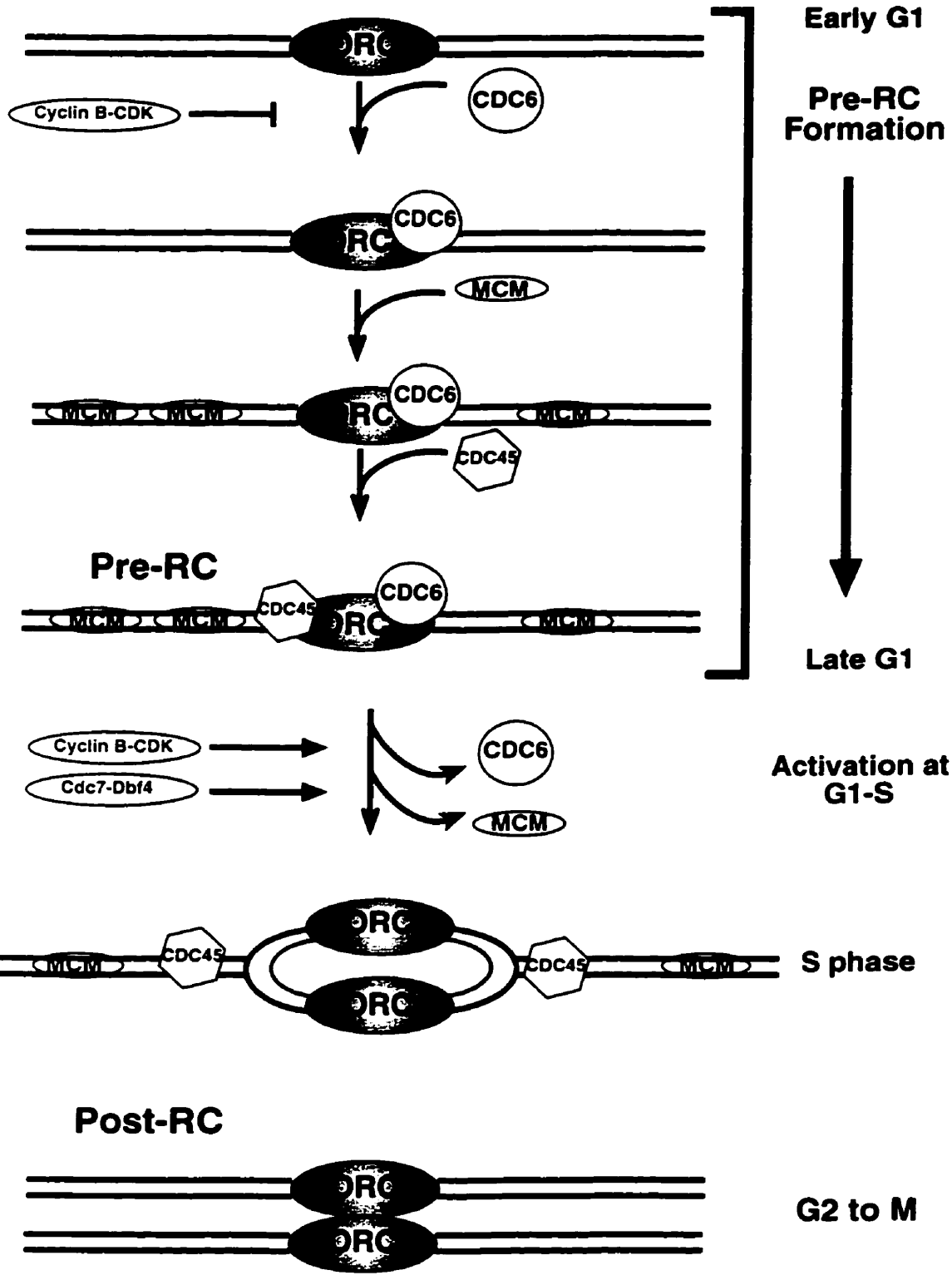
The activity of the Cdc7-Dbf4 kinase is also required for the initiation of DNA replication (Figure 1.9). The CDC7 gene encodes a protein kinase whose activity is dependent on the Dbf4 protein. Mutations in the CDC7 gene block initiation of DNA replication. The targets of the CDC7-Dbf4 complex are unknown, but the MCM proteins have been implicated (Botchan, 1996). CDC7 interacts with ORC, and Dbf4 interacts with the yeast ARS (Stillman, 1996 and references therein).

(xi) Remaining questions concerning regulation of initiation

Despite the identification of several proteins that may participate in pre-RC formation in some eukaryotes, many questions remain. The identies of all the proteins that make up the pre-RC are currently unknown, and two essential requirements for initiation, origin unwinding and recruitment of the replication machinery, have yet to be satisfactorily attributed to the proteins identified to date as being necessary for pre-RC formation. Furthermore, as outlined above in section I,ii,B, there is little evidence supporting a role for ORC in the initiation of DNA replication in mammalian cells, leaving very little information available concerning the control of initiation at mammalian origins of replication.

Figure 1.9 Cell cycle regulation of DNA replication in *S. cerevisiae*

Prereplication complex (pre-RC) formation begins in early G1 phase with the association of CDC6 with ORC at the origin. Cyclin B-CDK activity blocks the formation of the pre-RC. By the end of G1 phase, the pre-RC consists at least of ORC, CDC6, MCM proteins, and CDC45. Cyclin B-CDK and Cdc7-dbf4 activities activate DNA replication at the beginning of S phase. Following initiation of DNA replication, the pre-RC, which is competent for initiation, is dismantled, forming the post-RC, thus prohibiting further initiations at that site until the passage through mitosis. MCM proteins are shown both bound to and dissociated from chromatin, following initiation, reflecting the two hypotheses presented to date (see text).


Early G1
Pre-RC Formation
ORC
Cyclin B-CDK
CDC6
ORC
CDC6
MCM
MCM
MCM
ORC
CDC6
MCM
CDC45
Pre-RC
CDC45
CDC6
MCM
MCM
ORC
MCM
Late G1
Cyclin B-CDK
Cdc7-Dbf4
CDC6
MCM
Activation at G1-S
ORC
CDC45
MCM
CDC45
MCM
ORC
S phase
Post-RC
ORC
ORC
G2 to M

IV. IRs and Cruciforms

(i) Incidence

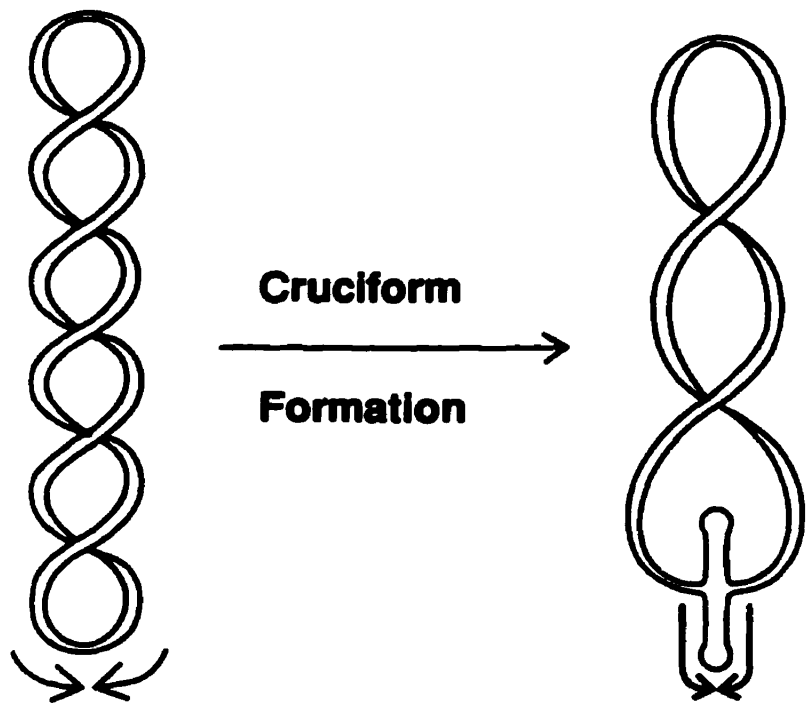
IRs are found extensively within the chromosomal DNA of prokaryotes and eukaryotes alike, including plants, yeast, *Neurospora*, *Physarum*, *Drosophila*, mouse, *Xenopus* and human (reviewed in Pearson *et al.*, 1996). IRs have been associated with regulation of gene expression in prokaryotes (Horwitz, 1989) and eukaryotes (Greenberg *et al.*, 1987; Shuster *et al.*, 1986; McMurray *et al.*, 1991; Spiro *et al.*, 1993). In addition, IRs are a common feature of replication origins of prokaryotes (Zyskind *et al.*, 1983); (Hiasa *et al.*, 1990), viruses (Muller and Fitch, 1982), eukaryotes (Campbell, 1986; Tschumper and Carbon, 1982) and mammals (Boulikas, 1993b; Hand, 1978; Landry and Zannis-Hadjopoulos, 1991; Zannis-Hadjopoulos *et al.*, 1984; Zannis-Hadjopoulos *et al.*, 1985).

(ii) Formation and structure

Cruciform structures, first proposed to exist in 1955, are four-way branched structures that may form at IRs under conditions of torsional strain by intra-strand base pairing. Cruciformation, or cruciform extrusion, at an IR relaxes superhelical strain. One superhelical turn is relaxed for every 10.5 bp of DNA that extrudes into a cruciform, thus lowering the free energy of the molecule (Figure 1.10). It is for this reason that cruciforms are energetically favoured in regions of negative supercoiling (reviewed in Pearson *et al.*, 1996).

Figure 1.10 Cruciform extrusion at inverted repeats

Cruciform extrusion occurs at inverted repeats (IRs, denoted by head to head arrows) under conditions of torsional strain by intra-strand base pairing. Cruciform formation relaxes superhelical tension, as illustrated by the reduction in the number of supercoils. Figure reproduced from Sinden, 1994 © Academic Press (http://www.apnet.com).


Cruciform
Formation

The cruciform structure consists of a four-way junction and stems containing single-stranded loops, with two possible conformations. The stacked X conformation, where the four helices adopt a compact, two-fold symmetric X-like shape, is probably the most stable cruciform structure in solution. Its formation is dependent on the presence of cations (Duckett *et al.*, 1990), and results in pairing and stacking of the bases at the junction (Sinden, 1994; Pearson *et al.*, 1996). In the absence of cations, the structure of the cruciform is most likely square planar, showing four fold symmetry with the base pairs at the junction unstacked (Pearson *et al.*, 1996 and references therein).

Holliday junctions (Holliday, 1964) are topologically similar to cruciforms in that they display intersecting helix-pairs and reciprocal strand exchange, but differ by their lack of single-stranded loops. Holliday junctions serve as one of the intermediates in homologous recombination, consisting of two homologous duplex DNA molecules linked by a single stranded crossover. The Holliday junction must be resolved by special enzymes, the resolvases, in order to form the recombinant duplex DNAs (Shinagawa and Iwasaki, 1996).

(iii) Cruciform structure *in vivo*

The calculated frequency of cruciforms in the human genome is ~1 in every 41,700 bp (Schroth and Ho, 1995), and it is now accepted due to the concurring results of many different experimental approaches that cruciform structures are capable of forming *in vivo* (reviewed by Sinden, 1994; Pearson *et al.*, 1996). These approaches have included monitoring the superhelical density in *E. coli* (Haniford and Pulleyblank, 1985), measuring transcriptional regulation at promoters containing IRs (Horwitz and Loeb, 1988), treatment of cells with chemical probes (see Pearson *et al.*, 1996 for references) or

chemical crosslinking agents such as psoralen to 'lock' the cruciform structure (Sinden and Ussery, 1992; Zheng *et al.*, 1991), cleavage by junction resolving enzymes (Panayotatos and Fontaine, 1987) and use of monoclonal antibodies directed towards cruciforms (Frappier *et al.*, 1989; Frappier *et al.*, 1987).

The monoclonal antibody against cruciforms, 2D3, recognizes conformational determinants specific only to cruciforms, and its binding site has been mapped to the four-way junction at the base of the cruciform (Frappier *et al.*, 1989; Steinmetzer *et al.*, 1995). Using these antibodies, the number of cruciforms in mammalian (monkey and human) cells was estimated to vary from 0.6×10^5 to 3×10^5 per cell, with the maximal number of cruciforms occurring at the onset of S phase (Ward *et al.*, 1990).

(iv) Cruciforms and DNA replication

Stem-loop or cruciform structures are known to be directly involved in the DNA replication of the ss phages ϕX174 and G4, the ds plasmids R1162 and pT181, mitochrondrial origins of replication, and the Epstein-Barr virus (EBV; reviewed in Pearson *et al.*, 1996). DNA supercoiling is known to affect the specific binding of regulatory proteins required for transcription, replication and recombination (Cozzarelli and Wang, 1990; Wang and Liu, 1990). In this regard, cruciforms may influence DNA replication by exerting an effect on the superhelical density of the chromatin since cruciform extrusion relaxes the DNA. This relaxation could affect the binding of proteins necessary for such processes as DNA replication (Horwitz, 1989). Cruciforms and DNA stem-loop structures bind poorly to histones or nucleosomes (Battistoni *et al.*, 1988; Kotani and Kmiec, 1994; Nickol and Martin, 1983; Nobile *et al.*, 1986). For this reason, it

is generally accepted that cruciforms exist in either the spacer regions between nucleosomes, or at right angles to the nucleosome surface. It is possible then, that cruciforms at origins of replication may influence replication by exposing nucleosome-free DNA, thus rendering it accessible to DNA binding proteins.

An alternative possibility is that cruciforms influence DNA replication by participating in DNA-protein complexes including specific proteins necessary for DNA replication (Zannis-Hadjopoulos *et al.*, 1988). This proposal is supported by the fact that introduction of the monoclonal anti-cruciform antibodies into permeabilized cells capable of DNA replication resulted in a 2-11 fold increase in DNA synthesis, including the replication of early replicating sequences such as *ors8*, *DHFR*, and c-myc (Zannis-Hadjopoulos *et al.*, 1988). These are the results which would be expected if cruciform structures at or near origins of replication are stabilized by the antibody and thus allow multiple rounds of replication at these sites.

The number of cruciforms in living cells has been monitored throughout the cell cycle (Ward *et al.*, 1990; Ward *et al.*, 1991) and, as mentioned above, the extrusion of DNA cruciforms appears to be cell-cycle regulated. A bimodal cruciform distribution was observed throughout S phase, with a maximum occuring at the G1/S boundary, and a second but lower peak appearing about 4 hours into S phase. The timing of these peaks correlates with that of the maximal the rates of DNA synthesis in S phase (McAlear *et al.*, 1989). Cruciforms were not detected in G2/M nuclei (Ward *et al.*, 1991).

Furthermore, the monoclonal anti-cruciform antibody has been used to successfully affinity purify active origins of DNA replication from CV-1 genomic DNA (Bell *et al.*, 1991), human genital fibroblasts, embryo lung fibroblasts (WI-38) and colorectal cancer

cells (SW48; Nielsen *et al.*, 1994), with the implication that there were, in each case, extruded cruciforms at or near the origins of replication. The early replicating sequence *ors8* was also recovered in this purification process, further supporting the proposal of the presence of an extruded cruciform within this sequence during replication.

(v) Cruciform binding proteins

Proteins that bind cruciforms may be classified into three groups: those that recognize the cruciform structure, those that recognize and cleave the structure, and those that recognize and cleave the structure in a site-specific manner (site-specific resolving enzymes). The proteins that cleave junctions are generally involved in recombination pathways (reviewed in Duckett *et al.*, 1992; Pearson *et al.*, 1996). The proteins that recognize but do not cleave junctions will be discussed further.

(a) HMG proteins

The eukaryotic high mobility group, or HMG, family of proteins interact with cruciform DNA as well as other DNA structures including negative supercoils, crossovers, and the axially kinked cis-platinated DNA (Ner *et al.*, 1994). The first identified member of this family, HMG1, is a very abundant non-histone nuclear protein, present in all mammalian cells and tissues. HMG1 has three structural domains: the amino-terminal A domain and the central B domain, which both bind DNA, and the carboxy-terminal C domain, which binds to histones. The A and B domains are repeats of a 70 amino acid sequence called the HMG box, which is also found in a large number of DNA-binding proteins, including transcription factors, sex-determining proteins, proteins involved in V-(D)-J recombination, and yeast proteins involved in mating-type control (Bustin and Reeves, 1996; Landsman and Bustin, 1993; Lilley, 1992). The HMG box itself is capable

of structure-specific binding to DNA junctions (Bianchi *et al.*, 1992). Some proteins that contain HMG boxes not only bind to bent DNA, but are themselves capable of bending DNA to large extents (Giese *et al.*, 1992). The HMG box-containing proteins are clearly involved in a wide array of biological functions, but the function of the HMG-1 protein has yet to be determined. What is clear is that HMG-1 interacts with chromatin, recognizing various structural elements. It has recently been proposed that HMG-1 may modulate the cytotoxicity of the DNA-damaging agent cisplatin (Hoffmann *et al.*, 1997).

(b) p53

The human p53 protein binds Holliday junctions, and although it does not itself cleave junctions, it facilitates their cleavage by a junction specific resolvase (Lee *et al.*, 1997).

(c) CBP

Based on the hypothesis that there exist specific cellular proteins that recognize DNA cruciforms, a cruciform-specific binding activity, CBP, has been identified and partially purified from HeLa cell extracts (Pearson *et al.*, 1994a). CBP sediments at 66 kDa on a glycerol gradient, is enriched in HeLa nuclei, and exhibits cruciform-specific binding but not nuclease activities. CBP does not exhibit any sequence-specific binding activity, nor does it bind linear or ss DNA (Pearson *et al.*, 1994a). Hydroxyl radical footprinting analyses show that CBP binds to the base of the four-way junction (Pearson *et al.*, 1995), in a manner similar to that observed for the monoclonal anti-cruciform antibody (Frappier *et al.*, 1987; Steinmetzer *et al.*, 1995). In fact, the monoclonal anti-cruciform antibody can compete against CBP for binding to cruciforms (Pearson, 1994), which could have biological relevance, since the anti-cruciform antibody can enhance

DNA replication (Zannis-Hadjopoulos *et al.*, 1988). The CBP interacts with cruciforms in a completely different fashion from other cruciform binding proteins studied to date (Pearson *et al.*, 1995). The CBP activity is regulated with respect to the cell cycle, with maximal activity appearing at the G1\S boundary (Pearson, 1994), which coincides with the point in the cell cycle where the maximum number of cruciforms appear (Ward *et al.*, 1990).

CBP has been identified as a member of the 14-3-3 family of proteins through microsequence analysis of CBP polypeptides that interact specifically with cruciform DNA (Todd *et al.*, 1998; Chapter 4).

V The 14-3-3 family of proteins

(i) Family overview

14-3-3 proteins were first described over 30 years ago by Moore & Perez in their systematic analysis of acidic, soluble brain proteins (Moore and Perez, 1967). The name is the result of their nomenclature, representing the protein's mobility on starch gel electrophoresis following fractionation by DEAE cellulose. At the time, the 14-3-3 proteins were observed to be very abundant in the brain, and were actually thought to be brain specific (Moore and Perez, 1967). 14-3-3 proteins are indeed very abundant in the brain, constituting up to 1% of the total soluble brain protein (Boston *et al.*, 1982), but they have now been shown to be expressed in most mammalian tissues. In addition, 14-3-3 proteins have been cloned from a wide range of eukaryotic organisms, including plants, both fission and budding yeasts, insects, nematodes, fish, and amphibians. Seven mammalian isoforms have been identified to date, (α,β,γ,δ,ε,ζ, and η) based on their order

of elution during reversed phase high-performance liquid chromatography (HPLC; Aitken *et al.*, 1992). The α and δ isoforms are post-translationally modified (by phosphorylation) products of the same gene that gives rise to the β and ζ isoforms (Aitken *et al.*, 1995a). Two additional isoforms, σ and τ, are expressed exclusively in epithelial and T-cells respectively. The 14-3-3 family is highly conserved at the amino acid level, with individual isoforms being either identical (such as for the α and β as well as the δ and ζ isoforms) or containing only a few conservative substitutions over a wide range of mammalian species.

(ii) Structure

14-3-3 proteins are acidic proteins (pI = ~5) of approximately 30 kDa. They possess a region (residues 134-150) that bears similarity to the primary structure of the C-terminus of members of the family of calcium- and lipid-binding proteins, the annexins. The annexin family plays a role in the regulation of protein kinase C (PKC; Aitken *et al.*, 1992). However, the function of this domain in 14-3-3 is not clear, as the inhibition of PKC by 14-3-3 proteins is not strongly affected by mutations in this region (Jones *et al.*, 1995b). Biochemical analyses have revealed that 14-3-3 proteins form both homo- and heterodimers *in vivo* and *in vitro* (Jones *et al.*, 1995a). The crystal structures of the 14–3–3 proteins confirmed their existance as dimers, and localized the dimerization domains to the N-termini (Liu *et al.*, 1995; Xiao *et al.*, 1995). The dimer structure appears to be necessary for function, at least for the role of 14-3-3 proteins in regulating Raf kinase activity (Tzivion *et al.*, 1998). Each monomer consists of nine antiparallel alpha helices which, when associated as a dimer, form an amphipathic groove. Interestingly, the highly conserved residues (about 45% of the approximately 240 residues) all lie within an

invariant surface which includes the dimerization domain and the lining of the groove. The non-conserved residues are located on the outer surface of the dimeric structure (Liu *et al.*, 1995; Xiao *et al.*, 1995). A large number of proteins have been found to interact with 14-3-3 proteins (see below), and the amphipathic groove of the 14-3-3 dimer is thought to mediate these associations. Indeed, 14-3-3 binding to known partners is disrupted by mutating amino acids which alter the hydrophobic property of the groove (Wang *et al.*, 1998). The amphipathic groove may infact be capable of binding two proteins simultaneously, which has implications for 14-3-3 function (see below; Petosa *et al.*, 1998). In addition, the x-ray crytal strucutres show enough space for two helices in the groove of the dimer (Liu *et al.*, 1995; Xiao *et al.*, 1995).

(iii) Cellular and chromosomal localization

The human gene encoding the η isoform has been localized by FISH analysis to chromosome 22, band q12 (Muratake *et al.*, 1996; Tommerup and Leffers, 1996). Also using FISH techniques, the human genes encoding 14-3-3 ζ and β have been localized to chromosome 2, band p25.1-p25.2, and chromosome 20 band q13.1 respectively.

Given the fact that 14-3-3 proteins associate with a number of cytoplasmic signalling proteins, it had long been assumed that 14-3-3 proteins are uniquely cytoplasmic. 14-3-3 proteins are abundantly found in the cytosol, but are also present in other cellular compartments. A small portion of rat brain 14-3-3 proteins are selectivly associated with a specific subset of membranes which include synaptic membranes, but not mitochrondrial or myelin membranes (Martin *et al.*, 1994). Also in rat brain fractions, 14-3-3 proteins were found in a particulate fraction which contains nuclei (Martin *et al.*,

1994). In adrenal chromaffin cells, 14-3-3 proteins stimulate the exocytosis of chromaffin granules and are associated with phospholipids and the actin cytoskeleton (Roth *et al.*, 1994). Association of 14-3-3 proteins with centrosomes and the spindle apparatus has also been reported in mouse leukemic cells (Pietromonaco *et al.*, 1996). Plant 14-3-3 isoforms have been directly localized to the nucleus using confocal microscopy techniques, in *Arabidopsis thaliana* and *Zia mays* cells (Bihn *et al.*, 1997). 14-3-3 proteins have also been detected in nuclear extracts from human MCF-7 breast carcinoma cells (Waterman *et al.*, 1998). We have shown by immunofluorescence the presence of 14-3-3 isoforms in HeLa nuclei (Todd *et al.*, 1998, Chapter 4).

(iv) Function

14-3-3 proteins were attributed a biological function twenty years after their first description, when Ichimura *et al.* (1987) reported the identity of 14-3-3 to be a known activator of tyrosine and tryptophan hydroxylases, which are involved in the biosynthesis of the neurotransmitters serotonin and dopamine in the brain. Other reported functions for the family included inhibition of PKC activity (Toker *et al.*, 1990), and stimulation of calcium-dependent exocytosis (Morgan and Burgoyne, 1992). Since then, there has been a veritable explosion of 14-3-3 publications reporting the involvement of 14-3-3 proteins in a wide array of biological processes (for reviews, see Aitken, 1995; Aitken *et al.*, 1992; Aitken *et al.*, 1995b; Burbelo and Hall, 1995; Marais and Marshall, 1995; Morrison, 1994; Ferl, 1996), owing primarily to the discovery that 14-3-3 proteins associate with a large number of unrelated proteins. Further excitement ensued when a number of 14-3-3-interacting proteins were reported that had roles in signal transduction pathways. Proteins

such as the Raf-1 kinase (Fantl *et al.*, 1994; Fu *et al.*, 1994), polyoma virus middle T antigen (Pallas *et al.*, 1994), and the Bcr-abl fusion protein (Reuther *et al.*, 1994) were among the first reported binding partners for 14-3-3 with signal transducing roles. Since these reports, the number of 14-3-3 interacting proteins identified has grown. The association of 14-3-3 with such a wide variety of proteins is explained, in part, by the recent discovery of two 14-3-3 consensus binding motifs, RSXpSXP and RXY/FXpSXP (where pS is phospho-serine) within the 14-3-3 binding partners (Muslin *et al.*, 1996; Yaffe *et al.*, 1997). These motifs are considered to be the minimal sequence criteria required for binding to 14-3-3 proteins, with additional contacts possibly dictating whether binding actually occurs, and conferring isoform specificity. Both phosphorylated and nonphosphorylated substrates appear to bind within the amphipathic groove. Crystal structures of 14-3-3 ζ complexed with a Raf-derived phosphoserine peptide or an unphosphorylated peptide indicate that the two peptides bind within the conserved amphipathic groove at overlapping but distinct sites (Petosa *et al.*, 1998).

The two yeast 14-3-3 homologues BMH1 and BMH2 are essential in S. *cerevisiae*, as demonstrated by the lethal effect of simultaneous disruption (van Heusden *et al.*, 1995). This double null mutant can be rescued by a 14-3-3 homologue from *Arabidopsis* (van Heusden *et al.*, 1996), indicating that there is overlap of function between isoforms and species. In the fission yeast *S. pombe*, there are two 14-3-3 homologues, rad24 and rad25, which are also essential for viability (Ford JC et al 1994, Science 265:533). In *Drosophila*, the 14-3-3 ζ homologue, *leonardo*, is highly expressed

in mushroom body neurons, and, its disruption causes olfactory learning defecits (Skoulakis and Davis, 1996).

To summarize, the 14-3-3 family of proteins consists of multiple isoforms that are conserved in primary and tertiary structure amongst themselves. Conservation also exists across species, as demonstrated by functional substitutions. They exist as dimers both *in vivo* and *in vitro*, and, and participate in a wide array of biological processes by acting as adapter or stabilizing molecules.

VI. Thesis goals

The following chapters further our understanding of the sequence and structural elements important for the initiation of DNA replication in mammalian cells. In addition, the identity of a human cruciform binding protein, CBP, is presented along with evidence supporting its participation in the process of DNA replication.

CHAPTER TWO

AUTONOMOUS REPLICATION *IN VIVO* AND *IN VITRO* OF CLONES SPANNING THE REGION OF THE *DHFR* ORIGIN OF BIDIRECTIONAL REPLICATION (oriβ)

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Abstract

Plasmids containing the origin of bidirectional replication (*oriβ*) of the Chinese hamster dihydrofolate reductase-encoding gene (*DHFR*) were tested for autonomous replication *in vivo* and *in vitro*. The results show that plasmids pX24 and p*neo*S13, that contain a 4.8- and a 11.5-kb fragment, respectively, spanning the *oriβ* region, are able to replicate autonomously in human cells and in a cell-free system that uses human cell extracts. Another plasmid, pX14, containing a 4.8-kb fragment that is immediately adjacent to the *oriβ* region, also replicated in these two assays.

Introduction

Several different methods have been employed for the isolation of mammalian DNA replication origins (*ori;* reviewed in Vassilev and DePamphilis, 1992). However, a major difficulty in their identification as functional *ori*, has been the lack of a simple, reliable and sufficiently sensitive functional assay. One of the best candidates for such an assay is the DpnI resistance assay (Peden *et al.*, 1980), which has been very useful in the functional assessment of viral *ori* (Vassilev and DePamphilis, 1992). This assay has been successfully employed by us (Bell *et al.*, 1991; Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991; Nielsen *et al.*, 1994) and others (Iguchi-Ariga *et al.*, 1993; McWhinney and Leffak, 1990; Virta-Pearlman *et al.*, 1993) to study the function of putative mammalian chromosomal *ori*. In addition, we recently developed an *in vitro* DNA replication system, which supports the specific initiation of one round of semiconservative replication in plasmids containing putative mammalian *ori* (Pearson *et al.*, 1991; Pearson *et al.*, 1994b). As we have shown, the *ori*-containing plasmids that are capable of autonomous replication *in vivo*, by transfection, can also replicate in the *in vitro* replication assay (Nielsen *et al.*, 1994; Pearson *et al.*, 1991). In this paper, we used the DpnI resistance assay, *in vivo* and *in vitro*, to functionally assess a series of plasmids containing the bidirectional replication origin, *oriβ,* of the dihydrofolate reductase-encoding gene *DHFR* (Burhans *et al.*, 1990).

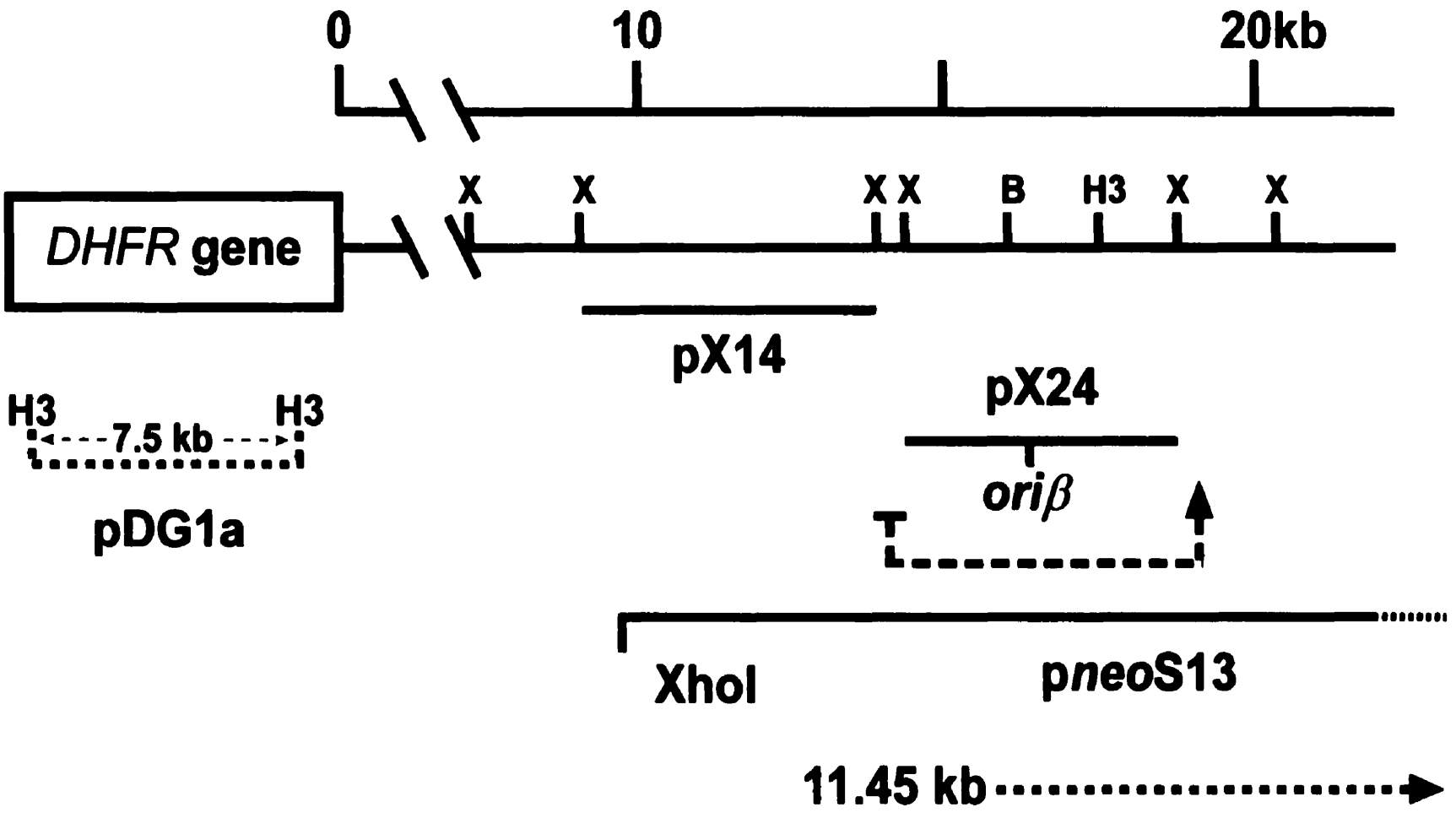
Experimental and Discussion

(a) Transient replication assays *in vivo* by transfection

The autonomous replication of a series of plasmids containing sequences from the coding (pDG1a; 7.5-kb HindIII fragment of the *DHFR* coding region cloned into pUC19) and 3' non-coding (pX14, pX24, p*neo*S13) region of the *DHFR* gene (gifts of Dr. M.L. DePamphilis, Roche Institute of Molecular Biology) was investigated (Figure 2.1).

Figure 2.1. Diagram of the *DHFR* gene and downstream noncoding regions

Diagram of the *DHFR* gene and downstream noncoding regions indicating the fragments contained in plasmids pDG1a, pX14, pX24, and p*neo*S13. The position of the *oriβ*, as mapped by Burhans *et al.* (1990) is also indicated. B, BamH1; H3, HindIII; X, XbaI


0
10
20kb
X
X
X X
B
H3
X
X
DHFR gene
pX14
pX24
H3
7.5 kb
H3
pDG1a
oriβ
XhoI
pneoS13
11.45 kb

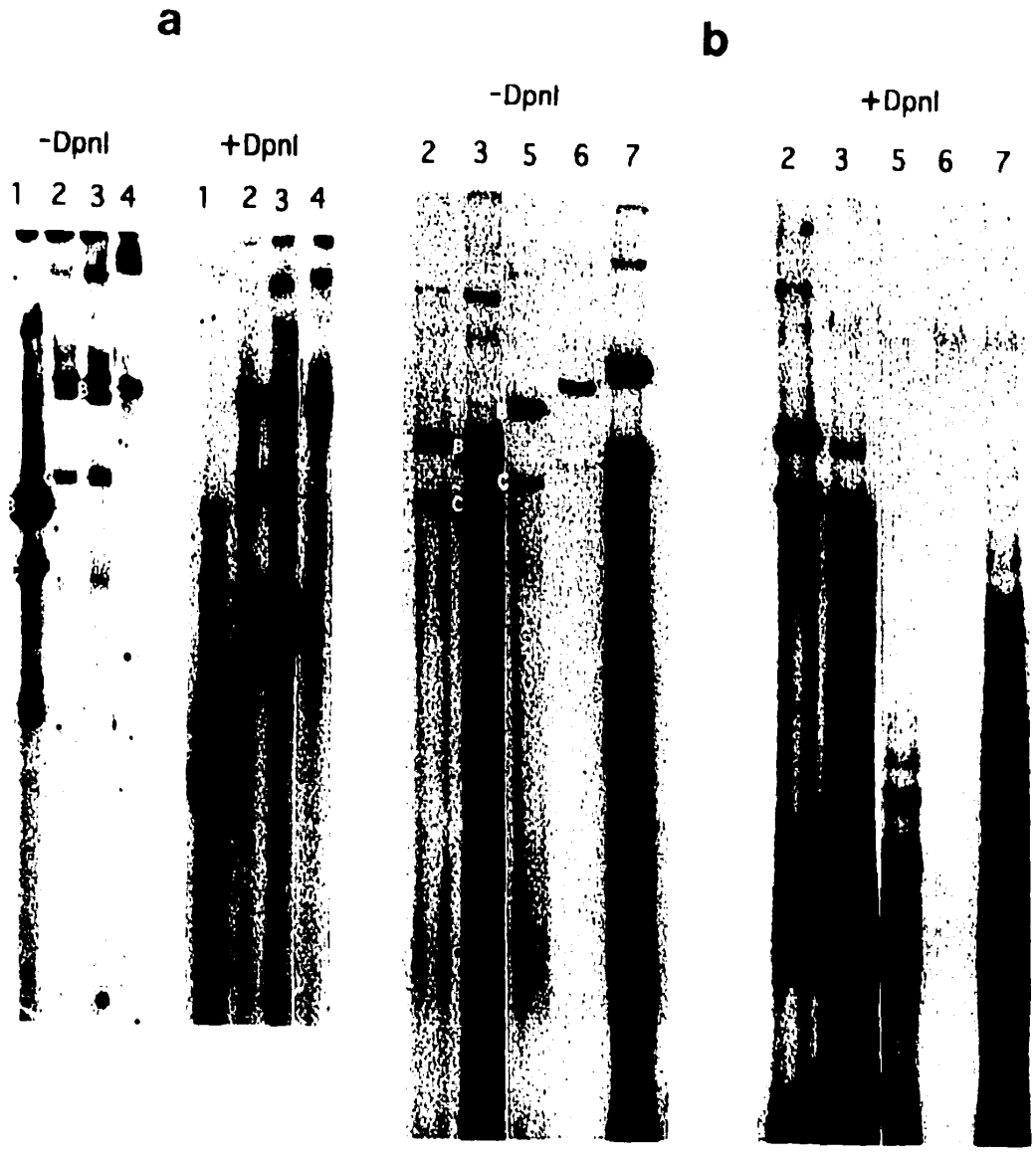
Appropriate control plasmids of equivalent size containing bacteriophage λ DNA sequences (pDGλ8.6, 6.6+2.0-kb HindIII fragments of λ DNA; and pDGλ6.6, 6.6-kb HindIII fragment of λ DNA; gifts of Dr. M.L. DePamphilis, Roche Institute of Molecular Biology) were also used. *Ors12*, a monkey autonomously replicating DNA sequence (Frappier and Zannis-Hadjopoulos, 1987; Rao *et al.*, 1990; Zannis-Hadjopoulos *et al.*, 1985) was used as a positive control. DNAs (5 μg) from these plasmids were transfected in HeLa cells by the Ca.phosphate co-precipitation method (Graham and Eb, 1973) and 48 h later Hirt supernatants (Hirt, 1967) were prepared. Plasmid DNA recovered in these supernatants was digested with DpnI (Peden *et al.*, 1980), which cleaves unreplicated (fully methylated) input DNA, but does not cleave DNA replicated (hemimethylated or unmethylated) in HeLa cells, and subjected to Southern blot analyses (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991; Wu *et al.*, 1993a). Blots were probed with either nick-translated pBR322 (Figure 2.2A) or a mixture of pDG1a and p*neo*S13 (Figure 2.2B), which are plasmids containing vectors pUC19 and pdMMTneo that carry the various DNA fragments used in the replication studies. As an internal control for full digestion by DpnI, 500 ng of methylated λ DNA (NE Biolabs, Baverly, MA, USA) were included in all reactions, and the digestion products were verified by ethidium bromide staining and Southern blot hybridization (data not shown). The recovered plasmid DNAs were of the correct size expected for p*ors12* (5.4 kb), pX14 (7.5 kb), pX24 (7.5 kb), pDG1a (10.2 kb), p*neo*S13 (18 kb), pDGλ6.6 (9.3 kb), and pDGλ8.6 (11.3 kb), as in previous assays (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991; Wu *et al.*, 1993a). The results (Figure 2.2) show that *ors12*, pX14, pX24, and p*neo*S13 generated DpnI-resistant bands, while pDG1a and the two control clones of bacteriophage λ DNA did not. *Ors12* has been shown previously to have autonomously replicating activity (Frappier and Zannis-Hadjopoulos, 1987). pX24 and p*neo*S13 both contain the *oriβ* region of *DHFR* (Burhans *et al.*, 1990). pX14 includes a region in which initiation was detected by 2D-gel electrophoresis, but at a lower

frequency than the fragments which contain the *oriβ* (Dijkwel and Hamlin, 1992). The production of *Dpn*I-resistant supercoiled (form I) DNA is variable and generally lower than that of relaxed circular (form II) and linear (form III) forms, as has also been observed previously (Landry and Zannis-Hadjopoulos, 1991). This may be caused either by variability in topoisomerase activity (Pearson *et al.*, 1991) or by the sensitivity of hemimethylated form I DNA to nicking (Sanchez *et al.*, 1992) that converts it to form II.

These results are consistent with previous observations that small (~100 bp - 2 kb) *ors* (Landry and Zannis-Hadjopoulos, 1991; Rao *et al.*, 1990; Zannis-Hadjopoulos *et al.*, 1985) or other selected sequences (the 5' c-*myc* origin: McWhinney and Leffak, 1990); cruciform-enriched human genomic fragments: (Bell *et al.*, 1991; Nielsen *et al.*, 1994); a sequence 3' to the mouse adenosine deaminase gene: (Virta-Pearlman *et al.*, 1993) of mammalian DNA can initiate autonomous replication *in vivo* (Bell *et al.*, 1991; Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991; McWhinney and Leffak, 1990; Virta-Pearlman *et al.*, 1993; Nielsen *et al.*, 1994) and *in vitro* (Nielsen *et al.*, 1994; Pearson *et al.*, 1991; Pearson *et al.*, 1994b). In contrast, some studies have reported that only large fragments (>10 kb) were observed to support autonomous DNA replication in human (Heinzel *et al.*, 1991; Krysan *et al.*, 1993) or rodent (Krysan and Calos, 1993) cells, including those that contain the *DHFR oriβ* (Caddle and Calos, 1992).

Figure 2.2. Autonomous replication assay of *oriβ*-containing plasmids

HeLa cells were transfected with 5 μg of each plasmid DNA. After 48 h the recovered plasmid DNA samples were split into two halves, one half left untreated (-DpnI) and the other digested with 1.5 units of DpnI for 2 h (+DpnI), electrophoresed on 1% agarose, blot-transfered, and probed with a mixture of nick-translated pDG1a and p*neo*S13 DNAs. Full digestion by DpnI was verified by including 500 ng of λ DNA in each reaction, as described previously (Frappier and Zannis-Hadjopoulos, 1987). Lanes: 1, *Ors12*; 2, pX14; 3, pX24; 4, p*neo*S13; 5, pDGλ6.6; 6, pDG1a,; 7, pDGλ8.6. Panels a and b represent two experiments, performed in the same manner excepting comb size and the plasmids tested. Supercoiled form I (A), relaxed circular form II (B), and linear form III (C) molecules are indicated in white. Bands migrating below form I represent DpnI digestion products; bands migrating above form II include catenated circular products of replication and replicative intermediates.

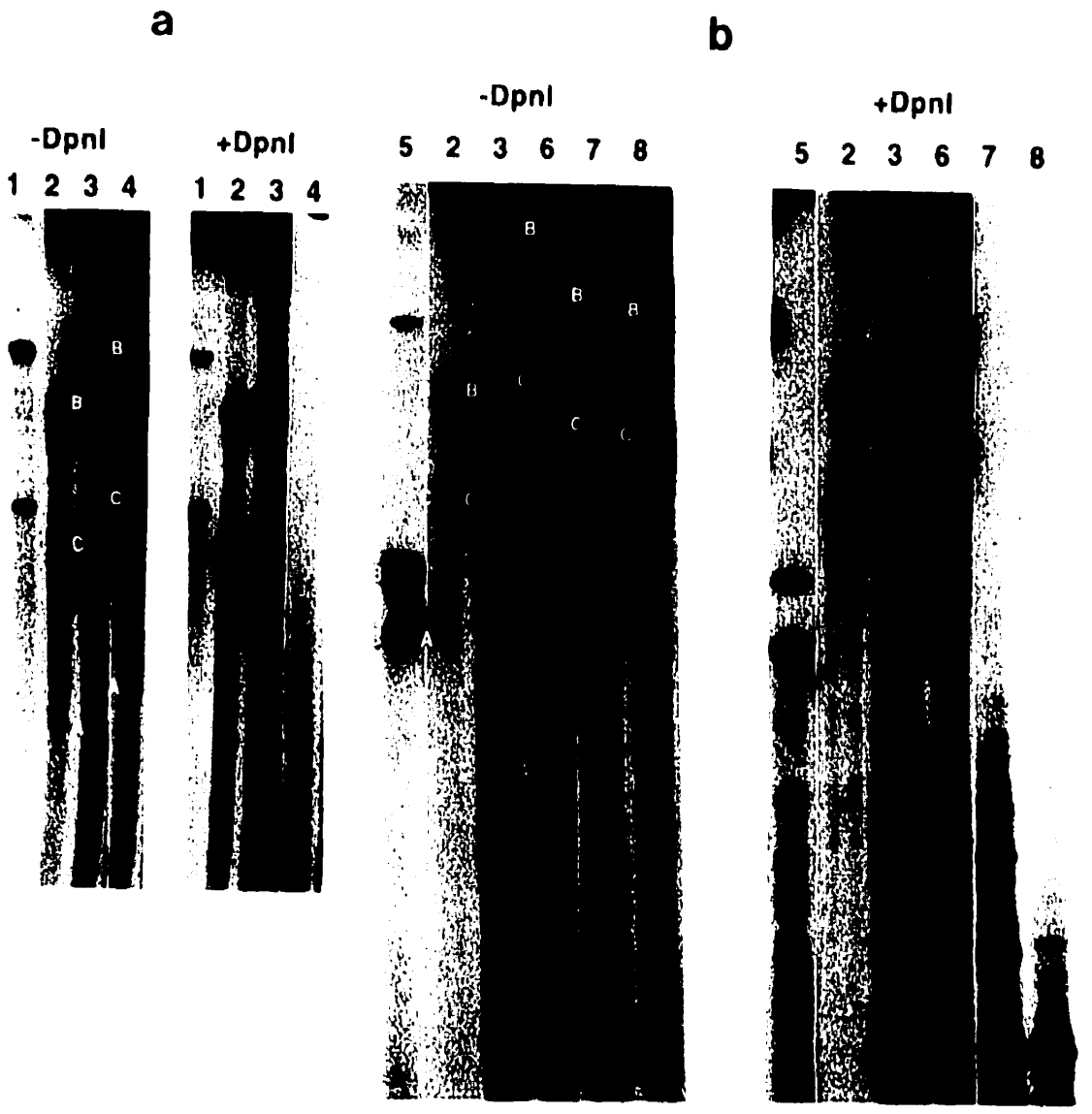

a
-DpnI
+DpnI
1 2 3 4
1 2 3 4
b
-DpnI
+DpnI
2 3 5 6 7
2 3 5 6 7
B
C
C

(b) *In vitro* replication assay

We have recently developed an *in vitro* replication system, in which monkey (*ors*) (Pearson *et al.*, 1991) and human (Nielsen *et al.*, 1994) autonomously replicating sequences can initiate and carry out one round of aphidicolin-sensitive semiconservative DNA replication (Pearson *et al.*, 1991). We next assessed the ability of this system to detect autonomously replicating plasmids among those used above, containing sequences both from within the coding region or downstream of *DHFR*. *In vitro* replication was carried out as described in (Pearson *et al.*, 1991), using equimolar amounts (approximately 100 ng) of each plasmid DNA as template. Since pNeo.Myc-2.4 (9.3 kb) can be successfully replicated in our *in vitro* system, consistent with *in vivo* transfection data of McWhinney and Leffak (1990), we used it and *ors*8 plasmid DNA (4.9 kb; Pearson *et al.*, 1991) as positive controls (Figure 2.3). We observed that the same clones (pX14, pX24 and p*neo*S13) that replicated *in vivo* after transfection were also capable of replicating *in vitro* , generating DpnI-resistant bands, while pDG1a and the two λ clones did not (Figure 2.3). All the unreplicated input DNAs, before their use either *in vivo* or *in vitro*, were DpnI-sensitive, indicating that they were fully methylated (Nielsen *et al.*, 1994; Peden *et al.*, 1980). As previously, in all plasmids we observed material which migrates slower than form II, indicative of catenated dimers and replicative intermediates (Pearson *et al.*, 1991). Control experiments in which template DNAs were not included in *in vitro* reactions yielded no products (Pearson *et al.*, 1991). Thus, as before (Pearson *et al.*, 1991; Nielsen *et al.*, 1994), we found that the *in vivo* and *in vitro* assays are both capable of detecting autonomous replicating activity of plasmids containing putative mammalian *ori*.

Figure 2.3. *In vitro* replication assay

Equimolar amounts of each plasmid DNA were incubated for 1 h at 30°C in reaction mixtures containing HeLa cell extracts, as previously described (Pearson *et al.*, 1991). The product DNAs were purified, concentrated, and divided into two halves; one half (~50 ng) of each reaction was left untreated (-DpnI) and the other was digested with 1 unit of DpnI for 1.5 h (+DpnI). All samples were then subjected to electrophoresis in 1% agarose. Lanes: 1, pNeo.Myc-2.4; 2, pX24; 3, pX14; 4, pDG1a; 5, *ors*8; 6, p*neo*S13; 7, pDGλ8.6; 8, pDGλ6.6. Panels a and b as well as the different replication products are as described in Figure. 2.2.


a
-DpnI
+DpnI
1 2 3 4
1 2 3 4
B
B
C
C
b
-DpnI
+DpnI
5 2 3 6 7 8
5 2 3 6 7 8
B
B
B
B
C
C
A

Burhans *et al.* (1990), using various transfection protocols and a similar *in vitro* system with a variety of mammalian extracts, had difficulty observing significant DpnI-resistant replication with either pX24 or pX14. Occasionally, however, under conditions that permitted borderline-detectable replication, both plasmids showed equivalent activity. Caddle and Calos (1992), transfecting 293S cells, observed autonomous replication of a 13.3-kb 3' *DHFR* fragment (incorporating the inserts of both pX24 and pX14), but did not observe any activity from a shorter construct with the same 4.3-kb *Xba*I fragment present in pX24.

(c) Conclusions

(1) Autonomous replication activity can be detected in transient episomal replication assays of plasmids carrying the *DHFR oriβ*, regardless of their size (4.8-kb fragment, pX24; 11.5-kb fragment, p*neo*S13).

(2) The *in vitro* replication assay is an acceptable alternative method to the *in vivo* assay for detection of autonomous replicating activity of plasmids containing mammalian putative *ori*, corroborating our previous observations (Nielsen *et al.*, 1994; Pearson *et al.*, 1991; Pearson *et al.*, 1994b).

(3) In both the *in vivo* and *in vitro* replication assays, control plasmids carrying λ DNA inserts are replication-negative, whereas the p*neo*S13 plasmid does replicate autonomously, as does the smaller plasmid, pX24 (discussed in Burhans *et al.*, 1990); both of the latter two plasmids carry inserts that contain *oriβ*. A third plasmid, pX14, containing a 4.8-kb insert from the *DHFR* downstream region immediately 5' to pX24, also displays autonomous replication; however, pDG1a, carrying 7.5 kb of the *DHFR* coding region, does not. The above results suggest that replication occurs only in plasmids containing mammalian inserts that carry an autonomously replicating sequence (a potential *ori*) and is not the result of random initiation from nicked, gapped or cut templates.

The observation that the inserts in pDG1a (7.5 kb) and pDGλ8.6 (8.6 kb) do not replicate in our assays, whereas those in pX14, pX24, pNeo.Myc-2.4 and *ors12* (4.8 kb, 4.8 kb, 2.4 kb and 0.8 kb inserts, respectively) do, implies that these results cannot be explained as simple fragment size dependent DNA replication (Heinzel *et al.*, 1991). While replication from the *oriβ* contained within pX24 is consistent with results obtained by many mapping techniques, including nascent DNA PCR (Vassilev *et al.*, 1990), and Okazaki fragment distribution (Burhans *et al.*, 1990), the possibility of origin activity from pX14 is supported by several lower resolution mapping techniques (reviewed in Burhans *et al.*, 1990), and by 2D-gel analysis (Vaughn *et al.*, 1990).

Acknowledgments

We would like to thank Dr. M.L. DePamphilis (Roche Institute of Molecular Biology) for providing the plasmids pX14, pX24, pDG1a, p*neo*S13, pDGλ8.6 and pDGλ6.6. We thank Dr. P. Leder (Harvard Medical School) for providing the lambda phage clone containing the human *c-myc* gene and flanking DNA, and Dr. M. Leffak (Wright State University), who subsequently made available a portion of this clone as pNeo.Myc-2.4. We also thank Drs. J.L. Hamlin (University of Virginia) and D. Gilbert (Roche Institute of Molecular Biology) for helpful advice. This work was funded by grants from the Medical Research Council (MRC) of Canada to M.Z.-H. (MT-7965) and the Cancer Research Society, Inc. to G.B.P. M. Z.-H. is an MRC Scientist. T.O.N. is a recipient of an MRC studentship. A.T. is a recipient of studentships from F.C.A.R. and Le Défi Corporatif Canderel.

Connecting Text

In the preceding chapter, plasmids bearing sequnces encompassing the *oriβ* origin of DNA replication from the *DHFR* locus were observed to replicate *in vivo* by transient transfection, and *in vitro.* In the next chapter, the same replication assays are used to show that the minimal 186-bp sequence required to support autonomous replication of a monkey early-replicating sequence includes bent DNA, an imperfect Oct-1 binding site, and an inverted repeat capable of extrusion into a cruciform *in vivo* (Bell *et al.*, 1991).

CHAPTER THREE

DELETION ANALYSIS OF MINIMAL SEQUENCE REQUIREMENTS FOR AUTONOMOUS REPLICATION OF *ORS8*, A MONKEY EARLY-REPLICATING DNA SEQUENCE

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ABSTRACT

We have generated a panel of deletion mutants of *ors8* (483 bp), a mammalian autonomously replicating DNA sequence, previously isolated by extrusion of nascent monkey (CV-1) DNA from replication bubbles active at the onset of S phase. The deletion mutants were tested for replication function by the DpnI resistance assay, *in vivo*, after transfection into HeLa cells, and *in vitro*. An internal fragment of 186-bp that is required for autonomous replication function of *ors8* was identified. This fragment, when subcloned into pBR322 and similarly tested, was capable of autonomous replication *in vivo* and *in vitro*. The 186-bp fragment contains several repeated sequence motifs, such as the ATTA and ATTTAT motifs, occurring three and five times, respectively, the sequences TAGG and TAGA, occurring three and seven times, respectively, two 5'-ATT-3' repeats, a 44-bp imperfect inverted repeat (IR) sequence, and an imperfect consensus binding element for the transcription factor Oct-1. A measurable sequence-directed DNA curvature was also detected, coinciding with the AT-rich regions of the 186-bp fragment.

Introduction

We have previously isolated and cloned, in pBR322, monkey (CV-1) DNA enriched 10^3- to 10^4-fold for nascent sequences that are replicated at the onset of S phase (Kaufmann *et al.*, 1985). Approximately 50% (17 of 30) of the origin enriched sequence (*ors*) clones that have been examined, act as episomal origins of replication, when transfected into HeLa cells, and tested by DpnI resistance, bromodeoxyuridine (BrdUrd) substitution, and electron microscopic examination of replication bubbles (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991). Seven of the *ors*, *ors1, 3, 8, 9, 10, 11* and *12* have also been shown to function as plasmid origins of DNA replication in an *in vitro* replication system that uses HeLa cell extracts (Pearson *et al.*, 1991; Price *et al.*, 1992; Zannis-Hadjopoulos *et al.*, 1992). Sequence analysis has revealed that the *ors* contain sequence and structural characteristics that have often been associated with replication origins, such as AT-rich regions, inverted repeat (IR) sequences, bent DNA, ARS consensus sequences of yeast, the consensus for scaffold attachment regions (SAR) of *Drosophila*, and various eukaryotic transcriptional regulatory elements (Rao *et al.*, 1990).

Ors8 (483 nucleotides in length; ≤5 copies per haploid CV-1 genome; Zannis-Hadjopoulos *et al.*, 1985) is replicated in the early part of S phase (Zannis-Hadjopoulos *et al.*, 1988). When present on plasmids, it has been shown to act as an episomal DNA replication origin when transfected into mammalian cells (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991), and *in vitro* (Pearson *et al.*, 1991); in both systems it has been mapped by EM as the initiation site of replication (Frappier and Zannis-Hadjopoulos, 1987; Pearson *et al.*, 1994b). In this study, we have identified by deletion mutagenesis the minimal sequence in *ors8* that is necessary for its function as an episomal origin of replication.

Experimental Procedures

Cells and plasmids

HeLa cells (monolayers) were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS). All plasmids were propagated in *Escherichia coli* HB101, as previously described (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991). Plasmid DNAs were isolated by the alkaline lysis method, as described by Pearson *et al.* (1991), and Maniatis *et al.*(1982).

Ors8 Deletion mutants by subcloning and Bal31 exonuclease digestion

The subfragments of *ors8* plasmid DNA: HinfI-HaeIII (96- bp), HaeIII-HinfI (438-bp), RsaI-HaeIII (192-bp), HaeIII-FokI (287-bp), HaeIII-NdeI (156-bp), and NdeI-HaeIII (378-bp) were excised and subcloned into the NruI site of pML-2 (a gift from Dr. J. Hassell), as described in Kaufmann *et al.* (1985). To generate the internal deletion mutants, *ors8*-pML2 plasmid DNA was linearized by digesting with NdeI, and then subjected to timed digestion by Bal31 (BRL; 44 units/ml) for 0.5, 1, 1.5 and 2 minutes. The aliquots that were withdrawn at the various time points were diluted 1:1 with an equal volume of stop solution (15 mM EDTA, 0.2% SDS). When all the time points had been collected, the samples were diluted with an equal volume of water, extracted with an equal volume of equilibrated phenol, then with ether, and precipitated by the addition of 2 volumes of absolute ethanol. The pellets of the digestion products were then resuspended in a ligation mixture (Maniatis *et al.*, 1982) containing 10 units of ligase (BRL), incubated for 16 hours at room temperature, and then used to transform *E. coli* HB101 cells, as described previously (Kaufmann *et al.*, 1985). Colonies of isolated

transformants were picked, grown as minipreps (Maniatis *et al.*, 1982) and screened by digestion with selected restriction enzymes for determination of the extent of the internal deletion. The size of the deletion mutant plasmids was determined by electrophoresis on polyacrylamide gels, by comparison to appropriate size marker standards, and verified by sequencing (Landry and Zannis-Hadjopoulos, 1991).

Finally, the NdeI-RsaI fragment (186-bp) of *ors8* was subcloned in the NruI site of pBR322, as before (Kaufmann *et al.*, 1985).

DpnI-resistance assay

Exponentially growing HeLa cells were transfected with 3 -5 μg of each plasmid DNA (intact *ors8* plasmid or the various deletion mutant plasmids) by the calcium phosphate coprecipitation method, as described previously (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991). 48 h later, low-molecular-weight DNA was isolated by the method of Hirt, (Hirt, 1967); the lysates were extracted, concentrated, digested with 2-3 units of DpnI for 1 hour at 37°C, and analyzed by Southern blot hybridization, as before (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991). As an internal control for full digestion by DpnI, 400 ng of methylated λ DNA (Pharmacia) were included in all reactions, and the digestion products were verified by ethidium bromide staining (data not shown). The DpnI-digested DNAs were also used to transform *E. coli* for detecting DpnI-resistant plasmid in Hirt supernatant DNA, as previously described (Landry and Zannis-Hadjopoulos, 1991; Vassilev and Johnson, 1988).

In vitro replication assays

In vitro replication was carried out as described in (Pearson *et al.*, 1991), with the following modifications: 100 ng of *ors8* plasmid DNA was used as the template in the control reaction, while all the *ors8*-deletion plasmids and the vector (pML-2) plasmid were used on an equimolar basis relative to *ors8*. For quantitative analysis, each reaction was treated as published in (Guo *et al.*, 1989) with some modifications. Briefly, the products of the *in vitro* reactions were first linearized by digestion with SalI, then digested with DpnI and separated electrophoretically on 1% agarose gels; 200-300 ng of each respective plasmid in linear form (III) was included as marker. Full length linear forms were visualised by ethidium bromide staining and excised. The gel slices were dissolved in 1 ml of 4 M Urea for 15 min at 95°C before addition of 15 ml of Universol (ICN) and measurement of radioactivity in a scintillation counter (Guo *et al.*, 1989).

Sequence-directed curvature (bent DNA) assays

The *ors8* DNA used in these assays was generated by PCR amplification of *ors8* plasmid, as described previously (Mah *et al.*, 1993). The *ors8* portion of the plasmid was amplified using external (pBR-specific) primers (+ and -, sequence of nucleotide positions 954 to 968 of the top pBR322 strand, and 994 to 979 of the bottom pBR322 strand, respectively). The PCR-generated *ors8* DNA, which included 21 bp and 20 bp of pBR322 sequence on either side of the cloning site (NruI, pBR322 nucleotide position 972), was digested with the enzymes DdeI, MboII or RsaI, for the bent DNA assays. The deletion mutants B and C, which were subcloned in the NruI site of the vector pML-2, were also generated by PCR amplification using the same primers described above. The presence of anomalously migrating

fragments within various subfragments of *ors8* (see Figure 3.5) and in deletion mutants B and C was tested by two methods: 1) *ors8* was digested with either DdeI, MboII or RsaI, and the resulting fragments were separated on a 2% agarose gel at room temperature. The lanes were then excised, reoriented at a 90° angle relative to the first dimension, cast in a 7% polyacrylamide gel and electrophoresed at 9°C (Anderson, 1986); 1 μg of a 123-bp ladder DNA marker (Gibco/BRL) was included with each sample. The arc of DNA fragments was visualized by staining with ethidium bromide; and 2) the digested DNA was also loaded onto two parallel 4% polyacrylamide gels and electrophoresed at 9°C and 25°C, respectively; the relative mobility of each fragment was measured with reference to the 123-bp ladder marker.

RESULTS

Description of *ors8* sequence and the deletion mutants

The various landmarks of *ors8* are shown at the top diagram of Figure 3.1. *Ors8* is a 483bp-long DNA sequence (GenBank Accession No. M26221; Rao *et al.*, 1990) which contains an AT-rich sequence domain, a 44-bp inverted repeat (IR) sequence (12-bp stem, 20 base loop; Rao *et al.*, 1990), a GCS consensus sequence characteristic of transcriptional elements upstream of β-globin genes (Rao *et al.*, 1990), a scaffold attachment region (SAR-B) consensus sequence (Gasser and Laemmli, 1986), and a region of perfect homology (11/11-bp match) with the yeast ARS (autonomously replicating sequences) consensus (Palzkill and Newlon, 1988).

Selected subfragments of *ors8* were generated by restriction digestion of the DNA with the appropriate enzymes (shown in Figure 3.1) and subsequently subcloned into the NruI site of the plasmid pML-2, as described in the Experimental Procedures, giving rise to the deletion

mutants A-F (Figure 3.1). In addition, a panel of internal deletion mutants (1, 6, 10, and 12) (Figure 3.1) was generated by timed digestion with Bal31 of *ors8* that had been previously linearized with NdeI (see Experimental Procedures).

Autonomous Replication Assay of the Deletion Mutants.

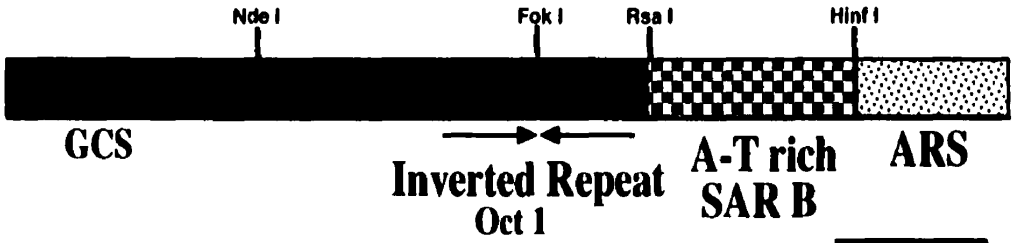
Supercoiled plasmid DNA from each of these constructs was transfected into HeLa cells and its ability to undergo autonomous DNA replication was assayed by the DpnI resistance assay (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991), in order to distinguish input plasmids from plasmids replicated in HeLa cells. The DpnI-digested DNA samples were divided in two halves, one of which was used to transform *E. coli* (Table 1), as described previously (Landry and Zannis-Hadjopoulos, 1991; Vassilev and Johnson, 1988), and the other was subjected to Southern blot analysis (Figure 3.2). Both assays showed that deletion mutants B, F, and 12 yielded DpnI resistant material, while mutants A, C, D, E, 1, 6, and 10 did not (Figs. 3.1, 3.2, and Table 1). These results suggested that an internal region of *ors8*, delimited by the NdeI restriction site on one side and RsaI on the other, was necessary for autonomous replicating function.

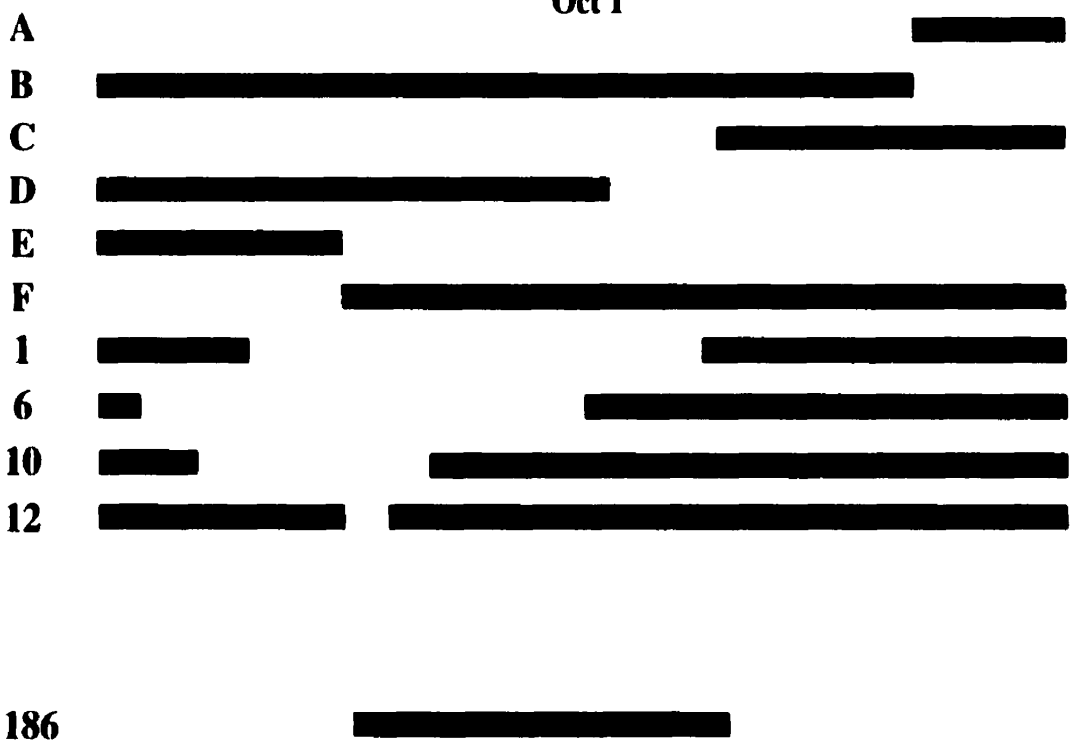
The NdeI-RsaI subfragment (186-bp) of *ors8*, heretofore called clone 186, was then subcloned into the NruI site of pBR322 and was subjected to the same analyses as above. The results (Figure 3.2 and Table 1) showed that clone 186 at 48 h post-transfection yielded DpnI-resistant DNA (Figure 3.2A, lane 7), which transformed *E. coli* with an efficiency that was approximately 5-fold higher than that of the intact *ors8* plasmid (Table 1).

Figure 3.1. Schematic diagram of *ors8*, (483 bp)

The sequence is divided into regions containing the GCS consensus, the inverted repeat and imperfect Oct-1 binding site, the AT-rich region and the yeast ARS consensus (see text for details). Shown below is the panel of deletion mutants of *ors8*. The autonomous replication of the deletion mutants, both *in vivo* and *in vitro*, is shown for *ors8* and each of the deletion mutants. Replication was assayed *in vivo* by DpnI resistance of transfected plasmids on Southern blots (S) or by transformation of *E. coli* (T; see Experimental Procedures).

Ors 8





	Autonomous Replication		
	In vivo		In vitro
	S	T	
A	-	-	-
B	+	+	-
C	-	-	-
D	-	-	-
E	-	-	-
F	+	+	-
1	-	-	-
6	-	-	-
10	-	-	-
12	+	+	-
186	+	++	++

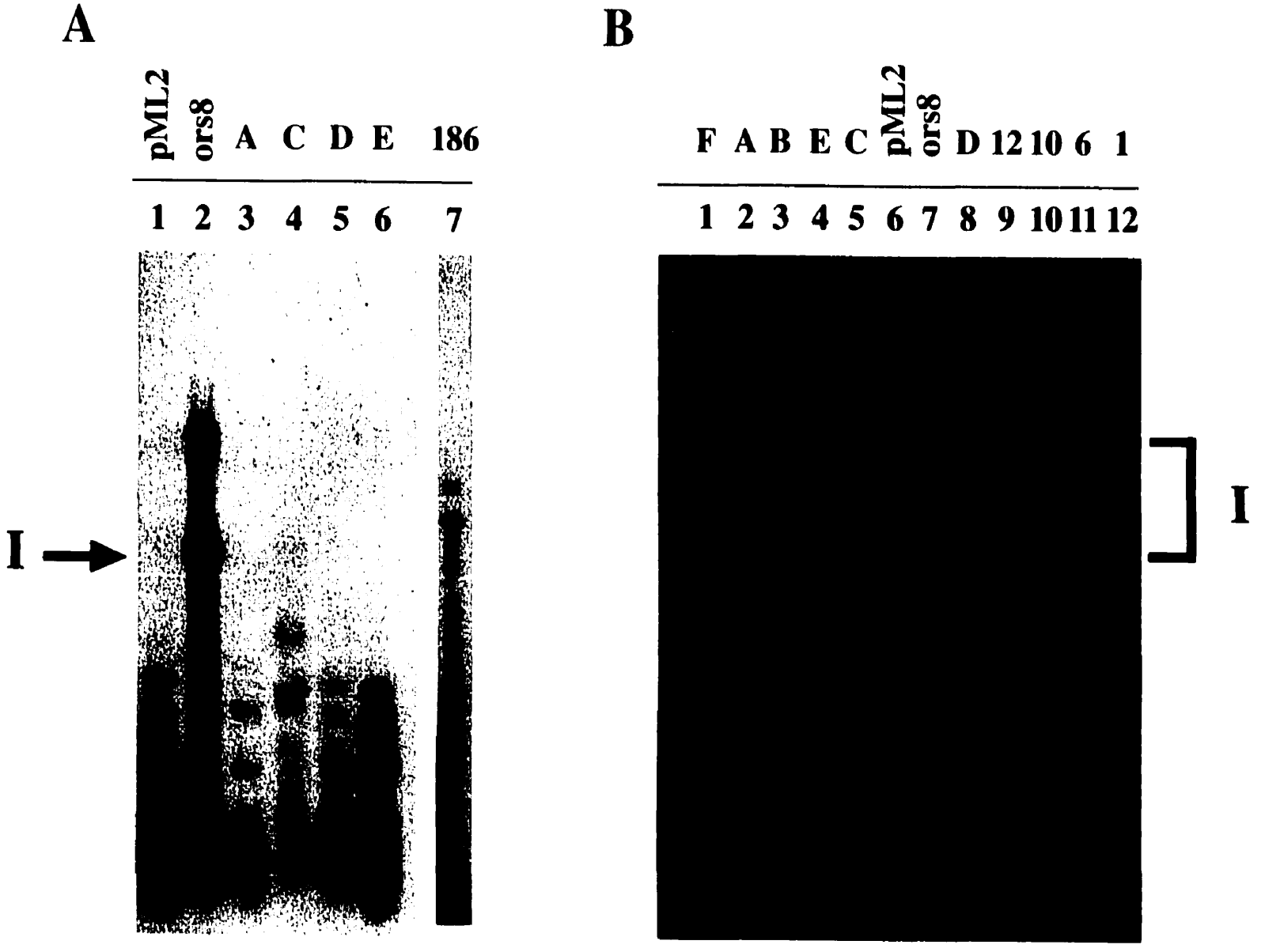
Table 3.1

Ampicillin resistant colonies arising from transformation of *E. Coli* HB101 with DpnI digested plasmids

Plasmid	Total no. of DpnI colonies per 3μg of transfected plasmid DNA	% DpnI-resistant colonies relative to *ors8* plasmid
ors8	16	100
A	0	0
B	11	69
C	0	0
D	0	0
E	0	0
F	25	156
1	0	0
6	0	0
10	0	0
12	11	69
186	91	568
pML2	0	0

Figure 3.2 Autonomous replication assay of the deletion mutants by DpnI resistance

Southern blot analysis (see Experimental Procedures). Form I is indicated by the arrow and bracket. The amount of DNA loaded to produce the blot in panel B is approximately one third of that in panel A.


A
pML2
ors8
A C D E 186
1 2 3 4 5 6 7
I
B
F A B E C pML2 ors8 D 12 10 6 1
1 2 3 4 5 6 7 8 9 10 11 12
I

***In vitro* Replication of the Deletion Mutants.**

Analysis of deletion mutant DNA replication *in vitro* (Figure 3.3) yielded results similar to those obtained *in vivo*, except that *in vitro* all the deletion mutants were reduced to approximately the same level (20-40%) of replication relative to the intact *ors8* plasmid, while clone 186 replicated with approximately 2-fold higher efficiency than the intact *ors8* plasmid. The results were the same, regardless of whether *in vitro* reactions were carried out using equimolar (Figure 3.3), or equal mass amounts of each template DNA (data not shown).

Nucleotide Sequence Features of Deletion Mutant 186.

The nucleotide sequence of the 186-bp fragment was re-examined (Figure 3.4) in light of its apparent content of sequences essential for the replication origin function. The presence of several repeated sequence motifs was noted, such as the ATTA and ATTTAT motifs (Boulikas, 1992; Boulikas, 1993a), which occurred three and five times, respectively, the 5'-ATT-3' sequence (Mastrangelo *et al.*, 1993) repeated twice, and the sequences TAGG and TAGA, which occurred three and seven times, respectively. The inverted repeat (IR) sequence present in *ors8* (Rao *et al.*, 1990), which may assume a cruciform configuration *in vivo* (Bell *et al.*, 1991), is included in the 186-bp fragment, as is an imperfect consensus element for Oct-1 (ATTTATGCAT; Iguchi-Ariga *et al.*, 1993, and references therein), in which the bases AT are inserted in the middle of the consensus.

Figure 3.3 ***In vitro*** **replication activity of the** ***ors8*** **deletion mutants**

All data have been plotted relative to the incorporation of the complete *ors8* plasmid. Each bar represents multiple experiments (see Experimental Procedures for assay).

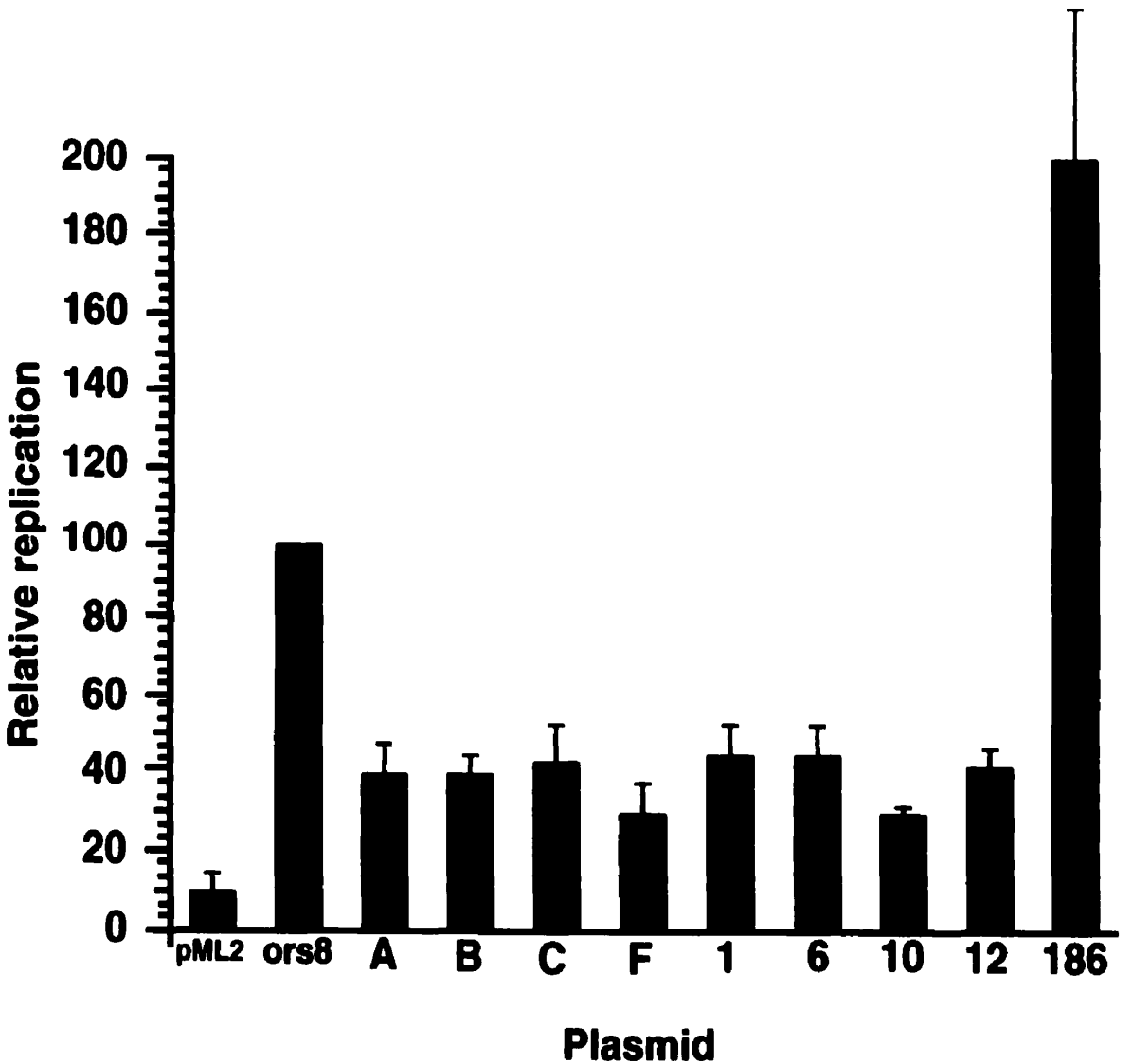
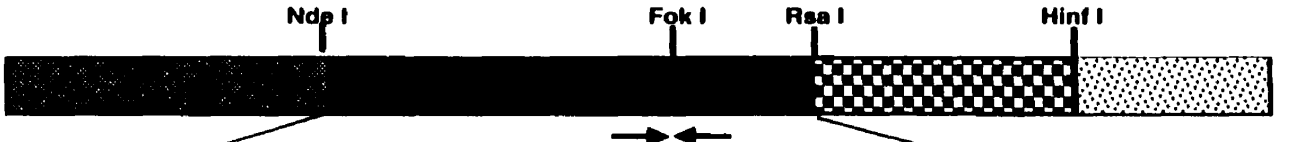

Relative replication
200
180
160
140
120
100
80
60
40
20
0
pML2
ors8
A
B
C
F
1
6
10
12
186
Plasmid

Figure 3.4 Sequence detail of the 186-bp deletion mutant of *ors8*

The inverted repeat (arrows), sequence motifs TAGG, and TAGA (solid underline), ATTA and ATTTAT (bold), the imperfect Oct 1 binding site (dashed box), the 5'-ATT-3' repeats (dotted underline) and the five $d(A)_3$ bend elements that are in phase with the helix periodicity (10 - 11 bp apart, boxed) are indicated.

ORS 8



186 bp

ATATGTAGGAAGAT**ATTA**AGATAGATGGTAATAGATATAGACACGAT

AGAGACTTTTGG**ATTA**GGCATGGTAG**ATTTAT**GCAT**ATTTAT**

GATAGAG**ATTTAT**AGGTAGAG**ATTTAT**CAGTAGGG**ATTTAT**GGAT

GCTCTTTCTTCATCTCCACTATAGCCAT**ATTA**TTACTTTCGTGTGCC

CTGG

Bent DNA

Ors8 DNA was digested with the restriction enzymes DdeI, MboII and RsaI, and the resulting fragments (Figure 3.5) were analyzed for anomalously migrating fragments either by a 2-dimensional (2-D) gel assay (Anderson, 1986) or by electrophoresis on parallel polyacrylamide gels at 9°C and 25°C. The relative mobilities of each fragment (Figure 3.5) were calculated in relation to the 123 bp ladder marker at 9°C and 25°C. In each digest, bands with a different migration rate at 9°C, by comparison to that at 25°C, were detected and the average percent difference in migration between the two temperatures was calculated (Figure 3.5). The average range of difference in relative fragment migration under cold electrophoresis conditions varies from 1.8% (Figure 3.5, C) to 4.6% (Figure 3.5, DdeI), depending on the restriction fragment tested. Thus, the 409-bp DdeI fragment of *ors8* migrates 4.6% slower at 9°C than at 25°C, while the RsaI fragment migrates 3% faster at 9°C than at 25°C. The occurrence of increased mobilities of fragments containing poly (dA)·(dT) tracts has been reported (Anderson, 1986, and references therein). Two of the deletion mutants, B and C, were similarly tested for the presence of bent DNA (Figure 3.5). The anomalous migration of several of these fragments (Figure 3.5[b]) was verified on 2-D agarose-polyacrylamide gels. In this assay, restriction fragments are separated on the basis of their size in the first dimension (agarose gel). The lanes are then excised from the gel, rotated 90° and run in 7% polyacrylamide gels at 9°C (Figure 3.6). DNA fragments of the 123-bp ladder form a smooth arc in the second dimension, whereas fragments with bent DNA migrate anomalously relative to molecular weight and therefore are located off the smooth arc formed by random DNA fragments. The 409 bp DdeI and 332 bp RsaI fragments of *ors8* exhibit anomalous migration when subjected to the 2-D gel assay (Figure 3.6). Comparative analysis of the results obtained

with the various fragments and deletion mutants (Figure 3.5) indicates that the 235 bp fragment, contained between the DdeI and RsaI sites, accounts for most of the apparent curvature (anomalous migration) observed in *ors8*. Macroscopically bent DNA arises from oligo (dA) tracts of 3-6 bp in length, repeated in phase with the helix periodicity of 10-11 bp (known as bend elements; Eckdahl and Anderson, 1990, and references therein). The 409 bp DdeI and 332 bp RsaI fragments of *ors8* contain five $d(A)_3$ bend elements, separated by 10 or 11 bp (nucleotide position 209-265, Figure 3.4). These fragments contain the 186-bp NdeI-RsaI fragment.

Discussion

We have previously demonstrated that *ors8* (483 bp), obtained by short nascent strand extrusion of monkey (CV-1) DNA from replication bubbles active at the onset of S phase (Kaufmann *et al.*, 1985; Zannis-Hadjopoulos *et al.*, 1985), can function as an origin of DNA replication in autonomously replicating plasmids (Frappier and Zannis-Hadjopoulos, 1987) and in a mammalian cell-free replication system (Pearson *et al.*, 1991). *Ors8* is present in less than 5 copies per haploid genome (Zannis-Hadjopoulos *et al.*, 1985), and is contained within a 1.2 kb PstI fragment of CV-1 genomic DNA (Mah *et al.*, 1993). Primary sequence analysis (Rao *et al.*, 1990) has shown that it contains extensive AT-rich regions that coincide with areas of detectable anomalous migration of the DNA indicative of altered structure, an IR potentially capable of extruding into a cruciform (Bell *et al.*, 1991), AP3 and SAR-B consensus, and a perfect (11/11 bp) yeast ACS (Palzkill and Newlon, 1988). Here, we have generated a panel of deletion mutants and tested them for origin function by the DpnI-resistance assay, *in vivo* (by transfection) and *in vitro*.

Figure 3.5 DNA curvature of *ors8*

The indicated restriction fragments and deletion mutants of *ors8* were run on parallel polyacrylamide gels at 9°C and 25°C (see Experimental Procedures). Fragments exhibiting anomalous migration are shown along with the percent differences in relative mobility between the two temperatures. Narrow bars at the ends of fragments are pBR322 sequences, resulting from PCR amplification using pBR322 primers.

[a]Data from several experiments. [b]Confirmed by two-dimensional gel data.

Relative mobility of ors8 and its subfragments on 1-D gel electrophoresis at 9°C and 25°C

		Size (bp)	Relative Mobility 25°C	Relative Mobility 9°C	Average % Difference
Ors 8		528	530	550	3.7[a]
Dde I		42	-	-	-
		77	-	-	-
		409[b]	420	440	4.6[a]
Rsa I		332[b]	335	325	3.0[a]
		196	198	198	0[a]
Mbo II		106	113	113	0.9[a]
		47	125	123	1.8[a]
		375	265	260	2.7[a]
B		449	430	440	2.3
C		218	230	226	1.8

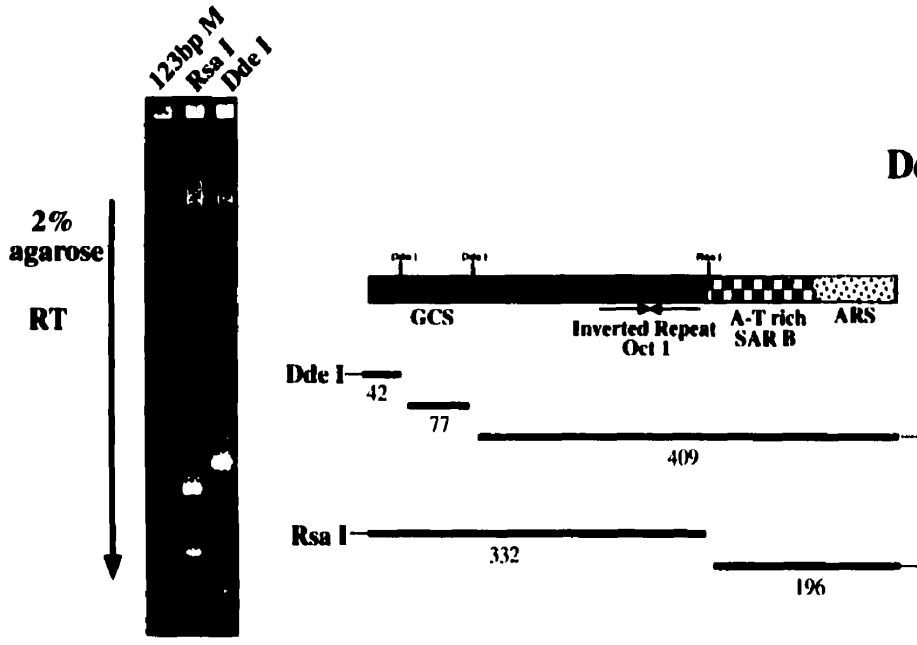
Figure 3.6 2-D gel assay for DNA curvature

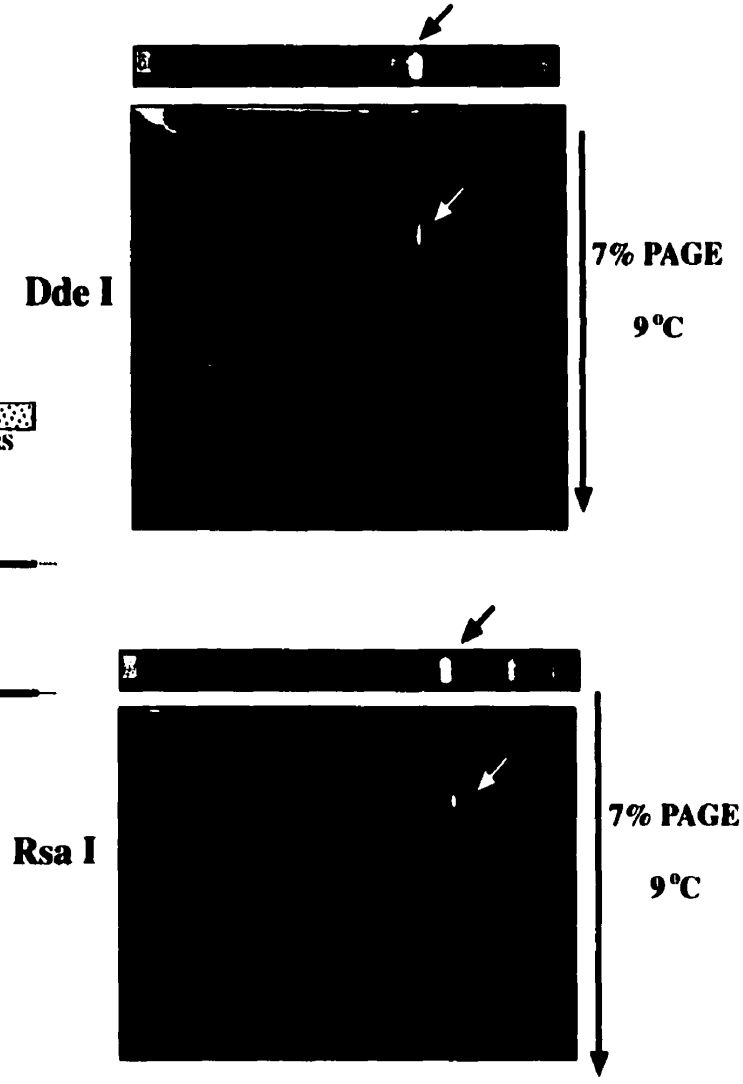
Anomalous migration of the 409 bp DdeI and 332 bp RsaI fragments of *ors8* on 2-D agarose polyacrylamide gels. The DdeI and RsaI digests of *ors8* PCR products (see Experimental Procedures) were separated together with the 123-bp ladder in the same lane (left panel). Electrophoresis in the first dimension was in 2% agarose. The lanes were then excised, rotated 90°, and subjected to 7% polyacrylamide electrophoresis at 9°C in the second dimension (right panel). The 2-D migration pattern was visualized by staining with ethidium bromide. The 409-bp DdeI and 332-bp RsaI fragments are indicated (arrows).

2-D Bent DNA Assay

First Dimension: Size separation

Second Dimension: Shape separation





Our analyses indicate that an internal region of 186-bp that comprises the 44-bp IR (Rao *et al.*, 1990), several direct repeats, such as the ATTA, ATTTAT, 5'-ATT-3', TAGG and TAGA sequence motifs, an imperfect Oct-1 consensus (ATTTATGCAT), and areas of apparent DNA curvature, is crucial for *in vivo* and *in vitro* replication function of *ors8*. This 186-bp subfragment, when subcloned into pBR322 and similarly tested, was found capable of autonomous replication *in vivo* as well as *in vitro* . The results suggest that a minimal *ori* lies within the 186-bp (NdeI-RsaI) fragment of *ors8*, and that the sequences lying outside this minimal *ori* seemingly contain elements that exert a negative effect on the replication of the intact plasmid. Interestingly, deletions B, F and 12, when tested by the DpnI-resistance assay *in vivo*, yielded products of equal intensity to the intact *ors8* plasmid. These same deletion mutants, when tested for replication *in vitro* were reduced to the same extent (20 - 40%) as the other deletion mutants relative to the intact *ors8* plasmid. This difference most likely reflects the different requirements for replication between the two systems as also previously observed (Nielsen *et al.*, 1994; Pearson *et al.*, 1991).

The ATTA and ATTTA motifs, which constitute the core elements recognized by the homeobox domain from species as divergent as flies and humans, frequently occur in the matrix attachment sites of several genes, as well as in several eukaryotic and viral origins of DNA replication (Boulikas, 1992), including the mammalian *ors17*, *ors24* and *ors25* (Landry and Zannis-Hadjopoulos, 1991), the replication origin of the human *c-myc* gene (Iguchi-Ariga *et al.*, 1993), and the replication origin of the Chinese hamster *DHFR* gene (Caddle *et al.*, 1990b). Recently, we showed that replicating genomic *ors8* is enriched on the nuclear matrix in early S phase (Mah *et al.*, 1993). Similar AT-rich repeat motifs have also been found in the minimal replication origin of the 200 kb *Halobacterium* plasmid pNRC100 (Ng and DasSarma,

1993) and other prokaryotes (Eckdahl and Anderson, 1990). It has been postulated that one possible function of such repeats could be in binding of replication proteins, with the formation of a melted replication complex being facilitated by the AT-rich regions (Eckdahl and Anderson, 1990; Kornberg and Baker, 1992; Ng and DasSarma, 1993).

The presence of transcriptional regulatory elements are a common feature of eukaryotic replication origins and are thought to be implicated in the temporal regulation of replication (reviewed in DePamphilis, 1993b). The 186-bp fragment contains an imperfect consensus binding site for the transcription factor Oct-1; the octamer transcriptional element has been recently suggested as a putative origin for cellular DNA replication (Iguchi-Ariga *et al.*, 1993). Not included in the 186-bp fragment, but immediately upstream of it, lies the β-globin upstream transcriptional control sequence, CACCC, which is also contained in the portion of the SV40 enhancer that binds AP3 (Rao *et al.*, 1990).

Among the sequence features present in the 186-bp fragment of *ors8* is a 44-bp imperfect IR, whose two branches are 20 bp apart from each other and whose estimated energy of formation is -7.4 kcal (Rao *et al.*, 1990). IRs, a common feature of prokaryotic and eukaryotic replication origins (Boulikas, 1993b; Campbell, 1986; Muller and Fitch, 1982), have been shown to be functionally important for the initiation of DNA replication in plasmids (Masukata and Tomizawa, 1984; Noirot *et al.*, 1990), prokaryotes (Brantl and Behnke, 1992; Hiasa *et al.*, 1990; Zyskind *et al.*, 1983) and eukaryotic viruses (Deb *et al.*, 1986a; Frisque, 1983; Lockshon and Galloway, 1986; Prives *et al.*, 1987; Reisman *et al.*, 1985; Stow and McMonagle, 1983; Weller *et al.*, 1985). We have previously reported that IRs are enriched in monkey (Rao *et al.*, 1990; Zannis-Hadjopoulos *et al.*, 1984; Zannis-Hadjopoulos *et al.*, 1985) and human (Nielsen *et al.*, 1994) *ors*. IRs have the potential to form cruciform structures under

conditions of torsional strain on the DNA (Panayotatos and Wells, 1981), and the occurrence of cruciforms *in vivo* has been demonstrated in DNA of prokaryotes (Dayn *et al.*, 1992; Panayotatos and Fontaine, 1987) and the eukaryotic virus SV40, at the viral origin of replication (Hsu, 1985). We have previously obtained evidence that the IR present in *ors8* is capable of extruding into a cruciform *in vivo* (Bell *et al.*, 1991) and *in vitro* (Price *et al.*, 1992; Zannis-Hadjopoulos *et al.*, 1992). The existence of cruciforms in mammals has been associated with the process of initiation of DNA replication (Hand, 1978; Zannis-Hadjopoulos *et al.*, 1988; Zannis-Hadjopoulos *et al.*, 1984).

Finally, it is interesting to note that the perfect (11/11 bp) yeast ACS (Palzkill and Newlon, 1988) present in *ors8* , which is essential for ARS function in yeast (Van Houten and Newlon, 1990), is not included in the 186-bp fragment of *ors8*. In the plasmid (B) that had the region of *ors8* containing the yeast ACS deleted, the replication efficiency, both *in vivo* and *in vitro* , was only diminished by approximately 30%.

To assess the importance of the various sequence elements that have been identified in the 186-bp fragment of *ors8*, we are currently investigating the protein-DNA interactions between this *ors8* subfragment and replication proteins that are purified from HeLa cells.

ACKNOWLEDGEMENTS

We would like to thank Dr. G.B. Price for his helpful comments about the manuscript. This research was supported by the Medical Research Council of Canada (MT-7965). A.T. is recipient studentships from Le Défi Corporatif Canderel and the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche; S.L. of a studentship from The Cancer Research Society, Inc.; and C.E.P. of a Graduate Faculty Award (Faculty of Medicine, McGill University.)

Connecting Text

In the preceding chapter, the 186-bp minimal orign of *ors8* was shown to contain an inverted repeat, which is capable of extrusion into a cruciform structure *in vivo* (Bell *et al.*, 1991). This data, together with the fact that cruciform structures are implicated in the regulation of initiation of DNA replication led to an investigation of a human cellular protein that binds specifically to cruciform DNA. In the next chapter, the previously described cruciform binding activity, CBP, is shown to belong to the 14-3-3 family of proteins.

CHAPTER FOUR

HUMAN CRUCIFORM BINDING PROTEIN BELONGS TO THE 14-3-3 FAMILY

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Abstract

Cruciform DNA has been implicated in the initiation of DNA replication. Recently, we identified and purified from human (HeLa) cells a protein, CBP, with binding specificity for cruciform DNA. We have reported previously that the CBP activity sediments at approximately 66 kDa in a glycerol gradient. Here, photochemical crosslinking studies and Southwestern analyses confirm that a 70 kDa polypeptide interacts specifically with cruciform DNA. Microsequence analysis of tryptic peptides of the 70 kDa CBP reveals 100% homology to the 14-3-3 family of proteins and shows that CBP contains the epsilon, beta, gamma and zeta isoforms of 14-3-3. In addition to polypeptides of the characteristic molecular weight of 14-3-3 proteins (30 and 33 kDa), CBP also contains a polypeptide of 35 kDa which is recognized by an antibody specific for the epsilon isoform of 14-3-3. Cruciform-specific binding activity is also detected in 14-3-3 proteins purified from sheep brain. Immunofluorescene studies confirm the presence of the epsilon, beta, and zeta isoforms of 14-3-3 proteins in the nuclei of HeLa cells. The 14-3-3 family of proteins has been implicated in cell cycle control, and has been shown to interact with various signaling proteins. Cruciform-binding is a new activity associated with the 14-3-3 family.

Introduction

Inverted repeat sequences (IRs) are a common feature of prokaryotic and eukaryotic regulatory regions, including promoters (Greenberg *et al.*, 1987; Horwitz, 1989; McMurray *et al.*, 1991; Shuster *et al.*, 1986; Spiro *et al.*, 1993), terminators (Rosenberg and Court, 1979), and origins of DNA replication in prokaryotes (Hiasa *et al.*, 1990; Zyskind *et al.*, 1983), viruses (Muller and Fitch, 1982), eukaryotes (Campbell, 1986; Tschumper and Carbon, 1982) and mammalian organisms (Boulikas, 1993b; Hand, 1978; Landry and Zannis-Hadjopoulos, 1991; Zannis-Hadjopoulos *et al.*, 1984; Zannis-Hadjopoulos *et al.*, 1985), as well as in amplified genes (Fried *et al.*, 1991). They have been shown to be functionally important for the initiation of DNA replication in plasmids, bacteria, eukaryotic viruses and mammalian cells (reviewed in Pearson *et al.*, 1996). IRs have the potential to form cruciform structures through intra-strand base pairing and under conditions of torsional strain on the DNA (reviewed in Pearson *et al.*, 1996 and Sinden, 1994). Cruciform formation *in vivo* (reviewed in Pearson *et al.*, 1996) has been demonstrated in prokaryotes (Dayn *et al.*, 1992; Noirot *et al.*, 1990; Panayotatos and Fontaine, 1987; Zheng *et al.*, 1991) and in mammalian cells (Ward *et al.*, 1990; Ward *et al.*, 1991; Zannis-Hadjopoulos *et al.*, 1988).

We and others have previously demonstrated the involvement of cruciforms in the initiation of DNA replication (Noirot *et al.*, 1990; Ward *et al.*, 1990; Ward *et al.*, 1991; Zannis-Hadjopoulos *et al.*, 1988; reviewed in Pearson *et al.*, 1996). In support of the hypothesis that a cellular cruciform-specific binding protein may be involved in the regulation of DNA replication, we recently identified and isolated from human cell (HeLa) nuclei a cruciform-specific binding activity, CBP (Pearson *et al.*, 1994a). This activity was enriched from HeLa cells and appears as a 66 kDa protein with binding specificity for cruciform-containing molecules (Pearson *et al.*, 1994a). Hydroxyl radical footprinting studies demonstrated that the CBP binds at the base of four-way junctions (Pearson *et al.*,

1995), interacting with them in a different manner from other proteins known to bind such junctions (Pearson *et al.*, 1994a; Pearson *et al.*, 1995).

In this study, we report the identity of CBP as 14-3-3 proteins. The 14-3-3 family of proteins is highly conserved through plants, invertebrates, and higher eukaryotes with several diverse functions, which include involvement in neurotransmitter biosynthesis, signal transduction pathways, and cell cycle control (Aitken, 1996; Aitken, 1995).

Experimental Procedures

Bandshift assays

pRGM21 X pRGM29 is a stable cruciform formed by heteroduplexing the 200 bp HindIII-SphI fragments of the plasmids pRGM21 and pRGM29 as described previously (Pearson *et al.*, 1994a; Figure 4.1a). C1 X C2 was made by annealing (Wall *et al.*, 1988) the synthetic oligonucleotides C1, 69 base pairs (bp), and C2, 68 bp. The 200 bp pRGM21 X pRGM29 cruciform has identical stem loop structure to the C1 X C2 cruciform, but longer flanking arms. These cruciforms were used to assay, purify, and compete CBP. Bandshift analyses of 14-3-3 proteins purified from sheep brain were carried out as described previously for CBP (Pearson *et al.*, 1994a) using 4μg of 14-3-3 proteins purified from sheep brain to homogeneity by a combination of anion-exchange and hydrophobic-interaction chromatography steps as described in Toker *et al.* (1990).

UV Cross-linking

Protein-DNA complexes were formed for photochemical cross-linking (in volumes of 20-50μl) essentially as for use in bandshift assays described elsewhere (Pearson *et al.*, 1994a) with the following modifications: 1) protein-DNA complexes were formed in 20mM Tris-HCl (pH 7.5), 1mM DTT, 3% glycerol, in the absence of 1mM EDTA; 2) the cruciform DNA was uniformly labeled with α^{32}P-dCTP; 3) protein-DNA complexes were photochemically cross-linked for 5 minutes on ice, with reaction volumes placed on an aluminum foil-covered glass plate 2 cm from the UV light source; 4) the UV light source used was a 15-W germicidal lamp (Fotodyne G15T8); 5) following cross-linking, protein-

DNA complexes were digested with 10U deoxyribonuclease I (DNase) and 15-20U micrococcal nuclease (both from Boehringer-Mannheim) in the presence of 10mM $CaCl_2$ for 60 minutes at 37°C. The resulting nuclease-digested protein-DNA complexes were then reduced using 5% β-mercaptoethanol, and separated on 10% SDS-PAGE. After electrophoresis, the gels were dried and exposed for autoradiography. The pRGM 21 X pRGM 29 cruciform was used as specific competitor , and the HindIII-SphI fragment of the pRGM 21 or pRGM 29 plasmid was used as non-specific competitor. Quantitation of ^{32}P-labeled bands was carried out using a Bio Image densitometer (MillGen/Biosearch).

CBP purification.

Briefly, an Affi-gel heparin column (BioRad) flow through (F_{TH}; Pearson *et al.*, 1994a) from log phase HeLa S3 extracts was concentrated using Centriprep concentrators (30,000 mw cutoff, Amicon) and lyophilization. This protein concentrate was used in several analytical and preparative scale bandñshift reactions using the pRGM21 X pRGM29 cruciform, and run on 4% polyacrylamide gels. After electrophoresis, both protein-cruciform DNA complexes in which the cruciforms are specifically bound (Pearson *et al.*, 1994a) were eluted from the gel as described previously (Pearson *et al.*, 1995). The combined eluates were precipitated using 4 volumes of ethanol, and the protein pellet was resuspended in distilled water to a concentration of 1-5 mg/ml.

Southwestern analysis

Southwestern assays were carried out using a modification of the procedure described by Philippe (1994). Approximately 5μg of CBP purified by elution of protein-cruciform DNA complexes was separated on a 10% SDS-PAGE gel in the presence of 5% β-mercaptoethanol. The gel was electroblotted to Immobilon-P (Millipore) PVDF membrane in 192mM glycine, 25mM Tris base, 0.01% SDS. Following transfer of protein to the membrane, all manipulations were carried out at 4°C with gentle rocking. The membrane was blocked overnight in binding buffer (25mM Hepes KOH, pH 7.7, 25mM NaCl, 5mM $MgCl_2$, 1mM DTT), 5% skim milk, and 0.05% NP40. Protein on the

membrane was denatured using two 10 minute incubations of 6M guanidium hydrochloride followed by renaturation with five 10 minute incubations of guanidine hydrochloride, each time halving the concentration (i.e. 3M, 1.5M, 0.75M, 0.375M, 0.187M). The final incubation was followed by two 10 minute incubations in binding buffer, a one hour incubation in binding buffer supplemented with 5% skim milk and 0.05% NP40, and a 30 minute incubation in binding buffer supplemented with 1% skim milk and 0.05% NP40. 500-800ng of ^{32}P-labeled pRGM21 X pRGM29 cruciform DNA was added at 2 x 10^5 cpm/ml in 5ml hybridization buffer (20mM Hepes pH 7.7, 75mM KCl, 0.1mM EDTA, 2.5mM $MgCl_2$, 1% skim milk, 0.05% NP40) containing 0.1mg/ml double stranded poly (dI-dC) (Pharmacia), and a 20 fold molar excess of cold linear HindIII-SphI fragment of pRGM21 DNA. Hybridization was carried out overnight followed by three 15 minute washes with hybridization buffer and exposure of the membrane to a Fuji phosphoimager plate between 2 layers of plastic wrap.

Microsequence Analysis

CBP purified by elution of protein-cruciform DNA complexes was run on a 7% SDS-PAGE gel under reducing conditions, and electroblotted to Problott^{TM} membrane (Perkin-Elmer). The membrane was stained with 0.2% Ponceau-S in 1% acetic acid. Two bands, of apparent molecular weight of 50 and 70 kDa (see Results), were excised from the membrane and sequence analysis was performed at the Harvard Microchemistry Facility by collisionally activated dissociation (CAD) mass spectroscopy on a Finnigan TSQ 700 triple quadrupole mass spectrometer. The peptide sequences obtained were subjected to homology searches using the BLAST program (Altschul *et al.*, 1990).

Western blotting

3μg each of CBP purified by elution of protein-cruciform DNA complexes and purified sheep brain 14-3-3 (Toker *et al.*, 1990) were separated per lane on a 10% SDS-PAGE gel in the presence of 5% β-mercaptoethanol. The gels were electroblotted to Immobilon-P (Millipore) PVDF membrane in 99mM glycine, 12mM Tris base, 10%

methanol. Membranes were blocked for 1 hour in Blotto (5% skim milk, TBS (10mM Tris-HCl, pH 8.0, 150 mM NaCl), 0.05% Tween 20), incubated with primary antibody in Blotto for 45 minutes, washed twice for 7 minutes in TBS-T (TBS, 0.05% Tween 20), incubated in a 1:2000 dilution in Blotto of horseradish peroxidase conjugated goat anti-rabbit secondary antibody (Bio Rad) for 30 minutes, followed by six 5 minute washes in TBS-T. Bands were visualized using enhanced chemiluminescence as prescribed by the ECL kit (Amersham). Specific antisera against isoforms of 14-3-3 proteins were raised in rabbits as described previously (Martin *et al.*, 1993). The antibodies used were: β_T 2042, specific for the 14-3-3 β isoform, was raised against the acetylated N-terminal sequence of the alternative start β isoform: Ac.TMDKSELV (diluted 1:2000); ECT 1116, specific for the C-terminus of the 14-3-3 ε isoform, was raised against the C-terminal 17 residues of ε: GEEQNKEALQDVEDENQ (diluted 1:2000); γ1006, specific for the 14-3-3 γ isoform, was raised against an acetylated N-terminal peptide of the γ isoform: Ac.VDREQLVQKAC (diluted 1:6000); and ζ 1002, reactive with the 14-3-3 ζ isoform with slight cross reaction with the 14-3-3 β isoform, was raised against an acetylated N-terminal peptide of the ζ isoform: Ac. MDKNELVQKAC (diluted 1:2000).

Immunolabelling

HeLa cells were grown in log phase in alpha-MEM supplemented with 10% FCS, 2mM glutamine, 2mM asparagine, and 50U/ml of penicillin/streptomycin on 22mm coverslips (whole cell preparations) or 90mm cell culture dishes (nuclear preparations). Cells grown for whole cell and isolated nuclei preparations were washed and fixed with paraformaldehyde as described previously (Krude, 1995). Cells grown for preparations of nuclei were further processed as described previously (Ward *et al.*, 1990). Coverslips with whole cells were washed in PBS, fixed and transferred to separate wells in a 6 well cell culture plate prior to immunostaining. Both nuclei and whole cell preparations were immunostained as described previously (Krude, 1995), and mounted with antifade (Oncor). The use of primary antibodies is described above for Western blotting, with the

following exceptions: ε_2 2025, (Martin *et al.*, 1993), specific for the N-terminus of the 14-3-3 ε isoform, raised in rabbits against an acetylated N-terminal peptide of the ε isoform: Ac.MDDREDLVYQAK (diluted 1:2000), and anti actin antibody, H-196, from Santa Cruz (diluted 1:100). The secondary antibody used was fluorescein-linked goat anti-rabbit antibody (Oncor), diluted 1:50.

Results

CBP had previously been partially purified from HeLa cell extracts using chromatographic techniques (Pearson *et al.*, 1994a). The CBP activity is assayed by performing band-shift reactions with stable cruciform DNA molecules and partially purified HeLa cell extracts (Figure 4.1b, 4.3a, and Pearson *et al.*, 1994a). Two stable cruciforms, pRGM21 X pRGM29 (Frappier *et al.*, 1987; Pearson *et al.*, 1994a) and C1 X C2 (see Experimental Procedures), which differ in the lengths of their flanking arms (Figure 4.1a), were used in the assays and purification of CBP. The band-shift patterns are the same for both cruciforms (Figure 4.1b).

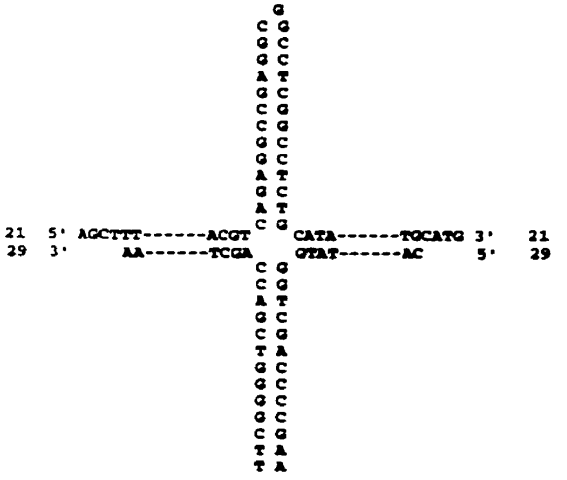
UV-crosslinking

Photochemical crosslinking studies were carried out on the CBP-cruciform DNA complex. Uniformly ^{32}P-labeled cruciform (C1 X C2) DNA was complexed with CBP and irradiated with UV light to form covalent protein-DNA adducts. The mixture was then digested with nuclease in order to remove flanking DNA that was not covalently attached to photochemically cross-linked proteins. The resulting ^{32}P-labeled proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), (Figs. 4.2a and 4.2b). Polypeptides of approximately 70 kDa and 30 kDa were crosslinked to the cruciform DNA (Figure 4.2a, lane 1). The ^{32}P -labeled products obtained were UV-and protein-dependent, as there are no nuclease resistant bands present in the absence of either UV irradiation or protein (Figure 4.2a, lanes 3 and 5, respectively), or both (Figure 4.2a lane 7).

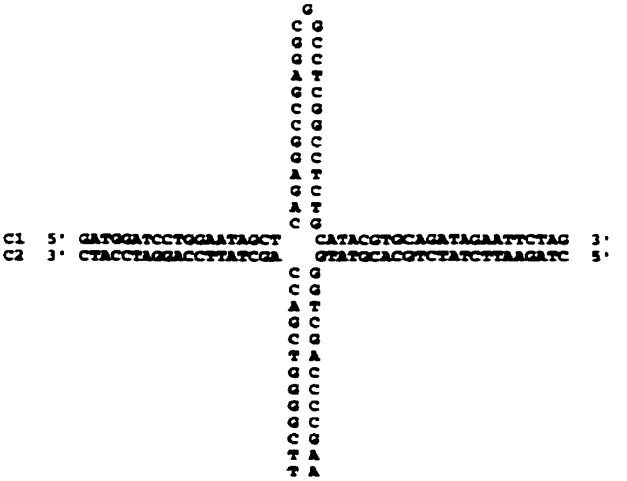
Figure 4.1 Stable cruciforms used to assay and purify the CBP activity

(a) Structure of the stable cruciform molecules pRGM21 X pRGM29 and C1 X C2. The 202 bp pRGM21 X pRGM29 cruciform has identical stem loop structure to the 68 bp C1 X C2 cruciform, but longer flanking arms. (b) Both pRGM21 X pRGM29 and C1 X C2 cruciforms share the same CBP band shift pattern. Left panel: migration of band-shift assay using the CBP activity recovered from a heparin column flow through (F_{TH}, see Experimental Procedures) and the pRGM21 X pRGM29 cruciform in a 4% polyacrylamide gel. Right panel: migration of band-shift assay using the same CBP as above with the C1 X C2 cruciform in a 6% polyacrylamide gel.

(a)



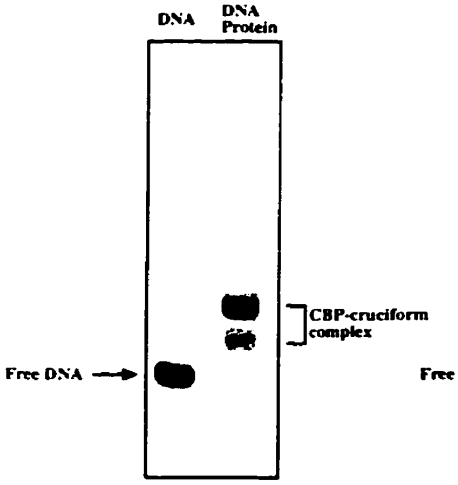
pRGM21 X pRGM29



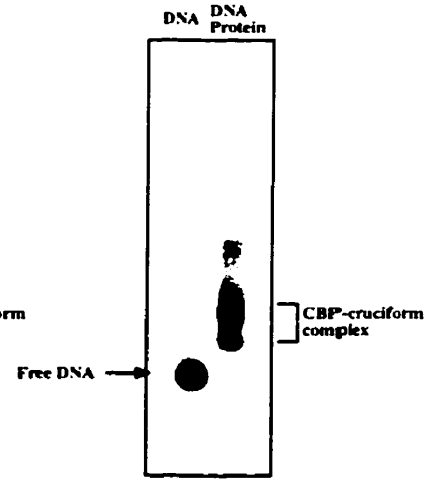
C1 X C2

(b)

pRGM21 X pRGM29 Cruciform



C1 X C2 Cruciform



When 100- and 200-fold molar excess of cold pRGM21 X pRGM29 cruciform DNA was included in the cross-linking reaction as a specific competitor, the intensity of the 70 kDa band was reduced by 20 and 48%, respectively (Figure 4.2b lanes 4 and 5 and Figure 4.2c). In contrast, 100-fold molar excess of cold pRGM21 or pRGM29 linear DNA of the same length and sequence did not compete for the ^{32}P-labeled 70 kDa band (Figure 4.2b, lane 8 and Figure 4.2c), while 200-fold molar excess of the same competitor DNA competed for only 13% of it (Figure 4.2b lane 9 and Figure 4.2c). A crosslinked polypeptide of approximately 30 kDa is also seen in this experiment (Figure 4.2a, lane 1 and Fig 4.2b), which appears to be competed by specific competitor. However, when the data of several experiments is averaged, less than 25% of the 30 kDa polypeptide can be competed away using 200 fold molar excess cruciform, as compared to an average 50% competition for the 70 kDa polypeptide. The 30 kDa polypeptide is also not detected by Southwestern analysis (Figure 4.3b). Therefore, we do not believe the 30 kDa polypeptide is interacting specifically with the cruciform DNA. These results indicate that the 70 kDa polypeptide is specifically crosslinked to the cruciform DNA and are consistent with the CBP activity sedimenting at approximately 66 kDa in glycerol gradient, as reported previously (Pearson *et al.*, 1994a).

CBP Purification and Sequencing

As a first step towards the identification of CBP, we further purified the activity by performing preparative scale band-shift assays using the pRGM21 X pRGM29 cruciform (Figure 4.1a) on polyacrylamide gels and elution from the gel of the two protein-cruciform DNA complexes (Figure 4.3a, bracket) in which the cruciforms are specifically bound (Pearson *et al.*, 1994a).

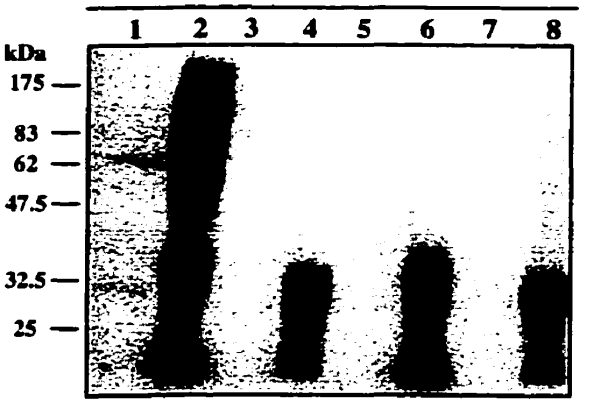
Aliquots of CBP purified by elution from protein-cruciform DNA complexes were subjected to electrophoresis on reducing SDS-PAGE, electroblotted onto membrane and probed with ^{32}P-labeled pRGM21 X pRGM29 cruciform in a Southwestern assay (Figure 4.3b) to identify the polypeptides involved in the binding of cruciform DNA.

Figure 4.2 A polypeptide of 70 kDa specifically crosslinks to cruciform DNA.

(a) and (b) Autoradiographs of UV-irradiated C1 X C2 cruciform-protein complexes (see Experimental Procedures) that have been separated on a 10% SDS-PAGE gel under reducing conditions. (b) The pRGM 21 X pRGM 29 cruciform was used as specific competitor, and the HindIII-SphI fragment of the pRGM 21 or pRGM 29 plasmid was used as non-specific competitor. The fold excess of each competitor is indicated. (c) The percent radioactivity of the 70 kDa polypeptide from the autoradiograph in panel (b) is expressed as a percentage relative to the crosslinking reaction without competitor (panel (b), lane 1).

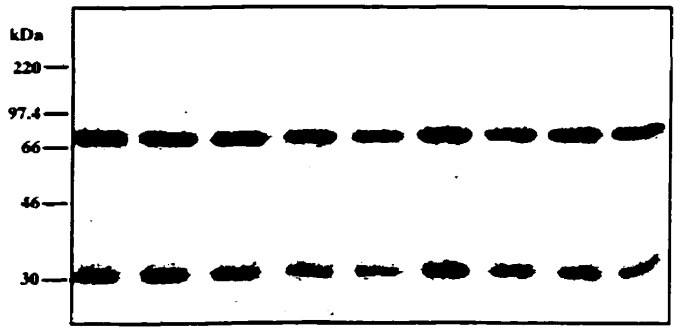
(a)

UV	+	+	-	-	+	+	-	-
^{32}P-labeled cruciform	+	+	+	+	+	+	+	+
Protein	+	+	+	+	-	-	-	-
DNase/MN digestion	+	-	+	-	+	-	+	-

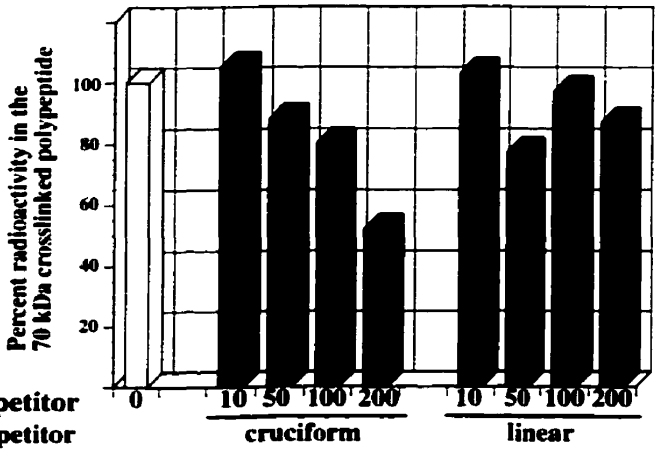


(b)

Cruciform DNA competitor	-	+	+	+	+	-	-	-	-
Linear DNA competitor	-	-	-	-	-	+	+	+	+
Fold molar excess competitor	0	10	50	100	200	10	50	100	200
	1	2	3	4	5	6	7	8	9



(c)



Among several protein bands present in CBP eluted from protein-cruciform DNA complexes (see Figure 4.4a), a polypeptide of approximately 70 kDa (CBP1) was identified in this manner as interacting directly with the labeled cruciform DNA (Fig 4.3b, arrow). A second band of approximately 50 kDa (CBP2) was also found to interact with cruciform DNA on some Southwestern assays (not shown). The CBP1 and CBP2 polypeptides were subjected to tryptic digestion and microsequence analysis by collisionally activated dissociation mass spectrometry (CADMS). The HPLC profiles of the tryptic digests of CBP1 and CBP2 suggested that the two are related, with the possibility of CBP2 being a degradation product of CBP1. The sequences obtained (Fig 4.3c) indicate that both the 50 (CBP2) and 70 (CBP1) kDa polypeptides share common sequence with the 14-3-3 family of proteins. The peptide sequences of neither CBP1 nor CBP2 share homology with any other proteins outside the 14-3-3 family of proteins. One of the tryptic peptides common to both CBP1 and CBP2 (CBP1-PT41 and CBP2-PT48, respectively, Figure 4.3c) is identical to the beta (β) and zeta (ζ) isoforms of 14-3-3. The β and ζ isoforms are identical in sequence to the alpha (α) and delta (δ) isoforms, respectively, which are post-translationally modified by phosphorylation at Ser^{185} (Aitken *et al.*, 1995a). Electrospray mass spectrometry ESMS) data show no indication of phosphorylation of CBP1-PT41 or CBP2-PT48 peptides, which include Ser^{185}, suggesting that these peptides originate from the unmodified β and /or ζ isoforms (data not shown). Phosphorylation at this site does not cause a shift in apparent molecular weight on SDS-PAGE (Aitken, 1995; Toker *et al.*, 1992). The presence of the epsilon (ε) isoform in the CBP is confirmed by the sequence of the CBP1-PT28 and CBP2-PT32 peptides, which are 100% identical to a unique region of the ε sequence. Finally, a third peptide obtained from CBP1 (CBP1-PT47) is identical to a region common to all known 14-3-3 isoforms.

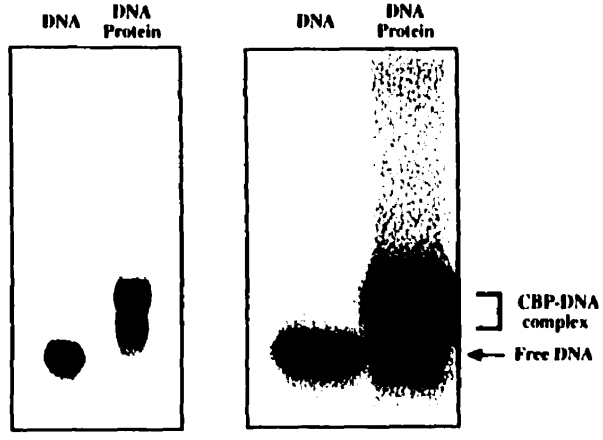
Figure 4.3 Identification of CBP

(a) Left panel: Migration of analytical scale band-shift assays using the CBP activity recovered from a heparin column flow through (F_{TH}, see Experimental Procedures) and the pRGM21 X pRGM29 cruciform in a 4% polyacrylamide gel. Right panel: Migration of preparative scale band-shift assays using the CBP activity recovered in the F_{TH} and the pRGM21 X pRGM29 cruciform in a 4% polyacrylamide gel. The position of the cruciform bound complex is bracketed. Band-shift assays were performed as described previously (Pearson *et al.*, 1994a) using the amounts of ^{32}P-labeled DNA and protein indicated. (b) Southwestern analysis of CBP eluted from cruciform DNA-protein complexes. The 70 kDa cruciform-DNA binding polypeptide is indicated by the arrow. (c) Sequence of tryptic peptides of the 70 kDa (CBP1) and 50 kDa (CBP2) cruciform DNA-binding polypeptides. The position of Ser^{185} is indicated (see text). All peptides show 100% identity with the indicated 14-3-3 protein isoforms.

(a)

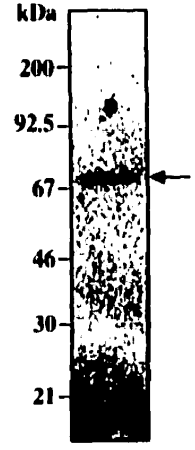
Band shift

Analytical **Preparative**



(b)

Southwestern



(c)

Tryptic peptides

Peptide (PT)	Amino Acid Sequence	14-3-3 Isoforms
CBP1-PT28 CBP2-PT32	Glu Ala Ala Glu Asn Ser Leu Val Ala Tyr Lys	ε only
CBP1-PT41 CBP2-PT48	Tyr Tyr Glu Ile Leu Asn Ser[89] Pro Glu Lys	β and ζ only
CBP1-PT47	Asn Leu Leu Ser Val Ala Tyr	all isoforms

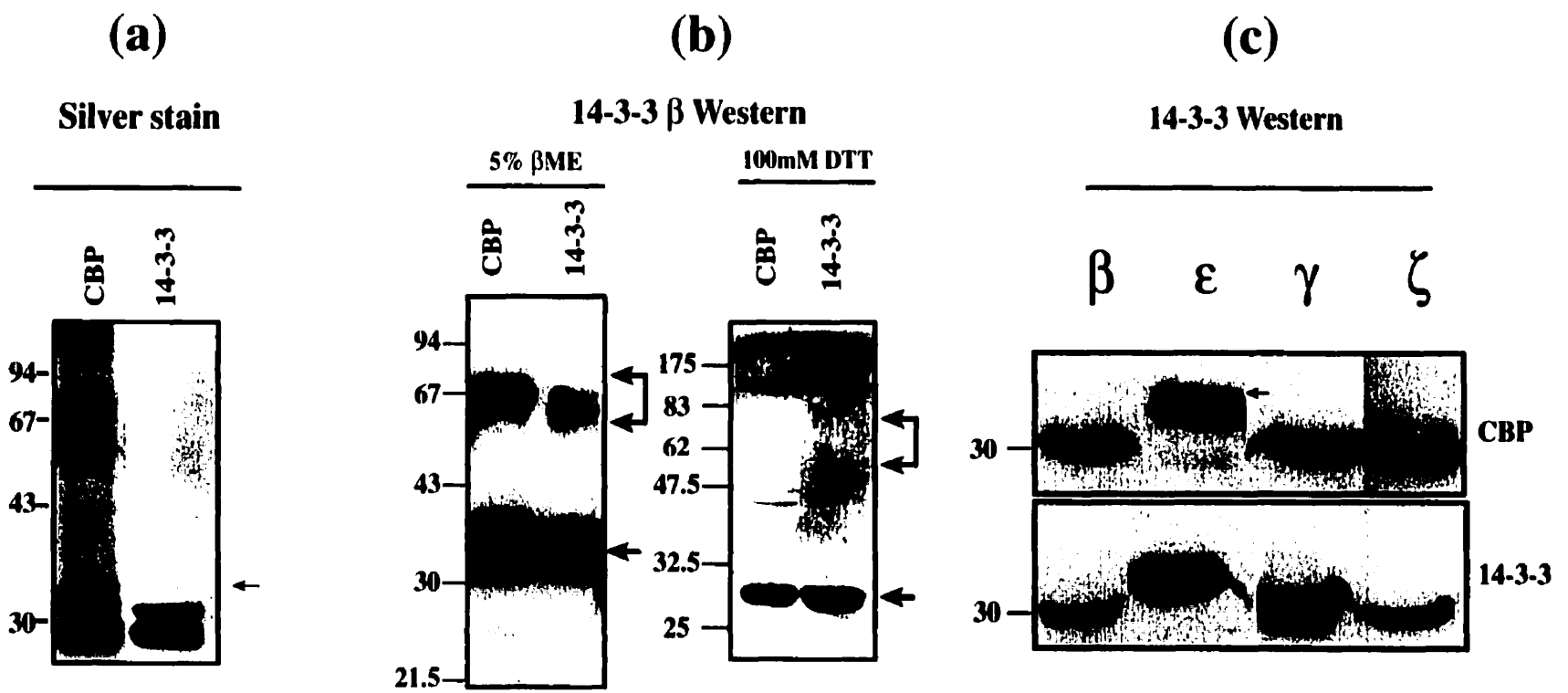
Comparison of sheep brain 14-3-3 to CBP

CBP was eluted from protein-cruciform DNA complexes, and compared by electrophoresis on 10% polyacrylamide gels (Figure 4.4a) to purified sheep brain 14-3-3 proteins, which is a mixture of all isoforms (Toker *et al.*, 1990). In addition to bands with apparent mobilities of 30 kDa and 33 kDa, which are characteristic of brain 14-3-3 monomers, CBP reveals a band with an apparent mobility of 35 kDa (Figure 4.4a, arrow) not seen in purified sheep brain 14-3-3 proteins. There is also a series of higher molecular weight bands visible in CBP corresponding to the dimer weight of 14-3-3 proteins (approximately 60-70 kDa, Figure 4.4a). These latter bands are recognized by 14-3-3 antibodies on an anti-14-3-3 β western blot (Figure 4.4b left panel). Bands of similar size are present in the 14-3-3 proteins purified from sheep brain (Figure 4.4b), but are not visible by silver stain (Figure 4.4a). These bands corresponding to the dimer molecular weight can be completely reduced when 100mM DTT is used in place of 5% β-mercaptoethanol (Fig 4.4b, right panel). The CBP was identified as 14-3-3 based on the sequence of proteins of approximate molecular weights of 50 and 70 kDa which interact with labeled cruciform DNA (see Figs. 4.3b and 4.3c). 14-3-3 proteins are known to form both homo and heterodimers *in vivo* (Jones *et al.*, 1995a) and *in vitro* (Jones *et al.*, 1995a; Liu *et al.*, 1995; Toker *et al.*, 1992; Xiao *et al.*, 1995). Chemical cross-linking of 14-3-3 proteins also produces bands which migrate in this region on SDS-PAGE gels (Toker *et al.*, 1992).

In Western analyses of SDS-PAGE gels run under reducing conditions, the CBP purified by elution from protein-cruciform DNA complexes is recognized by antibodies specific for the β, γ , and ε isoforms, as well as an antibody which recognizes the ζ isoform and cross-reacts with the β isoform (Figure 4.4c CBP, lanes β, ε, γ, and ζ). Furthermore, the 35 kDa band (Figure 4.4a, arrow) is recognized by an antibody that is specific for the C-terminus of the ε isoform (Figure 4.4c, CBP, lane ε, arrow).

Figure 4.4 The CBP contains a band of 35 kDa apparent molecular weight (arrow) not present in sheep brain 14-3-3.

(a) Separation of 3μg each of purified sheep brain 14-3-3 and CBP purified by elution from protein-cruciform DNA complexes (see text) on a 10% SDS-PAGE gel using 5% β-mercaptoethanol as reducing agent. The arrow indicates a 35 kDa band present in CBP but not clearly seen in sheep brain 14-3-3 proteins. (b) Anti-14-3-3 β Western blot of CBP purified by elution from protein-cruciform DNA complexes and 14-3-3 proteins purified from sheep brain. Left panel: The SDS-PAGE was carried out using 5% β-mercaptoethanol (5% βME) as reducing agent. Right panel: 100mM DTT was used as the reducing agent. The position of the 14-3-3 monomer species are indicated by arrows, and the dimer species are indicated by brackets. (c) Western blots of CBP purified by elution from protein-cruciform DNA complexes and purified sheep brain 14-3-3 proteins. The Greek characters β, ε, γ and ζ denote the antisera specific for each isoform of 14-3-3 proteins used on each lane of CBP and 14-3-3 proteins. The arrow indicates the presence of a 35 kDa band found in CBP but not in the sheep brain 14-3-3 proteins.


(a)
Silver stain
CBP
14-3-3
94
67
43
30
(b)
14-3-3 β Western
5% βME
100mM DTT
CBP
14-3-3
94
67
43
30
21.5
175
83
62
47.5
32.5
25
(c)
14-3-3 Western
β
ε
γ
ζ
30
CBP
30
14-3-3

A band with an apparent mobility of 35 kDa is not seen in the sheep brain 14-3-3 proteins (Figure 4.4c, 14-3-3, lane ε), nor in 14-3-3 from a large number of other tissues (Ichimura *et al.*, 1991). However, a faint band at 35 kDa can often be seen on SDS-PAGE of heavily loaded purified brain protein after HPLC purification (unpublished results). There is a specific variant of the epsilon isoform found in haematopoietic cells of a wide range of mammalian species, which contains a single amino acid change from the epsilon isoform found in sheep, rat, and human placenta and brain (Pietromonaco *et al.*, 1996).

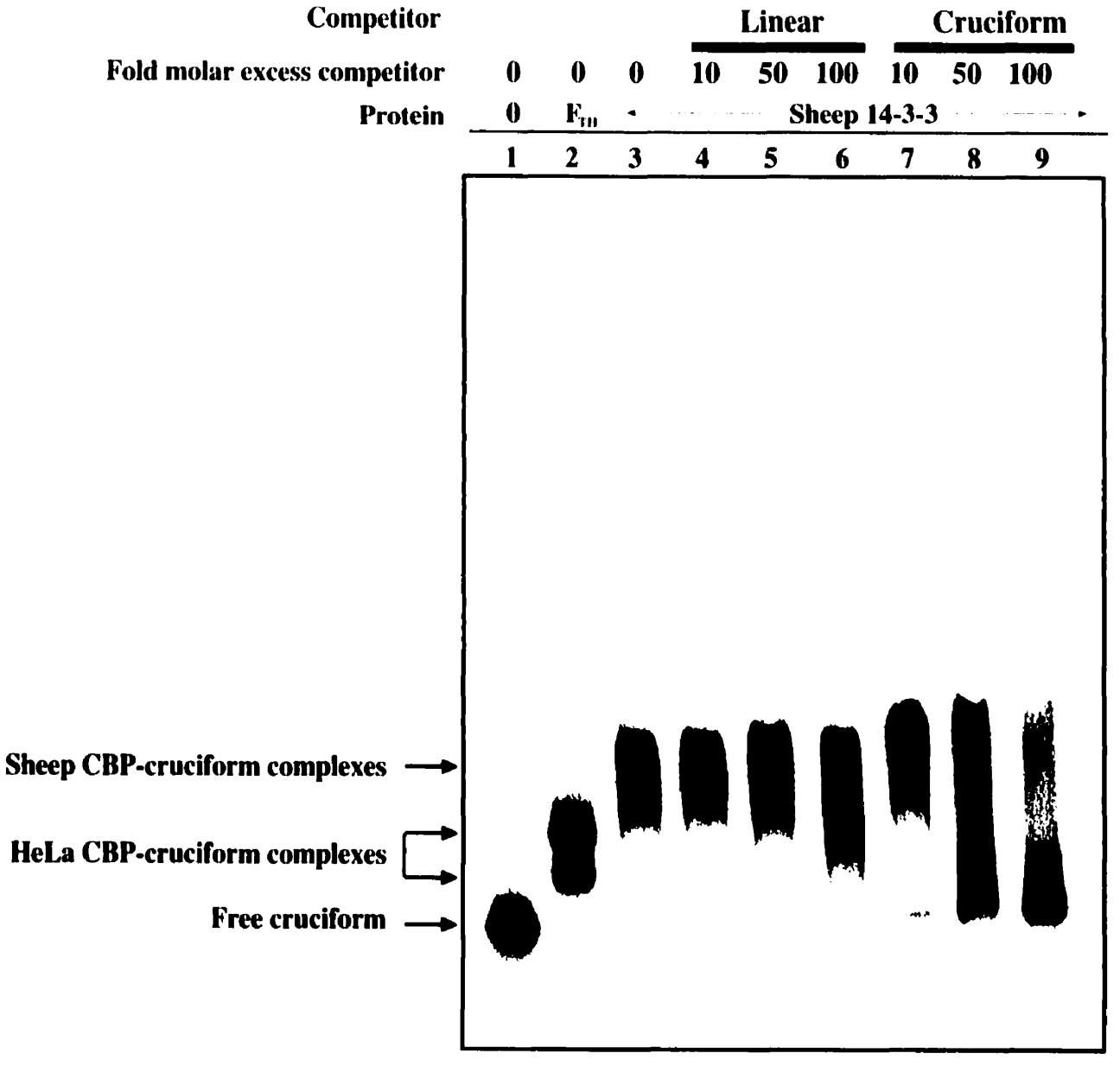
Band-shift analysis of purified sheep brain 14-3-3 proteins with the pRGM21 X pRGM29 stable cruciform reveals cruciform-specific binding activity (Figure 4.5). When 50-and-100 fold molar excess of cold specific (cruciform) competitor is added to the sheep brain 14-3-3 bandshift reaction, the cruciform-specific binding activity is competed by 66 % and 87 %, respectively (Figure 4.5 lanes 8 and 9). In contrast, 50-and-100 fold molar excess cold linear competitor has no effect on the sheep brain 14-3-3 cruciform binding activity (Figure 4.5 lanes 5 and 6). The migration of the sheep CBP-cruciform complexes is retarded relative to the HeLa CBP-cruciform complexes, possibly reflecting the species and tissue differences in the protein sources.

Detection of 14-3-3 isoforms in HeLa cell nuclei

The subcellular distribution of 14-3-3 was analyzed by immunofluorescence studies performed on whole HeLa cells (Figure 4.6a) and on isolated HeLa cell nuclei (Figure 4.6b). Rabbit polyclonal antibodies specific for the β, ε, and γ isoforms, as well as an antibody that recognizes the ζ isoform and cross reacts with β, show uniform labeling of whole HeLa cells fixed in paraformaldehyde (Figure 4.6a, β, ε, γ, and ζ respectively). Due to the possibility that the intensity of the signal generated by the cytosolic 14–3–3 proteins may be obscuring the nuclear signal, islolated HeLa nuclei were stained in a similar manner. Antibodies specific for the β, and ε isoforms, as well as an antibody that recognizes the ζ isoform and cross reacts with β show nuclear labeling in isolated HeLa nuclei (Figure 4.6b, β, ε and ζ respectively)

Figure 4.5 Sheep brain 14-3-3 proteins have CBP activity

Migration on a 4% polyacrylamide gel of 14-3-3 proteins subjected to competition band-shift analysis. Lane 1 shows the migration of labeled pRGM 21 X pRGM 29 cruciform in the absence of protein. CBP activity recovered from a heparin column flow through (F_{TH}, see Experimental Procedures) is shown in lane 2. Lanes 4-7 show the CBP activity present in 14-3-3 proteins purified from sheep brain. The HindIII-SphI fragment of the pRGM 21 or pRGM 29 plasmid was used as linear non-specific competitor (lanes 4-6) and the pRGM 21 X pRGM 29 cruciform was used as specific competitor (lanes 7-9). The migration positions of free cruciform, HeLa CBP-cruciform complexes and sheep CBP-cruciform complexes are indicated.


Competitor
Linear
Cruciform
Fold molar excess competitor
0 0 0 10 50 100 10 50 100
Protein
0 F_{III} Sheep 14-3-3
1 2 3 4 5 6 7 8 9
Sheep CBP-cruciform complexes
HeLa CBP-cruciform complexes
Free cruciform

Antibodies directed towards the γ isoform may also show faint nuclear labeling (Figure 4.6b, γ); the detection of the γ isoform in the cruciform-protein complex (Figure 4.4c) lends support to the likelihood of a nuclear presence. The control antibody directed towards the cytoplasmic protein actin shows no labeling in the isolated HeLa nuclei (Figure 4.6b, panel A).

Discussion

Cruciform structures, which can form transiently *in vivo* from IR sequences (Dayn *et al.*, 1992; Noirot *et al.*, 1990; Panayotatos and Fontaine, 1987; Ward *et al.*, 1990; Ward *et al.*, 1991; Zannis-Hadjopoulos *et al.*, 1988; Zheng *et al.*, 1991) have been shown to be involved in the initiation of DNA replication (Noirot *et al.*, 1990; Pearson *et al.*, 1996; Ward *et al.*, 1990; Ward *et al.*, 1991; Zannis-Hadjopoulos *et al.*, 1988).

We have previously isolated and partially purified from HeLa cell extracts a novel cruciform-specific binding protein (CBP) which sedimented on a glycerol gradient with an apparent molecular weight of 66 kDa (Pearson *et al.*, 1994a). Hydroxyl radical analysis of the protein-DNA interaction revealed binding of the protein at the base of the four-way junction, making contacts with the sugar phosphate backbone, and inducing structural alterations in the DNA (Pearson *et al.*, 1995).

In this study, we have estimated the molecular weight of the CBP to be approximately 70 kDa by photochemically cross-linking partially purified HeLa cell extracts to uniformly labeled cruciform DNA (Figure 4.2). The 70 kDa band is specifically crosslinked to cruciform DNA, as it can be competed with 200-fold molar excess of cold cruciform DNA, but not with an equivalent amount of cold linear DNA of the same sequence (Figure 4.2b and 4.2c). We have purified CBP based on 2 functional assays. The first assay is a bandshift assay where the cruciform-CBP complexes (Figure 4.3a, bracket) are eluted from the native bandshift gel. In the second assay, the protein eluted from the bandshift gel is again subjected to a cruciform binding assay in the form of a Southwestern

assay. The Southwestern identifies a polypeptide of 70 kDa that is interacting with ^{32}P-labeled cruciform DNA in the presence of excess non-specific competitor DNA (Figure 4.3b). A second polypeptide of approximately 50 kDa in size is also occasionally found to interact with cruciform DNA in the Southwestern assay, and is thought to be a degradation product of the 70 kDa polypeptide by virtue of its identical amino acid sequence in two different peptides (Figure 4.3c). Sequence analyses of the tryptic peptides of the two CBP polypeptides of approximately 70 and 50 kDa reveal 100% identity to isoforms of the 14-3-3 family of proteins (Figure 4.3c).

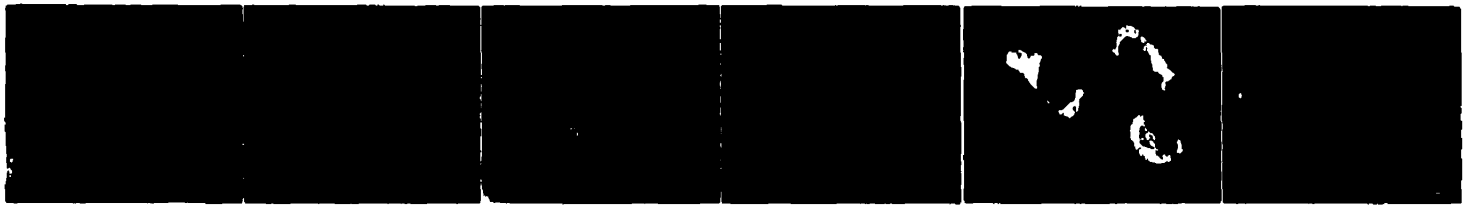
Electrophoretic analysis of the CBP isolated from the CBP-cruciform complex on reducing SDS-PAGE gels reveals three bands corresponding to the monomer size of 14-3-3 proteins (30, 33 and 35 kDa, respectively, see below) as well as a series of bands of 60-70 kDa, corresponding to the dimer molecular weight of the 14-3-3 proteins (Figure 4.4a). These dimer bands, although not visible by silver staining, are also present in a lesser amount in 14-3-3 proteins purified from sheep brain (Figure 4.4b). In addition to the monomer and dimer 14-3-3 bands, CBP contains a series of bands of approximately 50-60 kDa which are not recognized by 14-3-3 antibodies (Figure 4.4a and 4.4b). These bands are thought to be degradation products of the dimeric 14-3-3 (such as the 50 kDa CBP2 polypeptide) and therefore may not be recognized by the 14-3-3-β antibody used in Fig 4.4b, since it is directed against the acetylated N-terminal of the 14-3-3 protein.

Western analyses of the CBP isolated from two different CBP-cruciform complexes (using either the pRGM21 X pRGM29 or C1 X C2 cruciforms, Figure 4.1a) reveal the presence of the β, ε, γ, and ζ isoforms of 14-3-3 (Figure 4.4c). Our observation of at least 4 different isoforms of 14-3-3 interacting with cruciform DNA is not suprising given the high degree of conservation between isoforms of 14-3-3, both in primary structure (Aitken, 1996; Aitken, 1995) and at the level of three dimensional structure (Liu *et al.*, 1995; Xiao *et al.*, 1995)

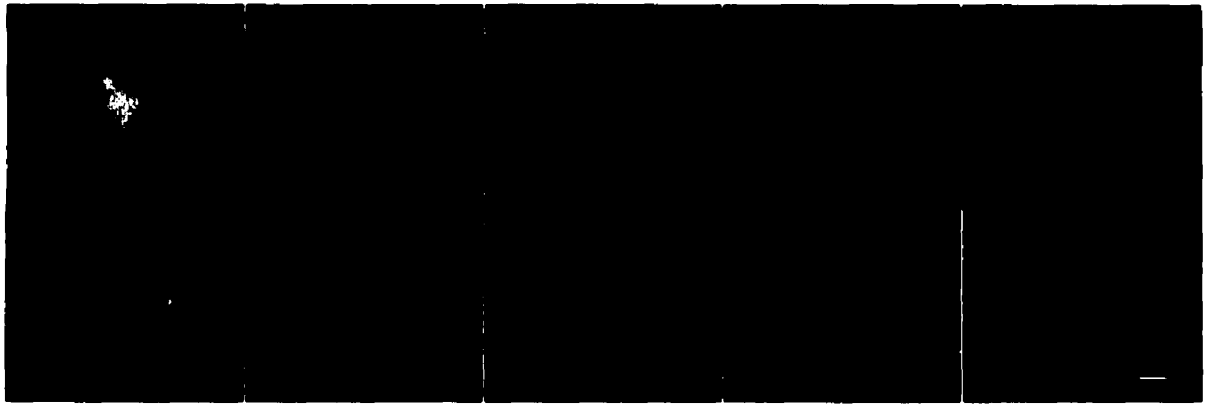
Figure 4.6 Nuclear localization of 14-3-3 proteins.

HeLa whole cell preparations (a) and isolated nuclei (b) were immunostained using polyclonal rabbit antisera specific for four 14-3-3 isoforms (indicated along the tops of each panel), and for the cytoplasmic protein actin (labeled as A). C in panel (a) denotes a negative control prepared without primary antibody. White bars in boxes C (panel a) and A (panel b) represent 1μm, respectively.

(a)



(b)



In addition, the phosphoserine binding affinity for phosphorylated Raf-1 peptides is the same for several isoforms (Muslin *et al.*, 1996). Other 14-3-3 interacting proteins, such as the apoptotic promoter BAD, lack isoform selectivity and interact with multiple isoforms of 14-3-3 (Zha *et al.*, 1996). The apoptoptic inhibitor A20 also interacts with several isoforms, although there is preference for the η isoform (Vincenz and Dixit, 1996). Furthermore, the ε and ζ isoforms show redundancy in their effects on Ras signaling in *Drosophila* (Chang and Rubin, 1997). This overlap in function between isoforms appears to transcend species, since four isoforms of 14-3-3 from the plant *Arabidopsis thaliana* are able to complement the lethal double disruption of the *Saccharomyces cerevisiae* 14-3-3 homologs ***bmh1*** and ***bmh2*** (van Heusden *et al.*, 1996).

The results presented here show that the CBP, although containing identical sequence, differs in some aspects from the purified sheep brain 14-3-3 proteins (Figs. 4.4a and 4.4c). One notable difference is the presence in CBP of a polypeptide of 35 kDa apparent molecular weight (Figure 4.4a, arrow), which is recognized by antibodies specific for the epsilon isoform of 14-3-3 (Figure 4.4c, arrow) and is not usually seen in sheep brain 14-3-3, although a faint band at 35 kDa is often observed on HPLC of heavily loaded purified brain protein (unpublished results).

Bandshift competition analysis of 14-3-3 proteins purified from sheep brain (Toker *et al.*, 1990) reveals cruciform-specific binding activity (Figure 4.5). The migration of the sheep brain 14-3-3-cruciform complex is retarded relative to the CBP-cruciform complex from HeLa cells, reflecting potential differences between CBP from HeLa cells and 14-3-3 proteins from sheep brain (Figure 4.4). The purification protocols for the two proteins also differ (Pearson *et al.*, 1994a; Toker *et al.*, 1990). The non-proliferative status of brain tissue might also contribute to the differences we have noted between the purified sheep brain 14-3-3 proteins and the CBP (Figs. 4.4 and 4.5).

Preliminary studies indicate that the CBP activity is regulated with the cell cycle and is maximal at the G1/S boundary but absent at G_0 (unpublished data). This pattern correlates with the profile of cruciform distribution in the cell cycle (Ward *et al.*, 1990).

The crystal structures of 14-3-3 proteins (Liu *et al.*, 1995; Xiao *et al.*, 1995) reveal highly helical proteins that dimerize. The dimer forms a large channel which is lined by amino acids that are very highly conserved throughout the family. Interestingly, the described structure of members of the 14-3-3 family (Liu *et al.*, 1995; Xiao *et al.*, 1995) is similar to the model previously proposed for the CBP-cruciform complex (Pearson *et al.*, 1995).

This is the first report of a mammalian 14-3-3 protein with DNA binding activity. 14-3-3 protein homologues in the plants *Arabidopsis thaliana* and maize participate in DNA-protein complexes (de Vetten *et al.*, 1992; Lu *et al.*, 1992). These plant 14-3-3 homologues are part of a complex that binds a G-box promoter element of inducible genes and are thought to regulate transcription. However, they do not bind directly to DNA. The sequence of the plant G-box promoter, 5'-CCACGTGG-3', is not present in either of the cruciform molecules used in the isolation of CBP, nor does CBP bind DNA in a sequence dependent manner (Pearson *et al.*, 1994a). The nuclear localization of the plant 14-3-3 homologs have been confirmed in *Arabidopsis thaliana* and *Zea mays* by scanning laser microscopy and immunocytochemistry (Bihn *et al.*, 1997).

Mammalian 14-3-3 proteins have previously been described as cytosolic with a small proportion of brain 14-3-3 proteins tightly associated with some membranes (Martin *et al.*, 1994). The ε and γ isoforms have also been shown to bind the cytoskeleton (Roth *et al.*, 1994), as well as centrosomes and spindle apparatus (Pietromonaco *et al.*, 1996). The association of various isoforms of 14-3-3 proteins with several different signaling molecules known to interact at or near the cell membrane (see Burbelo and Hall, 1995; Morrison, 1994 for reviews) also suggests a cytoplasmic location. Cell fractionation studies of rat brain have provided indirect evidence for the nuclear localization of 14-3-3

proteins (Martin *et al.*, 1994). Furthermore, the presence of 14-3-3 in mammalian nuclei is supported by a recent report which documents p53 protein interacting with 14-3-3 following exposure to ionizing radiation in nuclear extracts from several cell lines (Waterman *et al.*, 1998). The authors also show that the p53-14-3-3 interaction increases the affinity of p53 for sequence specific DNA binding. The CBP activity is enriched at least 4-fold in HeLa cell nuclei as determined by quantitative band-shift assays (not shown). Immunostaining analyses performed here demonstrate the presence of the β, ε, ζ, and possibly γ isoforms in HeLa cell nuclei, both in whole cells, and isolated HeLa nuclei (Figure 4.6). These immunolocalization studies now provide direct evidence for the presence of at least three isoforms of 14-3-3 proteins in HeLa cell nuclei. The nuclear localization of several 14-3-3 isoforms supports the hypothesis that the HeLa cruciform specific binding protein, CBP, recognizes cruciform structures in nuclei.

14-3-3 proteins have been ascribed a wide array of unrelated functions (Aitken, 1995; Aitken *et al.*, 1992; Burbelo and Hall, 1995; Morrison, 1994). These proteins associate with a number of oncogene and proto-oncogene products including middle T antigen of polyoma, Raf-1 and Bcr-Abl (Aitken, 1995; Burbelo and Hall, 1995; Morrison, 1994). Although 14-3-3 proteins have been convincingly shown to associate with signaling molecules, the functional role of these proteins in these signaling pathways remains unsolved. However, the recent description of two 14-3-3 consensus binding motifs, RSXpSXP and RXY/FXpSXP, where pS is phosphoserine, may explain why so many diverse proteins associate with 14-3-3 proteins (Muslin *et al.*, 1996; Yaffe *et al.*, 1997). Many of the 14-3-3 interacting proteins contain one or more of these consensus binding motifs, and 14-3-3 has been documented to interact with many proteins through one or more of these phosphoserine containing consensus sites. Some of these 14-3-3-interacting proteins are involved in processes such as apoptosis, through interactions with the BAD protein (Datta *et al.*, 1997; Zha *et al.*, 1996); cell cycle regulation, by interacting with the Cdc25 (Conklin *et al.*, 1995; Kumagai *et al.*, 1998a; Peng *et al.*, 1997) and A20 proteins

(Vincenz and Dixit, 1996); and signal transduction by interacting with the Raf-1 protein, among others (Freed *et al.*, 1994; Fu *et al.*, 1994). The nature of interaction with 14-3-3 is determined by the extent to which the binding motif(s) matches the consensus, and the nature of this interaction determines whether 14-3-3 will act as an adapter, chaperone or sequestering molecule (Yaffe *et al.*, 1997). In addition, a second site for binding to 14-3-3 exists and confers isoform specificity of interaction. We have shown, for example, that phosphorylation of 14-3-3 ζ Thr 233 affects interaction with Raf (Dubois *et al.*, 1997). These findings, along with the crystallographic data (Liu *et al.*, 1995; Xiao *et al.*, 1995) points strongly towards a role of scaffold or stabilzing protein for 14-3-3. A 14-3-3 protein that functions in such a manner would be ideally suited for recognizing cruciforms at origins of replication, to serve as a mediator in signaling pathways essential to DNA replication and cell cycle progression. 14-3-3 participates in cell cycle control at the level of the DNA damage checkpoint in the budding yeast *Schizosaccharomyces pombe*, (Ford *et al.*, 1994). The gene products of the *rad 24* and *rad 25* genes are 14-3-3 homologues, and participate in the radiation-damage control checkpoint which prevents cells from entering mitosis after radiation-induced DNA damage. Cells carrying mutations in the *rad 24* gene enter mitosis prematurely, suggesting a link between the DNA damage control checkpoint and the cell cycle machinery (Ford *et al.*, 1994). 14-3-3 proteins are also involved in cell cycle regulation at the G2 checkpoint through the interaction with human cdc25 phosphatases *in vivo* and *in vitro* (Conklin *et al.*, 1995). Recent evidence shows that 14-3-3 σ can inhibit G2/M progression in a p53 dependent manner (Hermeking *et al.*, 1997).

In summary, we have demonstrated here that the human cruciform binding protein (CBP) is a nuclear 14-3-3 protein, composed of permissible combinations of four of the known 14-3-3 isoforms, but exhibits different characteristics from purified sheep brain 14-3-3 proteins.

Acknowledgments

We would like to thank Ms. Claude Lamoureux for technical assistance.

Connecting Text

In the preceding chapter, the cruciform binding protein, CBP, has been identified as a member of the 14-3-3 family of proteins. In the following chapter, it is demonstrated that 14-3-3 binding to cruciform structures is necessary for the *in vitro* DNA replication of the 186-bp minimal origin of *ors8*, which provides further evidence supporting the involvement of DNA cruciforms in the process of DNA replication.

CHAPTER FIVE

14-3-3 PROTEINS ARE REQUIRED FOR *IN VITRO* DNA REPLICATION

To be submitted as a short communication (1999)

Abstract

Cruciforms can form *in vivo* at inverted repeats, a common feature of origins of DNA replication, and have been implicated in the initiation of DNA replication of mammalian cells. We have previously reported the identity of a cruciform-binding protein, CBP, purified from HeLa cells, to be 14-3-3 proteins. Here, we show that anti-14-3-3 antibodies interfere with complex formation between cruciform DNA and CBP/14-3-3 protein. The same antibodies are capable of reducing the *in vitro* replication activity in a cell-free replication system that uses HeLa cell extracts to replicate supercoiled plasmid templates, providing additional evidence for the participation of cruciform structures in DNA replication.

Introduction

Metazoan genomes are considerably more complex than those of simple organisms such as bacteria, yeast and viruses. Consequently, the simple modular structure of the origins of DNA replication found in these simple organisms, which often include consensus-binding sequences for initiator proteins, has yet to be observed in higher eukaryotic origins. Rather, the origins of higher eukaryotes have been found to share common sequence and structural elements, which include AT rich DNA, DUEs, MARs, regions of bent DNA, and the presence of IR sequences. IR sequences have been found in all of the early replicating sequences enriched for replication origins isolated from monkey (CV-1) DNA (Kaufmann *et al.*, 1985; Landry and Zannis-Hadjopoulos, 1991; Rao *et al.*, 1990; Zannis-Hadjopoulos *et al.*, 1984; Zannis-Hadjopoulos *et al.*, 1985) as well as other known origins of replication. IR sequences have the potential to form cruciforms under conditions of torsional strain. Cruciforms are known to form *in vivo*, and these structures have been implicated in the regulation of DNA replication in prokaryotes and eukaryotes (reviewed in Pearson *et al.*, 1996).

The generation of a monoclonal anti-cruciform antibody (Frappier *et al.*, 1989; Frappier *et al.*, 1987) has allowed the quantitation of the number of cruciforms throughout the cell cycle. Cruciform numbers show a bimodal distribution, with maximal numbers appearing at the onset of S phase, coinciding with the maximal rates of DNA synthesis. The same anti-cruciform antibodies have been used to affinity-purify active origins of DNA replication from genomic DNA (Nielsen *et al.*, 1994), providing further evidence for the involvement of cruciform structures in DNA replication.

Recently, we have identified a cruciform-specific binding activity from HeLa cell nuclei to be 14-3-3 proteins (Todd *et al.*, 1998). Here, we show the effect of anti-14-3-3 antibodies on the 14-3-3-cruciform binding complex, and on the *in vitro* DNA replication activity of a mammalian sequence containing the minimal replication origin of *ors8*, that comprises an inverted repeat.

Experimental Procedures

Bandshift assays

Bandshift assays were carried out as described previously (Todd *et al.*, 1998) using 0.25 ng of stable pRGM21 X pRGM29 cruciform molecules, and 5 μg of a CBP/14-3-3-enriched fraction from a heparin column flow-through. The proteins from the heparin column flow through were preincubated with 1-30 μg antisera on ice for 5 hours prior to the addition of cruciform DNA, followed by a further 20 minute incubation on ice, and separation on 4% polyacrylamide gels. The antibodies used were: 1) ζ 1002, reactive with the 14-3-3 ζ isoform with slight cross reaction with the 14-3-3 β isoform, was raised against an acetylated N-terminal peptide of the ζ isoform, Ac-MDKNELVQKAC; 2) γ 1006, specific for the 14-3-3 γ isoform, was raised against an acetylated N-terminal peptide of the γ isoform, Ac-VDREQLVQKAC; and 3) normal rabbit serum.

In vitro replication assays

In vitro replication assays were carried out as described previously (Pearson *et al.*, 1991) with the following modifications: 28 μg of HeLa extracts that were preincubated with approximately 20 μg anti 14-3-3 antisera, normal rabbit serum, or hypotonic buffer (20 mM Hepes (pH 7.8), 5 mM potassium acetate, 0.5 mM $MgCl_2$, 0.5mM DTT) for 20

minutes on ice were used to replicate 50 ng of clone 186 plasmid template DNA (Todd *et al.*, 1995). Reaction products were digested with 1.5 units of DpnI for 90 minutes and separated on 1% agarose gels. Bands were visualized and quantified using a Bio Image densitometer (MillGen/Biosearch).

Results

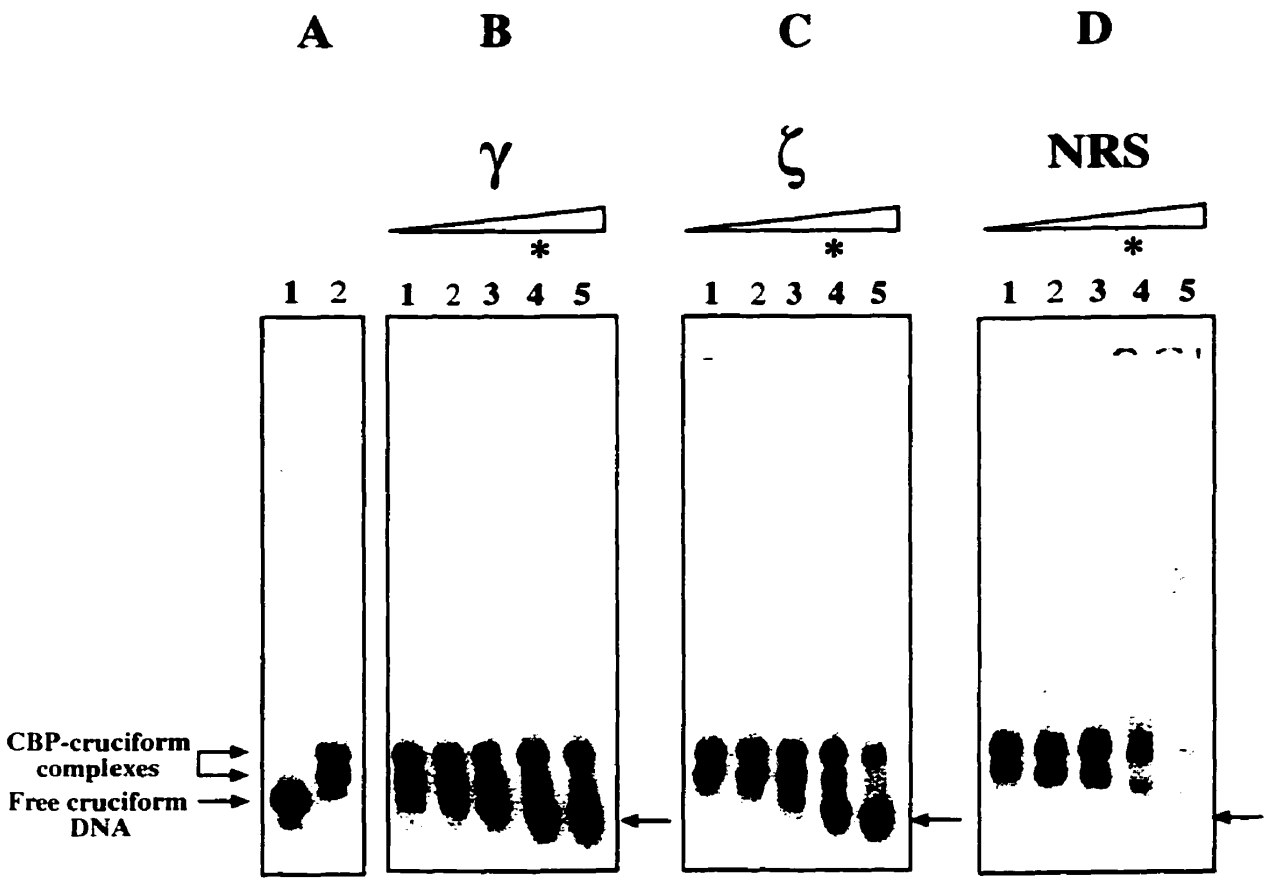
Anti-14-3-3 antibodies interfere with CBP/14-3-3-cruciform complex formation.

We have previously identified the 70 kDa CBP as 14-3-3 proteins by microsequence analysis of a 70kDa polypeptide which interacts specifically with cruciform DNA by Southwestern assay and found in CBP-cruciform complexes eluted from bandshift gels (Todd *et al.*, 1998). Western analysis of CBP eluted from the CBP-cruciform complexes revealed the presence of the $\beta, \zeta, \varepsilon$ and γ isoforms of 14-3-3. Furthermore, a mixture of 14-3-3 isoforms purified from sheep brain possesses cruciform-specific binding activity (Todd *et al.*, 1998).

Preincubation of a CBP/14-3-3-enriched protein fraction with anti-ζ and γ 14-3-3 antibodies prior to the addition cruciform DNA in a bandshift assay shows a reduction in the CBP/14-3-3-cruciform complexes (Figure 5.1). Addition of increasing amounts of anti-14-3-3 antibodies to the CBP/14-3-3-enriched fraction of the bandshift assay results in an increase in the amount of free cruciform molecules (Figure 5.1, arrow). In contrast, the addition of increasing amounts of normal rabbit serum results only in non-specific interactions with the cruciform DNA (Figure 5.1, NRS panel). The data suggest that the binding of the anti-14-3-3 antibodies to the 14-3-3 in the protein extract is interfering with complex formation between CBP/14-3-3 and cruciform DNA

Figure 5.1 Anti-14-3-3 antibodies interfere with 14-3-3 binding to cruciforms.

Panel A shows the migration in a 4% polyacrlamide gel of free cruciform and cruciform-14-3-3 complexes in the absence of preincubation with antisera (lanes 1 and 2 respectively; see Experimental Procedures). Panels B, C, and D. shows the migration of 14-3-3-cruciform complexes formed following preincubation of protein extracts with increasing amounts of 14-3-3 γ (panel B), 14-3-3 ζ (panel C) or normal rabbit serum (NRS, panel D). Panels B, C, and D: lane 1-5 indicate preincubation with 0.3, 0.5, 1, 3, and 5 μl of antisera, the arrows point to the position of free cruciform DNA.


A
B
C
D
γ
ζ
NRS
*
*
*
1 2
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
CBP-cruciform complexes
Free cruciform DNA

This result is consistent with the fact that we have been unable to supershift the CBP/14-3-3-cruciform complex using the same anti-14-3-3 antibodies (data not shown), suggesting that once CBP/14-3-3 has bound the cruciform DNA, the antibody epitope is either masked or altered.

Anti-14-3-3 antibodies inhibit *in vitro* DNA replication.

Preincubation of the HeLa extracts used in the *in vitro* DNA replication assay with 20 μg of anti-14-3-3 antisera reduces levels of replication to 20-25% of control reactions where HeLa extracts were preincubated with buffer. In contrast, HeLa extracts preincubated with the same amount of normal rabbit serum replicated the template DNA to levels similar to the control (Figure 5.2), indicating that the effect of the anti-14-3-3 antibodies is a result of their anti-14-3-3 binding activity.

Discussion

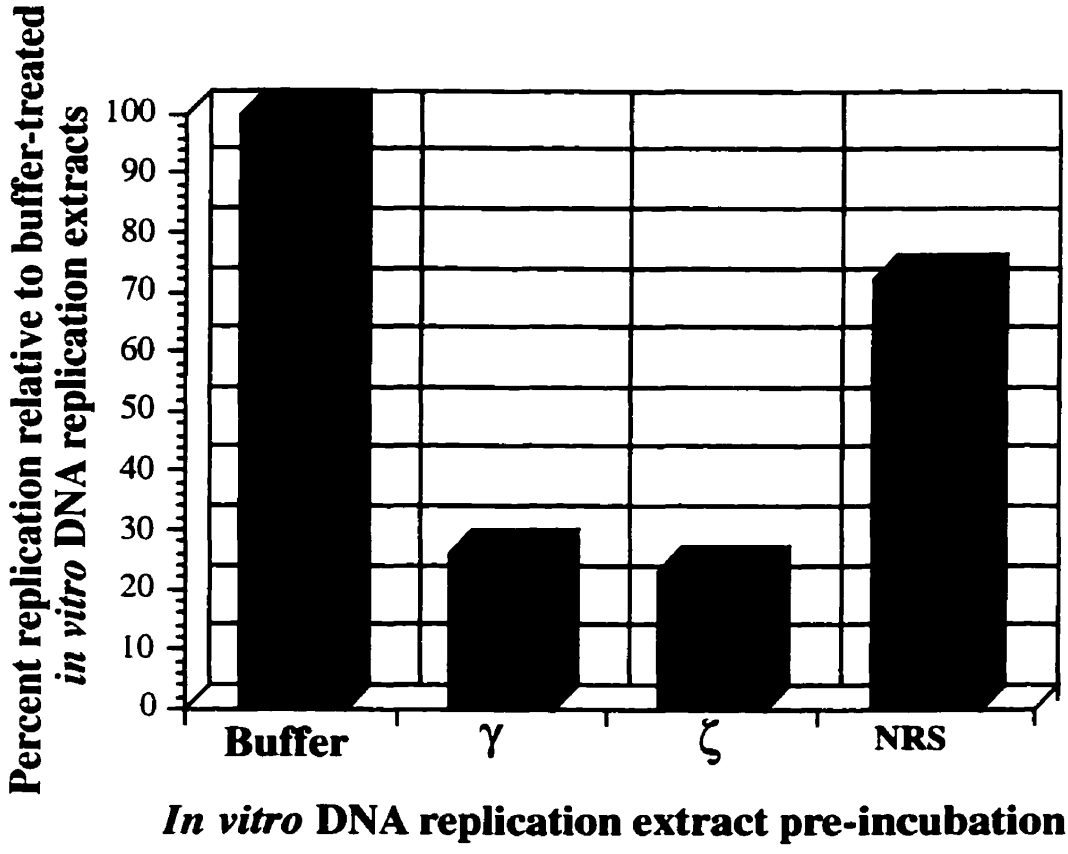
Cruciform structures, which can form transiently from IR sequences *in vivo*, play a role in the initiation of DNA replication (reviewed in Pearson *et al.*, 1996).

We have shown previously that a cruciform-specific binding activity from HeLa nuclei belong to the 14-3-3 family of proteins, consisting of at least the β, ε, γ and ζ isoforms of 14-3-3 (Todd *et al.*, 1998). We have confirmed the presence in HeLa nuclei of the β, ε, ζ, and possibly γ 14-3-3 isoforms, and demonstrated that a mixture of sheep brain 14-3-3 isoforms possess cruciform-specific binding activity.

Here, we have investigated the effect of anti-14-3-3 antibodies on the CBP/14-3-3-cruciform binding complex and on the *in vitro* replication of the minimal origin of *ors8*.

Figure 5.2 Anti-14-3-3 antibodies inhibit *in vitro* DNA replication

HeLa extracts preincubated with approximately 20μg anti-14-3-3 γ, anti-14-3-3-ζ or normal rabbit serum (NRS); the same amount used in lane 4 of Panels B, C, and D, Figure 5.1, were used to replicate the minimal 186-bp origin of *ors8 in vitro*. Rates of replication are indicated, relative to the buffer-treated extract, which is taken as 100%


Percent replication relative to buffer-treated *in vitro* DNA replication extracts
100
90
80
70
60
50
40
30
20
10
0
Buffer
γ
ζ
NRS
In vitro DNA replication extract pre-incubation

The anti-14-3-3 ζ and γ antibodies are each capable of interfering with the ability of CBP/14-3-3 to bind cruciforms, implying that the epitopes of the antibodies overlap with sites within 14-3-3 important for cruciform binding. Equivalent amounts of normal rabbit serum (NRS) does not result in the appearance of free cruciform DNA, rather it appears from the smear in the NRS panel of Figure 5.1 that components of the rabbit serum are interacting non-specifically with the cruciform DNA. This result is consistent with our inability to supershift the CBP/14-3-3-cruciform complex with anti-14-3-3 antibodies (data not shown). Alternatively, the reduction of the CBP/14-3-3-cruciform complexes might be due to the disruption of the 14-3-3 dimers caused by the anti-14-3-3 antibodies. The antibodies used were raised against the acetylated N-terminal 11 amino acids of each isoform, a region which is known to participate in the dimerization of the 14-3-3 proteins; residues 5-21 in one monomer forms contacts with residues 58-89 in the opposing monomer. Disrupting the 14-3-3 dimer could abrogate its ability to bind cruciforms. Our data support the evidence obtained previously that 14-3-3 binds to cruciforms as a dimer, namely that the CBP/14-3-3 activity sediments at approximately 66 kDa in a glycerol gradient (Pearson *et al.*, 1994a), and that the 70 kDa CBP/14-3-3 specifically interacts with cruciform DNA by photochemical crosslinking and Southwestern assays (Todd *et al.*, Chapter 4). The dimer has also been found to be absolutely necessary for the regulation of Raf activity (Tzivion *et al.*, 1998).

Use of the anti-14-3-3 γ and ζ antibodies, at a concentration that interferes with 14-3-3-cruciform complex formation, in preincubations of the HeLa extracts used in the *in vitro* replication assay reduces DNA replication of the supercoiled template DNA to

approximately 25% of the control reactions which were preincubated without antibodies. In contrast, preincubation of the HeLa extracts with the equivalent amount of normal rabbit serum produces little or no effect on the *in vitro* replication of the template DNA. The template used was the minimal 186-bp of *ors8* which are capable of supporting autonomous replication both *in vivo* and *in vitro* (Todd *et al.*, 1995). The 186-bp sequence contains an IR which has been shown previously to be capable of extruding into a cruciform structure *in vivo* (Bell *et al.*, 1991) and *in vitro* (Price *et al.*, 1992; Zannis-Hadjopoulos *et al.*, 1992). The reduction of replication activity through the action of anti-14-3-3 antibodies provides further evidence for the cruciform structure's involvement in DNA replication, and for the first time implicates 14-3-3 proteins in the process of DNA replication, by virtue of their binding to cruciform structures.

Previously, it has been shown that an anti-cruciform antibody is capable of modulating levels of DNA replication in permeabilized cells, through the stabilization of the cruciform structures formed transiently *in vivo* (Zannis-Hadjopoulos *et al.*, 1988). The mode of binding of this antibody to cruciforms is similar to that of CBP/14-3-3, as revealed by hydroxyl radical footprint analyses of each of these interactions (Pearson *et al.*, 1995; Steinmetzer *et al.*, 1995) and we have evidence that the anti-cruciform antibody is capable of competing with CBP/14-3-3 for cruciform binding (unpublished results), indicating that the antibody's effect on permeabilized cells could be attained through an amplification of the natural signal mediated by the CBP/14-3-3 protein at cruciforms.

In summary, we have shown that the CBP/14-3-3 protein's ability to bind to cruciform structures is of importance to the *in vitro* replication of a supercoiled plasmid template containing an IR-containing origin-enriched sequence.

CHAPTER SIX

GENERAL DISCUSSION

Mammalian origins of DNA replication do not appear to share the simple, modular structure of origins of simple organisms such as bacteria, viruses and yeast (Chapter 1). The aim of this thesis, therefore, has been to further define the sequence and structural elements of the more complex mammalian origins of DNA replication, and to identify the protein, CBP, which binds specifically to DNA cruciforms, a DNA secondary structure that has been implicated in the initiation of DNA replication.

To achieve the goal of closely examining origins of DNA replication, replication activity was measured *in vivo* by transient transfection of plasmids bearing DNA sequences of interest into mammalian cells, and measurement of the autonomous episomal replication of the plasmid by resistance to DpnI (Bell *et al.*, 1991; Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991; Nielsen *et al.*, 1994). *In vitro* replication activity was assessed using the same potential origin-bearing plasmids in a cell-free replication system that requires HeLa cell extracts (Pearson *et al.*, 1991; Pearson *et al.*, 1994b). These assays were first used to test plasmids bearing the previously characterized *oriβ* origin of bi-directional replication from the *DHFR* locus of Chinese hamster cells. We detected autonomous replication activity of plasmids bearing the *DHFR oriβ*, regardless of their size, in agreement with recently published data (Chapter 2; Kobayashi *et al.*, 1998 and references therein). Previous studies had shown that initiation occurs at two primary initiation sites in the *DHFR* locus: *oriβ* and *oriγ*, which are located 17 and 40 kb downstream from the *DHFR* gene, respectively (Kobayashi *et al.*, 1998 and references therein). Although the 55 kb non-coding region downstream of the *DHFR* gene

had been exhaustively tested for the presence of replication origins by many methods (Chapter 1), there had been only one attempt at testing plasmids bearing short (4.8kbp-11.5kbp) sequences from the *DHFR* locus in transient episomal replication assays (Burhans *et al.*, 1990). In this study, Burhans *et al.* reported the replication activity of plasmids bearing sequences encompassing *oriβ* to be at levels similar to a negative control plasmid. Recently, however, Kobayashi *et al.* (1998) published the position of a previously undetected initiation site within the *DHFR* locus, *oriβ'*. This site was included in the negative control plasmids used by Burhans *et al.*, thus indicating that Burhans *et al.* had indeed been observing replication of the plasmids bearing *oriβ*, but had not considered the result relevant because their negative control, which contained *oriβ'*, was also replicating. The position of *oriβ'* was detected by measuring the abundance of nascent strands using probes in a region that had not been tested previously (Kobayashi *et al.*, 1998), which raises the possibility that there are other, as yet undetected, initiation sites within the *DHFR* locus in regions that have not been closely examined by this method. We also observed replication activity in plasmids bearing a 4.8 kb sequence immediately 5' (pX14) to the sequence encompassing *oriβ* (pX24), in which initiation had been previously detected by 2D-gel electrophoresis (Dijkwel and Hamlin, 1992).

We have shown in this study, therefore, that these replication assays are suitable for the detection of replication activity of sequences that have been shown by others to function as mammalian origins in their chromosomal context.

To further study the sequence and structural features of mammalian origins, our laboratory has utilized a novel method to isolate DNA sequences that are enriched for

origins of DNA replication. Short sequences of monkey (CV-1) DNA that replicate early in S-phase, were extruded from replication bubbles, and cloned into plasmids under the assumption that at or near the centre of these sequences would lie an origin of bidirectional replication (Zannis-Hadjopoulos *et al.*, 1984; Zannis-Hadjopoulos *et al.*, 1985; Zannis-Hadjopoulos *et al.*, 1981). As a result of these experiments, a library of origin enriched sequences (*ors*) was generated. One of these early replicating sequences, the 483 bp *ors8*, has been shown to act as an episomal origin of DNA replication when transiently transfected into mammalian cells (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991) and when used as a template in the *in vitro* replication assay (Pearson *et al.*, 1991). In each of these assays, the site of initiation was localized within *ors8* by electron microscopy (Frappier and Zannis-Hadjopoulos, 1987; Pearson *et al.*, 1994b). Sequence analysis of *ors8* and the other *ors* sequences revealed the presence of sequence and structural elements common to known origins of replication which include AT rich DNA, MAR sequences, IRs, transcription factor binding sites, and matches for the yeast ARS consensus sequence (Rao *et al.*, 1990). Some of these elements have the potential of forming alternate DNA structures. For example, IRs have the potential to form cruciform structures, and AT rich sequences can give rise to bent DNA. Deletion analysis was carried out on *ors8* in order to determine which of these elements were included in the minimal sequence required to support autonomous replication activity. Testing various deletion mutants of the *ors8* sequence revealed an internal sequence of 186 bp that was capable of supporting autonomous replication when cloned into a plasmid and submitted to transient transfection *in vivo*, and to *in vitro* replication assays (Chapter 3). In addition, assays were carried out to assess the bent DNA character

of *ors8*, showing the majority of the bent character to be found within the 186-bp minimal origin. The 186-bp sequence contains an IR, which may extrude into a cruciform structure *in vivo* (Bell *et al.*, 1991). Also found within the 186-bp minimal origin of *ors8* is an imperfect binding site for the transcription factor, Oct-1. The Oct-1 protein binds to this site, and can modulate the replication of the plasmid bearing this sequence *in vitro* (Matheos *et al.*, 1998). The minimal 186-bp sequence of *ors8* can support autonomous replication with higher efficiency than the entire *ors8* sequence, implying that there are negative regulatory sequences that lie outside the 186-bp sequence. In addition, the 186-bp sequence of *ors8* has been used to purify from HeLa extracts an origin binding activity which has been identified as the Ku autoantigen (Ruiz *et al.*, 1995); Ruiz et al 1998, submitted). The sequence elements found within the 186-bp sequence of *ors8* are also present in other eukaryotic and prokaryotic origins of replication (Chapter 1).

Deletion analysis of the 812-bp *ors12*, another of the early replicating monkey sequences that was recently shown to serve as a chromosomal replication origin (Pelletier *et al.*, submitted), has revealed that a 215-bp internal fragment can serve as the minimal origin both *in vivo* by transient transfection assays of plasmids bearing this sequence, and by the *in vitro* replication assay (Pelletier *et al.*, 1997). The 215-bp minimal origin contains two IR sequences, as well as simple repeated sequence motifs.

Interestingly, *ors8* and *ors12* contain matches (11/11bp and 11/12, respectively) for the yeast ACS which lie outside the respective minimal origins required to support autonomous replication (Chapter 3; Pelletier *et al.*, 1997). This suggests that the ACS is not a required element in this system, in contrast with the yeast system, in which the ACS

is essential for origin activation. ORC binds to the ACS in yeast cells to serve as a 'landing pad' for other proteins required for initiation to occur. The fact that the ACS is not included in the minimal origin sequences of *ors8* and *ors12*, which are activated early in the S phase of monkey cells, suggests that mammalian cells use a different mechanism to that used in yeast for the initiation of DNA replication.

The presence within the minimal 186-bp sequence of *ors8* of an IR which can extrude into a cruciform structure *in vivo* (Bell *et al.*, 1991) lends support to the idea that cruciforms play a role in the initiation of DNA replication (Chapter 1). Indeed, previous studies have shown that an anti-cruciform antibody can modulate DNA replication in permeabilized cells (Zannis-Hadjopoulos *et al.*, 1988), and the same antibody is capable of affinity-purifying active origins of DNA replication from genomic DNA (Nielsen *et al.*, 1994). Based on the hypothesis that specific cellular proteins exist which recognize cruciform structures, a cruciform-specific binding activity, CBP, was identified in HeLa cell nuclei and characterized (Pearson *et al.*, 1994a; Chapter 1). CBP is devoid of nuclease activity, and was first reported to sediment at 66 kDa in a glycerol gradient. In photochemical crosslinking assays CBP has an apparent molecular weight of 70 kDa, which is in agreement with the size observed by Southwestern analysis (Chapter 4). Microsequence analysis of the 70 kDa CBP identified it as a member of the 14-3-3 family of proteins (Chapter 4). Subsequently, Western analysis confirmed that CBP consists of at least the ε, β, γ, and ζ isoforms of 14-3-3

The divergent roles, in a wide range of cellular processes, attributed to 14-3-3 proteins by virtue of their association with many different proteins is now understood to

be a single role: that of chaperone or adapter proteins (Chapter 4 and references therein). Their binding to a phosphoserine consensus binding site has been compared to the binding of SH3 domains to proline-rich regions, and of SH2 domains to phosphotyrosine (Yaffe *et al.*, 1997). Acting as adapters, 14-3-3 proteins are of prime importance, such as in the regulation of Raf kinase maturation. 14-3-3 binding to phosphorylated Raf stabilizes the kinase-competent form of Raf, which would otherwise be lost through the action of a phosphatase on the site protected by 14-3-3 binding (Thorson *et al.* 1998). 14-3-3 proteins are indeed of prime importance, since, in *S. Pombe*, the two 14-3-3 homologues, which play a role in the DNA damage control checkpoint, have been found to be essential (Ford *et al.*, 1994). Recently, the mechanism of 14-3-3 action in this pathway has been described in *S. Pombe* and *Xenopus*. In the presence of unreplicated or damaged DNA, Cdc25 becomes phosphorylated by the Chk1 or Cds1 kinases. This phosphorylation promotes the association between 14-3-3 and Cdc25 through the phospho-serine consensus binding motif within Cdc25. The 14-3-3-Cdc25 association abrogates the ability of Cdc25 to activate Cdc2, resulting in G2 arrest (Kumagai *et al.*, 1998b; Zeng *et al.*, 1998). This pathway has also been found to be mediated by the increase in p53 levels which follow DNA damage by ionizing radiation. p53 was found to transcriptionally activate 14-3-3σ expression by binding to the 14-3-3σ promoter, leading to increased association between Cdc25 and 14-3-3 and subsequent G2 arrest as described above (Hermeking *et al.*, 1997). The p53 protein has also been found to interact directly with 14-3-3 proteins following ionizing radiation (Waterman *et al.*, 1998). The authors of this study report that 14-3-3 association with p53 after ionizing radiation enhances the sequence-specific DNA binding activity of p53 *in vitro*, suggesting that p53 activation

may be regulated by a positive feedback loop involving 14-3-3 proteins, in light of the fact that p53 also induces the expression of 14-3-3σ following ionizing radiation (Hermeking *et al.*, 1997)

We have observed that antibodies directed against 14-3-3 isoforms can inhibit the *in vitro* replication of a plasmid, clone 186, bearing the minimal origin of *ors8*, which contains an IR capable of cruciform extrusion *in vivo*. This effect is presumably the result of interference by the anti-14-3-3 antibody with the ability of 14-3-3 to interact with cruciforms, and is consistent with the observation that a monoclonal anti-cruciform antibody can increase levels of DNA replication in permeabilized cells (Chapter 5; Zannis-Hadjopoulos *et al.*, 1988). The proposed mechanism for the anti-cruciform antibody induced increase in DNA replication was a stabilization of the extruded cruciform structures within the nucleus, thus allowing multiple rounds of replication to occur at sites where only one initiation event would normally occur. This hypothesis was supported by an observed increase in the copy number of several single copy loci (Zannis-Hadjopoulos *et al.*, 1988). Given the ability of 14-3-3 proteins to act as adapter or stabilizing molecules, it would be attractive to hypothesize that in binding to cruciform structures that act as signals for the initiation process to occur, 14-3-3 proteins could recruit other necessary proteins to the site of initiation. 14-3-3 proteins may also be serving to stabilize the cruciform structure in a fashion similar to that proposed for the anti-14-3-3 antibody, which is supported by their similar modes of cruciform binding revealed by hydroxyl radical footprinting studies (Steinmetzer *et al.*, 1995; Pearson *et al.*, 1995).

Interestingly, results presented recently (unpublished data presented by Alan Wolffe at the Fifth McGill University International Conference on the Regulation of Eukaryotic DNA Replication, St Sauveur, Quebec, October 1998) showed that 14-3-3 proteins co-purify with a histone acetyl transferase activity. This result is not surprising, since DNA crossovers, in which helices cross without exchanging strands, occur at the site where DNA enters and exits the nucleosome. Proteins, such as histones H1 and H5, which can bind DNA crossovers have also been documented to bind DNA cruciforms (Varga-Weisz et al 1994). This result raises the possibility that 14-3-3 may also exert a regulatory role at the level of chromatin structure, since histone acetylation may serve to maintain the chromatin in a structure that is accessible to DNA binding proteins, as would be required for DNA replication (Wolffe, 1996).

Several questions remain to be addressed, including determining whether 14-3-3 proteins interact with proteins important for DNA replication, and determining whether the interaction of 14-3-3 proteins with cruciforms is regulated by other proteins. A better understanding of the sites of contact between the cruciform and 14-3-3 are also needed to better understand the interaction and the possibility of interactions with other proteins. 14-3-3 has been crystallized while bound to a phosphorylated peptide corresponding to the consensus binding motif (Yaffe *et al.*, 1997). The phosphorylated peptide binds within the conserved amphipathic groove of 14-3-3, and mutations within the groove can disrupt binding (Zhang *et al.*, 1997; Wang *et al.*, 1998). Furthermore, the binding site of unphosphorylated peptides has been found to lie within the conserved groove at a site that

overlaps, but is distinct from, that where the phosphorylated peptide binds (Petosa *et al.*, 1998).

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Contributions to original knowledge

The following results, presented in this thesis, are original:

1. The demonstration of the autonomous replication of plasmids bearing sequences encompassing the *oriβ* mammalian origin of DNA replication from the DHFR locus.

2. The demonstration that the minimal origin of the monkey origin enriched sequence, *ors8*, consists of an internal 186-bp fragment containing an inverted repeat, bent DNA and an imperfect Oct-1 binding site.

3. The identification of a human cruciform binding protein, CBP, as a member of the 14-3-3 family of proteins, consisting of at least the β, ε, γ and ζ isoforms.

4. The demonstration that 14-3-3 proteins purified from sheep brain possess cruciform-specific binding activity.

5. The demonstration that the β, ε, γ and ζ isoforms of 14-3-3 are present in the nuclei of HeLa cells.

6. The demonstration that antibodies directed against 14-3-3 isoforms can interfere with 14-3-3-cruciform binding, and inhibit the *in vitro* replication of the 186-bp minimal origin of *ors8*.