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DNA REPLICATION AND METHYLATION

By

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in partial fulfillment of the requirements of the degree of Doctor of Philosophy

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To my parents, Valéria and José
To Debora, Mariana and Aparecida

Abstract

One of the main questions of modern biology is how our cells interpret our genetic and epigenetic information. DNA methylation is a covalent modification of the genome that is essential for mammalian development and plays an important role in the control of gene expression, genomic imprinting and X-chromosome inactivation (Bird and Wolffe, 1999; Szyf et al., 2000). Furthermore, changes in DNA methylation and DNA methyltransferase 1 (DNMT1) activity have been widely documented in a number of human cancers (Szyf, 1998a; Szyf et al., 2000).

In *Escherichia coli*, timing and frequency of initiation of DNA replication are controlled by the levels of the bacterial methyltransferase and by the methylation status of its origin of replication (Boye and Lobner-Olesen, 1990; Campbell and Kleckner, 1990). In mammalian cells, however, the importance of methyltransferase activity and of DNA methylation in replication is only now starting to emerge (Araujo et al., 1998; Delgado et al., 1998; DePamphilis, 2000; Knox et al., 2000).

The work described in this thesis focuses mainly on understanding the functional relationship between changes in DNA methylation and DNMT1 activity on mammalian DNA replication. In higher eukaryotes, DNA replication initiates from multiple specific sites throughout the genome (Zannis-Hadjopoulos and Price, 1999). The first part of the thesis describes the identification and characterization of novel *in vivo* initiation sites of DNA replication within the human *dnmt1* locus (Araujo et al., 1999). Subsequently, a study of the temporal relationship between DNA replication and the inheritance of the DNA methylation pattern is presented. We also demonstrate that mammalian origins of replication, similarly to promoters, are differentially methylated (Araujo et al., 1998). We then tested the hypothesis that DNMT1 is a necessary component of the replication

machinery. The results presented indicate that inhibition of DNMT1 results in inhibition of DNA replication (Knox et al., 2000). Finally, results are presented, demontrating that the amino terminal region of DNMT1 is responsible for recognizing hemimethylated CGs, DNMT1's enzymatic target. Taken together, the results presented in this thesis demonstrate that DNMT1 is necessary for proper DNA replication and that DNA methylation may modulate origin function.

Résumé

Une des grandes questions de la biologie moderne est comment nos cellules interprètent nos informations génétiques et épigénétiques. La méthylation de l'ADN est une modification covalente du génome qui est essentielle au développement et joue un rôle important dans le contrôle de l'expression de nos gènes, l'impression génomique et l'inactivation de chromosome X (Bird et Wolfe, 1999; Szyf et al., 2000). De plus, des changements dans la méthylation de l'ADN at l'activité de l'ADN méthyltransférase 1 (DNMT 1) ont été largement documentés dans un grand nombre de cancers humains (Szyf, 1998a; Szyf et al., 2000)

Dans *Escherichia coli*, le moment et la fréquence d'initiation de la réplication de l'ADN sont contrôlés par la quantité de méthyltransférase bactérienne et par la méthylation de son origine de réplication (Boye et Lobner-Olesen, 1990; Campbell et Kleckner, 1990). Cependant, dans les cellules mammifères, l'appréciation de l'importance de la méthylransférase et de la méthylation de l'ADN dans la réplication ne fait que débuter (Araujo et al., 1998; Delgado et al., 1998; DePamphilis, 2000; Knox et al., 2000)

Les travaux présentés dans cette thèse se concentrent principalement sur la compréhension des effets potentiels de la méthylation de l'ADN et des changements dans l'activité de la DNMT 1 sur la réplication de l'ADN mammifère. Dans les eukaryotes supérieurs, la réplication de l'ADN est initiée à de nombreux sites spécifiques à travers le génome (Zannis-Hadjopoulos et Price, 1999). La première partie de cette thèse décrit l'identification et la caractérisation de nouveaux sites d'initiation de réplication de l'ADN *in vivo* à l'intérieur du locus ***dnmt 1*** humain (Araujo et al., 1999). Ultérieurement, une étude de la transmission du patron de méthylation relatif à la réplication de l'ADN est

présenté avec des résultats démontrant que les origines de réplication mammifères, comme les promoteurs, sont méthylés différemment (Araujo et al., 1998). Ensuite, l'hypothèse voulant que la DNMT 1 soit une composante nécessaire du mécanisme de réplication est testée, et les résultats présentés indiquent que l'inhibition de la DNMT 1 provoque l'inhibition de la réplication de l'ADN (Knox et al., 2000). Finalement, des résultats démontrant que la région N-terminale de la DNMT 1 est responsable de la reconnaissance des CG hémiméthylés (cible enzymatique de la DNMT 1) sont présentés. Pris ensemble, ces résultats démontrent que la DNMT 1 est nécessaire pour le bon accomplissement de la réplication de l'ADN et que la méthylation de l'ADN peut moduler la fonction des origines de réplication.

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Acknowledgments

I would like to thank Dr. Maria Zannis-Hadopoulos for giving me the opportunity to come to McGill and study in her laboratory. Maria provided a laboratory environment with positive energy and constant support. I am grateful to Dr. Gerald B. Price for his supervision and for helping me particularly in the initial stages of my degree when I resided in his laboratory.

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I would like to give credit to Dr. J. David Knox, who worked with me in almost all of my projects. Dave is also a great friend. I am also indebted to Andrew D. Slack, Snezana Milutinovic, Richard Pelletier, Shyam Ramchandani, Olivia Novac, Pascal Bigey, and Sylvie Croteau for collaborating with me and for being good friends.

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would like to thank my grandmother Aparecida, my sister Mariana and my parents Valéria and José, since without them none of this would have been possible.

Preface

This thesis is presented in manuscript-based form, under the terms listed by the Faculty of Graduate Studies and Research in their *"Guidelines of Thesis Preparation"* (Section 2*, cited below).

"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 be bound as an integral part of the thesis.

If this option is chosen, connecting text that provides logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the *"Guidelines of Thesis Preparation"*. The thesis must include: A table of contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and 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 the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these

cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers."

Contribution of authors

Chapter II: Identification of Initiation Sites for DNA Replication in the Human *dnmt1* (DNA Methyltransferase) Locus. (1999) *Journal of Biological Chemistry.* Felipe D. Araujo, J. David Knox, Shyam Ramchandani, Richard Pelletier, Pascal Bigey, Gerald Price, Moshe Szyf and Maria Zannis-Hadjopoulos.

I performed the autonomous replication assays and the competitive PCR assays with help from Richard Pelletier, the gel shift assays and, together with David Knox, the bisulfite mapping analysis. Shyam Ramchandani and Pascal Bigey provided the plasmids and sequences containing the human*dnmt1* locus fragments.

Chapter III: Concurrent replication and methylation at mammalian origins of replication. (1998) *Molecular and Cellular Biology.* Felipe D. Araujo, J. David Knox, Moshe Szyf, Gerald B. Price and Maria Zannis-Hadjopoulos.

I performed the nascent DNA preparation and the competitive PCR assays, as well as the nearest neighbor assays. Together with David Knox I performed the bisulfite mapping analysis.

Chapter IV: Inhibition of DNA Methyltransferase inhibits DNA Replication. (2000) *Journal of Biological Chemistry.* J. David Knox, Felipe D. Araujo, Pascal Bigey, Andrew D. Slack, Gerald B. Price, Maria Zannis-Hadjopoulos, and Moshe Szyf.

I performed all of the competitive PCR assays, David Knox performed all of the tissue culture experiments, isolation of nascent DNA, and together with Andrew Slack he performed the DNMT1 inhibition assays.

Chapter V: The DNMT1 target recognition domain resides in the N-terminus. (2000) *Journal of Biological Chemistry.* Felipe D. Araujo, Sylvie Croteau, Andrew D.

Slack, Snezana Milutinovic, Pascal Bigey, Gerald B. Price, Maria Zannis-Hajopoulos and Moshe Szyf.

I performed, together with Sylvie Croteau and Andrew Slack, the *in vitro* translation assays and competition experiments. Pascal Bigey designed the hairpin oligonucleotides and subcloned together with Sylvie Croteau the constructs utilized. Snezana Milutinovic performed the PCNA disruption assay.

Moshe Szyf, Gerald B. Price and Maria Zannis-Hadjopoulos supervised all of these projects.

List of abbreviations

ACS – ARS consensus sequence

ARS – Autonomously replicating sequence

ATP – Adenosine triphosphate

bp – Base pair

BrdU – Bromodeoxyuridine

CHO – Chinese hamster ovary

CsCl – Cesium chloride

Dam – Deoxyadenosine methylase

DHFR – Dihydrofolate reductase

DNMT – DNA methyltransferase

DUE – DNA unwinding element

FTR – fork targeting region

HDAC – Histone deacetylase

kb – Kilo base pair

Ku – Ku antigen

MAR – Matrix attachment region

MBD – Methyladed binding domain

MeTase – Methyltransferase

OBA – *Ors*-binding activity

ORC – Origin recognition complex

ori – Origin of replication

Ors – Origin-rich sequence

PCR – Polymerase chain reaction

Chapter I

General Introduction

I. DNA replication

(i) General principles

DNA replication is the most fundamental molecular event of living organisms. Several parameters have to be in place such that replication takes place only once per cell cycle, and at the precise time within the cell cycle. The investigation of prokaryotic and lower eukaryotic DNA replication has led to the identification of several properties that may have been conserved throughout evolution. In bacteria, a basic model has long been proposed where in order to initiate DNA replication a *trans*-acting initiator protein is required to bind to a specific *cis*-acting DNA sequence, termed replicator element (Jacob, 1963). A large body of experimental evidence has verified this model, known as the replicon model (Ritzi and Knippers, 2000; Zannis-Hadjopoulos and Price, 1999).

Classic experiments have determined that DNA replication is bidirectional (Cairns, 1966; Huberman and Riggs, 1968) and semiconservative, since each of the two parental DNA strands is utilized as a template for replication (Meselsohn, 1958). The rate of replication is set both by the pace of nascent chain elongation and by the frequency of initiation. It has been estimated that eukaryotic DNA replication initiates from approximately 10^4-10^6 sites throughout the genome (Hand, 1978; Martin, 1981). DNA replication is a semidiscontinous process. While one daughter strand is polymerized in a continuous fashion, the other strand is polymerized in a discontinuous fashion, called the leading and lagging strands respectively. This semidiscontinuity exists because polymerization occurs solely in the 5'-3' direction.

Even though much progress has been made at understanding detailed aspects of the initiation step of DNA replication in model systems such as bacteria and yeast, the same cannot be said for our current understanding of initiation of DNA replication in higher eukaryotes.

(ii) Replication proteins

The identification of novel mammalian DNA replication proteins and their respective roles is a major interest of our laboratory. Several proteins are required for proper DNA replication. DNA polymerases are responsible for the addition of new nucleotides onto free 3'- OH groups at growing nascent strands. There are several DNA polymerases in mammalian cells. DNA polymerase alpha (pol-α) has the ability to initiate replication, since it associates with an oligoribonucleotide primase activity that lays down RNA primers which are later utilized by pol-α (Loeb et al., 1986; Waga and Stillman, 1994). DNA polymerase delta (pol-δ), in a complex with the replication factor C (RF-C) and the proliferating cell nuclear antigen (PCNA), is thought to be responsible for the elongation step of replication (Tsurimoto and Stillman, 1989; Tsurimoto and Stillman, 1991). Pol-δ has both proofreading and processivity capabilities (Tsurimoto and Stillman, 1989; Tsurimoto and Stillman, 1991). DNA polymerases beta and gamma (pols βand γ) are involved in DNA repair and mitochondrial DNA replication respectively, and polymerase epsilon (pols-ε) is not as well defined.

PCNA is best known as a DNA pol-δ accessory protein, but it also functions in cell cycle control, DNA repair, as well as DNA replication. PCNA is commonly referred to as the sliding clamp, since it is able to encircle DNA and slide along the strand, conferring processivity to the protein complex containing pol-δ (Bymes et al., 1976). The observation that PCNA is capable of a mutually exclusive interaction with the cyclin-dependent kinase inhibitor p21 (cip1/waf1/sdi1) and with DNA methyltransferase 1 (DNMT1) (Chuang et al., 1997), supports the hypothesis that PCNA is directly involved in the control of the cell cycle progression. PCNA's interaction with p21 obstructs its interaction with both DNMT1 and pol-δ, thus inhibiting PCNA-dependent DNA replication (Chuang et al., 1997; Flores-Rozas et al., 1994). Recently, it has been shown that the proto-oncogene *c-myc* can also directly bind p21, relieving its inhibitory

effect on DNA synthesis directed by the PCNA-dependent pol-δ complex (Kitaura et al., 2000). RF-C is the protein responsible for loading PCNA onto DNA in an ATP-dependent fashion (Lee and Hurwitz, 1990).

DNA helicases define the growing point of the replication fork through its ATP-dependent capacity to unwind the double-helix (Matson and Kaiser-Rogers, 1990). As DNA helicases unwind the DNA, duplex helical torsional stress builds up. Subsequently, DNA topoisomerases introduce momentary DNA nicks, allowing the strands to relax. Single-stranded DNA binding proteins (RP-A, or RF-A, or HSSB), as the name indicates, bind to single stranded DNA and maintain it in a conformation that is required both for initiation of replication and nascent DNA chain elongation (Wobbe et al., 1987). In order to coordinate the replication of the lagging strand several proteins have to come together to degrade RNA primers, lay down short DNA stretches and ligate the several short Okazaki fragments. The proteins responsible for these functions, among others, are the maturation factor 1, RNAse H1, and DNA ligase 1.

(iii) Ku antigen

The finding that Ku 86/OBA (ors binding activity) can be purified through its ability to bind specifically to origin rich sequences (ors) has suggested that it may be involved in the initiation step of mammalian DNA replication (Ruiz et al., 1999; Ruiz et al., 1995).

Ku is a multifunctional DNA binding heterodimer, comprised of 70 kDa and 86 kDa subunits (Mimori et al., 1981), and it functions as the DNA-dependent protein kinase holoenzyme DNA binding subunit (Gottlieb and Jackson, 1993). It has been widely demonstrated that Ku functions in DNA repair and recombination, and one study has shown that Ku 86 knockout mice are approximately 50% the size of normal mice, indicating a function of Ku 86 in growth control (Nussenzweig et al., 1996). In yeast,

cross-linking experiments have shown that Ku is bound to telomeric DNA, indicating a possible role in the establishment of a normal DNA end structure (Gravel et al., 1998).

Recently, it was revealed that Ku is a genomic caretaker, suppressing genomic rearrangements and, therefore, preserving genomic integrity (Difilippantonio et al., 2000). Ku binds nonspecifically to broken DNA, on the other hand, several studies have indicated that Ku binds specifically to certain sequences, among them NRE1 (negative regulatory element 1) (Giffin et al., 1996). NRE1 is an element in the long terminal repeat of mouse mammary tumour virus (MMTV) that is utilized for repressing improper viral expression. It has been shown that specific binding of Ku to NRE1 represses glucocorticoid-induced MMTV transcription (Giffin et al., 1996). It has also been shown that Ku has helicase activity and is able to bind to human origins of replication (Araujo et al., 1999; Ruiz et al., 1999; Toth et al., 1993; Tuteja et al., 1994), indicating a possible function in the initiation of DNA replication. Currently, evidence for *in vivo* binding of Ku to specific origin sequences is being investigated in our laboratory through cross-linking experiments (Novac, manuscript in preparation).

(iv) DNA structures that facilitate replication

Specific structures, such as bent DNA, cruciforms and DNA unwinding elements (DUE), have been commonly found within origins of DNA replication (Zannis-Hadjopoulos and Price, 1998; Zannis-Hadjopoulos and Price, 1999). A number of studies indicate that these structures have diverse regulatory roles in DNA replication. The importance of bent DNA is unclear, but it has been suggested that like DUEs, it facilitates DNA unwinding, allowing replication to occur (Zannis-Hadjopoulos and Price, 1999).

Cruciform DNA originates from intra-strand base pairing of inverted repeat sequences (Schmid et al., 1975), and experimental evidence has indicated that cruciform formation is enriched at the G1/S border (Ward et al., 1990). It has been suggested that

cruciform-binding proteins may regulate replication (Zannis-Hadjopoulos and Price, 1998). Experimental evidence has demonstrated that the epsilon, beta, gamma, and zeta isoforms of the 14-3-3 family of proteins, which are important in cell cycle regulation, specifically interact with cruciforms (Todd et al., 1998).

Chromatin structure is another important parameter in DNA replication, as well as in transcription. Several reports have indicated that tightly packed chromatin structures inhibit DNA replication both in yeast and in mammalian cells (Forrester et al., 1990; Simpson, 1990). Microscopy studies have demonstrated that DNA replication and gene transcription are segregated in distinct nuclear domains (Wei et al., 1998). Nevertheless, several transcription factors, such as the ubiquitous transcription factor Oct-1, have been found to have a positive effect in DNA replication (Matheos et al., 1998). These observations indicate a possible relationship between transcription and replication not only in terms of chromatin structure, but also in sharing of specific factors.

(v) *Escherichia coli* replication

Experiments performed more than two decades ago have determined that initiation of DNA replication in *E. coli* starts from a very defined place within the 4.7 X 10^6 bacterial genome (Oka et al., 1980). These experiments demonstrated that the minimal origin of DNA replication is contained within 245 bp, termed *oriC*, and subsequent electron microscopy experiments verified that plasmid replication begins at or near *oriC*, from which it progresses bidirectionally to completion (Kaguni et al., 1982). Shortly after, it was shown that soluble protein fractions from *E. coli* dnaA+ cells, but not from dnaA temperature-sensitive cells, replicated plasmids containing *oriC* (Fuller and Kornberg, 1983). Furthermore, DNase I footprinting experiments indicated that dnaA binding sites share a common 9 bp sequence, 5'-TTATCACACAA, repeated at four conserved positions within *oriC* (Fuller et al., 1984). Taken toghether, these results demonstrate that dnaA is the *E. coli* replication initiator protein. In addition, it was

shown that DNA supercoiling, in the absence of replication proteins, prompted localized unwinding within the same sequence opened by the dnaA (Kowalski and Eddy, 1989). Three AT-rich 13-mer sequences were found to be responsible for DNA unwinding, and these *cis*-acting sequences, whose helical instability is required for origin function, were the first characterized DUEs (Kowalski and Eddy, 1989).

Within *oriC* there are eleven GATC sites that are recognized by the bacterial dam-methyltransferase, and the limiting levels of the enzyme determine the DNA methylation pattern in *E.coli* (Szyf et al., 1984). It is now known that timing and frequency of initiation of DNA replication is controlled by the methylation status of these eleven GATC sites (Boye and Lobner-Olesen, 1990; Campbell and Kleckner, 1990; Russell and Zinder, 1987). This was first demonstrated in a study where an accumulation of hemimethylated daughter plasmids was observed following transformation of dam- strains of *E.coli* by fully methylated plasmids, suggesting that hemimethylation prevents initiation of DNA replication (Russell and Zinder, 1987). Subsequently, it was shown that *oriC* is sequestered from dam methyltransferase by the bacterial plasma membrane, preventing remethylation of the GATC sites within *oriC* until replication is complete (Campbell and Kleckner, 1990). Analogously, the dnaA promoter region was observed to be sequestered (Campbell and Kleckner, 1990). These observations led to the hypothesis that hemimethylation of these two regions function coordinately to ensure that initiation of DNA replication occurs only once per cell cycle (Campbell and Kleckner, 1990). Additional experiments, where a temperature-inducible promoter controlled the cellular level of dam-methyltransferase, indicated that controlled initiation of replication only occurred at a narrow temperature range, further supporting previous findings (Boye and Lobner-Olesen, 1990) (please see Figure 1).

Taken together, these observations have led to the hypothesis that DNA methylation could also function in a similar fashion to control timing and frequency of

initiation of replication in higher eukaryotes. However, as discussed in the following sections, no such mechanism appears to exist in mammalian cells.

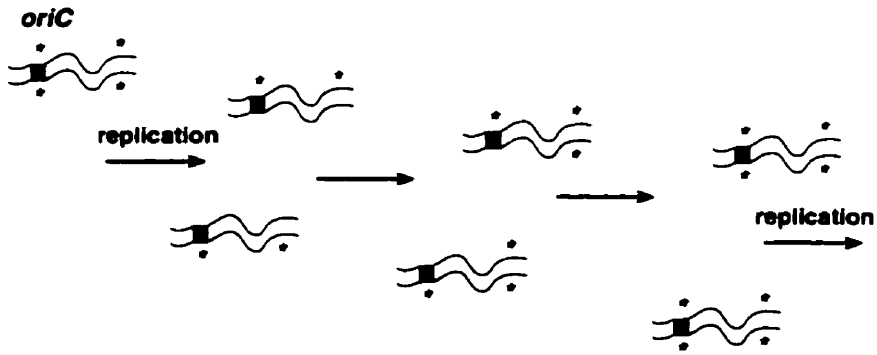

Fig 1. *E. coli* DNA replication is controlled by DNA methylation. DNA replication initiates from the bacterial origin, *oriC* (depicted by the black box). The bacterial origin is fully methylated at the start of replication (depicted by the asterisc), and as replication proceeds the origin becomes hemimethylated, as does the rest of the bacterial genome. However, *oriC* remains hemimethylated until replication is complete, whereas the rest of the genome becomes fully methylated as replication takes place.

(vi) Yeast replication

Investigation of the control mechanisms of initiation DNA replication in eukaryotes has its foundations in yeast genetics (Ritzi and Knippers, 2000). DNA methylation has not been detected in yeast. Since the identification of *in vivo* specific initiation sites of DNA replication within the human *dnmt1* locus is the topic of Chapter II, it is appropriate to briefly discuss the current knowledge of yeast DNA replication.

In *Saccharomyces cerevisiae* initiation sites for replication are contained within 150 bp comprising an essential AT-rich consensus region, the A element, and three stimulatory elements, the B elements (Ritzi and Knippers, 2000). Yeast origins of replication are often called ARS, for autonomous replication sequences, since when cloned into plasmids they promote episomal DNA replication. *S. cerevisiae* origins also contain an ARS consensus sequence (ACS), however, studies have indicated that not all ARS are actually utilized as *bona fide* chromosomal origins (Ritzi and Knippers, 2000). In *Schizosaccharomyces pombe* studies have shown that origins are contained within 500-1000 bp, and although several AT-rich elements have been shown to aid initiation of replication, no consensus sequence has been found (Kim and Huberman, 1998) (Chuang and Kelly, 1999).

A major progress in our understanding of the mechanism of initiation of *S. cerevisiae* DNA replication came from experiments that first identified a multiprotein complex that specifically recognizes *S. cerevisiae* ARS in an ATP-dependent manner (Bell and Stillman, 1992). This protein complex is composed of six distinct proteins and is known as the origin recognition complex (ORC) (Bell and Stillman, 1992). The current hypothesis is that ORC functions as a landing pad for the stepwise formation of a larger protein complex, known as the pre-replication complex, that makes chromatin competent for DNA replication. Experimental evidence has revealed that other replication proteins, such as Cdc6 and Mcm proteins, do indeed interact with ORC in a

stepwise manner (Ritzi and Knippers, 2000). Homologs of individual proteins from ORC have been identified in human cells, *S. pombe*, *C. elegans* and *Drosophila*, indicating the possibility of functional evolutionary conservation (Gavin et al., 1995; Gossen et al., 1995). Not only individual ORC homologs have been identified, but endogenous ORC homologous complexes containing all subunits have been identified as well in *S. pombe*, *Xenopous* egg extracts, and *Drosophila* (Chesnokov et al., 1999; Moon et al., 1999; Tugal et al., 1998). However, complexes containing all ORC components have not yet been identified in humans or in mice.

(vii) Mammalian replication

The existence of mammalian sequence-specific origins of DNA replication has been a long lasting debate. It has been suggested that initiation of replication in mammalian cells may not have precise sequence requirements, which target initiation to specific locations (Krysan and Calos, 1991). However, the fine mapping of a growing number of initiation sites of mammalian DNA replication indicates that replication does not initiate at random and that specific sequences are indeed required (Zannis-Hadjopoulos and Price, 1999). The development of quantitative PCR analysis coupled to novel methods of isolation of nascent DNA has facilitated the study of mammalian DNA replication (Gerbi and Bielinsky, 1997; Zannis-Hadjopoulos and Price, 1999) (please see Figures 2 and 3).

A number of specific initiation sites of mammalian DNA replication have been identified and mapped in our laboratory (Araujo et al., 1999; Pelletier et al., 1999; Tao et al., 2000; Wu et al., 1993b). Recently, detailed origin mapping experiments performed at the human *c-myc* locus have revealed that initiation of DNA replication occurs specifically from two major sites (Tao et al., 2000; Trivedi et al., 1998; Waltz et al., 1996). Furthermore, experiments utilizing site-specific recombination strategies demonstrated that DNA sequences previously identified as initiation sites of DNA

replication can initiate replication when transferred to new chromosomal locations, confirming the requirement of specific sequences (Aladjem et al., 1998; Malott and Leffak, 1999).

Specificity of initiation has been confirmed in mapping experiments performed in a well-defined region downstream of the dihydrofolate reductase locus (DHFR) (Pelizon et al., 1996). However, the study of this locus has generated controversy, since different results have been obtained depending on the assay utilized by the different groups (Dijkwel and Hamlin, 1996; Pelizon et al., 1996; Wang et al., 1998). The current consensus is that, similarly to the *c-myc* paradigm, multiple specific sites initiate replication, but there is preference for two main sites (Dijkwel and Hamlin, 1996; Pelizon et al., 1996; Wang et al., 1998).

Autonomous replication assays have provided further support for site specificity of initiation, since specific chromosomal initiation sites have been shown to support autonomous replication of plasmids, whereas random sequences of similar sizes have not (Pelletier et al., 1997; Wu et al., 1993a; Wu et al., 1993b; Zannis-Hadjopoulos et al., 1994). A more detailed discussion of mapping of initiation sites of DNA replication is included in Chapter II, where the identification of such sites within the human *dnmt1* locus is presented.

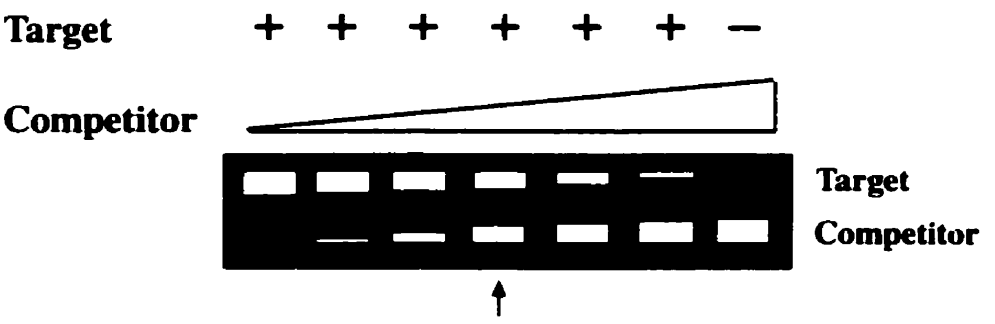

Fig 2. Competitive PCR assay. Constant amounts of a target DNA template are quantified with increasing amounts of a specific competitor. The competitor DNA template is amplified by the same set of primers as the target template, but the product sizes are different. The PCR products are resolved on an agarose gel where each reaction can be quantified. The quantity of target initially added to the reaction can be estimated from the quantity utilized in the reaction where equal amounts of both templates are present (ratio 1:1).

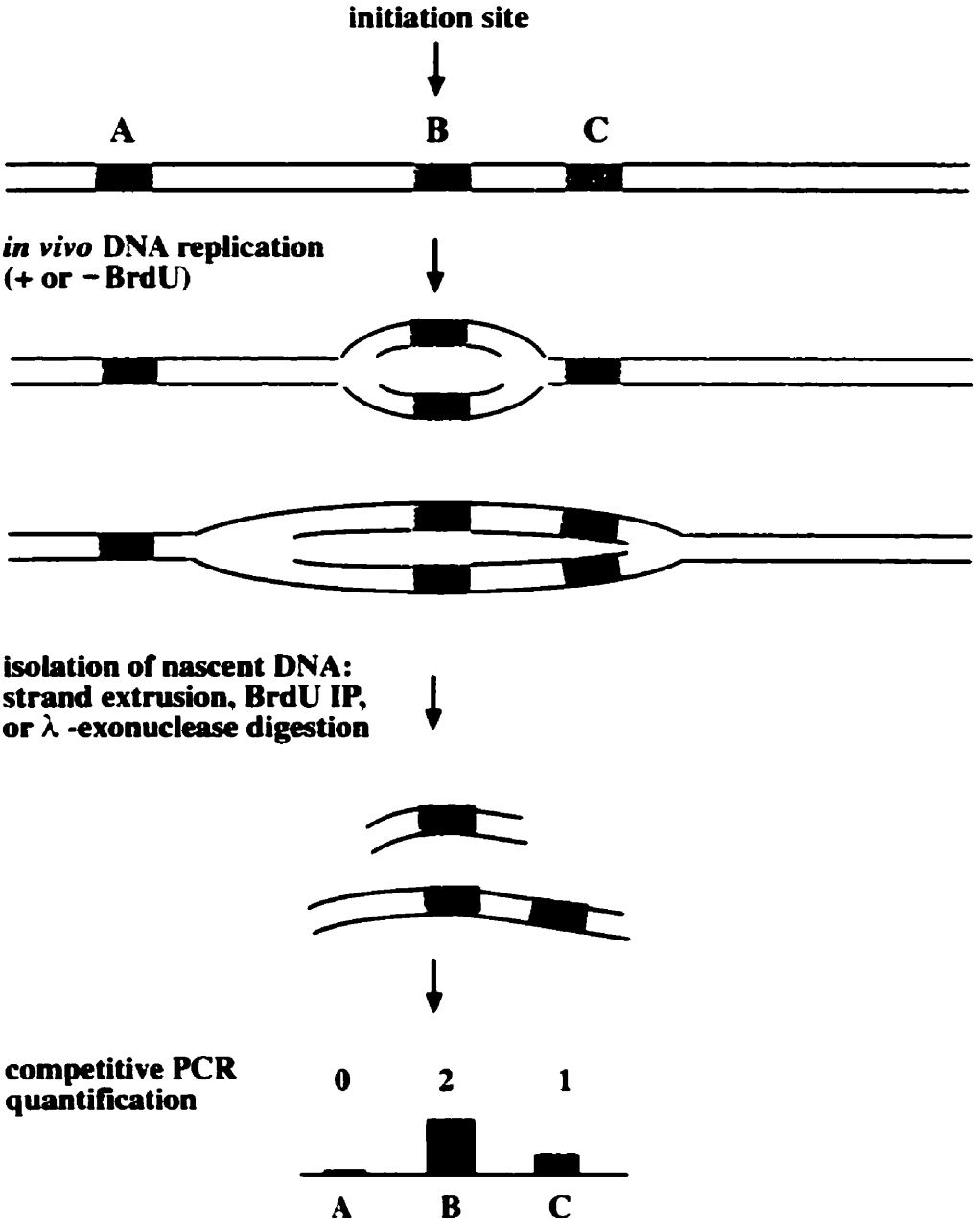

Fig 3. Nascent DNA abundance quantification by competitive PCR. Newly synthesized DNA can be isolated by several distinct methodologies and utilized for subsequent competitive PCR quantification.

II. DNA methylation

(i) DNA methyltransferases and general principles

DNA methylation is catalyzed by a family of conserved DNA methyltransferases (DNMTs) and is widespread in many, but not all, organisms from bacteria to humans (Colot and Rossignol, 1999; Razin and Szyf, 1984). Mammalian DNMTs methylate the 5'position of cytosines, mostly in the CG dinucleotide context (Gruenbaum et al., 1981), but cytosine methylation in other dinucleotide contexts has been reported (Clark et al., 1995; Tasheva and Roufa, 1994; Tasheva and Roufa, 1995; Toth et al., 1990; Woodcock et al., 1987).

The first mammalian cDNA encoding a DNMT, now termed DNMT1, was cloned and sequenced in 1988 (Bestor et al., 1988). The carboxy-terminal part of the protein was found to contain 570 amino acid residues with striking similarity to bacterial type II DNA cytosine methyltransferases (Bestor et al., 1988). This region was postulated to represent the catalytic methyltransferase domain, whereas the amino-terminal portion of the enzyme was hypothesized to act as a regulatory domain, since it was demonstrated that antibodies directed against a peptide sequence located within the amino region inhibits enzymatic activity *in vitro* (Bestor et al., 1988). Sequence analysis now indicates that eukaryotic DNA methyltransferases fall into at least five structurally distinct subfamilies (Colot and Rossignol, 1999).

The kinetic and catalytic properties of the DNMTs have been extensively studied (Wu and Santi, 1985; Wu and Santi, 1987). Reports utilizing bacterial DNMTs have suggested that the DNA methylation reaction proceeds in processive mechanism. First, there is a nucleophilic attack by DNMT to the C5 of cytosines, leading to the formation of a transient covalent adduct between the enzyme and DNA. This is followed by S-adenosylmethionine (AdoMet) binding and methyl transfer, then S-

adenosylhomocysteine (AdoHcy) dissociates from the complex, followed by DNMT dissociation from the methylated DNA (Wu and Santi, 1985; Wu and Santi, 1987).

It is now believed that DNMT1 is the predominant maintenance DNMT in mammalian cells. Recent deletion analyses have indicated that that a large part of the DNMT1 N-terminal domain is required for methylating activity since the C-terminal domain alone was not sufficient for this activity (Margot et al., 2000). Moreover it is known that the 5' most part of the N-terminal domain, including the major nuclear import signal and tissue-specific exons, is not required for enzymatic activity (Margot et al., 2000). Interestingly, the functional subdivision of DNMT1 correlates well with the structure of the DNMT1 gene in terms of intron/exon size distribution as well as sequence conservation. Taken together these data indicate the possibility that the *dnmt1* gene has evolved from the fusion of at least three genes (Margot et al., 2000). The genomic structure of DNMT1 was only determined a decade after cloning of its cDNA (Ramchandani et al., 1998). Direct sequence analysis demonstrated that the gene consists of at least 40 exons and 39 introns spanning a distance of approximately 60 kilobases. The region within DNMT1 responsible for targeting the enzyme to hemimethylated CGs remains unclear, however, results presented in Chapter V, now indicates the the N-terminus of the enzyme is responsible for target recognition. A more detailed discussion of the structure-function of DNMT1 is also included in Chapter V.

A long lasting point of contention had been how many DNMTs were present in mammalian cells. One issue that revolved around this question was whether *de novo* methylation and maintenance methylation activities were encoded by a single or multiple distinct genes. To address these questions a null mutation of the only known mammalian DNMT gene, through homologous recombination, was performed in mouse embryonic stem cells (Lei et al., 1996). The homozygous null embryonic stem cells were found to be viable and contain low but stable levels of methylation and DNMT activity, indicating

the existence of other DNMTs in mammalian cells (Lei et al., 1996). These observations led to the identification of novel mammalian DNMTs, *dnmt3a* and *dnmt3b*, which are essential for *de novo* methylation and mouse development (Okano et al., 1999), since inactivation of both genes blocks *de novo* methylation in embryonic stem cells and early embryos. Importantly, no effect on maintenance methylation was observed. The results also demonstrated that *dnmt3a* and *dnmt3b* display non-overlapping roles in development, with *dnmt3b* specifically needed for methylation of centromeric minor satellite repeats (Okano et al., 1999).

Even though DNA methylation is widespread among protists, plants, fungi and animals, its apparent absence in *Drosophila* has generated controversy. One report has shown, employing a Southern blotting procedure, that *Drosophila* genomic DNA hybridizes with a 4423-bp C-terminal fragment of the murine DNMT1 gene (Vanyushin and Poirier, 1996). This finding indicates that the *Drosophila* genome has sequence homology to the mammalian DNMT1 gene. A later report has presented evidence for two CG DNMT-like proteins expressed in *Drosophila* cells (Hung et al., 1999). One of these proteins, DmMTR1, contains peptide epitopes immunologically related to the catalytic domain of the mammalian DNMT1. Interestingly, DmMTR1, like the mammalian DNMT1, also interacts *in vivo* with PCNA (Hung et al., 1999). Immunofluorescence data indicates that DmMTR1 may play an essential function in the cell cycle regulated condensation of the *Drosophila* chromosomes (Lyko et al., 2000). The same report, through genomic database search, also identified another polypeptide, DmMT2, that displays sequence homology to the mammalian DNMT2 and the yeast CG DNMT homolog *pmt1*. Subsequent results have shown that DmMT2 is a component of a transposon-like element (Lyko et al., 2000). Since DNA methylation is apparently absent in *Drosophila*, it provides a useful tool to analyse the function of DNMT proteins in a heterologous system. Experiments were performed demonstrating that DNMT

expression in *Drosophila* inhibited the viability of transgenic flies, implying that DNA methylation has functional consequences for *Drosophila* development (Lyko et al., 1999).

(ii) Demethylases

During embryo development a sequence of genome-wide demethylation and *de novo* methylation events occur in order to establish the gene-specific methylation pattern in mammalian cells (Kafri et al., 1993). The question that still generates debate is whether DNA demethylation occurs by a passive mechanism, either through DNA replication or repair in the absence of a DNMT, or by an active mechanism, facilitated by a DNA demethylase. It has been suggested that the strength of the covalent bond between the methyl group and the cytosine ring makes it virtually impossible for an active enzymatic demethylation reaction (Cedar and Verdine, 1999). On the other hand, the rapid kinetics of DNA demethylation events taking place before the very first round of DNA replication in the paternal genome in zygotes following fertilization points towards an active mechanism (Oswald et al., 2000).

Recently, a demethylase activity was purified from human lung cancer cells (A549) (Ramchandani et al., 1999), and, subsequently, it was demonstrated that MBD2 (Methyl-CG-Binding Domain 2), a protein that can also function as a transcriptional repressor, contained this activity (Bhattacharya et al., 1999). Furthermore, it was observed that DNA demethylation by MBD2 occurs in a processive fashion, similarly, but opposite, to DNA methylation by DNMT1 (Cervoni et al., 1999).

A DNA demethylase should in theory function as transcriptional activator, but the fact that MBD2 can function as a transcriptional repressor indicates the possibility of a dual role for this protein. This duality has been previously shown for other proteins involved in the control of gene expression (Wotton et al., 1999). For example, in the TGFβ signal transduction pathway, it has been shown that Smad protein complexes that

function as TGFβ signal transducers, can either form a transcriptional coactivator complex with proteins such as such as p300/CBP, or form a transcriptional repressors complex with TGIF and HDACs (Wotton et al., 1999). It is possible that similar diverse protein-protein interactions might determine the fate of MBD2.

(iii) Methylation and transcription

(a) Direct repression

DNA methylation is now known to have several cellular functions, some of which coexist within the same organism (Colot and Rossignol, 1999; Razin and Szyf, 1984). It is clear that DNA methylation supresses gene expression (Bird and Wolffe, 1999; Boyes and Bird, 1991; Busslinger et al., 1983; Razin and Cedar, 1991; Razin and Riggs, 1980; Slack et al., 1999b; Szyf, 1994), but the mechanisms behind this function are still being elucidated. Genomic methylation analysis has revealed a nonrandom pattern of methylation, whereby active genes are not methylated and inactive genes are (Yisraeli and Szyf, 1984). Using a modified nearest neighbor assay it has been demonstrated that 70-80% of cytosines within CGs are methylated in mammalian cells (Razin et al., 1984).

One way by which cytosine methylation inhibits gene expression is by preventing binding of transcription factors to their cognate sequence. This is the case with the cyclic AMP-dependent activator CREB, where DNA methylation inhibits expression of a human proenkephalin-CAT fusion gene when it is transiently expressed in CV-1 cells or stably expressed in C6-glioma cells (Comb and Goodman, 1990). A similar mechanism occurs for the binding and transactivation of the adenoviral promoter of E2A by AP-2, where the unmethylated promoter is expressed, but methylation of a single CG inactivated transcription (Langner et al., 1984). Yet another example of this phenomenon is the inhibition of Myc/Myn binding by methylation of the core CG in the E-box recognition site (Prendergast et al., 1991; Prendergast and Ziff, 1991). However, it is clear that other transcription control mechanisms exist since binding of certain

transcription factors to their cognate sequence is unaffected by DNA methylation. This is the case with Sp1, where methylation of its target sequence, a GC-rich sequence known as a "GC-box" has no effect on binding or transactivation (Harrington et al., 1988).

(b) Indirect repression

It has been observed that promoters and other regulatory DNA regions are associated with chromatin that contains hyperacetylated histones, which in turn are associated with high density unmethylated CG regions, termed CG islands (Bird and Wolffe, 1999; Kass et al., 1997b; Kass and Wolffe, 1998). On the other hand, methylated DNA is usually associated with chromatin containing hypoacetylated histones (Bird and Wolffe, 1999; Kass et al., 1997b; Kass and Wolffe, 1998). In 1989 the first methyl-CG binding protein (MeCP) was characterized (Meehan et al., 1989). Experimental evidence indicated that fifteen or more symmetrically methylated CG sites per MeCP were necessary for strong binding (Meehan et al., 1989).

Importantly, methyl-CG-binding proteins, as well as DNMT1, are able to associate with histone deacetylases (HDACs) and chromatin remodeling complexes (Fuks et al., 2000; Jones et al., 1998; Nan et al., 1998) to control gene expression. First, it was demonstrated that methylated DNA, assembled into chromatin, was able to bind the methyl-CG binding protein MeCP2, which in turn cofractionated with the chromatin remodeling complex containing Sin3A and an HDAC. The transcription inhibition by MeCP2 and methylated DNA was diminished by trichostatin A (TSA), an inhibitor of HDACs (Jones et al., 1998; Nan et al., 1998). Shortly after these findings, it was demonstrated that methyl-CG binding proteins other than MeCP2 were also able to associate with HDACs and chromatin remodeling complexes (Ng et al., 1999). A complex termed MeCP1, which contains MBD2, was shown to be part of a larger complex comprising HDAC1 and HDAC2 (Ng et al., 1999). MBD2 is closely related to another methyl-CG-binding protein, MBD3 (Bird and Wolffe, 1999), which is associated

with yet another chromatin remodeling complex, the Mi2/NuRD deacetylase complex (Wade et al., 1999; Zhang et al., 1999) (please see Figure 4 for a schematic view of the currently known mammalian DNMTs and MBD proteins).

Recently, it has been demonstrated that DNMT1, the enzyme believed to be the major maintenance DNMT, can establish a repressive transcription complex with HDAC2 and a novel protein, DMAP1 (for DNMT1 associated protein) (Rountree et al., 2000). DMAP1 is targeted to replication foci through its interaction with DNMT1 throughout S-phase, whereas HDAC2 joins DNMT1-DMAP1 at late S-phase (Rountree et al., 2000). This indicates the possibility that DNMT1 not only replicates the DNA methylation pattern, but might also control the inheritance of transcriptionally repressive chromatin.

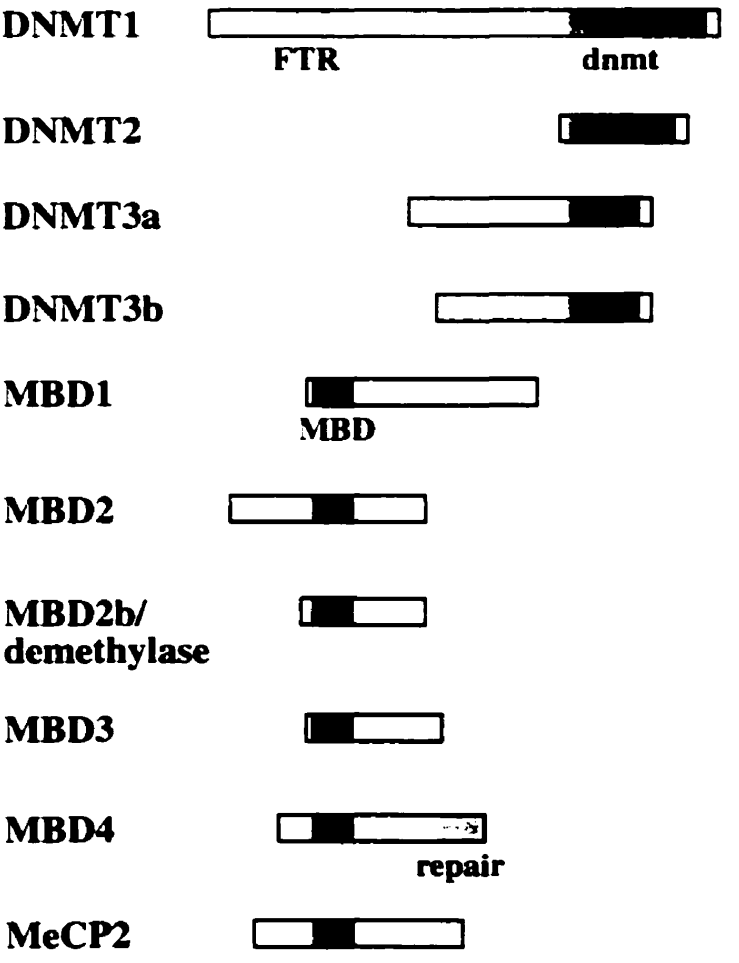

Fig 4. Proteins of the mammalian DNA methylation machinery. DNMT proteins contain a "DNMT" homologous region and MBD proteins share a methyl-CG-binding domain, the "MBD" domain. "FTR" refers to the replication foci targeting region of DNMT1 and "repair" refers to the T-G mismatch glycosylase domain of MBD4. For greater detail of DNMT1 structure-function please see Figure 1 of Chapter V.

(c) Methylation independent repression

Recent results also indicate that DNMT1 is not only responsible for maintaining the methylation pattern, but that it also can function as a transcriptional co-repressor in a manner that is independent of DNA methylation patterns (Milutinovic et al., 2000; Robertson et al., 2000). One study has shown that DNMT1 inhibition leads to a transcriptional upregulation of the tumor suppressor p21 (Milutinovic et al., 2000). Interestingly, the p21 locus is not methylated prior to DNMT1 inhibition, indicating that the transcriptional upregulation observed following inhibition of DNMT1 is not a result of promoter demethylation, as expected. Another study has shown that DNMT1 co-purifies with the retinoblastoma (Rb) tumour suppressor protein, E2F1, and HDAC1 and that, together, this complex can repress transcription from promoters containing E2F-binding sites (Robertson et al., 2000). These results are an indication of the multiple mechanisms by which DNMT1 can modulate gene expression even in the absence of DNA methylation.

Further support for a methylation-independent mechanism of action of DNMTs and MBD proteins comes from experiments performed in *Drosophila*, where DNA methylation has not been detected. The first evidence for this hypothesis came from a report identifying two CG DNMT-like proteins expressed in *Drosophila* cells (Hung et al., 1999), as mentioned previously. A subsequent study then indicated that a *Drosophila* gene, dMBD2/3, that codes for two MBDs could repress transcription effectively in *Drosophila* cells (Roder et al., 2000). It was suggested that the striking similarity of the MBD, as well as DNMT-related proteins, in *Drosophila* and vertebrates points towards diverse roles for these proteins in eukaryotic cellular functions (Roder et al., 2000).

(iv) Silencing of foreign genes

A considerable percentage of genomic methylation occurs in parasitic sequence elements in vertebrate genomes (endogenous retroviruses and transposons) (Doerfler, 1991; Orend et al., 1991). Therefore, it has been hypothesized that the primary function of DNA methylation is to defend cells against parasitic sequence elements (Bestor, 1998), and that genomic parasites can be recognized by *de novo* DNMTs because of their high copy number. These findings have implications for the successful development of gene therapy, since *de novo* methylation of a newly introduced gene would preclude the therapy from working. One example of this scenario was described in studies of gene transfer and expression of the human glucocerebrosidase cDNA by a Moloney murine leukemia virus (MoMuLV)- based retroviral vector in a murine gene transfer/bone marrow transplant (BMT) model, where expression was suppressed due to methylation of the viral LTR (Challita and Kohn, 1994). Similar findings further demonstrated that *de novo* DNA methylation may be a hurdle to surpass if long term gene expression from viral promoters is to be achieved (Barletta and Greer, 1992).

(v) Genomic imprinting and insulation

Genomic imprinting refers to the fact that in mammals there is unequal gene expression between the two parental alleles. The existence of imprinting emerged from nuclear transplantation studies, where it was observed that the maternal and paternal contributions to the embryonic genome were not equivalent (McGrath and Solter, 1984). These experiments showed that a diploid genome derived from only one of the two parental sexes was incapable of supporting complete embryogenesis (McGrath and Solter, 1984). Results from experiments performed with transgenic mice indicated that the pattern of DNA methylation of exogenous DNA sequences could be modified by switching their gamete of origin in successive generations (Sapienza et al., 1987),

suggesting that DNA methylation could be the cause of imprinting (Sapienza et al., 1987).

In the last decade many imprinted genes have been characterized, and the best-studied example of genes controlled by parental imprinting is the H19/Igf2 pair. H19 is expressed exclusively from the maternal chromosome, and it encodes an RNA of unknown function. In mice, H19 lies 90 kb downstream from the Igf2 gene, which encodes the insulin-like growth factor II, and is expressed primarily from the paternally inherited chromosome (Bartolomei et al., 1993). A male-specific DNA methylation domain, 7- to 9-kb in length, surrounding the H19 gene and its promoter has been identified (Bartolomei et al., 1993), and this region is now known as the differentially methylated domain (DMD). It was proposed that this allele-specific methylation was responsible for transcription suppression of the H19 paternal allele (Bartolomei et al., 1993). Consistent with this hypothesis, the H19 promoter contained an open chromatin conformation only on the unmethylated active maternal allele. Moreover, in DNMT homozygous knockout mice, gene expression of H19 and Igf-2 is altered (Li et al., 1993). Surprisingly however, the normally active paternal allele of the Igf-2 gene became repressed, whereas the normally silent paternal allele of the H19 gene was activated, as expected.

Recently, while investigating the transcriptional control of the H19/Igf2 gene pair, independent reports uncovered a novel mechanism by which DNA methylation can modulate gene expression. It had been previously reported that deletion of the DMD, resulted in loss of imprinting at both H19 and Igf2 genes, similarly to the results obtained with the DNMT deficient mice (Thorvaldsen et al., 1998). Recent results now show that the DMD region contains an insulator element that blocks enhancer activity by its ability to bind to CTCF, a zinc-finger protein involved in vertebrate boundary function (please see Figure 5) (Bell and Felsenfeld, 2000; Hark et al., 2000; Lobanenkov et al., 1990). An

insulator is a DNA sequence that can act as a boundary to the effects of adjacent cis-acting elements, such as enhancers (Bell et al., 1999; Michel et al., 1993). The binding of CTCF prevents the downstream enhancer from activating Igf2. Recent observations now demonstrate that CTCF does not bind the insulator when the CGs within it are methylated (Bell and Felsenfeld, 2000; Hark et al., 2000). Therefore, at the paternally inherited chromosome, where the insulator is methylated, CTCF does not bind and the downstream enhancer is active and Igf2 is expressed. At the maternally inherited chromosome the opposite occurs, CTCF binds the unmethylated insulator region preventing the Igf2 enhancer activity and allowing H19 gene expression (please see Figure 5) (Bell and Felsenfeld, 2000; Hark et al., 2000). These observations provide a molecular explanation for the parentally inherited, mutually exclusive expression of Igf2, and its dependence on parental origin specific methylation.

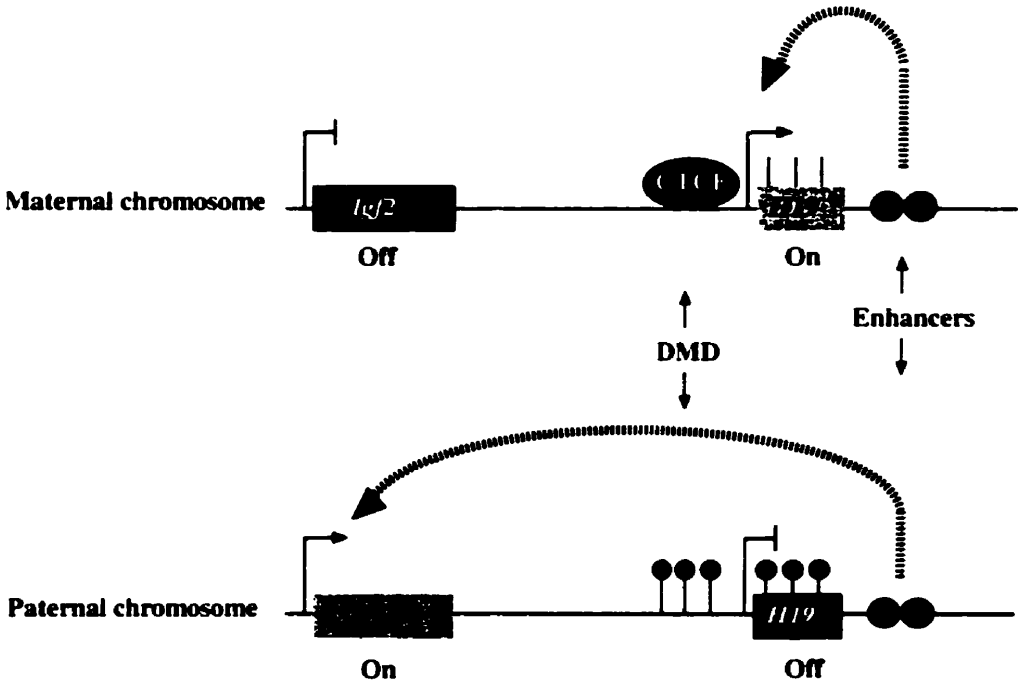

Fig 5. CTCF insulation is controlled by DNA methylation. *Igf2* and *H19* are expressed either from the maternal or paternal chromosome, as indicated. The two genes share an enhancer element whose effect is controlled by the methylation status of the DMD (differentially methylated domain). CTCF cannot bind to the methylated DMD. Black lollipops represent methylated CGs and vertical lines on the opposite chromosome represent unmethylated CGs.

(vi) X-chromosome inactivation

In mammalian cells the X-chromosome is unique in its ability to completely inactivate gene expression, resulting in a dosage compensation for X-linked genes in females. A number of mechanisms are needed for the initiation of this developmental event and the maintenance of the inactive state. In recent years research has ratified the long-held theory that X inactivation events are controlled by the presence in *cis* of a locus, 'X-inactivation center' (Xic), and by the gene Xist, which is located at or near the X-inactivation center. Xist encodes an untranslated RNA specifically from the inactive X chromosome in both humans and mouse, suggesting that it is involved in X inactivation. Moreover, truncation of Xist RNA by gene targeting is lethal for female embryos and prevents the inactivation of the X-chromosome carrying the deletion (Jaenisch et al., 1998). Recent results now indicate that another element, tsix, is able to antagonize Xist expression, determining which X-chromosome is inactivated (Lee, 2000; Lee and Lu, 1999). However, what controls tsix expression is unclear.

The proposal that DNA methylation might be responsible for the epigenetic changes behind X-chromosome inactivation precedes the purification and identification of eukaryotic DNMTs (Riggs, 1975). Xist expression is also silenced by DNA methylation, indicating that DNA methylation is important for preventing the active X-chromosome from becoming inactivated (Jaenisch et al., 1998).

Even though it is clear that DNA methylation prevents gene expression, the mechanism that initiates DNA methylation on the inactive X-chromosome is unclear. A new hypothesis to explain X inactivation comes from plant methylation studies where RNA-DNA interactions can serve as a signal that triggers *de novo* DNA methylation (Wassenegger, 2000). So far, RNA-directed DNA methylation mechanisms have only been demonstrated in target transgenes, but it is possible that methylation of endogenous sequences could also be directed by RNA (Wassenegger, 2000). In this RNA-directed

scenario, methylation takes place in DNA regions that are complementary to the directing RNA, so it has been suggested that a putative RNA-DNA duplex directs methylation (Wassenegger, 2000).

(vii) DNA repair and recombination

A mechanism that utilizes DNA methylation for DNA repair and recombination is well established in *E. coli*, where the involvement of hemimethylated GATC sequences in mismatch correction by the mutH, mutL, and mutS proteins is understood in great detail (Lahue et al., 1987). However, a similar type of mechanism is yet to be found in higher eukaryotes. In mammalian cells results have indicated that DNA methylation has no detectable effect on either DNA end-joining or homologous recombination (Liang and Jasin, 1995). Nevertheless, it was postulated that DNA methylation might influence the accessibility of the repair/recombination machinery to chromosomal DNA by altering local chromatin structure (Liang and Jasin, 1995). Despite these findings, the fact that the mammalian DNMT1 can interact with the proliferating cell nuclear antigen (PCNA), an auxiliary protein for DNA replication and repair, indicates the possibility that DNA methylation may be important in mammalian DNA repair (Chuang et al., 1997). On the other hand, experimental evidence has indicated that methylation of deoxycytidine incorporated by DNA excision-repair is slow and incomplete (Kastan et al., 1982). These results have led to the hypothesis that DNA damage and repair may cause heritable loss of methylation at certain sites (Kastan et al., 1982).

DNA methylation is also prominent cause of mutations. In humans, the mutation rate from 5-methylcytosine to thymine is 10-50 fold higher than other transitions. This is thought to be the reason why CG dinucleotide sequences are under-represented in mammalian genomes (Jones et al., 1992). Over one-third of germline point mutations associated with human genetic disease and many somatic mutations leading to cancer involve loss of CGs. These mutations appear to be due to the hydrolytic deamination of

methylated cytosines, giving rise to G x T mispairs. A recent study has suggested that TG mismatches might be repaired by the thymine DNA glycosylase, TDG (Hendrich et al., 1999). This study has shown that MBD4, a methyl-CG binding protein, can remove thymine or uracil from mismatched CG sites *in vitro*, indicating that this enzyme may function to minimize mutation at methyl-CGs (Hendrich et al., 1999).

On the other hand, it has been suggested that DNA methylation, rather than being mutagenic, actually confers genomic stability in mammalian cells (Chen et al., 1998). Elevated mutation rates at both the endogenous hypoxanthine phosphoribosyltransferase (HPRT) gene and an integrated viral thymidine kinase (TK) transgene has been observed in murine embryonic stem cells nullizygous for the *dnmt1* gene (Chen et al., 1998). Furthermore, experiments performed in the fungus *Ascobolus immersus* has indicated that the methylation associated with DNA repeats in many eukaryotes might serve to inhibit homologous recombination and play a role in preserving genome integrity (Maloisel and Rossignol, 1998). These experiments have demonstrated that the frequency of crossing-over between two specific sequences was reduced by several hundredfold when the sequence was methylated on the two homologs (Maloisel and Rossignol, 1998), indicating that methylation inhibits homologous recombination. It was proposed that DNA methylation inhibits pairing between the two homologs before recombination initiation and/or inhibits the normal processing of recombination intermediates (Maloisel and Rossignol, 1998).

Another interesting phenomenon relating DNA methylation and recombination occuring in *Ascobolus immersus* is the transfer of methylation between alleles in homologous chromosomes (Colot et al., 1996). Transfer of methylated DNA to the unmethylated allele was observed when both methylated and an unmethylated alleles of the *Ascobolus* b2 spore color gene were placed together in single meiotic cells (Colot et al., 1996). The methylation transfer occurred by a gene conversion mechanism, 5' to 3'

along the b2 gene, indicating that methylation transfer and recombination are mechanistically related (Colot et al., 1996).

III. Methylation and cancer

Numerous studies have determined that changes in genomic DNA methylation as well as changes in DNMT levels can contribute to oncogenesis (Szyf, 1996; Szyf, 1998a; Szyf et al., 2000). Hypermethylation of specific tumor suppressors genes as well as genome-wide hypomethylation have been observed in cancer cells (Szyf, 1996; Szyf, 1998a; Szyf et al., 2000). Multiple tumorigenic pathways are known to influence *dnmt1* gene expression, whereas inhibitors of DNMT1 repress oncogenesis and tumor progression. Moreover, methylated CGs are known to be hotspots for deleterious mutations that can contribute to oncogenesis (Jones et al., 1992). Taken together these data point out to several possible ways by which DNA methylation and the proteins involved in methylation can contribute to cancer formation. However, there is still debate over whether the changes observed in methylation and DNMT levels are a consequence or cause of oncogenesis. Furthermore, there is debate over how to interpret data that are seemingly contradictory, such as hypermethylation versus hypomethylation.

(i) Methylation of tumor suppressors

A number of groups have demonstrated that inactivation of p16/INK4 and p15 by methylation is a common event in the pathogenesis of acute leukemia (Gonzalez et al., 2000; Guo et al., 2000). These two proteins are inhibitors of cyclin-dependent kinases 4/6 (CDK4/6) and are encoded by genes located on chromosome 9p21. Inactivation of p16 leads to the abrogation of the RB/p16 tumor suppressor pathway, which is a frequent event in many human cancers. Inactivation of p16/INK4 has also been documented in prostate carcinoma, where a very low incidence of point mutations has been reported, but deletions of 9p21 and promoter methylation are observed more frequently (Halvorsen et al., 2000). The same phenomenon has been studied in primary breast cancers and in

Wilms tumors, and the results indicate that the reduced p16/INK4a expression, due to *de novo* methylation, is a frequent event (Arcellana-Panlilio et al., 2000; Hui et al., 2000). Other tumor suppressors, such as BRCA1 and E-cadherin, have also been shown to be inactivated by methylation in a number of neoplasias (Esteller et al., 2000; Magdinier et al., 2000; Melki et al., 2000; Tamura et al., 2000).

An interesting observation is that methylation can also participate in loss of heterozygosity (LOH) events. For instance, following mutation of one of the p16 alelles, silencing of the remaining wild type alelle by methylation leads to total p16 inactivation (Kanai et al., 2000). Methylated DNA has also been shown to be a hotspot for mutations in many genes, including p53 (Pfeifer, 2000), where a disproportionately high number of mutations are found at methylated CG dinucleotides (Pfeifer, 2000). These observations have led to the description of a novel phenotype, the CG island methylator phenotype (CIMP), which results in multiple methylation events in a subset of cancers. This phenotype has been associated with the majority of sporadic colon cancers characterized by microsatellite instability, as well as most tumors with *k-ras* mutations (Toyota and Issa, 2000). These studies have clearly demonstrated a correlation between methylation of tumor suppressors and cancer, however, it is still not clear if these events are early events in cancer development or if they are late events that are selected by neoplastic cells for growth advantage. It is possible that in certain cases methylation is the cause and in other cases the consequence of oncogenesis.

(ii) DNMT1 hyperactivation

Since hypermethylation is a prevalent phenomenon in oncogenesis, it stands to reason that higher levels of DNMT1 should also take place in oncogenesis. DNMT1 level is regulated with the cell cycle (Singer-Sam et al., 1990; Szyf et al., 1991; Szyf et al., 1985), and time course experiments have indicated that the increase in methyltranferase activity corresponds to S-phase entry (Szyf et al., 1985). Furthermore,

dnmt1 transcript levels have been found to be over an order of magnitude higher throughout spermatogenesis than in non-dividing liver cells (Singer-Sam et al., 1990).

Dnmt1 gene expression has also been measured in studies where expression was compared betweeen normal human cells, virally transformed cells, and human cancer cells (el-Deiry et al., 1991). The results from these experiments indicate that in normal cells DNMT1 level is low, while in virally transformed cells the levels are about 50 fold higher and in human cancer cells the levels are several hundred fold higher. However, other experiments have demonstrated only a limited upregulation of DNMT1 in colon cancer cells (Lee et al., 1996).

A number of studies indicate that *dnmt1* is a downstream effector of multiple oncogenic pathways (MacLeod et al., 1995; Rouleau et al., 1995). A set of experiments has demonstrated that DNA methylation in an adrenocortical tumor cell line, Y1, is regulated by the Ras signaling pathway (MacLeod et al., 1995). In addition, transient transfection experiments verified that the expression of *dnmt1* promoter in Y1 cells is regulated by Ras and AP-1, establishing a molecular connection between a major signaling pathway involved in tumorigenesis and DNMT1 levels (MacLeod et al., 1995). Employing deletion analysis and site-specific mutagenesis to map the 5' regulatory region of the *dnmt1* gene, it was shown that three AP-1 sites are responsible for induction of *dnmt1* promoter activity by the Ras and AP-1 signalling pathway (Rouleau et al., 1995). Recently, these results have been verified by representational difference analysis, showing that the *Fos* and *Jun* oncoproteins form dimeric complexes that stimulate transcription of *dnmt1* (Bakin and Curran, 1999). In *fos*-transformed cells *dnmt1* expression was reported to be three times higher than in normal fibroblasts, and *fos*-transformed cells were shown to have approximately 20 percent more methylcytosines than normal fibroblasts (Bakin and Curran, 1999). Moreover, ectopic expression of DNMT1 induced cellular transformation, while inhibition of DNMT1 led to reversion of

transformation (Bakin and Curran, 1999). Interestingly, HDAC inhibition also caused transformation reversion, indicating the importance of chromatin structure (Bakin and Curran, 1999). Another recent study has demonstrated that DNMT1 has a causal role in cellular transformation induced by SV40 T-antigen (Slack et al., 1999a). T-antigen has been shown to cause transformation partly due to abrogation of Rb function (Riley et al., 1994). In this study it was shown that T-antigen expression leads to higher levels of *dnmt1* mRNA and protein. The T-antigen induced *dnmt1* upregulation occurs mainly at the post-transcriptional level by modulation of mRNA stability (Slack et al., 1999a). As with *fos*-transformed cells, inhibition of *dnmt1* resulted in inhibition of cellular transformation by T-antigen, indicating that *dnmt1* upregulation is a fundamental event of the T-antigen induced oncogenic pathway (Slack et al., 1999a).

Even though DNMT1 upregulation appears to be a critical step in transformation, how it contributes to oncogenesis is still a matter of debate. It would seem logical to argue that higher levels of DNMT1 would quickly translate into higher levels of genomic DNA methylation, however, only specific regions become hypermethylated. In fact, the genome in many transformed cells is globally hypomethylated (Feinberg, 1988; Feinberg et al., 1988; Feinberg and Vogelstein, 1983a; Feinberg and Vogelstein, 1983b; Feinberg and Vogelstein, 1987; Goelz et al., 1985). DNMT1 is very inefficient in *de novo* methylation and experiments directed at studying the correlation between DNMTs' upregulation and CG island hypermethylation have been controversial, with reports of both positive and no correlation between the two events being found (Eads et al., 1999; Vertino et al., 1996).

One possible way by which DNMT1 upregulation might contribute to oncogenesis is by facilitating entry into the S-phase of the cell cycle, through protein-protein interactions with DNA replication proteins such as PCNA and DMAP1 (Chuang et al., 1997; Rountree et al., 2000). Moreover, DNMT1's ability to control gene

expression as part of the transcriptional repressor complex with Rb and E2F points towards alternative ways by which changes in DNMT1 levels may contribute to cellular transformation. Recent experiments indicate that in colorectal cancer cells the cell cycle regulation of DNMT1 levels is disrupted (De Marzo et al., 1999). In normal colonic epithelial cells DNMT1 expression occurs concomitantly with proliferation markers, such as PCNA. In these DNMT1 positive cells the tumor supressor p21, which competes for PCNA binding with DNMT1, is largely absent. On the other hand, in adenomatous polyps, cells expressing p21 also expressed DNMT1, indicating how critical the cell cycle control of DNMT1 levels is.

Exactly when and how tumor supressors become *de novo* methylated in cancer cells is still a controversy, yet it seems probable that DNMT1 upregulation may lead to uncontrolled DNA replication and cellular proliferation prior to tumor suppressors hypermethylation.

(iii) Genome-wide hypomethylation

Interestingly, numerous reports have clearly demonstrated that in spite of relatively high levels of DNMT1, many tumor cells have their genomic DNA hypomethylated (Feinberg et al., 1988; Feinberg and Vogelstein, 1983a; Feinberg and Vogelstein, 1983b; Feinberg and Vogelstein, 1987; Goelz et al., 1985). In fact, when DNA methylation levels were analyzed in human cancer cells, substantial hypomethylation was observed in cancer cell genes versus their normal counterparts (Feinberg and Vogelstein, 1983a). Almost two decades ago the first demonstration of alterations in methylation of specific cellular oncogenes in human cancer was reported (Feinberg and Vogelstein, 1983b). It was shown that, while tumor supressors become hypermethylated, the oncogenes *c-Ha-ras* and *c-Ki-ras* become specifically hypomethylated in cancer cells (Feinberg and Vogelstein, 1983b). We now know, as mentioned before, that DNMT1 is a downstream target of the *ras* oncogenic pathway

(MacLeod et al., 1995; Rouleau et al., 1995). Another example of gene-specific hypomethylation in cancer occurs at the multidrug resistance 1 gene (MDR1), which encodes the cell surface P-glycoprotein, and in human cells when overexpressed can confer resistance to vincristine or doxorubicin. Several hematological malignancies, including acute myeloid leukemia (AML), have been shown to be closely associated the overexpression of the MDR1 gene (Holmes et al., 1989; Kemnitz et al., 1989). These findings led to the hypothesis that MDR1 promoter hypomethylation may be a condition for the establishment of the MDR1 P-glycoprotein-mediated multidrug resistance in AML patients (Nakayama et al., 1998). Additionally, hypomethylation of pericentromeric DNA in breast adenocarcinomas has been reported to often occur in conjunction with hypermethylation of E-cadherin (Narayan et al., 1998). In breast cancer cells, the global DNA methylation pattern was also examined versus tumor progression, and the results indicated that although DNA hypomethylation is a consistent feature of breast cancer cells, its variations do not correlate with tumor progression (Bernardino et al., 1997).

Yet another example of the correlation between genomic hypomethylation and cancer takes place in Long Interspersed Element-1 (LINE-1) retrotransposons from *Homo sapiens* (L1Hs). L1Hs are not expressed in normal somatic but are transcriptionally active and hypomethylated in malignant cells (Alves et al., 1996). These results indicate that activation of oncogenes is a clear way by which DNA hypomethylation can contribute to cellular transformation. However, hypomethylation may cause cancer by promoting genomic instability rather than by activating oncogenes (Chen et al., 1998). A mutation rate analysis of two specific genes, the endogenous hypoxanthine phosphoribosyltransferase (HPRT) gene and an integrated viral thymidine kinase (TK) transgene was performed in normal cells versus murine embryonic stem cells nullizygous for *dnmt1*, and the results demonstrated significantly elevated mutation rates at both loci

(Chen et al., 1998). These results are seemingly in contrast with the notion that methylated DNA is hypermutable. One possible way to explain this paradox is that, in spite of the hypermutability of individual 5-methylcytosines, methylated CGs, when complexed with a tightly packed chromatin, may supress genomic rearrangements as previously discussed.

In contrast with the reports above, there is evidence to suggest that genomic hypomethylation can be beneficial and supress cancer formation. Experiments, in which Apc-Min mice heterozygous for the *dnmt1* locus were given a weekly dose of the DNA methyltransferase inhibitor 5-aza-deoxycytidine, indicated that the average number of intestinal adenomas was reduced from 113 in the control mice to only 2 polyps in the treated heterozygotes (Laird et al., 1995). This result is apparently inconsistent with an oncogenic effect of DNA hypomethylation (Laird et al., 1995). In order to reconcile these paradoxical observations, researchers will have to determine the enzymatic activity not only of DNMT1 but also of the other DNMTs and MBDs. Furthermore, it will be important to determine the relative enzymatic activity of each of these proteins versus cell cycle parameters and the enzymatic activity of other cell cycle regulators.

(iv) Inhibitors of DNMT1 as anticancer agents

Since it has been widely reported that cancer cells have relatively high levels of DNMT1 and that tumor supressors are hypermethylated, it stands to reason that DNMT1 inhibitors could reverse those parameters, thus inhibiting oncogenesis. The obvious problem with this theory is that global genomic hypomethylation, as well as hypomethylation of oncogenes, are also a feature of neoplastic cells. Therefore, by inhibiting DNMT1, one might actually induce cellular transformation rather than reverse it. Taking all this into consideration, several studies set out to determine if inhibition of DNMT1 could become a useful tool in cancer treatment. The most common inhibitors of DNMT1 are the nucleoside analogs 5-azacytidine (5-azaC) and 5-aza-2'-deoxycytidine

(5-aza-dC), which get incorporated into the genome during DNA replication and are able to form irreversible adducts with DNMT1 when methylation occurs (Wu and Santi, 1985). It has been observed that cellular treatment with either 5-azaC or 5-aza-dC leads to induction of silent genes and genomic hypomethylation (Jones, 1985). Since it was demonstrated that tumor suppressor genes could be reactivated by these nucleoside analogs, they became often used in cancer therapies (Momparler and Bovenzi, 2000). The major problem with this strategy is the pleiotropic effects observed in cells following treatment with either 5-azaC or 5-aza-dC. In *Aspergillus*, where DNA methylation is negligible, 5-azaC is able to induce novel developmental phenotypes, indicating that major effects can occur independently of methylation (Tamame et al., 1983). Furthermore, 5-azaC has been shown to be highly mutagenic in both mammalian and yeast cells, the latter also lacking genomic methylation (Amacher and Turner, 1987; Zimmermann and Scheel, 1984).

These concerns prompted the development of alternative DNMT1 inhibition strategies. One of the strategies used was *dnmt1* antisense mRNA expression (MacLeod and Szyf, 1995). Transient transfection of a murine adrenocortical tumor cell line Y1 with antisense oligonucleotides resulted in DNA demethylation, distinct morphological alterations, inhibition of anchorage-independent growth, and decreased tumorigenicity in syngeneic mice (MacLeod and Szyf, 1995). Subsequent experiments indicated that injection of *dnmt1* antisense oligonucleotides intra-peritoneally inhibits the growth of Y1 tumors in syngeneic LAF1 mice, reduces the DNMT1 levels, and induces demethylation of the adrenocortical-specific gene C21 and its expression in tumors *in vivo* (Ramchandani et al., 1997). Recently, it was demonstrated that out of 16 known tumor suppressors and cell cycle regulators, the cyclin- dependent kinase inhibitor p21 was the only tumor suppressor induced in the human lung cancer cell line, A549, following DNMT1 inhibition. In this study both antisense oligonucleotides as well as novel

hemimethylated hairpin oligonucleotides that function as DNMT1 antagonists were used. As mentioned before, the CG sites upstream of the endogenous p21 gene are unmethylated, indicating that the induction of p21 expression does not involve DNA methylation (Milutinovic et al., 2000). Modified *Dnmt1* antisense oligonucleotides (MG98), currently generated at MethlyGene Inc. in Montreal (Fournel et al., 1999), are undergoing phase I clinical trials. Thus, it appears that DNMT1 is a good target for anticancer therapy, but questions as to the molecular mechanisms by which these potential drugs act will be the focus of research for years to come.

IV. Immunodeficiency, Centromere instability and Facial anomalies (ICF) syndrome

ICF syndrome is a rare recessive autosomal condition that can be recognized in patients by the presence of a variable immunodeficiency, instability of the pericentromeric heterochromatin, specifically of chromosomes 1, 9, and 16, genomic DNA hypomethylation, and a number of facial anomalies (Smeets et al., 1994). Recently, inactivation of the *dnmt3b* gene indicated that it is specifically required for methylation of centromeric minor satellite repeats (Okano et al., 1999; Xie et al., 1999). Moreover, experimental evidence now indicates that mutations of human *dnmt3b* gene are found in patients with ICF syndrome, linking loss of enzymatic activity with the syndrome (Xu et al., 1999).

V. Rett syndrome

Rett syndrome is a progressive neurodevelopmental disorder and one of the most common causes of mental retardation in females, with an incidence of 1 in 10.000-15.000 (Amir et al., 1999). Rett patients seem to develop normally until 6-18 months of age, then progressively lose speech and intentional hand use, and develop microcephaly, seizures, autism, ataxia, intermittent hyperventilation and stereotypic hand movements. The patients frequently survive into adulthood since after the initial stages the condition

stabilizes. The fact that this syndrome almost never occurs in males led to the hypothesis that Rett is caused by an X-linked dominant mutation with lethality in hemizygous males. Using a systematic gene screening approach, mutations in the MeCP2 gene, located in the X-chromosome, was identified as the cause of certain cases of Rett. These findings provide a clear indication of the critical importance of epigenetics in human physiology and pathology.

VI. Replication and methylation in mammalian cells

A potential link between DNA methylation and replication in mammalian cells was first observed when densely methylated islands (DMIs) were described in two distinct origins of replication. One DMI was reported to reside at the *oriβ* site, at the dihydrofolic acid reductase (DHFR) locus, and the other DMI was reported to reside at *oriS14*, located within the coding sequence for ribosomal protein S14 on chromosome 2q (Tasheva and Roufa, 1994; Tasheva and Roufa, 1995). Using a sodium bisulfite mapping method (please see Chapter II for a detailed description of the methodology), it was observed that every cytosine contained within the DMIs was methylated. This was the first report of such an island. The DMI within *oriS14* was reported to be 127 bp long, and the DMI within *oriβ*516 bp long. Interestingly, when cell growth and DNA replication were arrested, methylation of CA, CT, and CC dinucleotides was lost and the islands display only a subset of their originally methylated CG dinucleotides (Tasheva and Roufa, 1994; Tasheva and Roufa, 1995). This report indicated several possible roles for methylation in DNA replication. However, subsequent experiments performed by several independent groups, including ours, failed to reproduce those findings (Antequera and Bird, 1999; Araujo et al., 1998; Delgado et al., 1998; Rein et al., 1999; Rein et al., 1997a; Rein et al., 1997b). One of these reports, while examining the same *oris* previously studied, indicated that only the CGs were methylated (Rein et al., 1997a). It was therefore suggested that high-density clusters of methylated CG dinucleotides may

play a role in either the establishment or the regulation of mammalian origins (Rein et al., 1997b). On the other hand, experiments performed by another group revealed that unmethylated CG islands, but not their flanks, were abundantly present in very short nascent strands, suggesting that they are replication origins (Delgado et al., 1998). Furthermore, it was demonstrated that the bulk of the CG islands replicated coordinately early in S-phase, implying that unmethylated CG islands are initiation sites for both transcription and DNA replication, and may represent genomic footprints of replication initiation sites (Delgado et al., 1998). Results presented in chapter III, addressing this same issue, indicated that, while no DMI is observed, certain origins are methylated on CG dinucleotides, while others are not (Araujo et al., 1998).

It is possible that unmethylated origins represent early firing origins located mainly at actively transcribed genes, whereas methylated origins represent late firing origins at nontranscribed regions. In support of this hypothesis it has been demonstrated that the X-linked, fragile mental retardation (*FMR1*), locus replicates late when is methylated and early when is not (Torchia et al., 1994). Moreover, housekeeping genes, which are mostly not methylated, are usually replicated relatively early in S-phase (Zannis-Hadjopoulos and Price, 1998). Further methylation studies will be necessary in order for us to understand the potential functions of methylated CGs in origin usage and replication timing.

(i) DNMT1 and replication

Although the role of methylated CGs in replication remains unclear, it seems likely that DNMT1 is an important player in regulating initiation of replication. One interesting observation, as mentioned before, is that in *Drosophila*, in which methylated bases are not detected, a DNMT1 like enzyme is found and results indicate that it is able to associate with PCNA (Hung et al., 1999). The ability of DNMT1 to associate with PCNA was previously reported in mammalian cells, where it competes with the tumor

suppressor p21 for PCNA binding (Chuang et al., 1997). Interestingly, p21 and DNMT1 gene expressions are inversely correlated (Chuang et al., 1997). As previously mentioned, when p21 interacts with PCNA, the latter is no longer able to interact with pol-δ, thus replication is blocked. DNMT1 is targeted to replication foci at the G1-S phase border and replication and methylation occur concurrently (Araujo et al., 1998; Leonhardt et al., 1992). Recent results indicate that at replication foci, DNMT1 is able to interact with HDAC2 at late S-phase and with a novel protein, DMAP1, throughout S-phase (Rountree et al., 2000). The fact that HDAC2 interacts with DNMT1 at late S-phase indicates the possibility that this interaction is needed to replicate the methylated/hypoacetylated population of genes.

These observations have led to the hypothesis that DNMT1 hyperactivity can induce transformation by overriding the cell cycle stop signals and deregulate DNA synthesis rather than by methylating tumor suppressors (Szyf et al., 2000). In support of this hypothesis, it has been shown that within adenomatous polyps, although DNMT1 expression coincides with the expression of other cell proliferation markers, many DNMT1-expressing cells also expressed p21, indicating that DNMT1 is indeed able to override p21's cell cycle arrest signal (De Marzo et al., 1999). Moreover, the results presented in chapter IV, indicate that inhibition of DNMT1 leads to inhibition of DNA replication in lung cancer A549 cells (Knox et al., 2000). The recent observation that DNMT1 interacts with Rb and E2F to repress E2F responsive genes (Robertson et al., 2000) indicates the possibility that DNMT1 works on multiple methylation-independent mechanisms that affect cell cycle progression. Consequently, DNMT1 inhibition will result in induction of E2F responsive genes, such as p21, independent of the methylation status of the gene (Hiyama et al., 1998; Milutinovic et al., 2000).

The following chapters study the relationship between DNMT1 and DNA replication. We have identified and mapped mammalian initiation sites of DNA

replication and their physical relationship with methylated regions. Furthermore, experiments illustrating the importance of DNMT1 in DNA replication and in the inheritance of the methylation pattern are shown.

Chapter II

Identification of Initiation Sites for DNA Replication in the Human *dnmt1* (DNA Methyltransferase) Locus

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Abstract

Vertebrates have developed multiple mechanisms to coordinate the replication of epigenetic and genetic information. *Dnmt1* encodes the maintenance enzyme DNA methyltransferase that is responsible for propagating the DNA methylation pattern and the epigenetic information that it encodes during replication. Direct sequence analysis and bisulfite mapping of the 5' region of *dnmt1* have indicated the presence of many sequence elements associated with previously characterized origins of DNA replication. This study tests the hypothesis that the *dnmt1* region containing these elements is an origin of replication in human cells. First, we demonstrate that a vector containing this *dnmt1* sequence is able to support autonomous replication when transfected into HeLa cells. Second, using a gel retardation assay, we show that it contains a site for binding of OBA (Ors Binding Activity), a recently purified replication protein. Finally, using competitive PCR we show that replication initiates in this region *in vivo*. Based on these lines of evidence, we propose that initiation sites for DNA replication are located between the first intron and exon 7 of the human *dnmt1* locus.

Introduction

Mammalian DNA replication initiates from multiple sites throughout S phase (DePamphilis, 1993). These sites are determined both by cis-acting DNA sequences, known as replicators, and by trans-acting elements, defined by initiator proteins that bind to the replicator (DePamphilis, 1993; Stillman, 1994). Due to the complexity of the mammalian genome, initiation of replication has been studied in greater detail in prokaryotes and lower eukaryotes. However, a number of techniques, including methods for the isolation of newly synthesized DNA in combination with competitive PCR techniques, have led to the identification of new mammalian replication initiation regions as well as characterization of additional initiation sites at established origins (Delgado et al., 1998; Giacca et al., 1994; Kobayashi et al., 1998; Pelizon et al., 1996; Tao et al., 1997; Vaughn et al., 1990). The best example of such a region is the extensively studied initiation region mapped downstream of the Chinese hamster dihydrofolate reductase gene (*dhfr*) (Kobayashi et al., 1998; Pelizon et al., 1996; Vaughn et al., 1990) where multiple initiation sites have been shown distributed over a 55 kb region.

Metazoan chromosomal origins of replication comprise specific sequence motifs. Replication initiation elements can be moved to new chromosomal sites and still initiate replication (Aladjem et al., 1998; Handeli et al., 1989; Orr-Weaver, 1991), whereas deletions of specific sequences in these elements abolish their ability to initiate replication (Aladjem et al., 1995; Aladjem et al., 1998). The ability of specific mammalian DNA sequences to support autonomous replication of plasmids into which they have been inserted has been used in the past as an assay for the presence of an origin of replication in that sequence (Frappier and Zannis-Hadjopoulos, 1987; Heinzel et al., 1991; Kipling and Kearsey, 1990; Landry and Zannis-Hadjopoulos, 1991; McWhinney and Leffak, 1990; Pelletier et al., 1997; Todd et al., 1995; Zannis-Hadjopoulos et al., 1994). In several instances, the ability of certain specific sequences to support

autonomous replication of plasmids has been directly validated by mapping of the same sequences as chromosomal initiation sites for replication in living cells (Zannis-Hadjopoulos et al., 1994).

Whereas only a few origins of replication have been identified in mammals, certain sequence motifs have been found to be common in most of these origins (Zannis-Hadjopoulos and Price, 1998). These motifs include A/T rich regions, ATTA and ATTTA nuclear matrix attachment motifs and yeast ARS consensus sequence (ACS) elements (WTTTATRTTTW), identified in autonomously replicating sequences in *Saccharomyces cerevisiae* (Kipling and Kearsey, 1990; Palzkill and Newlon, 1988). Furthermore, a origin binding activity (OBA) was recently purified from HeLa cells (Ruiz et al., 1995) through its ability to bind to the 186-bp minimal replication origin of *ors 8* (Frappier and Zannis-Hadjopoulos, 1987), and a 36 bp sequence (A3/4) that is found in a number of mammalian replication origins. OBA sediments at approximately 150 kDa in a glycerol gradient, and it cofractionates with DNA polymerases α and δ, topoisomerase II, and replication protein A (RP-A) (Ruiz et al., 1995).

In addition to genetic elements, epigenetic components, such as DNA modification by methylation and chromatin structure, have been proposed to be characteristic determinants of origins of replication (Delgado et al., 1998; Rein et al., 1997b; Tasheva and Roufa, 1994). We have previously shown that origins of replication like regulatory regions of genes, are differentially methylated (Araujo et al., 1998). Furthermore, it was recently shown that non-methylated CpG islands are enriched in early replicating nascent DNA (Delgado et al., 1998). However, due to the limited number of mammalian origins of replication that have been characterized thus far, it is still too early to draw general conclusions regarding the critical genetic and epigenetic determinants of origin function and its differential regulation. Additional origins have to

be characterized to allow for understanding of the general rules governing origin function in mammals.

DNA methylation is a post-replicative covalent modification of DNA that is catalyzed by the DNA methyltransferase enzyme (DNMT1) (Ramchandani et al., 1998; Razin and Riggs, 1980; Yisraeli and Szyf, 1984). In vertebrates, 60 - 80% of cytosines in CpG sequences are methylated; the non-methylated CpGs are distributed in a non-random manner generating a pattern of methylation that is gene- and tissue-specific (Yisraeli and Szyf, 1984). Recent breakthroughs have provided conclusive molecular evidence for the hypothesis that DNA methylation of a gene represses its expression (Kass et al., 1997b; Razin and Riggs, 1980). DNA methylation can repress gene expression directly, by inhibiting binding of transcription factors to regulatory sequences (Kass et al., 1997a), or indirectly, by signaling the binding of factors specific for methylated DNA (Becker et al., 1987; Razin and Cedar, 1991). Two methylated DNA binding proteins, MeCP2 and MeCP1 (Kass et al., 1997a; Nan et al., 1997), that can repress transcription in a methylation-dependent manner, have been recently characterized. The carboxy-terminal half of MeCP2 contains a repressor domain, which associates with the transcription repressor mSin3A and histone deacetylase (Jones et al., 1998; Nan et al., 1998). Since DNA replication and methylation are catalyzed by different enzymes, it is likely that vertebrates have developed multiple mechanisms to coordinate the replication of the genetic and epigenetic information (Szyf, 1996). In support of this hypothesis, we have shown that the expression of the *dnmt1* gene, encoding the maintenance DNMT1 enzyme, is regulated with the growth state of cells (Szyf et al., 1991) and that methylation of DNA occurs concurrently with replication (Araujo et al., 1998). Another mechanism that coordinates methylation of nascent DNA with its replication is the binding of DNMT1 to PCNA at the replication fork during the S-phase of the cycle (Chuang et al., 1997). P21, a cyclin kinase inhibitor that triggers cell

arrest, competes with DNMT1 for PCNA binding and could dislodge it from the replication fork (Chuang et al., 1997).

We have previously resolved the genomic structure of the gene encoding the human DNMT1 and showed that it is composed of at least 40 exons and 39 introns spanning a distance of 60 kb (Ramchandani et al., 1998). While characterizing the 5' regulatory regions of *dnmt1*, we identified an intronic sequence immediately upstream of exon 2 that comprised many of the sequence elements previously described for replication initiation regions (Zannis-Hadjopoulos and Price, 1998). In this study, we test the hypothesis that this region of the *dnmt1* locus contains initiation sites for DNA replication, thus physically linking the regulatory region of the gene encoding the DNMT1 enzyme with an origin of replication in human cells. Using several lines of evidence based on autonomous replication assays, OBA binding assays, and *in vivo* mapping by competitive PCR, we propose that the 5' region of the *dnmt1* gene comprises functional initiation sites for DNA replication.

Materials and methods

Cell culture and Plasmid Construction- HeLa cells (monolayers) were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum. The c1 construct (fig. 1A) was generated using genomic subclones of the *dnmt1* pFTR1 that were previously described (Ramchandani et al., 1998). pFTR1 was digested with *Xba I* and a 2.38 kb fragment containing intronic sequence immediately upstream of exon 2 was isolated and inserted into a *Xba I* digested pBluescript SK-plasmid[2].

DNA sequencing - The 2.38 kb insert was fully sequenced by the dideoxy chain termination method using a T7 DNA sequencing kit (Pharmacia).

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[2] The nucleotide sequence for the dnmt1 c1 construct has been deposited in the GenBank database under GenBank Accession Number AF119248.

Gel retardation assay - The following oligonucleotide duplexes containing putative OBA binding sites were used: A3/4 oligonucleotides: 5'-CCTCAAATGGTCTCCAATTTTCCTTTGGCAAATTCCI (sense); 5'-GGAATTTGCCAAAGGAAAATTGGAGACCATTTGAGG (antisense); A3/4 homologous (*d n m t 1*) oligonucleotides: 5'-TTGTTATGGGCTGTTGTCAGACCCAACTGG (sense); 5'-TCCAGTTGGGTCTGACAACAGCCCATAACAA (antisense). The sense oligonucleotides were radiolabeled in a mixture containing 5 µl of oligonucleotide, 50 µCi of (γ^{32}P)-ATP (3000 Ci/mmol, Amersham), and 10 units of T4 Polynucleotide Kinase (Boeringer-Mannheim) in a final volume of 50 µl and purified on a Sephadex G25 microcolumn (Pharmacia). The antisense strand was then added (1 µg), and the duplex was annealed following 5 minutes of boiling and gradual cooling of the reaction mixture to room temperature. The DNA binding reaction contained 10 ng of labelled duplex DNA, 1 µl poly dI-dC (1 µg/µl), 2 µl of 3A buffer (10mM Tris-HCl, 80 mM NaCl, 10 mM EDTA, 10 mM b-mercaptoethanol, 1% triton X-100, 40% glycerol), 2 µl of 40nM ATP and 200 ng of purified OBA in a 20 µl final volume. In the competition experiments, 10 µg of the respective cold oligonucleotide competitor were added. The mixtures were incubated for 30 minutes on ice, and then subjected to electrophoresis on a 4% Polyacrylamide Gel Electrophoresis (PAGE) for 1 hour and 30 minutes at 180 V. The gel was then dried and exposed for autoradiography.

Autonomous replication assay - 5 µg of the c1 construct and pBluescript (SK) were each separately transfected into HeLa cells by the calcium phosphate coprecipitation method and the autonomous replication assay based on BrdU incorporation and density shift was performed as previously described (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991; Pelletier et al., 1997).

Competitive PCR assay - The following primers were used for the *dnmt1* locus (bp # 1 was assigned to the first 5' bp of the c1 construct): c1 region, 5'-AGAGACATCCTGAAGAATGAGTT (p1, sense, starting at bp 240), 5'-AGTGAGCCGTGATTGCATCA (p2, antisense, starting at bp 643), 5'-GATTGCATCAGGGGCAGGTCATATAGTTGG (primer used to design competitor at c1); c2 region, 5'-AGACGTAGAGTTACATCCAG (p3, sense, ~ 6.3 kb downstream of p2), 5'-GCTCTTTCAGGTTCTTCTGC (p4, antisense, ~ 7 kb downstream of p2), 5'-TTACATCCAGAATCAGGAACGCGCACTGAA (primer used to design competitor at c2); c3 region, 5'-CTACAGGCTCAAGCCACCAT (p5, sense, starting at bp 2906), 5'-TTCCCAAGCTATTCACTAGT (p6, antisense, starting at bp 3300), 5'-ATTCACTAGTCAGGCAATGCTGTCTCAGTC (primer used to design competitor at c3); c4 region, 5'-CACTCTAGACTGCGGGG (p7, sense, starting at bp 1718), 5'-TCATGCCATTGCACTCTAGC (p8, antisense, starting at bp 2159), 5'-GCACTCTAGCACCACCCAACTATTAGCAGC (primer used to design competitor at c4); c5 region, 5'-CTCCCGAGTTCAAGCAATTC (p9, sense, ~ 4 kb upstream of p1), 5'-GCTGTACAGGGGAAGAGCTG (p10, antisense, ~ 3.5 kb upstream of p1), 5'-CAAGCAATTCGACTGGGTTTTGCCATGTTG (primer used to design competitor at c5). Approximate distances between some of the *dnmt1* primers were calculated from southern blot analysis (Yisraeli and Szyf, 1984). The following primers were used for the *c-myc* locus (GenBank accession no: J00120): MO region, 5'-TGCCGTGGAATAACACAAAA (p11, sense, starting at bp 761), 5'-CTTTCCAGGTCCTCTTTCCC (p12, antisense, starting at bp 1134), 5'-TAACACAAAAGATCATTTCAGGGAGCAAAC (primer used to design competitor at MO); MF region, 5'-GGTTCTAAGATGCTTCCTGG (p13, sense, starting at bp 7848), 5'-ATGGGTCCAGATTGCTGCTT (p14, antisense, starting at bp 8299), 5'-TGCTTCCTGGGAGAAGGTGAGAGGTAGGCA (primer used to design competitor at

MF). The PCR reactions for c1 and c2 regions were performed as follows: 3 minutes at 94 °C, then 30 cycles of 1 minute at 94 °C, 1 minute at 55 °C, 1 minute at 72 °C, followed by 5 minutes at 72 °C. The PCR reactions for the remaining regions were performed as follows: 3 minutes at 94 °C, then 30 cycles of 1 minute at 94 °C, 1 minute at 60 °C, 1 minute at 72 °C, followed by 5 minutes at 72 °C. Isolation of nascent DNA, *c-myc* PCR primer design, and competitor construction were done as previously described (Araujo et al., 1998; Tao et al., 1997; Vassilev et al., 1990; Zannis-Hadjopoulos et al., 1981).

Bisulfite mapping analysis- The following primers were used for the *dnmt1* site (bp # 1 was assigned to the first 5' bp of the c1 construct): 5'-TTATGTTGTTTAGGGTTGGATT (Morif 1, sense, starting at bp 6); 5'-TTTATAAGTTATTTTTTTATTATAGTT (Morif 2, sense, starting at bp 38) 5'-AAAACAACCAACTAATATTCCT (Birom 1, antisense, starting at bp 415); 5'-TCAAAATAATAACCCAAAACCA (Birom 2, antisense, starting at bp 451). Bisulfite mapping was performed as previously described (Araujo et al., 1998).

Results

Sequence elements associated with characterized origins of DNA replication are present in the first intron of the dnmt1 locus - Sequence analysis of a 2.38 kb construct (c1 construct) (Fig. 1A, 1B) containing intronic sequence immediately upstream of exon 2 revealed several sequence elements that are associated with characterized origins of replication (Zannis-Hadjopoulos and Price, 1998). These elements include: a 536 bp stretch which is 77% A/T-rich; at least nineteen ATTA and ATTTA nuclear matrix attachment motifs (Zannis-Hadjopoulos and Price, 1998); a perfect match of the 11 bp ARS consensus sequence (WTTTRTATTTW) of the yeast *S. cerevisiae* (Palzkill and Newlon, 1988), a methylated CpG cluster (Araujo et al., 1998; Rein et al., 1997b); and a

region homologous to the binding site of OBA, a human origin binding activity (Ruiz et al., 1995).

The c1 construct is able to support autonomous replication - In order to ascertain whether the *dnmt1* sequence indeed contained an origin of replication, we tested first the ability of the c1 construct to support autonomous replication using a density shift assay following bromodeoxyuridine (BrdU) incorporation into nascent DNA, as previously described (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991; Pelletier et al., 1997). The results (Fig. 2A), demonstrate a density shift of the c1 construct, corresponding to incorporation of BrdU into one (heavy-light) or both of the nascent DNA strands (heavy-heavy), indicating one and two or more rounds of replication, respectively. The SK plasmid vector, on the other hand, did not exhibit a similar shift, and all plasmid DNA was recovered in the unreplicated (light-light) form of input DNA (Fig. 2B). The linearity of each gradient was verified by measuring the refractive index (RI) of every other fraction.

The dnmt1 A3/4 homologous region is able to bind OBA- Sequence analysis revealed a 30 bp stretch that is 86 % homologous to the 36 bp A3/4 sequence, identified as part of the binding site for OBA[1]. An oligonucleotide of this 30 bp *dnmt1* sequence was synthesized and its binding activity to OBA was assayed by gel retardation in the presence of excess nonspecific competitor poly (dI-dC). The results (Fig. 3) show that the *dnmt1* oligonucleotide is able to bind to and form a complex with OBA (lane 3) of the same size as the complex formed when the 36 bp A3/4 oligonucleotide is used (lane 6). This binding is specific, since the *dnmt1* oligonucleotide was able to compete for the OBA binding (lanes 4 and 8), as effectively as the 36 bp A3/4 oligonucleotide when it was used as competitor (lanes 5 and 7).

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[1]Ruiz, M., Matheos, D., Price, G. B., and Zannis-Hadjopoulos, M. Manuscript submitted

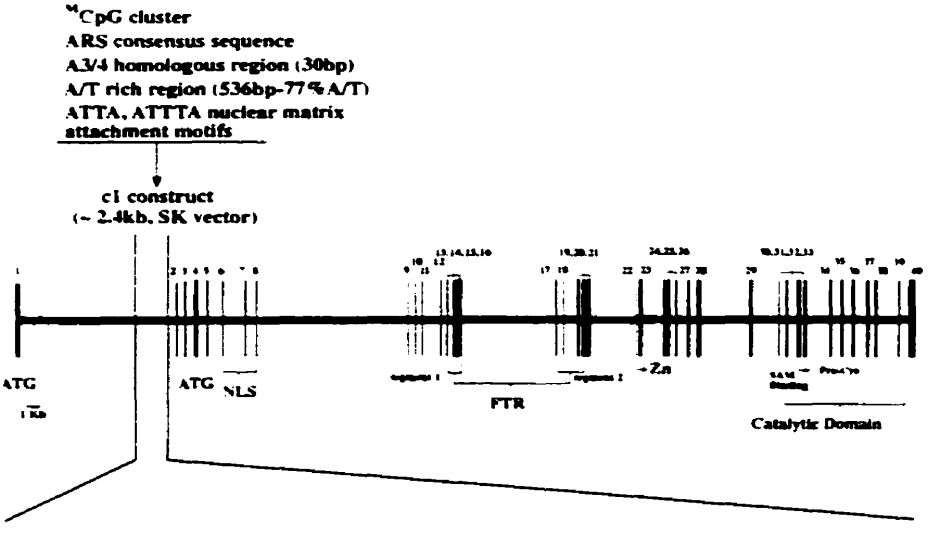
Initiation sites for DNA replication are present within the dnmt1 locus- In order to quantitatively analyze the replication origin activity of the c1 construct and map the initiation site(s) in it, a series of competitive PCR experiments were performed in order to measure and compare nascent DNA abundance of five different regions within the *dnmt1* locus (Fig. 4). The top gel (Fig. 4AI) of each of the regions (c1 - c5, MO, and MF) corresponds to the competitive PCR amplification of HeLa genomic DNA, used to standardize the differences among primers and competitors with respect to their amplification efficiencies. The linearity of each of the competitive PCR analysis was verified by plotting the ratio of competitor DNA concentration over target DNA concentration (y-axis) versus the concentration of competitor DNA (x-axis) (Fig. 4B). Small nascent DNA ranging in size between 800 bp to 1.3 kb was isolated by the nascent strand extrusion method (Araujo et al., 1998; Tao et al., 1997; Zannis-Hadjopoulos et al., 1981), followed by sucrose gradient sedimentation and further purification on agarose gel, as previously described (Zannis-Hadjopoulos et al., 1981). The bottom gel (Fig. 4AII) of each of the regions (c1 - c5, MO, and MF) corresponds to the competitive PCR amplification of HeLa nascent DNA, and the bar graphs of the *c-myc* locus (Fig. 4C) and the *dnmt1* locus (Fig. 4D) represent the nascent DNA abundance obtained by the respective competitive PCR assays. Two regions, MO and MF, within the well characterized initiation region associated with the *c-myc* gene (Vassilev et al., 1990) were used as control for the quality of the nascent DNA preparation (Fig. 4C). The results (Fig. 4C) show that a previously characterized *c-myc* origin of replication (MO) is present in the nascent DNA preparation at 6 x 10^{3} copies of nascent DNA, whereas no signal is observed for a region located 7 kb downstream from the origin of replication (MF), indicating that the nascent DNA used did not contain degraded parental DNA (Araujo et al., 1998; Tao et al., 1997). The same preparation of nascent DNA was used in order to determine the relative abundance of five regions within the *dnmt1* locus. The results

(Fig. 4D) indicate that two regions of DNA located between the first intron and the seventh exon of *dnmt1* (c1 and c3) are highly abundant in the nascent DNA fraction, containing approximately 3 x 10^{-4} copies of nascent DNA each. In contrast, the relative abundance of regions of *dnmt1* residing approximately 3 kb upstream of the c1 region (c5) or 5 kb downstream of the c3 region (c2) are far less abundant, containing 6 x 10^{-3} copies of nascent DNA (c5) and no nascent DNA (c2) respectively. The data are consistent with the hypothesis that at least two major initiation sites for DNA replication are localized between the first intron and exon 7 of the *dnmt1* gene.

A methylated CpG cluster is associated with the initiation sites for DNA replication- A number of origins of replication, among them *oriβ* at the *dhfr* locus have been previously shown to be associated with a methylated CpG cluster (Rein et al., 1997b), whereas other origins of replication have been shown to be unmethylated in the associated CpG sequences (Araujo et al., 1998; Delgado et al., 1998), suggesting differential methylation of replication origins (Razin and Riggs, 1980). To determine whether *dnmt1* is associated with either a methylated or unmethylated CpG cluster, we performed a bisulfite methylation mapping analysis of a CpG cluster located within the c1 construct region (Fig. 5A). Twelve clones were selected and the eight CpGs residing in this region were analyzed in each clone with respect to their methylation status. Six of them were methylated in all clones tested, one CpG was unmethylated, and, interestingly, one CpG was methylated in six out of the twelve clones tested (Fig. 3A, 3B). A sample result (Fig. 5B) of the bisulfite analysis indicates the methylated CpGs and their respective positions. The results show that the *dnmt1* initiation region is associated with a methylated CpG cluster, as was previously demonstrated for the *dhfr oriβ* (Rein et al., 1997b).

A

Human DNA Methyltransferase locus



B

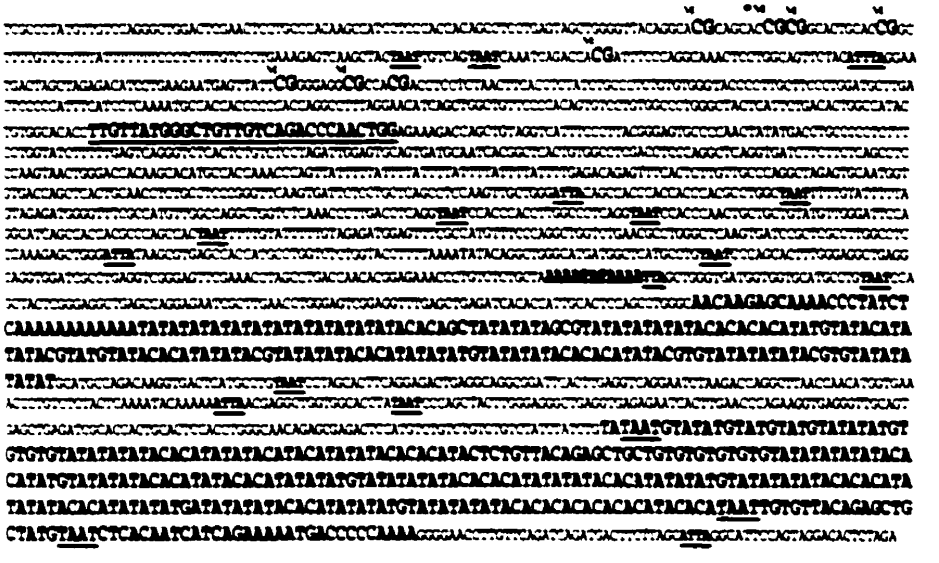
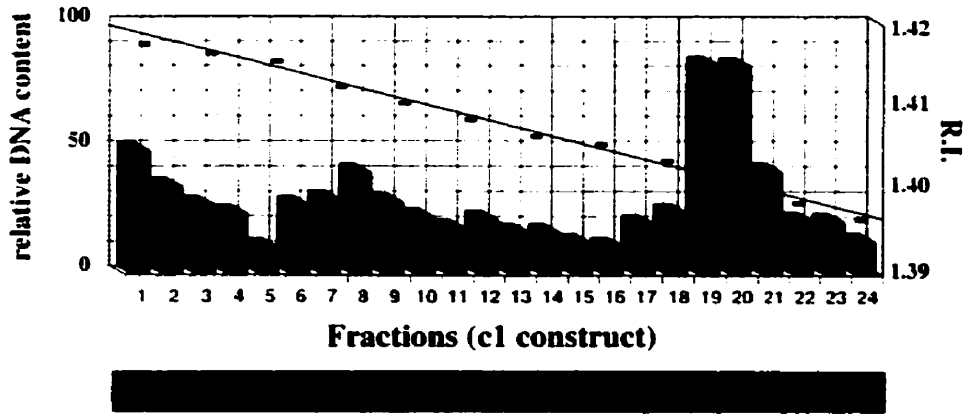


Fig. 1. Sequence elements, autonomous replication and OBA binding of the *dnmt1* c1 region. **A**, schematic diagram of the exon-intron structure of the human *dnmt1* locus. Exons are depicted as vertical bars and numbered above, introns as horizontal bars. Regions containing exons coding for specific functional domains are depicted, NLS = nuclear localization signal, FTR = replication foci targeting region, Zn = zinc binding domain, Sam binding = S-adenosyl methionine binding motif, Pro-Cys = proline-cysteine catalytic motif, Catalytic domain = region conserved in cytosine 5' methyltransferases. Exonal location of proposed initiation codons = ATG. **B**, Sequence analysis of a 2.38 kb construct (c1 construct consists of a Xba I fragment from ftr 1 phage [28] depicted in Fig. 1A) containing intronic sequence immediately upstream of exon 2 exhibited a remarkable 536 bp stretch 77% A/T rich. At least 19 ATTA and ATTTA nuclear matrix attachment motifs, as well as a perfect match of the 11 bp ARS consensus sequence (WTTTRTATTTW) reside in this region. A methylated CpG cluster and A3/4 homologous region are also present. Asterisk (*), CpG methylation observed in 50% of the clones tested.

A


relative DNA content
100
50
0
1.42
1.41
1.40
1.39
R.I.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Fractions (c1 construct)

B

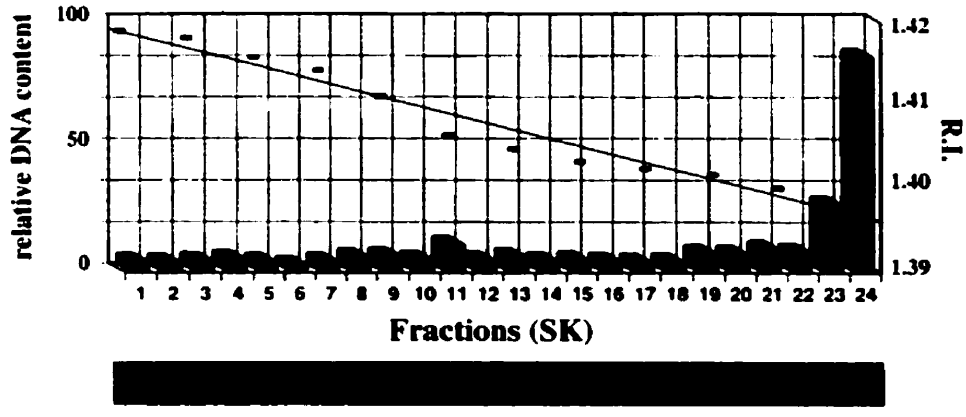

relative DNA content
100
50
0
1.42
1.41
1.40
1.39
R.I.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Fractions (SK)

Fig. 2. c1-bearing plasmid (**A**) and a pBluescript SK control (**B**) were transfected into HeLa cells and a density shift assay following BrdU incorporation were performed as previously described (19). The different fractions were spotted onto a Hybond N+ membrane and hybridized with a ^{32}P labeled probe bearing the vector sequence, and the intensity of the signal at each fraction (lower panels) was quantified by a phosphorimager and presented as relative DNA content (signal at specific spot/total signal). The linearity of each gradient was verified by measuring the refractive index (R. I.) of every other fraction and plotted as a linear graph.

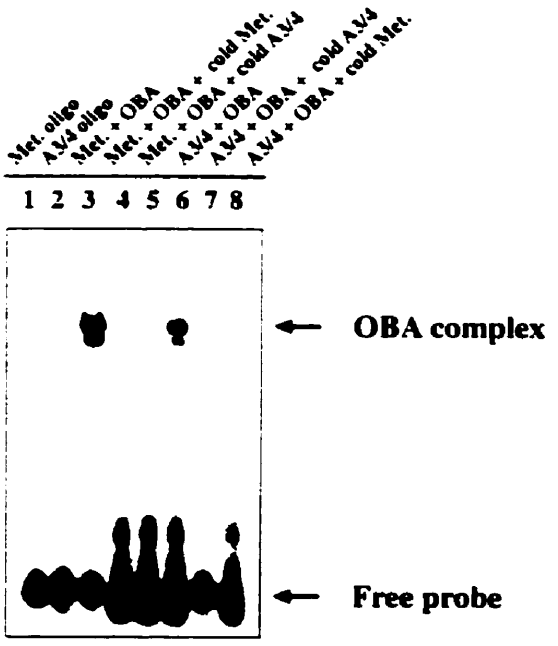

Met. oligo
A3/4 oligo
Met. + OBA
Met. + OBA + cold Met.
Met. + OBA + cold A3/4
A3/4 + OBA
A3/4 + OBA + cold A3/4
A3/4 + OBA + cold Met.
1 2 3 4 5 6 7 8
OBA complex
Free probe

Fig. 3. Sequence analysis of the first intron of the *dnmt1* exposed a 30 bp stretch 86 % homologous to the 36 bp A3/4 sequence. A ^{32}P-labeled double-stranded oligonucleotide bearing the 30 bp *dnmt1* sequence (Met.) or the consensus A3/4 sequence was incubated with 200 ng of purified OBA in the presence excess non-specific competitor poly dI-dC (1 μg). Specific competitors were added (10 μg of cold Met. or A3/4 oligonucleotide) in the competition experiments.

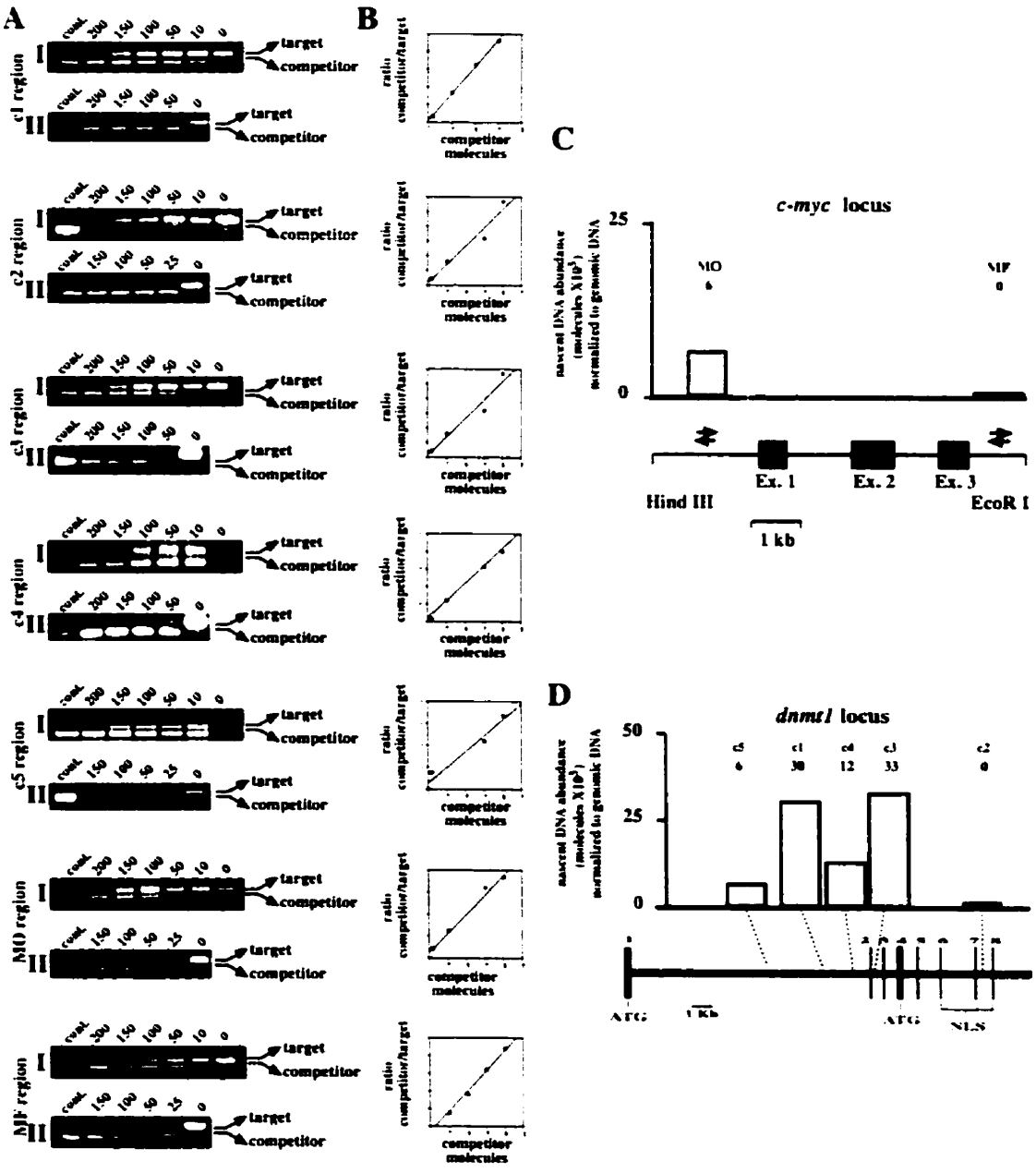
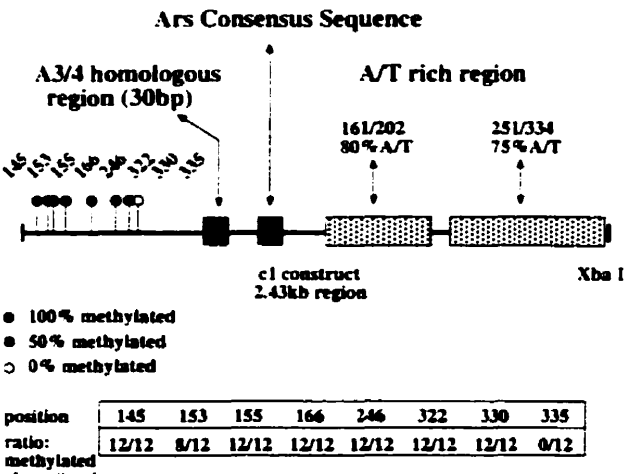

A
B
C
D
target
competitor
competitor molecules
ratio competitor/target
c1 region
c2 region
c3 region
c4 region
c5 region
MO region
MF region
c-myc locus
MO
MF
Ex. 1
Ex. 2
Ex. 3
Hind III
EcoR I
1 kb
dnmt1 locus
ATG
NLS

Fig. 4. Competitive PCR mapping of an *in vivo* origin of replication residing in the *dnmt1* locus. A series of competitive PCR experiments were performed in order to measure and compare the abundance of 5 different regions within the *dnmt1* locus (c1 - c5) in nascent DNA. In order to normalize the differences in primers and competitors amplification efficiencies, HeLa genomic DNA was used as template for the competitive PCR assay (4AI, top gel for each indicated regions). The linearity of each of the competitive PCR analysis was verified by plotting the ratio of competitor DNA concentration over target DNA concentration (y-axis) versus the concentration of competitor DNA (x-axis) (Fig. 4B). Small nascent DNA ranging in size between 800 bp to 1.3 kb was used as template for the competitive PCR assay (4AII, bottom gel for each of the indicated regions). The competitor molecule number used in each competitive PCR is displayed on top of every gel. As a control for the purity of the nascent DNA preparation, we determined the abundance of the *c-myc* origin of replication (MO) and a sequence residing 7 kb downstream (MF)(4C). The position of each of the tested regions is illustrated in the schematic diagrams of both *dnmt1* and the *c-myc* locus below the respective bar graphs. The bar graph results (4D) represent nascent DNA abundance for each indicated *dnmt1* region. ATG, exonal location of proposed initiation codons; *NLS*, nuclear localization signal.

A



position	145	153	155	166	246	322	330	335
ratio: methylated clones/total	12/12	8/12	12/12	12/12	12/12	12/12	12/12	0/12

B

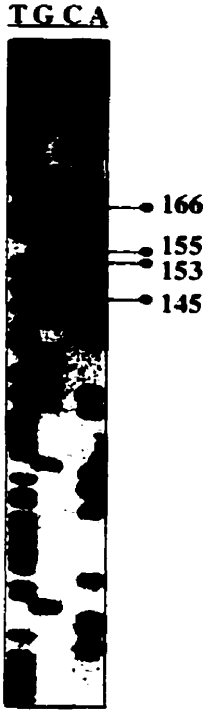


Fig. 5. A methylated CpG cluster is associated with the DNMT1 replication initiation region. A, Bisulfite mapping analysis of the state of methylation of a CpG cluster located upstream of the *dnmt1* A/T-rich region (Fig. 1B). The physical map of the c1 region exhibits the position of the 8 CpGs. DNA prepared from HeLa cells was subjected to bisulfite treatment, and the primers indicated were used to amplify the CpG cluster containing region. The amplified fragments were subcloned and 12 clones were sequenced. The state of methylation of each CpG is indicated in the table (5A). Methylated CpGs are indicated by a black lollipop, partially methylated CpGs by a checkered lollipop, and unmethylated CG by a white lollipop. **B,** A gel sample of the bisulfite analysis is shown; the positions of the methylated CpGs is indicated by the numbered lollipops.

Discussion

This paper describes the identification of initiation sites for DNA replication located between the first intron and exon 7 of the *dnmt1* locus. This finding is supported by several lines of evidence: first, a plasmid containing the c1 region of *dnmt1* is able to replicate autonomously; second, this region contains a binding recognition sequence and binds to OBA, a protein that binds to origins of replication; third, this region of *dnmt1* contains several sequence elements characteristic of known origins of DNA replication, such as, an A/T-rich region, matrix attachment motifs, a methylated CpG cluster, and a perfect match to the yeast ARS consensus sequence (Zannis-Hadjopoulos and Price, 1998); finally, this region is abundant in nascent DNA, as are other characterized origins of replication, as determined by competitive PCR. Two primary replication initiation sites were identified, one localized in the c1 region and another localized in the c3 region; these regions are approximately 2 kb apart from each other (Fig. 4C). This finding is similar to recently described observations in the CHO *dhfr* origin of replication, where initiation was shown to occur primarily from two sites, *oriβ* and *oriβ'* (Kobayashi et al., 1998).

The data presented in this paper further support the previous observations that regions of initiation of DNA replication share common sequence features (Zannis-Hadjopoulos and Price, 1998). A 536 bp, 77% A/T-rich element is present in the c1 region of the *dnmt1* locus, suggesting a possible function as a low melting region or as a DNA unwinding element (Zannis-Hadjopoulos and Price, 1998). This region also contains a perfect match for the yeast ARS consensus sequence, although previous reports have indicated that these sequences are not essential for mammalian origin function (Todd et al., 1995; Zannis-Hadjopoulos and Price, 1998). Nineteen ATTA and ATTTA matrix attachment motifs are present in the 2.4 kb c1 construct; these motifs constitute the core elements recognized by the homeobox domain from species as

divergent as flies and humans, and are frequently present in matrix attachment sites of several eukaryotic genes, in addition to numerous eukaryotic and viral origins of DNA replication (Boulikas, 1992).

A methylated CpG cluster is associated with the *dnmt1* replication initiation region. DNA methylation is now recognized as a fundamental mechanism of epigenetic regulation of genomic processes such as transcription, recombination, imprinting, development, carcinogenesis, and replication timing (Szyf, 1998a). Some origins of replication bear a cluster of heavily methylated CpG sites (Rein et al., 1997b), while other origins, such as *c-myc*, are associated with a non-methylated CpG cluster (Araujo et al., 1998). Taking these findings into account, it seems that origins of replication exhibit differential methylation patterns, similar to those observed in promoter elements of several genes (Szyf, 1998a). It remains unclear what role methylation plays in replication. It has been recently suggested that early-activated origins are associated with non-methylated CpG islands (Delgado et al., 1998). The identification of origins that are representative of either methylation profile will allow the testing of the hypothesis that methylation plays a role in differential activation of origins of replication.

The c1 construct containing all the structural elements mentioned above is able to support episomal autonomous replication when transfected into HeLa cells. Results from utilization of *in vivo* assays of autonomous replication have been controversial, since some studies have demonstrated that only large fragments (> 10 kb) could support autonomous replication (Heinzel et al., 1991), whereas several other studies showed that small specific sequences could successfully support autonomous replication (Frappier and Zannis-Hadjopoulos, 1987; McWhinney and Leffak, 1990; Zannis-Hadjopoulos et al., 1994). In addition, plasmids carrying *ori*β of the *dhfr* locus replicated autonomously both *in vivo* and *in vitro* regardless of their size, while plasmids of equivalent size inserts, but with random sequence, did not (Zannis-Hadjopoulos et al., 1994). Furthermore, the

capacity of several specific sequences to support autonomous replication of plasmids correlates directly with their chromosomal mapping sites (Zannis-Hadjopoulos et al., 1994).

Regarding the significance of the co-localization of initiation of replication with the regulatory region of the *dnmt1* gene, an attractive possibility would be that the physical association of the *dnmt1* regulatory region with initiation sites for DNA replication plays a role in coordinating the replication of the genetic information with that of the epigenetic information. Future experiments should resolve this question.

In summary, several lines of evidence, both structural and functional, demonstrate the presence of at least two major initiation sites for DNA replication residing in the region contained between the first intron and exon 7 of the *dnmt1* locus.

Acknowledgments

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Connecting text

In the preceding chapter, I have described the identification and mapping of *in vivo* initiation sites of DNA replication within the human *dnmt1* locus. The results also presented evidence for specific binding of OBA (KU 70/86) to a sequence located within *dnmt1*, indicating the possibility that KU 70/86 may be important for origin recognition during replication of mammalian cells.

The next chapter describes the relationship between DNA replication and the inheritance of the DNA methylation pattern in mammalian cells. Furthermore, the methylation status of sequences contained within different mammalian origins of DNA replication is examined.

Chapter III

Concurrent replication and methylation at mammalian origins of replication

Felipe D. Araujo, J. David Knox, Moshe Szyf, Gerald B. Price and Maria Zannis-Hadjopoulos

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Abstract

Observations made in *E. coli* have suggested that a lag between replication and methylation regulates initiation of replication. To address the question whether a similar mechanism operates in mammalian cells we have determined the temporal relationship between initiation of replication and methylation in mammalian cells both at a comprehensive level and at specific sites. First, newly synthesized DNA containing origins of replication was isolated from primate transformed and primary cell lines (HeLa, primary human fibroblast, African Green monkey kidney fibroblasts (CV-1) and primary African Green monkey kidney cells) by the nascent strand extrusion method followed by sucrose gradient sedimentation. Using a modified nearest-neighbor analysis the level of cytosine methylation residing in all four possible dinucleotide sequences of both nascent and genomic DNA was determined. The level of cytosine methylation observed in the nascent and genomic DNA were equivalent, suggesting that DNA replication and methylation are concomitant events. Okazaki fragments were also demonstrated to be methylated, suggesting that the rapid kinetics of methylation is a feature of both the leading and the lagging strands of nascent DNA. However, in contrast to previous observations, neither nascent nor genomic DNA contained detectable levels of methylated cytosines at dinucleotide contexts other than CpG (i.e., CpA, CpC and CpT are not methylated). The nearest-neighbor analysis also showed that cancer cell lines were hypermethylated in both nascent and genomic DNA relative to the primary cell lines. The extent of methylation in nascent and genomic DNA at specific sites was determined as well by bisulfite mapping of the Lamin B2 and *c-myc* origins of replication. The methylation pattern observed in the genomic and nascent clones were the same, confirming our hypothesis that methylation is concurrent to replication. Interestingly, the *c-myc* origin was found to be unmethylated in all clones tested. Consistent with the tight coordination of methylation and replication is the recent

observation that through its interaction with the proliferating cell nuclear antigen (PCNA), methyltransferase is positioned to methylate newly synthesized DNA.

Introduction

DNA methylation at cytosine residues at the CpG dinucleotide sequence is now recognized as an important mechanism of epigenetic regulation of genomic function (Razin and Cedar, 1991; Razin et al., 1985; Tate and Bird, 1993). Although methylated cytosines in distinction from other forms of epigenetic control, are part of the covalent structure of the genome, they are inherited by a post-replicative enzymatic transfer of methyl groups from S-adenosyl methionine which is catalyzed by DNA methyltransferase (MeTase) (Adams et al., 1979). An unresolved question is how the replication of epigenetic and genetic information is coordinated and whether DNA methylation plays a regulatory role in mammalian DNA replication. An interesting biological example of a role for DNA methylation in regulating DNA replication occurs at the origin of replication of *E. coli*, *oriC* (Boye and Lobner-Olesen, 1990; Campbell and Kleckner, 1990). Methylation of *oriC* by the Dam MeTase lags eight minutes behind its replication, maintaining it at a hemimethylated state throughout replication. The origin is sequestered by the bacterial plasma membrane (Campbell and Kleckner, 1990), making it inaccessible to the limiting levels of Dam MeTase available in the cell (Szyf et al., 1984). This hemimethylated state inhibits reinitiation from the origin before a full round of replication is completed. The challenge of preventing reinitiation from multiple origins of replication in eukaryotic cells is far greater than the one facing *E. coli*. It is possible that eukaryotic cells have developed a similar function for DNA methylation (Szyf, 1996). An additional control mechanism in *E. coli* that is dependent on a lag between replication and methylation is methyl-directed mismatch repair, whereby strand discrimination is based on the difference in methylation between the nascent and parental strands (MacLeod and Szyf, 1995; Roberts et al., 1985).

A recent report has identified cell cycle dependent "densely methylated islands" (DMI) at two chromosomal origins of replication, *oriβ* located ~17 kb downstream of the

dihydrofolate reductase locus (*dhfr*), in Chinese hamster ovary cells, and ori-RPS14 located at the 5' region of the ribosomal protein 14 (RPS14) locus (Tasheva and Roufa, 1995). These "densely methylated islands" were reported to contain cytosine residues, which were fully methylated at all four possible dinucleotide contexts (CpA, CpC, CpG, and CpT). Methylation of cytosines located in dinucleotide sequences other than CpG has been reported before (Woodcock et al., 1987), but the specific concentration of these methylated sequences in chromosomal origins of replication has raised the interesting possibility that they might be involved in regulating specific functions during replication such as attachment to nuclear matrix, licensing of specific origins and inhibiting re-activation of origins (Tasheva and Roufa, 1995). However, a more recent report has demonstrated the presence of a "high-density cluster" of cell cycle independent methylated CpG dinucleotides on the 5' region of both the *oriβ* and ori-RPS14 the (Rein et al., 1997b), but methylation of C in other dinucleotide sequences was not observed. It was postulated that methylated CpG clusters mark specific origins for replication through changes in chromatin structure (Rein et al., 1997b). Consistent with the hypothesis that methylation of these specific sequences is important for origin function, it was shown that *oriβ*is methylated after replication (Rein et al., 1997b). However, other origins of replication have not been examined.

The kinetics of methylation after replication of mammalian DNA has been previously examined (Gruenbaum et al., 1983; Woodcock et al., 1986), but the results appear to be conflicting. One report has suggested a lag between replication and methylation of up to 6 hours at some parts of the chromosome (Woodcock et al., 1986), while others have suggested a much shorter lag of approximately 1 minute (Gruenbaum et al., 1983). The identification of fork targeting sequences in the DNA MeTase directing it to sites of replication (Leonhardt et al., 1992) is consistent with the model that replication and methylation can occur concurrently. Another recent finding (Chuang et

al., 1997) showed that the DNA methyltransferase is able to associate with the proliferating cell nuclear antigen (PCNA) and compete with p21 for its binding site, further supporting the hypothesis that methylation patterns are inherited as replication is proceeding. However, the targeting of DNA MeTase to the replication fork and its ability to associate with PCNA does not exclude the possibility that origin sequences might be protected from methylation during replication, as they are in *E. coli*.

To elucidate the role of DNA methylation in replication and to understand how the pattern of methylation is inherited, one has first to determine the kinetics of methylation of newly synthesized DNA containing origins of DNA replication and its dinucleotide sequence specificity. To address this issue, we isolated newly synthesized DNA containing origins of replication from primary and transformed cell lines by extrusion of nascent DNA using a previously established protocol (Zannis-Hadjopoulos et al., 1981) and used it as substrate for both nearest-neighbor analyses and bisulfite mapping. This enabled us to look at the rate of methylation in the growing replication fork within a few hundred base pairs from the points of initiation. Since non-synchronized growing cells were used, these origin-enriched DNA samples include an accurate representation of all active origins in these cells. Using these approaches we directly measured the methylation status of active origins of replication and Okazaki fragments at the dinucleotide level in primary and transformed cell lines. This study establishes the temporal relationship between initiation of replication and propagation of genetic and epigenetic information encoded by the genome.

Materials and methods

Cell culture- HeLa and CV-1 cells were purchased from the American Type Culture Collection (ATCC); primary African Green monkey kidney cells, and human normal skin fibroblasts were purchased from BioWittaker. All cells were maintained as

monolayers and grown to approximately 30% confluence in alpha modified Eagle medium supplemented with 10 % fetal bovine serum (FCS, Flow Lab., McLean, VA).

Extrusion of nascent DNA- To isolate sequences of DNA located in close proximity to points of initiation of replication, nascent strands were extruded by branch migration, using previously described methods (Kaufmann et al., 1985; Tao et al., 1997; Zannis-Hadjopoulos et al., 1981) with slight modifications. The harvested (mid-log phase) cells were washed 3 times in 10 ml ice-cold phosphate buffer saline (PBS) and lysed in 4 ml Hirt lysis buffer (Hirt, 1967) with gentle shaking. The lysates were decanted, 0.1 mg/ml of proteinase K was added, and the samples were incubated at 37 °C overnight. Following phenol-chloroform extraction and ethanol precipitation the DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8). The nascent DNA strands were extruded by incubation at 50 °C for 16-18 hrs. To isolate sequences located at different distances from replication initiation points, the nascent DNA was size-fractionated on a 5-30% sucrose gradient (0.2M NaCl/10mM TE, pH 8/0.02% sodium azide) by centrifugation at 24,000 rpm in a SW27 rotor at 9 °C for 16-18 hrs, and then precipitated in ethanol, dissolved in TE, and analysed by electrophoresis on a 1% agarose gel (Fig. 1A). For both the bisulfite mapping and the competitive PCR amplification reactions the nascent DNA was further size-selected, 0.4-1.2 kb, by gel electrophoresis purification with a Sephaglas BandPrep Kit (Pharmacia Biotech).

Competitive PCR analysis- To verify that our method isolates highly enriched origin-DNA we determined whether our nascent DNA fractions contain non-origin derived DNA by the highly sensitive competitive PCR analysis as previously described (Tao et al., 1997). Both nascent (~ 30 ng in all reactions) and genomic HeLa DNA (~ 100 ng) were used as template for PCR amplification reactions. Two primer sets from human *c-myc* (GENE bank accession J00120) were used: the first set [5'-TGCCGTGGAATAACACAAAA-3' (sense, starting at 761), 5'-

CTTTCCAGGTCCTCTTTCCC-3' (antisense, starting at 1134), and 5'-TAACACAAAAGATCATTTCAGGGAGCAAAC-3' (primer used to design competitor at *c-myc* ori)] amplifies the region of the *c-myc* origin of replication (Tao et al., 1997; Vassilev and Johnson, 1990) and the second set [5'-GGTTCTAAGATGCTTCCTGG-3' (sense, starting at 7848), 5'-ATGGGTCCAGATTGCTGCTT-3' (antisense, starting at 8299) and 5'-TGCTTCCTGGGAGAAGGTGAGAGGTAGGCA-3' (primer used to design competitor downstream of *c-myc* ori)] amplifies a region located 6,711 bp downstream from the first set of primers. PCR conditions were as follows: 94 °C, 1 min, 55 °C 1 min, 72 °C 1min, 30 cycles.

Nearest neighbor analysis- Nearest neighbor analysis was performed as previously described (Razin et al., 1985; Szyf et al., 1995). 1μg of nascent DNA from each of the samples was incubated at 37 °C for 15 min with 0.1 units of DNase 1. Then, 1 μl of either [α-^{32}P] (10mCi/ml, Amersham) dATP, dCTP, dGTP, or dTTP, was added together with 1 unit of Kornberg DNA polymerase and incubated for 15 min at 30 °C. 30 μl of water were added to the reaction mixture, and the unincorporated nucleotides were removed by spinning through a Microspin S-300 HR column (Pharmacia Biotech Inc.). The labeled DNA was digested with 70 μg of micrococcal nuclease (Pharmacia) in the manufacturer's recommended buffer for 10 hrs at 37°C. The samples were loaded on TLC phosphocellulose plates (13255 cellulose, Eastman-Kodak), and the 3'-mononucleotides were separated in one dimension (iso-butyric acid:H_2O:NH_4OH, 66:33:1) and in the second dimension ($(NH_4)_2SO_4$:isopropanol:Na acetate, 80:2:18). Two labeled controls were used to indicate the relative migration of [^{32}P]-methyl-dCMP and [^{32}P]-dCMP. Fully methylated methyl-dC-dG or nonmethylated dC-dG double stranded oligomers were labeled with [α-^{32}P]-dGTP and digested to 3' methyl-dCMP or 3'dCMP as previously described (Szyf et al., 1984). The chromatograms were exposed to Fuji phosphoimaging plates, scanned in a BAS 2000 phosphorimager, and percentages of

corresponding cytosines and 5-methylcytosines were calculated after respective quantification. In general, the standard deviation of the assay was in the range of 1 -3%.

Okazaki fragment identification- Fractions 1-4 from the sucrose gradient contained DNA of Okazaki fragment size (Fig. 1A). The 5' ends of the Okazaki DNA fraction (100-250bp), containing RNA primers, were labeled with Polynucleotide Kinase (PNK) for 1 hr using 5 μl of [γ-^{32}-P]-ATP (10mCi/ml, Amersham). The sample was separated into two: one half was treated with 0.4 M NaOH, and the other half was left untreated. The two halves were fractionated by 5% polyacrylamide alkaline gel electrophoresis and analyzed by autoradiography (Fig. 1B).

Bilsulfite mapping- Sodium Bisulfite (ACS) grade from Sigma Cat# S-8890, FW= 104, was used. 3.6M Sodium Bisulfite was prepared fresh each time; 20mM stock of hydroquinone can be stored at -20°C. To make 7.49g of Sodium Bisulfite in 15 ml of ddH_2O, pH to 5 with 10N NaOH; we added 1ml of hydroquinone, and completed the volume to 20 ml with ddH_2O. 5 μg of DNA in 54 μl of ddH_2O in a 500 μl PCR reaction tube was utilized. 6 μl of 3N NaOH was added (final concentration of 0.3N) and incubated at 37°C for 15 minutes. 431 μl of 3.6M NaBisulfite/1mM hydroquinone was added. 100 μl of mineral oil was added to overlay the solution and the tube was heated at 55 °C for 12 hours. Bisulfite reaction was recovered from beneath the mineral oil and desalted using the Promega Wizard Prep (followed manufacturer's protocol), 6 μl of 3N NaOH (final concentration of 0.3N NaOH) was added and the tube was incubated for 15 minutes. 26 μl of 10M NH_4OAc (final contration of 0.3M NH_4OAc) was added plus 300 μl of 95% EtOH and incubated at -20°C for 20 minutes. Spin for 30 minutes at 4 °C in a microfuge. We removed the supernatant, lyophilized, and resuspended in 100 μl in ddH_2O. Approximately 50 ng of DNA were used in each of the PCR amplifications. PCR products were used as templates for susequent PCR reactions utilizing nested primers. The PCR products of the second reaction were then subcloned using the Invitrogen TA

cloning Kit (we followed the manufacturer's protocol) and the clones were sequenced using the ™Sequencing Kit (we followed the manufacturer's protocol, procedure C). The primers used for the *c-myc* origin (GenBank accession no. J00120) were: MYC(IN)1, 5'-CCTTTCCCAAATCCTCTTTCC-3' (position 1135-1116); MYC(in)2, 5'-GTGAGGGATTAAGGATGAGA-3' (position 721-730); MYC(OUT)1, 5'-AACCATTAACTCTTTCCTCC-3' (position 1178-1159); MYC(out)2, 5'-TTAAAATGTTTTTGGGTGAGG-3' (position 706-726). The primers used for the Human Lamin B2 origin (GenBank accesion no. M94363) were: HL.B2.IN.A, 5'-AAAAAAAAACCCTAACTTAACC-3' (position4372-4349); HL.B2.OUT.A, 5'-AAAAACTACAACTCCCACAC-3' (position 4502-4483); HL.B2.OUT.S, 5'-TTTTTAAGAAGATGTATGTTTAG-3' (position 3871-3893); HL.B2. IN.S, 5'-TTAATGATTTGTAATATATATTTTAT-3' (position 3852-3876). The primers used for the Human DNA Methyltransferase Gene (unpublished sequence, arbitrary no.) were: Morif1, 5'-TTATGTTGTTTAGGGTTGGATT-3' (position 1-21); Birom1, 5'-TCAAAATAAATAACCCAAAACCA-3' (position 423-445); Birom2 5'-AAAACAACCAACTAATATTCCT-3' (position 410-388); Morif2 5'-TTTATAAGTTATTTTTTTATTATAGTT-3' (position 32-58). PCR conditions were as follows: 95 °C for 1 minute, 56 °C for 2 minutes and 30 seconds, and 72 °C for 1 minute.

Results

Methylation of cytosines in CpG dinucleotides at origins of replication occurs concurrently with their replication- How fast are origins of replication methylated relative to initiation of replication? To answer this question, avoiding biases arising from particular methylation patterns associated with specific origins, such as for example *ori-β* and ori-RPS14, we chose first to examine a population of origins represented in origin-enriched DNA (Kaufmann et al., 1985; Tao et al., 1997; Zannis-Hadjopoulos et al., 1981). Nascent DNA, which can be extruded from bulk DNA due to the unique physical

properties of the replication bubble (Tao et al., 1997; Zannis-Hadjopoulos et al., 1981), was prepared from human and monkey cell lines in order to establish whether methylation at origins of replication represents a species-specific property. Logarithmically growing CV-1 and HeLa cells were used in order to obtain a representative sample of the population of functional origins of replication in these cells. Extrusion of nascent DNA methods (Kaufmann et al., 1985; Tao et al., 1997; Zannis-Hadjopoulos et al., 1981) was followed by size fractionation of the DNA in a neutral sucrose gradient. Recent reports have demonstrated that additional steps such as labeling the nascent DNA with BrdU followed by anti-BrdU immunoprecipitation are unnecessary (Kumar et al., 1996; Tao et al., 1997). Fractions 8-10, comprising newly synthesized DNA of 500 bp-1000 bp, were used in the nearest-neighbor analyses (Fig. 1A). The nascent DNA fraction containing 100-250 bp should contain mostly Okazaki fragments. In order to examine this supposition we alkali treated the corresponding DNA fractions (100-250 bp) in order to degrade the 5' RNA primers from the Okazaki fragments. The results from Okazaki identification (Fig. 1B) show that nearly all the labeled 100-250 bp fraction is sensitive to alkali treatment suggesting that >90% of this fraction is indeed composed of Okazaki DNA.

We verified by competitive PCR that our isolation protocol results in fractions that are highly enriched for nascent DNA located within ~ 500bp from the points of initiation of DNA replication, and demonstrated that the enriched nascent DNA does not contain non-origin related genomic DNA. We showed the presence of the *c-myc* origin of replication in the nascent DNA fractions (Fig. 2A) and the absence of a sequence located approximately 7kb downstream of the *c-myc* origin in the same fractions (Fig. 2B). Competitive PCR amplification were performed as described in the methods. This result also indicates that our nascent DNA is not contaminated with broken fragments of genomic DNA.

The state of cytosine methylation in CpG dinucleotides in the different DNA fractions was determined as described in Materials and Methods (Fig. 3A). The results (Fig. 3B) demonstrate that newly synthesized DNA located approximately 250-500 bp on either side of origins of replication is nearly fully methylated (79% for HeLa cells and 72% for CV-1 cells)(Fig. 3B, gray bars) when compared to the state of methylation of genomic DNA (80% for both HeLa and CV-1 cells) (Fig. 3B, black bars). The data suggests that, unlike in *E. coli*, methylation of origin sequences in mammals is initiated before the synthesis of ~ 250 bp is completed. This rapid rate of methylation of vertebrate DNA differs from previous published values and might be due to the fact that previous studies did not specifically look at origin enriched DNA (Gruenbaum et al., 1983; Woodcock et al., 1986).

Origins of replication are rapidly methylated irrespective of their state of transformation - Previous reports have suggested that CpG rich sequences are hypermethylated in cancer cells (Baylin et al., 1991; Merlo et al., 1995; Nelkin et al., 1991) and that this hypermethylation reflects an increase in the activity of the DNA methylation machinery (el-Deiry et al., 1991; Kautiainen and Jones, 1986). It has also been previously suggested that changes in the kinetics of DNA methylation of origins of replication might be involved in cellular transformation (Szyf, 1996).

We first determined whether the kinetics of DNA methylation of origins of replication is different in transformed human (HeLa) cells than in primary normal human skin fibroblast. Then, we compared the cytosine methylation state of CpG dinucleotides in monkey transformed (CV-1) cells and African Green monkey primary kidney cells. The state of methylation of sequences located ~ 500 bp from the point of initiation of replication was compared with the average state of methylation of genomic DNA. The results (Fig. 4A) show that nascent DNA from primary human cells is 68% methylated (gray bar) at CpG sequences in comparison with 72% of genomic DNA (black bar).

African Green monkey primary kidney cells have 52% of their CpGs methylated at the nascent DNA (gray bar) in comparison with 53% in genomic DNA (black bar). The small difference observed in primary human cells between cytosine methylation of CpG sequences in nascent (68%) and genomic DNA (72%) is close to the standard error of our assay. However, alternatively this small difference might suggest that few CpG sites remain nonmethylated in primary nascent DNA. These results imply that, similar to transformed cells (HeLa and CV-1), primary human and monkey cells initiate methylation immediately after replication. Methylation of cytosine residues residing in the dinucleotide CpG sequences is initiated before ~ 500 bp are synthesized following initiation of replication. Whereas the kinetics of methylation after replication seems to be similar in transformed and primary cells, the overall level of methylation of the primary cells in this study is lower. This result is consistent with the general hyperactivation of DNA MeTase activity observed in cancer cells (Belinsky et al., 1996; Wu et al., 1993c). Alternatively, in the case of HeLa cells versus primary human fibroblasts, the lower methylation might reflect the specific cell type of the untransformed cells.

Okazaki fragments are methylated- To determine whether rapid methylation is characteristic of sequences near or at the point of initiation of replication or whether all nascent DNA is methylated at a similar rate, we determined the state of methylation of Okazaki fragments. Okazaki fragments are replication intermediates synthesized on the lagging strand of DNA both near and distal to the point of initiation of DNA replication. To determine that the Okazaki fractions isolated by our procedure are indeed Okazaki fragments rather than sheared genomic DNA, we took advantage of the fact that Okazaki fragments contain RNA primers at their 5' end which are sensitive to NaOH treatment (Fig. 1B) whereas genomic DNA is not. Fractions 1-4 (Fig.1A and B), containing Okazaki fragment size DNA (100-250bp), were collected following sucrose gradient fractionation and the percentage of CpG methylation was determined, as described in

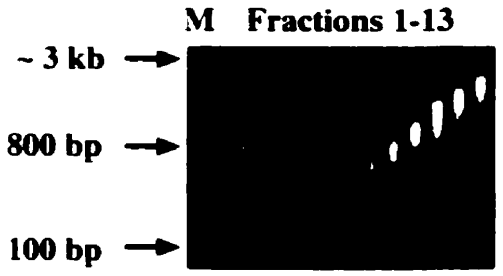
materials and methods. The results (Fig. 4B) show that Okazaki fragments are partially methylated suggesting that methylation of DNA is initiated before Okazaki fragments are ligated to form longer size DNA. Since the majority of Okazaki fragments are derived from the growing points of replication forks and not from origins of replication, this suggests that rapid kinetics of methylation is a feature of all nascent DNA. One interesting observation is that different cell lines show different level of methylation of Okazaki fragments. Human cells (HeLa and human skin fibroblast) exhibit a lower percentage of CpG methylation (30% and 35% respectively) than African Green monkey (CV-1 and primary kidney) cells (65% and 47% respectively) (Fig. 4B). These differences do not correlate with the state of transformation of the cells. One possible explanation for the partial methylation of Okazaki fragments as compared to origin-derived DNA might be a differential association of DNA MeTase with the leading and lagging strand replication machinery.

CpG is the only methylated dinucleotide sequence in origins of replication- A previous report has indicated that two Chinese hamster origins of replication, *ori-β* and *ori-S14* bear a high concentration of methylated cytosines that do not reside in the consensus CpG dinucleotide sequence (Tasheva and Roufa, 1994), but this observation has not been confirmed. Using the nearest-neighbor assay which allows determination of the state of cytosine methylation at each of the four possible CpX dinucleotide sequences, we addressed the question whether origins of replication, in general, have cytosines methylated in dinucleotide sequences other than CpG. Since this assay could detect less than one methylated cytosine in one hundred cytosines (Razin et al., 1985; Szyf et al., 1995), a cluster of methylated cytosines per origin would be easily detected. Nascent and genomic DNA prepared from HeLa cells were labeled with either [α-^{32}P] dCTP, dATP, dGTP or dTTP, digested to 3' mononucleotides and separated by TLC in one or two dimensions. The results show the absence of methylation of CpC or CpT (Fig 5A) and

CpA (Fig 5B). The open circle corresponds to the position of 5' methyl-cytosine (Fig 5B). The control experiment shows where the 5' methyl-cytosine migrates in a two dimensional TLC relative to the unmethylated cytosine (Fig 5C), where the open circle corresponds to the position of the unmethylated cytosine. These results support the conclusion that methylation of cytosines at CpG dinucleotide sequence is the main modification of DNA located at origins of replication and is probably carried out by the same enzymatic machinery responsible for methylation of the rest of the genome.

Specific CpG sites are rapidily methylated- To determine if specific sites were being methylated concurrently with replication we decided to perform bisulfite mapping of both the Lamin B2 and the *c-myc* origins of replication (Fig. 6 and Fig. 7) and compare the methylation patterns of genomic DNA with nascent DNA. The Lamin B2 origin was found to be partially methylated (Fig. 6A, 6B and Fig. 7) in all clones tested. Interestingly, one specific CpG was found methylated in all 5 genomic clones (Fig. 6A) and in all 4 nascent clones (Fig. 6B), supporting our hypothesis that replication and methylation occur concomitantly. The *c-myc* origin was found unmethylated in all clones tested, both genomic (Fig. 6C) and nascent (Fig. 6D). As a control we also sequenced this region using non-bisulfited DNA (Fig. 6E). This result suggests that not all active origins have to be associated with a high-density cluster of methylated CpG dinucleotides as previously suggested (Rein et al., 1997b), but rather that origins are differentially methylated. Some origins, like the DHFR ori-β and ori-RPS14, are heavily methylated (Rein et al., 1997b), other origins, such as Lamin B2 origin, are partially methylated, and others, such as the *c-myc* origin, are not methylated. As a positive control for methylated CpGs we performed bisulfite mapping of a heavily methylated region, the methyltransferase gene (Fig. 6F and Fig. 7).

A


M
Fractions 1-13
~ 3 kb
800 bp
100 bp

B

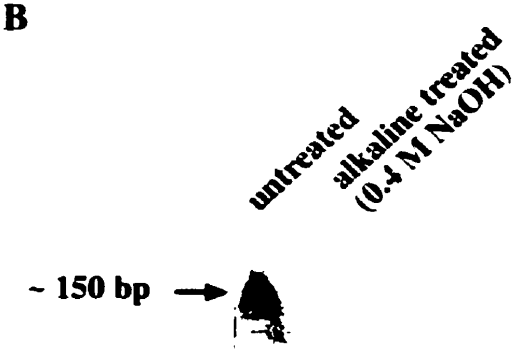

untreated
alkaline treated
(0.4 M NaOH)
~ 150 bp

Figure 1. (A) Fractionation of Nascent DNA from human normal skin fibroblast. DNA was prepared and extruded by branch migration as described in Materials and Methods from human normal skin fibroblasts and size fractionated on a sucrose gradient. The fractions were electrophoresed on a 1% agarose gel and ethidium bromide stained. Okazaki fragments (fractions 1-4) as well as nascent DNA of higher molecular weight (fractions 8-10) were used as substrates for the nearest neighbor analysis. Lane 1 (M) is a 100 base pair ladder marker. lanes 2-14 are nascent DNA fractions of increasing molecular weights. **(B) Fractions 1-4 contains mostly Okazaki fragments.** DNA from fractions 1-4 was 5' labeled with [γ-^{32}P]-ATP using polynucleotide kinase and subjected to alkaline treatment with 0.4 M NaOH. The alkaline treated and untreated samples were electrophoresed through a 5% polyacrylamide alkaline gel. An autoradiogram of the dried gel is shown. The lability of the 5' label in NaOH suggests that most of the DNA in these fraction bear ribonucleic acid nucleotides as expected from Okazaki fragments.

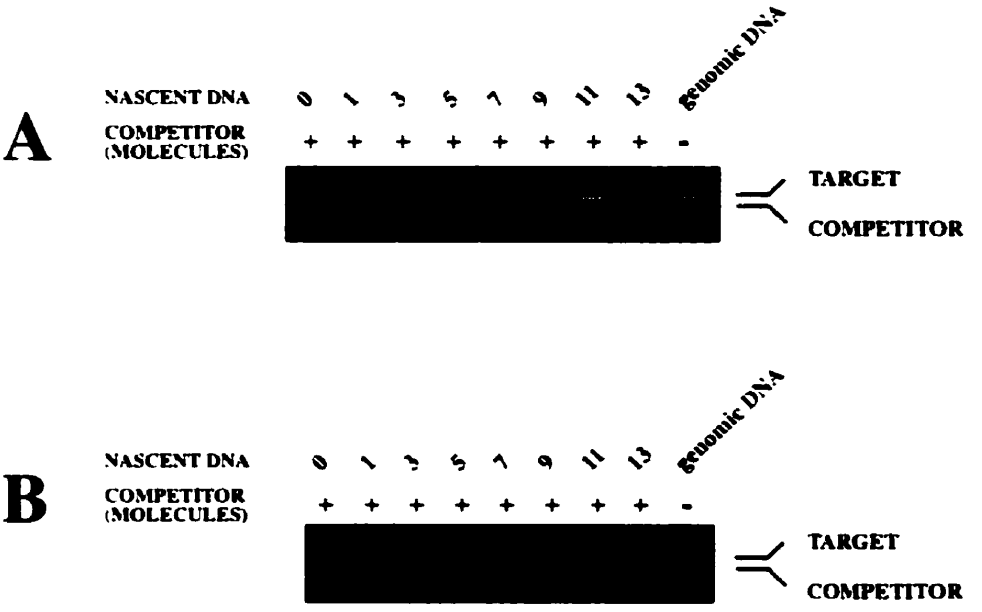

A
NASCENT DNA
0 1 3 5 7 9 11 13 Genomic DNA
COMPETITOR (MOLECULES)
+ + + + + + + + -
TARGET
COMPETITOR
B
NASCENT DNA
0 1 3 5 7 9 11 13 Genomic DNA
COMPETITOR (MOLECULES)
+ + + + + + + + -
TARGET
COMPETITOR

Fig. 2. Nascent DNA is highly enriched for sequences corresponding to sites of initiation of replication. (**A**) HeLa nascent DNA (0 to 13 µl as indicated, ~ 20ng/ µl) and 200 molecules of competitor were used as template for competitive PCR reactions with primers targeted to the *c-myc* origin of replication. (**B**) HeLa nascent DNA (0 to 13 µl as indicated, ~ 20ng/ µl) and 200 molecules of competitor were used as template for competitive PCR reactions, but with primers and competitors targeted to a region ~ 7 kb downstream of the *c-myc* origin.

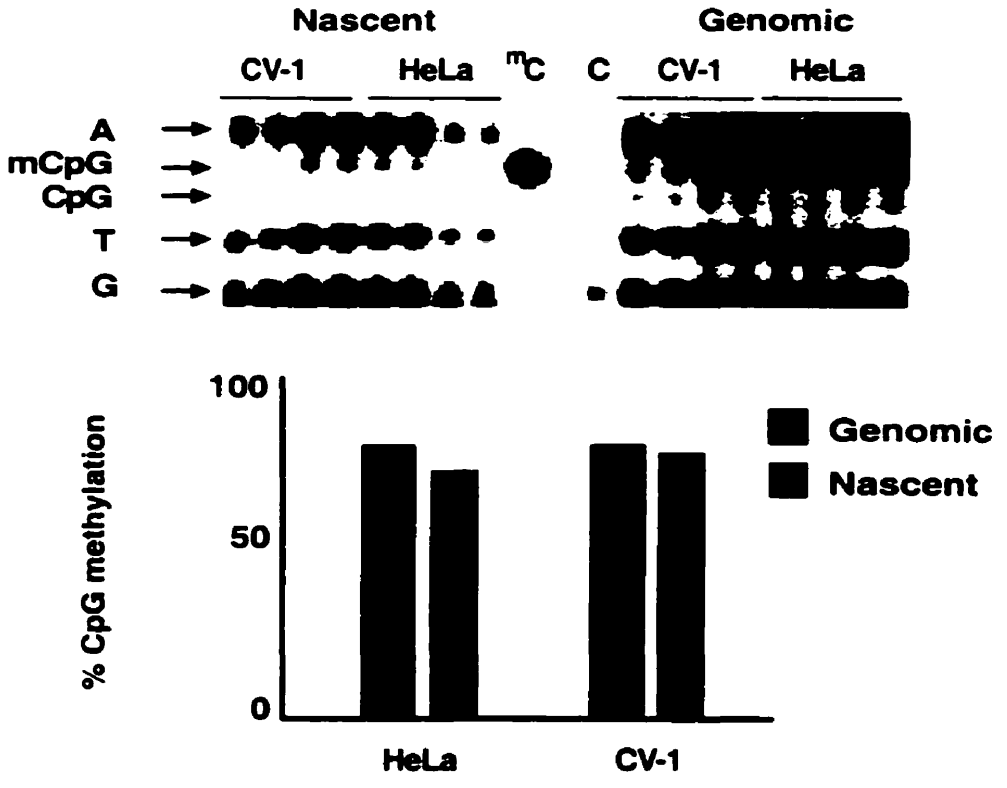

Nascent
Genomic
CV-1
HeLa
mC
C
CV-1
HeLa
A
mCpG
CpG
T
G
100
50
0
% CpG methylation
Genomic
Nascent
HeLa
CV-1

Figure 3. HeLa and CV-1 nascent DNA are rapidly methylated. (A) Nascent DNA fractions 8-10 (500 bp-1 kb) and genomic DNA prepared from HeLa and CV-1 cells as well as a poly-[methyl dCdG] control (which served as a control for migration of 3' methyl-dCMP) were used as substrates for nearest neighbor analysis using [α-32P]-dGTP as described in Materials and Methods. Two different reactions, each loaded twice (four lanes per sample) were performed from each cell type. The labeled DNA was digested to 3' mononucleotides which were then separated by thin layer chromatography. The position of migration of cold mononucleotide standards is indicated. **(B)** Quantification of a triplicate assay similar to the one presented in (A). The percentages of methylated cytosines relative to total cytosines was determined by phosphorimager quantification of the signals obtained for 5-methyl-dCMP and dCMP. The results are an average of three independent determinations ± S.D. Nascent DNA (filled boxes), genomic DNA (shaded boxes).

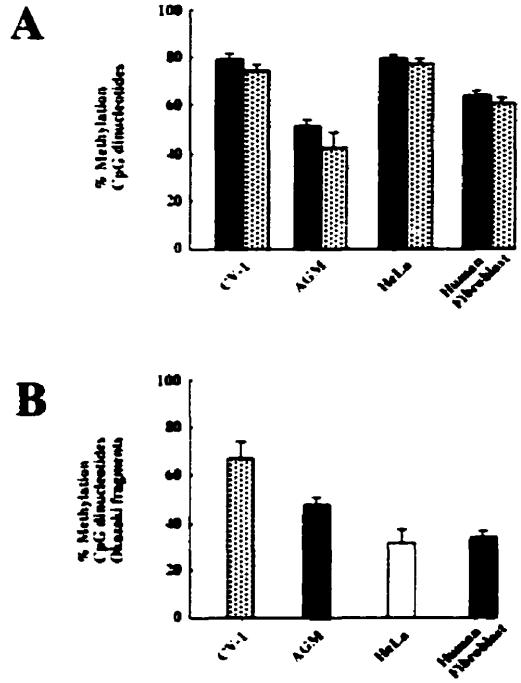
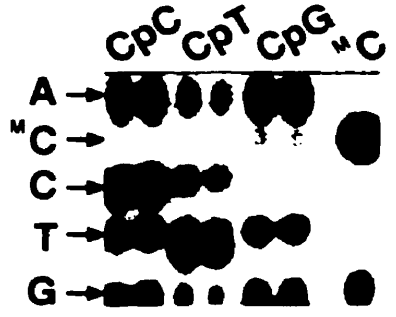
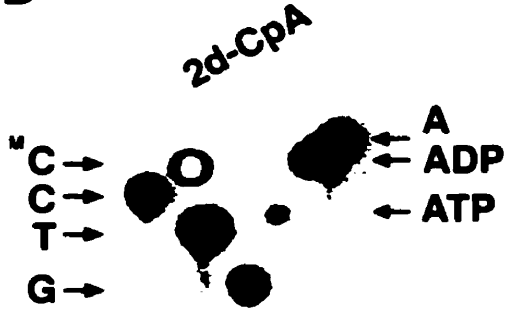

A
B
100
80
60
40
20
0
CV-1
AGM
HeLa
Human fibroblast

Figure 4. (A) Nascent DNA is rapidly methylated in transformed and untransformed cells. Nascent (shaded boxes) and genomic DNA (filled boxes) prepared from CV-1, African Green monkey (AGM), HeLa and human skin fibroblast cells were subjected to nearest neighbor analysis of DNA methylation at CpG dinucleotides as described in the methods. The results presented are an average of three determination ± S.D. **(B) Methylation of Okazaki fragments.** Okazaki fragment containing DNA was prepared as described in the Materials and Methods (fractions 1–4) and subjected to nearest neighbor analysis of DNA methylation at CpG dinucleotides. The results presented are an average of three independent determinations ± S.D.

A



B



C

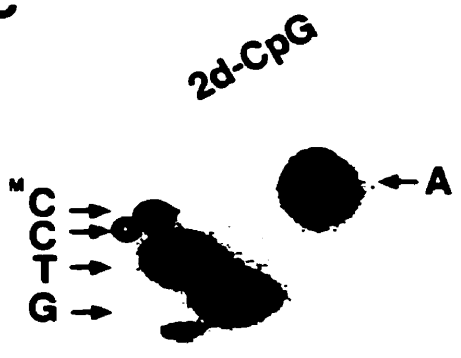
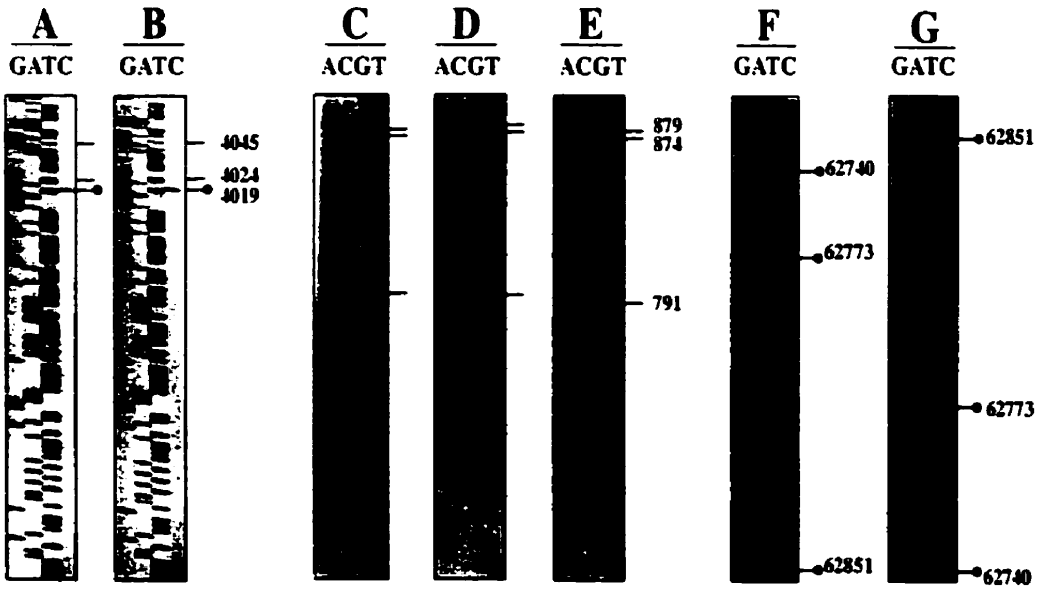


Fig 5. CpG is the only dinucleotide sequence methylated in nascent DNA. **(A)** HeLa nascent DNA prepared as described in the methods (500-1000 bp, fractions 8-10) was subjected to nearest neighbor analysis for CpC (labeled with [α-32P]-dCTP), CpT (labeled with [α-32P]-dTTP) and CpG (labeled with [α-32P]-dGTP) methylation. **(B)** To study methylation at dCpA sequences, the 3' mononucleotides were separated in two dimensions as described in the Materials and Methods. Two dimensions were used since dADP (a degradation product of the labeled dATP) comigrates with 5-methyl-dCMP. The open circle corresponds to the migration of 5' methyl-cytosine. **(C)** Two dimension analysis of dG neighbors showing 80% methylation at CpG dinucleotide sequences. The open circle corresponds to the migration of the unmethylated cytosine.


A
GATC
B
GATC
C
ACGT
D
ACGT
E
ACGT
F
GATC
G
GATC
4045
4024
4019
879
874
791
62740
62773
62851
62851
62773
62740

a

Fig 6. Specific methylated CpG sites are rapidly methylated, but not all active origins are methylated. **(A)** HeLa Genomic DNA was used as template for bisulfite mapping of sites within the Lamin B2 origin of replication, positions 3910 to 4100. Methylated CpGs are identified by lollipops and unmethylated CpGs by thin lines. **(B)** HeLa nascent DNA was used as template for the same assay mapping the same positions of the Lamin B2 origin. **(C)** HeLa Genomic DNA was used as template for bisulfite mapping of sites within the *c-myc* origin of replication, positions 850 to 980. **(D)** HeLa nascent DNA was used as template for the same assay mapping the same positions of the *c-myc* origin. **(E)** Non-bisulted HeLa genomic DNA was used as template for the same assay mapping the same positions of the c-myc origin, as a control for (C) and (D). **(F)** HeLa Genomic and **(G)** nascent DNA were used as template for bisulfite mapping of sites within the Methyltransferase gene as a positive control for methylated CpGs, positions 1 to 131.

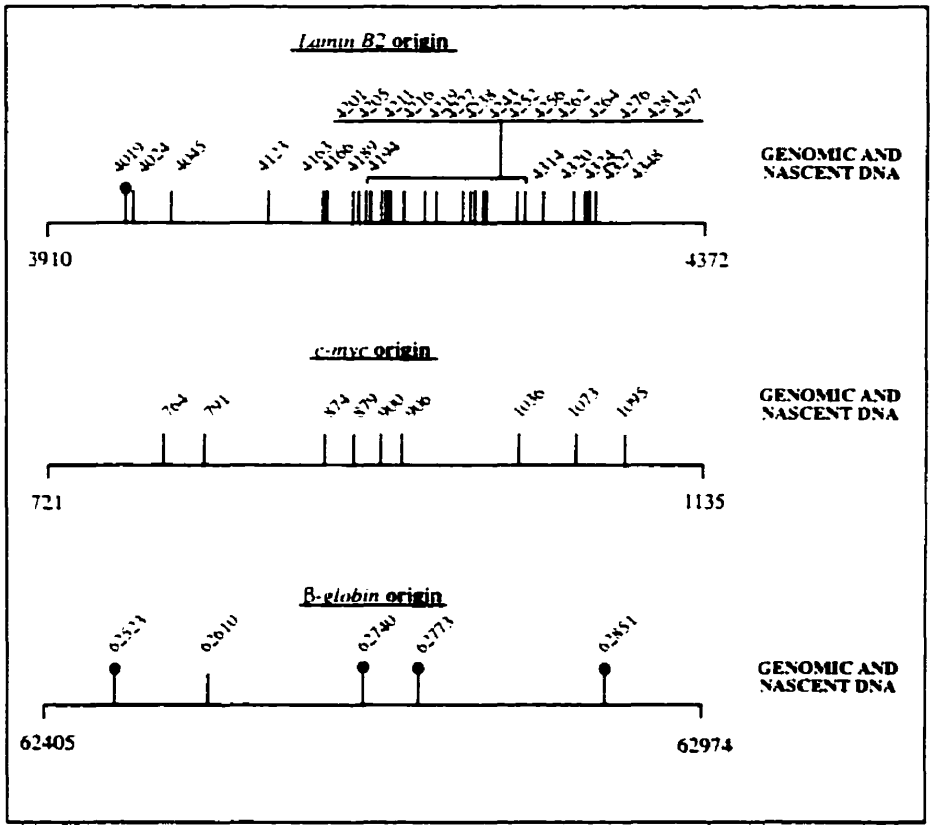

Lamin B2 origin
GENOMIC AND NASCENT DNA
3910
4372
c-myc origin
GENOMIC AND NASCENT DNA
721
1135
β-globin origin
GENOMIC AND NASCENT DNA
62405
62974

Fig 7. Methylation patterns at different sites. Lollipops symbols, CpG dinucleotides analyzed in this study (filled lollipops, CpG dinucleotides methylated in all clones tested; shaded lollipops, CpG dinucleotides methylated in 50% of clones tested); thin vertical lines, unmethylated CpG dinucleotides.

Discussion

Whereas DNA methylation is now accepted as an important epigenetic mechanism for regulation of genome function, the mechanism by which replication of the genome and its methylation are coordinated has been unknown. Different models have been proposed for the possible function that DNA methylation might play in replication (Boye and Lobner-Olesen, 1990; Campbell and Kleckner, 1990; Rein et al., 1997b; Tasheva and Roufa, 1994) but there have been no data to support or nullify these hypotheses. The study presented here defines some of the basic rules that govern the methylation patterns at the earliest events in replication, that is, methylation of origins of replication. We first show that methylation occurs immediately after initiation of replication, where the replication fork has advanced less than 500 bp after the point of initiation of replication. Second, tight coordination of initiation of replication and methylation is a characteristic of mammalian cells regardless of their state of transformation. Third, methylation occurs rapidly also at segments of the genome that are located distal to origins of replication, since methylation occurs before Okazaki fragments are ligated to form longer nascent strands. The level of methylation observed in the lagging strand is less than that occuring in the leading strand. This might reflect a difference between the kinetics of methylation at the origin of replication, which is fully methylated, and at sites distal to it. These sites may be either less tightly coordinated with replication or the lagging strand might be under a differential action of the MeTase. Methylation is complete before 3000 bp of DNA were synthesized (data not shown). Fourth, origins of replication are methylated only at the CpG dinucleotide sequence, as is the rest of the genome. This is consistent with the hypothesis that methylation of origin DNA occurs by the same enzymatic machinery that methylates the rest of the genome.

The data indicate a tight coordination between replication and methylation. This coordination is probably maintained to ensure that the pattern of methylation is

appropriately inherited. Upregulation (Wu et al., 1993c) and downregulation (Laird et al., 1995; MacLeod and Szyf, 1995; Ramchandani et al., 1997) of DNA MeTase activity have been previously shown to alter cellular phenotype. Furthermore, embryonic deficiency in DNA MeTase expression is lethal (Li et al., 1992). Several mechanisms are probably involved in ascertaining that these processes are coordinated. The expression of DNA MeTase is regulated at the posttranscriptional level with DNA synthesis (Szyf et al., 1991) and the DNA MeTase bears a replication fork targeting signal (Leonhardt et al., 1992). The simplest explanation for the data in this study is that the DNA MeTase is part of the DNA replication fork complex. This hypothesis is strengthened by the observation that the DNA MeTase associates with PCNA (Chuang et al., 1997). The fact that only CpG sequences are methylated is consistent with the conclusion that the known CpG specific DNA MeTase is the only DNA MeTase included in the replication fork complex. An interesting question is whether DNA MeTase is limited to the replication fork and, if so, how are repair patches methylated. It was previously shown that methylation of repair patches is an inefficient process (Wilson and Jones, 1983), probably because of the localization of DNA MeTase to replication forks. Alternatively, another population of DNA MeTase that is not targeted to the replication fork might exist.

Does DNA methylation play a role in regulating the activity of the replication fork? The data here suggest that differential methylation of active origins during the cell cycle does not play a role in regulating origin function, as it has been previously proposed (Szyf, 1996) based on the *E. coli* model (Boye and Lobner-Olesen, 1990; Campbell and Kleckner, 1990). However, recent observations suggest that clusters of methylated CpGs might be necessary to mark functional origins (Rein et al., 1997b). Thus, the number of activated origins in a cell might be regulated by methylation. It has been previously observed that ectopic expression of DNA MeTase can lead to cellular transformation (Wu et al., 1993c), while inhibition of DNA MeTase can reverse

transformation (Laird et al., 1995; MacLeod and Szyf, 1995; Ramchandani et al., 1997). The state of methylation of origins might be one mechanism through which hypermethylation might play a role in carcinogenesis.

In summary, the data presented here demonstrate that initiation of replication and methylation are tightly coordinated. Whether methylation of specific sites plays a role in regulating replication activity remains an open question.

Acknowledgements

This work was supported by grants from the Medical Research Council (MRC) of Canada to M.S. M. Z-H. and the Cancer Research Society Inc. (CRS) to G. B. P.

Connecting text

In the preceding chapter I have described that DNA replication and methylation are concurrent events and that mammalian origins of replication are differentially methylated.

The next chapter tests the hypothesis that DNMT1 is required for DNA replication. The results show that inhibition of DNMT1 leads to inhibition of DNA replication and slower cell cycle progression. We also present evidence that both methylated and unmethylated origins of replication are inhibited, suggesting that inhibition of DNMT1 results in a methylation-independent inhibition of replication.

Chapter IV

Inhibition of DNA Methyltransferase inhibits DNA Replication.

J. David Knox, Felipe D. Araujo, Pascal Bigey, Andrew D. Slack, Gerald B. Price, Maria Zannis-Hadjopoulos, and Moshe Szyf.

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Abstract

Ectopic expression of DNA methyltransferase transforms vertebrate cells and inhibition of DNA methyltransferase reverses the transformed phenotype by an unknown mechanism. We tested the hypothesis that the presence of an active DNA methyltransferase is required for DNA replication in human non-small cell lung carcinoma A549 cells. We show that the inhibition of DNA methyltransferase by two novel mechanisms negatively affects DNA synthesis and progression through the cell cycle. Competitive PCR of newly synthesized DNA shows decreased origin activity at three previously characterized origins of replication following DNA methyltransferase inhibition. We suggest that the requirement of an active DNA methyltransferase for the functioning of the replication machinery has evolved to coordinate DNA replication and inheritance of the DNA methylation pattern.

Introduction

Aberrant patterns of DNA methylation are observed in many cancer cells and these changes occur in parallel with hyperactivation of DNA methyltransferase (DNMT 1) (Baylin et al., 1991; el-Deiry et al., 1991). DNMT 1 is induced by nodal cancer signaling pathways (MacLeod et al., 1995; Rouleau et al., 1995; Slack et al., 1999a; Szyf, 1994) and a number of studies demonstrate that the hyperactivation of DNA methyltransferase plays a causal role in oncogenesis. For example, the expression of DNMT 1 in the antisense orientation reverses the tumorigenicity of Y1 adrenal carcinoma cells both in culture and in syngeneic mice (MacLeod and Szyf, 1995) and the intraperitoneal injection of DNMT 1 antisense oligonucleotides into LAF/1 mice bearing tumors derived from the syngeneic tumor cell line Y1 inhibits tumor growth (Ramchandani et al., 1997). In addition, the reduction of DNMT 1 caused by either 5-aza-cytidine treatment or by the mutation of one allele of the DNMT 1 gene reduces the frequency of the appearance of intestinal adenomas in *Min* mice bearing a mutation in the adenomatous polyposis coli gene (Laird et al., 1995).

The mechanism by which the over-expression of the DNMT 1 induces tumorigenesis remains unresolved. An attractive model is that the hyperactivation of DNMT 1 leads to the hypermethylation and inactivation of a large number of genes that suppress tumorigenesis (Merlo et al., 1995), tumor invasion (Yoshiura et al., 1995), and angiogenesis (Ahuja et al., 1997). An alternative hypothesis is that the DNMT 1 protein, through protein-protein interactions, is involved in controlling either the entry into the S-phase of the cell cycle or the activity of origins of replication and thereby progression through the cell cycle (Rein et al., 1997b; Szyf, 1998b).

To investigate how the inhibition of DNMT 1 results in the inhibition of tumorigenesis, we have developed phosphorothioate-modified hemimethylated oligonucleotides that, in the presence of a lipophilic carrier, can enter into the nucleus of

cancer cells in culture, form a stable complex with DNMT 1 and specifically inhibit its activity with an EC50 of approximately 60 nM (Bigey et al., 1999; Szyf, 1998a). We have also developed an inactive analog of this phosphorothioate hemimethylated inhibitor of the same sequence, which does not form a stable complex with DNMT 1 and does not inhibit its activity that can serve as an experimental control (Bigey et al., 1999; Szyf, 1998a). In addition, antisense oligonucleotides and an adenovirus expressing DNMT1 antisense mRNA were use to test the hypothesis that the inhibition of DNMT 1 directly affects the growth of A549 cells by inhibiting DNA replication.

Materials and methods

Oligonucleotide treatment and thymidine incorporation- A549 non-small cell lung carcinoma cells (ATCC: CCL 185) were treated with 100 nM of the relevant oligonucleotide which was mixed with 6.5 µL lipofectin (2mg/mL; BRL) and 1mL of OPTIMEM serum free medium as previously described (Bigey et al., 1999). The dose of oligonucleotide was determined by preliminary dose response assays to result in maximum inhibitory activity with essentially no non-specific toxicity (data not shown). The direct inhibitor used in our study is a phosphorothioate-modified hemimethylated hairpin of the sequence: 5' CTGAA(methyl)CGGAT(methyl)CGTTTCGATCCGTTCAG3' (3118), the control oligonucleotide is identical and is also phosphorothioate-modified but has been modified at all the 2'-O-methyl positions of the sugar backbone (3088). Both oligonucleotides were tagged with fluorescein at their 5' end. The antisense *DNMT1* oligonucleotide used in our study and the mismatch control are phosphorothioate modified: *DNMT1* antisense 5' AAGCATGAGCACCGTTCTCC 3' and mismatch control 5'AACGATCAGGACCCTTGTCC 3'. The oligonucleotide-containing medium was removed from the cells and replaced with regular growth medium after 4 hours. The treatment was repeated after 24 h. DNA synthesis was determined at the indicated time

points after initiation of the first treatment by measuring ^{3}H-thymidine incorporation into DNA following an 8 h pulse with 66 μCi per mL of ^{3}H-thymidine.

Adenoviral infection- *DNMT1* full-length cDNA was cloned into the AdEasy shuttle vector pAdTrack CMV in the Xba site in the antisense orientation. Adenoviral recombination and preparation of infectious particles in HEK 293 cells was performed as previously described (He et al., 1998). A549 cells were infected with either the control AdEasy virus or the AdEasy DNMT1 antisense at a MOI of 50 or 150. 100% percent of the cells were infected as determined by visualizing GFP fluorescence under a fluorescence microscope. 48 h after transfections the cells were pulsed with thymidine as described above and nuclear extracts were prepared for determination of DNA Methyltransferase activity (Bigey et al., 1999).

Mitotic Index- Cells were treated twice with hairpin oligonucleotides at 24 hour intervals. 48 hours after the start of the first treatment, the cells were treated with 1μg per mL colcemid (BRL). At the times indicated, the cells were fixed with -20^{0}C methanol, stained with 1 μg per mL of 4,6 diamidina-2-phenylindole (ICN Biomedicals), mounted and examined.

Isolation of Newly Synthesized DNA- A549 cells were treated twice with oligonucleotide DNA methyltransferase inhibitors at 24-hour intervals, as described above. Following the second treatment, the oligonucleotide-containing medium was aspirated and replaced with complete medium containing 20 μM Bromo deoxy-Uridine (BrdU) for 1 hour. The newly synthesized DNA was isolated from equal amounts of total DNA by immunoprecipitation with an anti-BrdU antibody as previously described (Contreas et al., 1992) followed by the gel isolation of strands 0.4-1.2kb in size. To verify our results, a second recently described method of enriching for nascent DNA, by selecting for 5'RNA-DNA chains from early replication bubbles, was used (Gerbi and Bielinsky, 1997). Equal amounts of total DNA extracted from the cells was treated with

λ-exonuclease, as previously described, to eliminate all the nicked 5'-phosphorylated DNA, leaving intact nascent DNA that has an RNA primer at its 5'position. The nascent DNA enriched samples were subjected to competitive PCR to quantify the amount of nascent DNA initiated from each origin.

Competitive PCR- Competitive PCR was performed as previously described, using the previously described primers and competitors for the ß-*globin*, *c-myc* and *dnmt 1* origins of replication (Araujo et al., 1999; Araujo et al., 1998; Tao et al., 1997).

Hydroxyurea treatment- Cells were serum-starved in OPTIMEM for 24 hours. The medium was then replaced with serum-free OPTIMEM containing 800μM hydroxyurea and incubated an additional 24 hours. To release the cells from the G1/S block, the cells were washed twice with warm PBS and then grown in Dulbecco's modified Eagle's medium (low glucose) supplemented with 10% fetal calf serum and 2mM glutamine. Oligonucleotide treatment was performed during the last four hours of the serum starvation and immediately prior to treatment with hydroxyurea as described above.

Results

Inhibitors of DNMT 1 slow cell growth, the progression through the cell cycle, and the rate of DNA replication- We have previously demonstrated that the treatment of A549 cells with direct inhibitors of DNMT 1 results in an inhibition of their anchorage-independent growth (Bigey et al., 1999). In Fig. 1A we show that the fluorescein-tagged inhibitor (3118) inhibits DNMT 1 activity from A549 cells in a dose dependent manner relative to the inactive analog (35% inhibition at concentration of 50 nM and 65% inhibition at a concentration of 100 nM) as determined by an *in vitro* DNMT 1 assay (Bigey et al., 1999).

To determine whether the DNMT 1 inhibitor inhibits DNA replication, we assayed the rate of ^{3}H-thymidine incorporation following either single or double

treatments. The results, shown in Figure 1B, demonstrate that the direct inhibitor of DNMT 1 causes a 50% inhibition in DNA synthesis 24 h after initiation of treatment relative to the inactive analog (30% versus 80% respectively). This level of inhibition of DNA synthesis remains similar 24 or 72 hours after a second oligonucleotide treatment (which corresponds to 48 and 96 hours after the start of the experiment).

To verify that the inhibition of DNA replication by 3118 is a consequence of inhibition of DNMT 1 activity and not a different cellular response triggered by 3118, we inhibited DNA methyltransferase by expressing a *DNMT1* antisense mRNA. A549 cells were infected with either an AdEasy adenovirus expressing the *DNMT1* cDNA in the antisense orientation or a control AdEasy virus expressing the green fluorescent protein GFP as described in materials and methods. DNMT 1 activity from A549 cells infected by the AdEasyDNMT1 antisense is inhibited 55% relative to A549 cells infected with the control virus as determined by an *in vitro* DNMT 1 assay shown in Fig. 1C (Bigey et al., 1999). To determine whether the inhibition of DNMT 1 by antisense *DNMT1* inhibits DNA replication, we assayed the rate of ^{3}H-thymidine incorporation following 48 h infection. The results, shown in Figure 1D, demonstrate that inhibition of DNMT 1 causes a 40% inhibition in DNA synthesis 48 h after infection relative to cells infected with the control virus. The fact that both antisense expression and the direct inhibitor 3118 inhibit replication supports the hypothesis that inhibition of DNMT 1 inhibits replication.

To verify that all the treated cells incorporate the direct inhibitor and its control we performed both fluorescence microscopy (Fig. 2A) and cell sorting (data not shown). Fluorescence microscopy demonstrated that the oligonucleotide becomes concentrated within the nucleus suggesting that the local inhibition of DNMT 1 activity may be greater than that observed in the *in vitro* experiments.

To determine whether inhibition of DNMT 1 affects the rate of progression through the cell cycle, we performed a mitotic index assay using the mitotic inhibitor colcemid (Fig. 2B-D) as previously described (Cahill et al., 1998), in the presence of either the direct inhibitor (3118) or the inactive analog (3188). The maximal mitotic index of cells treated with the inactive analog was 37% and was achieved 26 hours after the start of the colcemid treatment. The maximal mitotic index of the cells treated with the direct inhibitor was 24% and was achieved 34 hours after the start of the colcemid treatment (Fig. 2D). These results demonstrate that a direct inhibitor of DNMT 1 slows the progression through the cell cycle.

Inhibition of DNMT 1 inhibits origin activity- The rate of DNA synthesis is normally dependent upon the number of active origins. In order to determine whether the inhibition of DNA methyltransferase resulted in an inhibition of origin activity and whether this effect is dependent on the state of methylation of origins of replication, competitive PCR was used to quantify the abundance of two well characterized origins, *β-globin* and *c-myc* (Fig. 3), in newly synthesized DNA as previously described (Araujo et al., 1998). These origins are differentially methylated (Araujo et al., 1998) and are thought to replicate at different points in the S-phase. A549 cells were treated with either the direct inhibitor (3118) or the control (3188) for 48 h and then pulsed with BrdU for 1 h. Newly synthesized DNA was prepared by immunoprecipitation of BrdU pulse-labeled DNA with anti-BrdU antibodies followed by the gel isolation of strands 0.4-1.2kb in size. In order to standardize the experiment, due to differences in primers and competitor amplification efficiencies, competitive PCR of both *β-globin* and *c-myc* origins was performed using A549 genomic DNA (Fig. 3A-D). The results (Fig. 3E-G) show that the DNMT 1 inhibitor (3118) inhibits the activity of both *β-globin* and *c-myc* origins of replication to a similar extent, suggesting that inhibition of DNMT 1 inhibits origins of replication irrespective of their state of methylation.

Inhibition of DNMT 1 inhibits initiation of DNA replication- To further study how inhibition of DNMT 1 affects DNA replication, we used the DNA synthesis inhibitor hydroxyurea. Hydroxyurea, an inhibitor of ribonucleotide reductase, reduces the pool of deoxyribonucleotides in the cell, resulting in the blocking of progression of pre-existing replication forks and late origins but not initiation at early firing origins (Tsvetkov et al., 1997). Therefore, any added effect of the inhibitors would have to be achieved by a mechanism that is independent of the mechanisms affected by hydroxyurea. A549 cells were treated with lipofectin carrier alone, the direct inhibitor (3118) or the inactive analog (3188) for 4 h, followed by a 24 h treatment with 800 μM hydroxyurea (MO, MF 1-3). The cells were then washed twice with PBS and incubated in complete medium for 3 h (MO, MF 4-6). The rate of initiation of the *c-myc* origin of replication (MO1-6) and of a secondary initiation site located 7 kb downstream (MF1-6) was determined by competitive PCR of RNA-primed DNA that was resistant to λ-exonuclease as previously described (Gerbi and Bielinsky, 1997). Nascent DNA differs from genomic DNA by being RNA primed. Figure 4A shows that the λ-exonuclease treatment eliminates effectively all of the genomic DNA and the dephosphorylated plasmid DNA control. As shown in Figure 4B and quantified in Figure 4C and D, hydroxyurea treatment alone does not inhibit the firing of the c-*myc* origin of replication (MO1 and MF1), as expected. FACS analysis demonstrated that the treatment has indeed arrested all the cells at early-S, as expected (data not shown). If the direct inhibitor affects the elongation of nascent DNA strands rather than initiation, then the results (MO2 and MF2) should be the same as treatment with hydroxyurea alone (MO1 and MF1). However, as observed in Figure 4B and quantified in Figure 4C and D, the rate of initiation from the *c-myc* origin of replication is significantly inhibited by the direct inhibitor (MO2 and MF2) but not by the inactive analog (MO3 and MF3). To test whether the effect of the DNMT 1 inhibitor is reversible or whether it has a general toxic effect on the cell, we measured the nascent

DNA abundance following release from the hydroxyurea block and growth in regular medium for 3 h (samples 4-6). As shown in Fig. 4B and quantified in Fig. 4C and D, none of the cells, that is, those untreated (MO4, MF4), the cells treated with the direct inhibitor (MO5, MF5) or cells treated with the inactive analog (MO6, MF6) had any substantial inhibition of replication from the *c-myc* origin. These results (MO4-6 and MF4-6) demonstrate that the inhibitory effects observed on DNA replication by the DNMT1 inhibitor are reversible, and thus not toxic.

The data presented in Figs. 3 and 4 shows that inhibition of DNMT1 dramatically reduces the abundance of nascent strands near origins. However, this inhibition has a significantly less pronounced effect on overall DNA synthesis as measured by incorporation of ^{3}H-thymidine (Fig. 1). The discrepancy between the extent of inhibition of nascent strand abundance near origins and the extent of inhibition of ^{3}H-thymidine incorporation can most simply be explained by the hypothesis that inhibition of DNMT1 leads to inhibition of initiation, not to inhibition of ongoing replication fork movement.

To ascertain that the inhibition of origin activity observed with 3118 is a consequence of DNA methyltransferase inhibition, we measured origin activity following inhibition of DNA methyltransferase by a previously characterized DNMT1 antisense oligonucleotide (Fournel et al., 1999). A549 cells were treated with lipofectin carrier alone, the DNMT1 antisense oligonucleotide (MD88) or the mismatch control (MD208) for 4 h, followed with a 24 h treatment with 800 μM hydroxyurea . The antisense oligonucleotide (MD88) inhibits DNMT 1 activity from A549 cells relative to the mismatch control (50% inhibition at concentration of 80 nM) as determined by an *in vitro* DNMT 1 assay (data not shown). The rate of initiation of the *c-myc* origin of replication and of two initiation sites located in the *dnmt1* locus (Fig. 4), was determined by competitive PCR of RNA-primed DNA that was resistant to λ-exonuclease. As observed in Figure 4E and quantified in Figure 4F, the origin activity from both the *c-myc*

origin of replication and the two *dnmt1* initiation sites of replication is significantly inhibited by the DNMT1 antisense relative to the mismatch oligonucleotide.

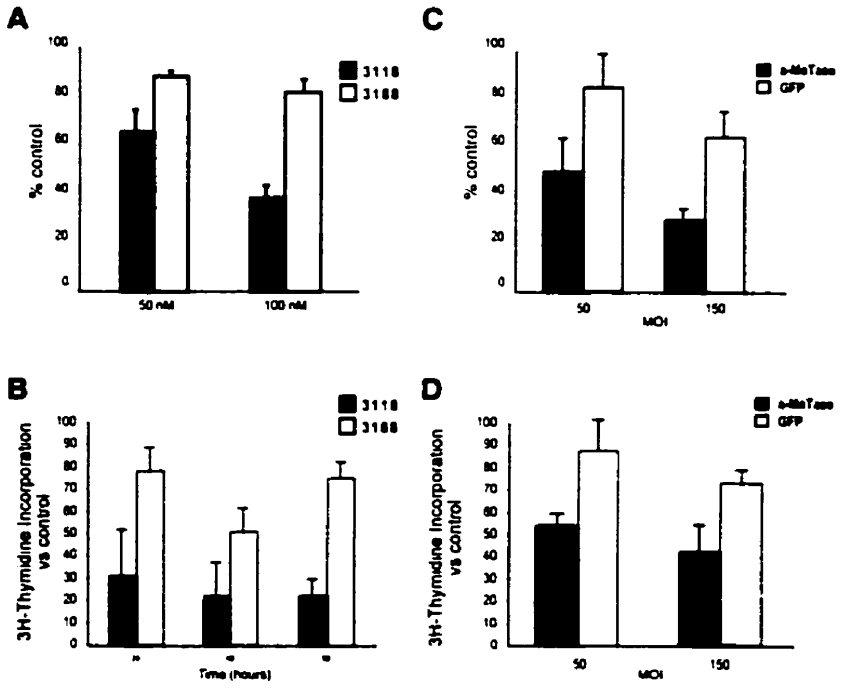

A
% control
3118
3188
50 nM
100 nM
C
% control
GFP
50
150
MOI
B
3H-Thymidine Incorporation vs control
3118
3188
Time (Hours)
D
3H-Thymidine Incorporation vs control
GFP
50
150
MOI

Fig 1. Inhibition of DNA methyltransferase by either a direct inhibitor 3118 or expression of antisense mRNA to DNMT1 inhibits DNA replication. **A,** Three μgs of nuclear extracts prepared from A549 cells were incubated with either no inhibitor, the direct inhibitor 3118 or the inactive analog 3188 at the indicated concentrations, and the DNMT 1 activity in the extract was determined using a hemimethylated substrate and S-[3H] adenosylmethionine as a methyl donor, as previously described (29). The results presented are an average of three determinations +/- S.D. The counts obtained were normalized relative to the counts obtained with untreated A549 cells (~20,000 dpm). **B,** Reduction in ^{3}H-thymidine incorporation by a direct inhibitor of DNMT 1 (3118). The bars represent the percent incorporation of ^{3}H-thymidine over an 8 h incubation period of cells treated with the direct inhibitor (3118) and cells treated with the inactive analog (3188) relative to cells treated with lipofectin only. Triplicate determinations of each time point were made and the results shown are the mean of two independent experiments ± the standard deviation. (The total counts obtained for untreated cells were ~35,000 dpm). **C.** A549 cells were infected with either AdEasyDNMT1 anti-sense or AdEasy control at a multiplicity of infection (MOI) of 50 or 150. Forty-eight hours later 3μg of nuclear extracts prepared from the control and infected cells were assayed for DNA methyltransferase activity as previously described (29). The results presented are an average of three determinations +/- the standard deviation. **D,** Reduction in ^{3}H-thymidine incorporation by AdEasyDNMT1 anti-sense. The bars represent the percent incorporation of ^{3}H-thymidine over an 8 h incubation period of cells treated with either AdEasy or AdEasyDNMT1 anti-sense relative to an uninfected control. Triplicate determinations of each infection were made and the results shown are the mean ± the standard deviation.

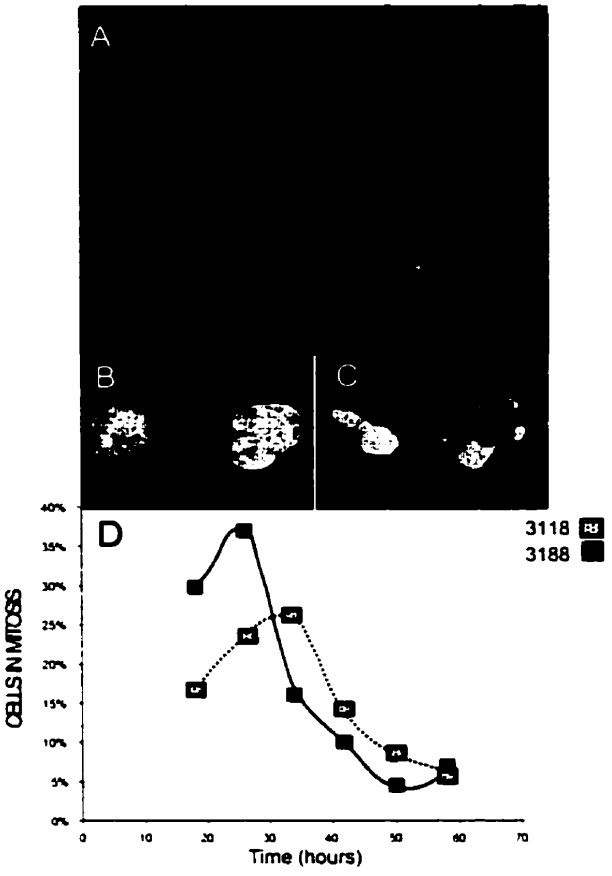

A
B
C
D
3118
3188
Time (hours)

Fig 2. Direct inhibitors of DNA methyltransferase cause reduction in progression of the cell cycle. A549 cells were treated with either 100nM of the inactive analog (3188) or the direct inhibitor (3118). **A,** Nuclear localization of hemimethylated hairpins. A549 cells were treated with the 5' fluorescein-tagged hemimethylated hairpin 3118 and the oligonucleotide was visualized by a fluorescence microscope 1 h after treatment. **B-C,** DAPI fluorescence indicative of nuclear and chromosomal DNA is detected in B and C. **B,** Two interphase nuclei. **C,** Two cells exhibiting the condensed chromosomes characteristic of a sustained mitotic block following colcemid treatment (8) **D,** Mitotic indices of cells treated with the direct inhibitor (3118, speckled) and inactive analog (3188, dark). The cells were treated with colcemid and for the indicated times, fixed, stained with DAPI, and analyzed by fluorescence microscopy. At least 300 cells were counted for each determination and the result shown is representative of those observed in two independent experiments.

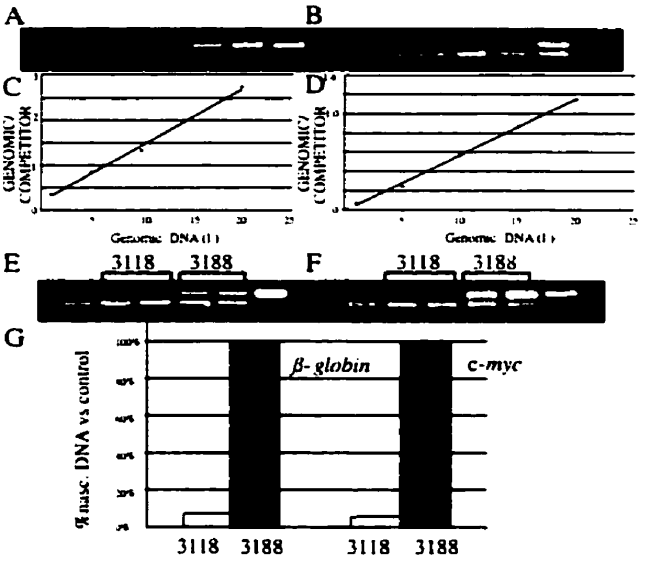
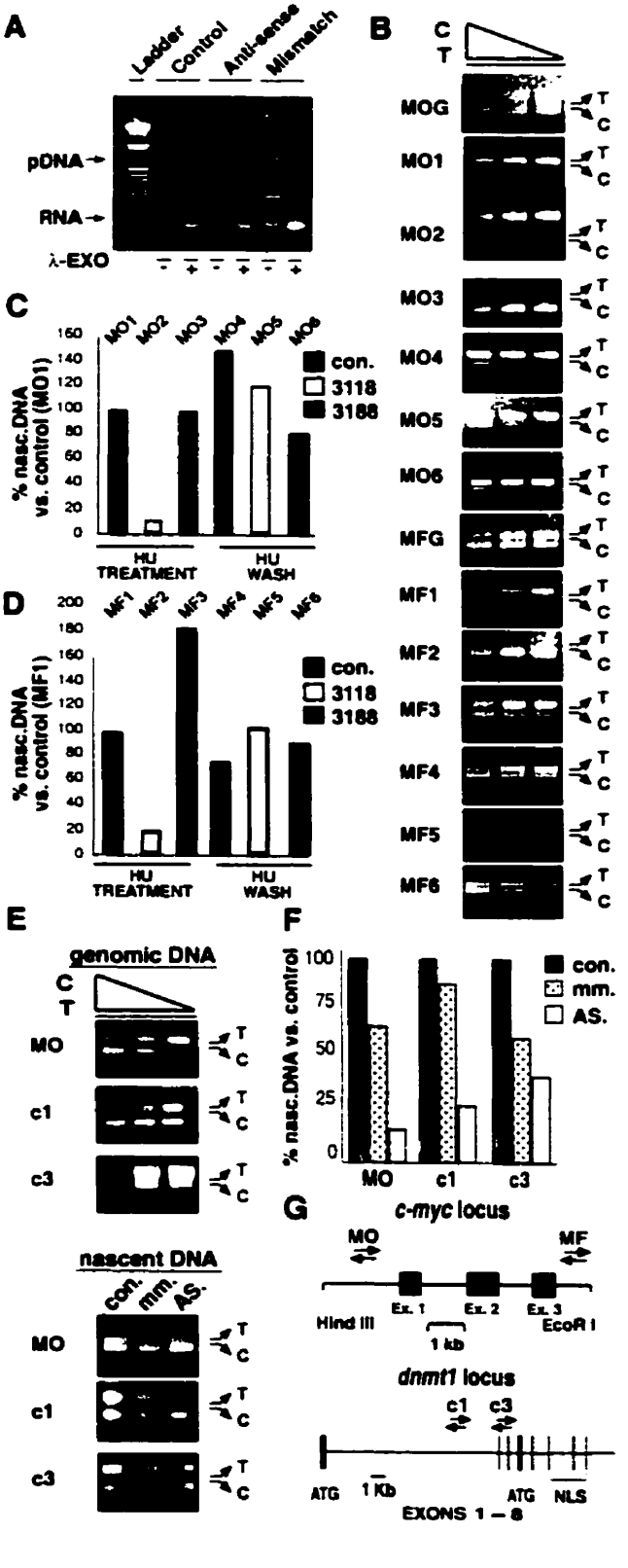

A
B
C
D
GENOMIC/ COMPETITOR
Genomic DNA (1)
E
3118
3188
F
3118
3188
G
% masc. DNA vs control
β-globin
c-myc
3118
3188
3118
3188

Fig 3. Direct inhibitors of DNA methyltransferase inhibit activity of origins of replication. **A**, A549 genomic DNA was used as template for competitive PCR analysis at the *β-globin* origin of replication. The first lane corresponds to PCR amplification of competitor DNA alone and the last lane to target DNA alone. In the intervening lanes increasing volumes of target DNA (A549 genomic DNA; 1, 5, 10, 15, and 20 μL) and a constant amount (100 x 10^3 molecules) of competitor DNA were used. **B**, An analogous competitive PCR analysis was done for the *c-myc* origin of replication. **C**, The linearity of the *β-globin* competitive PCR analysis was verified by plotting the ratio of the genomic DNA PCR signal over competitor DNA PCR signal (y-axis) versus the respective volumes of genomic DNA (x-axis) used in the PCR reactions. **D**, The competitive PCR analysis of the *c-myc* origin of replication was plotted as described in C. **E**, Newly synthesized DNA (1 μl and 5 μl) isolated from cells treated with direct inhibitor to DNA methyltransferase (3118), as well as newly synthesized DNA (1 μl and 5 μl) from cells treated with the inactive analog oligonucleotide (3188), were used as template for competitive PCR analysis at the *β-globin* origin of replication. **F**, A similar competitive PCR analysis was done for the *c-myc* origin of replication. **G**, Origin activity of both the *β-globin* and the *c-myc* origins of replication from cells treated with direct inhibitor to DNMT 1 (3118, white bar) relative to cells treated with the analog oligonucletide (3188, dark bar).


A
Ladder
Control
Anti-sense
Mismatch
pDNA
RNA
λ-EXO
B
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T
MOG
MO1
MO2
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MO5
MO6
MFG
MF1
MF2
MF3
MF4
MF5
MF6
C
% nasc.DNA vs. control (MO1)
con.
3118
3188
HU TREATMENT
HU WASH
D
% nasc.DNA vs. control (MF1)
E
genomic DNA
MO
c1
c3
nascent DNA
con.
mm.
AS.
F
% nasc.DNA vs. control
mm.
G
c-myc locus
Hind III
Ex. 1
Ex. 2
Ex. 3
EcoR I
1 kb
dnmt1 locus
ATG
1 Kb
NLS
EXONS 1 – 8

Fig 4. Two different DNMT 1 inhibitors in hydroxyurea-treated cells inhibit initiation from the c-myc origin of replication. A549 cells were treated with either 800 μM hydroxyurea alone (MO1 and MF1), hydroxyurea and the direct inhibitor, 3118 (MO2 and MF2), or hydroxyurea and the inactive analog, 3188 (MO3 and MF3), for 24 hours. The cells were then washed twice with PBS and incubated in complete medium for three hours (MO4-6 and MF4-6 correspond to washed MO1-3 and MF1-3 respectively). Nascent RNA-primed DNA was prepared by digesting equal amounts of genomic DNA with λ-exonuclease, as previously described (19). **A**, A549 genomic DNA isolated from cells treated with hydroxyurea alone, hydroxyurea and the antisense oligonucleotide, or hydroxyurea and the mismatch control oligonucleotide was phosphorylated and treated with either λ-exonuclease (+) or the incubation buffer alone (-) in the presence of dephosphorylated plasmid DNA (pDNA) to control for both phosphorylation and full digestion. The samples were fractionated on a 1% agarose gel and stained with EtBr. **B,** A competitive PCR assay was used in order to measure nascent DNA abundance. To normalize the differences in primer and competitor amplification efficiencies A549 genomic DNA (MOG, MFG) was used as template for competitive PCR analysis at the *c-myc* origin of replication using either the MO or MF primers. The competition was performed using a fixed amount of target DNA (nascent DNA with MO1-6 and MF1-6, and genomic DNA with MOG and MFG) while 3 different concentrations of competitors were used (5×10^3, 2.5×10^3, 1.6×10^3). T-target product, C-competitor product. The ratio of competitor DNA to target DNA was determined by densitometry and plotted as in Fig.3C,D (data not shown). The calculated percentage of nascent DNA vs. control at the c-*myc* origin (**C**) and a sequence ~7 kb away (**D**) were then plotted as bar graphs. Filled boxes represent samples treated with hydroxyurea alone, empty boxes represent samples treated with hydroxyurea plus the direct inhibitor (3118), and shaded boxes represent samples treated with hydroxyurea plus the inactive

analog (3188). The lines under the plot indicate the samples that were washed following hydroxyurea treatment (HU WASH) or not (HU TREATMENT). **E,** Competitive PCR was performed, again using genomic DNA (from A549 cells) as a control for variability in primer and competitor amplification efficiency (top panel, MO, c1, c3). Competitive PCR was also performed using nascent DNA from A549 cells treated either with antisense oligonucleotides (AS., bottom panel) directed to the DNMT1, or a mismatch oligonucleotide control (mm., bottom panel) or no treatment control (con., bottom panel). **F,** The percentage of nascent DNA versus untreated control was then plotted as in Fig.3C,D (data not shown) **G,** Physical maps of the c-*myc* and *dnmt1* loci with arrows indicating the regions amplified by the primers used for the PCR amplifications.

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sites of DNA replication (Leonhardt et al., 1992; Liu et al., 1998) where it interacts with PCNA (Chuang et al., 1997). Consistent with its localization during S-phase, we and others have previously shown that DNA replication and methylation are concomitant events (Araujo et al., 1998; Rein et al., 1999). The repression of DNA replication following the inhibition of DNMT 1 might be an additional mechanism. However, the results shown in Figure 3 are inconsistent with this hypothesis since they demonstrate that both methylated and nonmethylated origins of replication are similarly affected.

Yet another hypothesis is that the direct inhibitor as well as antisense treatment disrupts protein-protein interactions between DNMT 1 and other proteins of the replication complex, such as the previously demonstrated interaction with PCNA (Chuang et al., 1997), required for DNA replication. Interestingly, it has recently been reported that a protein related to DNMT 1 is expressed in *Drosophila* and associates with PCNA (Hung et al., 1999). Since *Drosophila* DNA does not bear methylated cytosines, this report supports the hypothesis that DNMT 1 might have additional functions in the replication fork. It is possible that the DNA methylation activity of DNMT 1 has evolved to coordinate the processes of DNA replication and inheritance of the DNA methylation pattern.

Additional experiments are required to establish the details of the mechanisms that are responsible for arresting DNA replication following the inhibition of DNMT 1. However, the inhibition of DNA replication by a direct inhibitor of DNMT 1 as well as an anti-sense oligonucleotide and an anti-sense adenoviral vector strongly suggests that DNMT 1 activity is essential for the activity of origins of replication in at least some cancer cell lines.

Acknowledgments

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Connecting text

In the preceding chapter, results demonstrating that inhibition of DNMT1 leads to inhibition of initiation of replication were discussed. Modified hemimethylated hairpin oligonucleotides were utilized as DNMT1 inhibitors, since they can bind DNMT1 and prevent from being functional.

In the following chapter, I describe experiments that were performed to map the region within DNMT1 that is responsible for recognizing the hemimethylated target—DNMT1's target recognition domain. Furthermore, we examined whether the interaction between DNMT1 and PCNA could be disrupted by hemimethylated oligonucleotides.

Chapter V

The DNMT1 target recognition domain resides in the N-terminus.

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Abstract

DNA-cytosine-5-methyltransferase 1 (DNMT1) is the enzyme believed to be responsible for maintaining the epigenetic information encoded by DNA methylation patterns. The target recognition domain of DNMT1 – the domain responsible for recognizing hemimethylated CGs – is unknown. However, based on homology with bacterial cytosine DNA methyltransferases it has been postulated that the entire catalytic domain, including the target recognition domain, is localized to 500 amino acids at the carboxy terminus of the protein. The N-terminal domain has been postulated to have a regulatory role and it has been suggested that the mammalian DNMT1 is a fusion of a prokaryotic methyltransferase and a mammalian DNA binding protein. Using a combination of *in vitro* translation of different DNMT1 deletion mutant peptides and a solid-state hemimethylated substrate, we show that the target recognition domain of DNMT1 resides in the N-terminus (amino acids 122-417) in proximity to the PCNA binding site. Hemimethylated CGs were not recognized specifically by the postulated catalytic domain. We have previously shown that the hemimethylated substrates utilized here act as DNMT1 antagonists and inhibit DNA replication. Our results now indicate that the DNMT1-PCNA interaction can be disrupted by substrate binding to the DNMT1 N-terminus. These results point out towards new directions in our understanding of the structure-function of DNMT1.

Introduction

Vertebrate genomes are modified by methylation of approximately 60-80% of the cytosines residing at the CG dinucleotide sequence (Razin and Szyf, 1984). The distribution of methylated cytosines is not random, resulting in gene and tissue specific patterns of methylation (Yisraeli and Szyf, 1984). A large body of evidence supports the hypothesis that both methylation patterns and activity of DNA methyltransferases (DNMTs) play critical roles in development and in controlling genome functions such as differential gene expression, chromosome imprinting and X-chromosome inactivation (Bird and Wolffe, 1999; Li et al., 1993; Siegfried and Cedar, 1997). It has also been suggested that DNMT1 is a downstream effector of many oncogenic pathways and a potential target for anticancer therapy (MacLeod et al., 1995; MacLeod and Szyf, 1995; Ramchandani et al., 1997; Slack et al., 1999a; Szyf, 1996; Szyf, 1998a). We have previously demonstrated that inhibition of DNMT1 leads to an inhibition of DNA replication (Knox et al., 2000). Recently, it has been shown that DNMT1 is able to form a complex with Rb, E2F and HDAC1 and repress E2F-responsive expression (Fuks et al., 2000; Robertson et al., 2000). Furthermore, it has been shown that DNMT1 can establish a transcriptional repressive complex with HDAC2 and DMAP1 at replication foci (Rountree et al., 2000). These data suggest that DNMT1 has multiple functions in the cell. However, since the DNMT1 target recognition domain is unknown it is not possible to determine how these multiple functions and protein-protein interactions relate to its target specificity.

If DNA methylation patterns contain significant information, there must be a mechanism that ensures its proper inheritance in cell lineages. It has been proposed that patterns of DNA methylation are inherited because DNMT1 is more proficient in methylating hemimethylated DNA than nonmethylated DNA (Razin and Riggs, 1980). This hypothesis has been verified by a number of experiments (Gruenbaum et al., 1982;

Stein et al., 1982). However, it is clear that other mechanisms must be responsible for the specificity of DNA methylation, since there is evidence that DNMT1 is also capable, albeit inefficiently, of de novo methylation (Flynn et al., 1996). Recently it has been shown that the DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation (Okano et al., 1999), thus it is possible that DNMT1 functions exclusively as a maintenance methyltransferase. During DNA replication DNMT1 localizes to replication foci (Leonhardt et al., 1992), where it associates with PCNA (Chuang et al., 1997), and maintenance methylation occurs concurrently with DNA replication (Araujo et al., 1998). Therefore, there is a mechanism that ensures that, as replication takes place, hemimethylated CG sites become fully methylated, whereas unmethylated CG sites remain unmethylated. Another level of specificity is the ability of DNMT1 to recognize CG sequences almost exclusively (Flynn et al., 1996; Yoder et al., 1997). Thus, DNMT1 exhibits both substrate and sequence specificity.

The mammalian DNMT1 is a protein postulated to be composed, based on its similarity to other cytosine DNA methyltransferases, of at least three structural components (Bestor et al., 1988; Bestor, 1988; Bestor, 1992; Kumar et al., 1994; Margot et al., 2000; Ramchandani et al., 1998). These domains are: A catalytic domain at the C-terminus, an N-terminal domain that is responsible for localization of the protein to the nucleus and replication foci, and another poorly characterized central domain. It is unclear as yet which segment is responsible for determining its specificity for hemimethylated CG sequences. Previous reports have shown that when the N-terminal domain is cleaved by proteolysis, the enzyme loses its ability to discriminate between hemimethylated and unmethylated DNA (Bestor, 1992). It has been suggested that the N-terminal domain performs a regulatory role by inhibiting the *de novo* methylation activity of the C-terminal domain (Bestor, 1992). Recently, it has been demonstrated that a mouse-prokaryotic methyltransferase hybrid, DNMT1-HhaI, containing the intact N-

terminus of DNMT1 and most of the coding sequence of HhaI has a 2.5-fold preference for hemimethylated DNA, whereas HhaI by itself has preference for unmethylated DNA (Pradhan and Roberts, 2000). This finding indicates that the N-terminus is responsible for binding specificity to hemimethylated DNA. Moreover, the fact that both *de novo* methyltransferases Dnmt3a and Dnmt3b, that recognize nonmethylated CGs, lack the N-terminal region present in DNMT1 (Bird and Wolffe, 1999; Okano et al., 1999) supports the hypothesis that the hemimethylated substrate specificity domain resides in the N-terminal region. However, three regions within the C-terminal catalytic domain have been proposed to be very near the CG site during methyl-transfer and, thus, to be involved in target recognition (Bestor, 1992; Kumar et al., 1994). DNA binding activity has been shown to reside within the N-terminal domain but this binding activity does not show sequence specificity and it has been suggested that it determines the distance traversed between replication and methylation (Chuang et al., 1996).

Since Dnmt1 is a gene spanning 40 exons over 55Kb of DNA (Ramchandani et al., 1998), targeted mutations by homologous recombination can inactivate only certain parts of the protein. To be able to analyze this data and to be able to correlate it to the different functions of the protein, one has to ascertain which function has been eliminated. A long line of experimental data has attempted to map the target recognition domain of many bacterial DNA methyltransferases (Kumar et al., 1994). It is generally believed that one can distinguish three functional regions in both the bacterial methyltransferases and the homologous catalytic domain of the mammalian enzyme: the evolutionarily conserved catalytic motif, the AdoMet binding regions and the variable target recognition region (Kumar et al., 1994; Malone et al., 1995). The majority of cytosine methyltransferases are composed of an AdoMet binding site at the N-terminal and a target binding region at the C-terminus (Malone et al., 1995). The catalytic domain is usually located between these two domains (Malone et al., 1995). Based on sequence

homology, the variable region, which is believed to contain the target recognition domain, has been localized downstream to the pro-cys catalytic center in the mammalian enzyme (Kumar et al., 1994). There is no biochemical evidence as of yet for this hypothesis.

In order to dissect the role that DNMT1 plays in different biological functions using structure-function analysis, one has to determine which domain of the DNMT1 is responsible for target recognition. Identifying the target recognition domain of the enzyme is also critical for developing direct inhibitors of this enzyme as potential anticancer agents. To map the target recognition domain, we utilized a solid-state hemimethylated DNMT1 substrate to test the binding affinities of *in vitro* translated DNMT1 deletion mutant peptides. This method enabled us to determine which segment of DNMT1 *per se* is responsible for target recognition. It has been previously demonstrated that these modified hairpin oligonucleotide substrates are able to form a stable complex with DNMT1 and inhibit its activity (Bigey et al., 1999). Furthermore, inhibition of DNMT1 with these modified hairpin oligonucleotides results in both inhibition of DNA replication (Knox et al., 2000) and trancriptional upregulation of the tumor supressor p21 (Milutinovic et al., 2000). However, the mechanism by which inhibition of DNA replication occurs remains unclear. In this manuscript, we show that the target recognition domain of DNMT1 does not reside in the previously defined catalytic domain but rather in the N-terminal region of DNMT1, between amino acids 122 - 417. This result demonstrates the fundamental disparity between mammalian and bacterial DNA methyltransferases and the inadequacy of amino acid sequence-sequence similarities *per se* in predicting the functional role of protein domains. The identification of the target recognition domain in the N-terminus points out towards new directions in our understanding of the structure-function relationship of mammalian DNA methyltransferases.

Materials and methods

Generating DNMT1 deletion mutant constructs- DNMT1 cDNA deletion mutants were generated by RT-PCR (Kawasaki and Wang, 1989) from 1 μg of total RNA prepared from the human small cell lung carcinoma cell line H446 (ATCC: HTB-171) using the following set of primers: 5'ccccatcggtttccgcgcgaaaa 3'(sense) and 5'gcatctgccattcccactct 3'.(antisense) for codons 1 to 125; 5'gcaaacagaaataaagaatc 3'. (sense) and 5'gtgatggtggtttgcctggt 3' (antisense) for codons 122 to 170 ; 5'ggcaagggaaaagggaaggg 3'(sense) and 5'gtccttagcagcttcctcct 3' (antisense) for codons 1113 to 1616 ; 5'ttatccgaggagggctacct 3' (sense) and 5'ccctttcccttttgtttccagggc 3' (antisense) for codons 122 to 1112 ; 5'ttatccgaggagggctacct 3' (sense) and 5'cgccggcgcttaaaggcgtt 3' (antisense) for codons 122 to 652. (Amplification conditions were: 95°C 0.5 min; 60°C 0.5 min. 68°C 5 min; for 30 cycles using Promega taq polymerase). The PCR products were cloned in a pCR3.1 vector (Invitrogen) and the sequence of the cDNAs was verified by dideoxy- chain termination method (Sanger et al., 1977) using a T7 DNA sequencing kit (Pharmacia) and alignment to the published human Dnmt sequence (Yen et al., 1992; Yoder et al., 1996). To generate construct M, we cleaved the pCR 3.1 bearing codons 1113-1616 with EcoRI, the fragment was blunted and ligated to a pCR3.1 vector bearing codons 122 to 1212 which was cleaved at the 3' EcoRV site. Construct FTR bears the RT-PCR product encoding codons 122-652 inserted in the EcoRI site of pCR 3.1. Constructs 5'FTR and 3' FTR were generated by digestion of construct FTR with BstEII and NotI. The resulting fragments were blunted and ligated separately into pCR 3.1. Construct N-FTR bears an RT-PCR product encoding codons 122 to 1112 inserted in the EcoRI site of pCR 3.1. To generate construct N-CAT, the blunted fragment bearing codons 1113-1616 was inserted into the EcoRV site of a pCR3.1 bearing codons 122-652. To generate construct CAT, we inserted the 1113-1616 coding fragment into the EcoRV site of a pCR3.1 bearing codons

122 to 168. To generate construct DNMT1, we cleaved construct M with XbaI and ligated it into a pCR3.1 vector bearing codons 1-122 which was cleaved at an internal XbaI site. To generate construct PS (the cysteine at the catalytic dipeptide proline-cysteine is replaced with a serine), the catalytic domain fragment encoding codons 1113 to 1616 was subcloned in the EcoRI site of the pAlter (Promega) site directed mutagenesis vector. Mutagenesis was performed according to the manufacturers protocol using a primer containing a single mismatch in the codon encoding the cysteine in the catalytic dipeptide: 5'AAGCCCTGGGAGGGCGGCC 3' (the underlined G is mismatched). The mutation was verified by sequencing and the mutated catalytic domain was cleaved with EcoRI, blunted and inserted into the EcoRV site of a pCR 3.1 vector bearing codons 122-1112. Construct DNMT1 corresponds to the DNA-cytosine-5methyltransferase initiated at the upstream ATG site and construct M to the one initiated at the downstream ATG site. The insertion of the catalytic domain into the EcoRV site of pCR 3.1 leaves a linker between the GK repeat and the ATG sequence (SRILQIIRLG). To ascertain that this linker does not impair the activity of the catalytic domain, it was inserted in the same position in the full length DNMT1 constructs M and DNMT1 with no observed effect on DNMT1 binding or enzymatic activities (data not shown).

Coupled in vitro transcription and translation- The peptides encoded by the constructs described above were transcribed and translated by coupled transcription-translation using the Promega TNT reticulocyte lysate kit (according to manufacturer's protocol), using 2 µg of each construct and 40 µCi of [^{35}-S]-methionine (1,000 Ci/mmol, Amersham) per 50 µl reaction volume.

Solid-state DNMT1 substrate and other oligonucleotides- All phosphorothioate hairpin oligonucleotides used for the binding study (see Table 1 for sequences) were synthesized at Hybridon Inc. using standard phosphoramadite chemistry as previously described (Ramchandani et al., 1997). Strepavidin coated magnetic beads (Dynabeads

M-280 streptavidin) were purchased from Dynal. The 5' biotinylated phosphorothioate hemimethylated hairpin oligonucleotides (B1 and B2 Table 1) (3 μM) were incubated with 5mg of beads in a buffer containing 10mM Tris-HCl EDTA [pH 7.4] and 1M NaCl for 10 minutes at room temperature. The beads were then washed and resuspended in 500 μl of the same buffer. The final concentration of the oligonucleotide bound to the beads was 150 pmol per mg. To assay binding to the hairpin bound beads, 3 μl of *in vitro* translated polypeptides were pre-incubated in a 30 μl reaction mixture including nonspecific or specific competitor oligonucleotides (100 μM), 10mM TrisHCl EDTA [pH 7.4] protease inhibitors (PMSF, Aprotinin, Sodium Vanadate, 1mg/ml) and 40 mM NaCl. For the experiments in which CAT and FTR peptides were concurrently present, 3 μl of each of the *in vitro* translated peptides was utilized where the plus sign (+) is indicated and 6 μl and 9 μl were used where indicated by a triangle. After 30 min. pre incubation with competitor oligonucleotide, the mixture was applied to 500 μg of hairpin bound beads (mix 1/1 of oligonucleotides B1 and B2 coated beads) for 5 min. The beads and supernatant were separated by a magnet and the beads were washed twice with 50 μl of 10mM Tris-HCl EDTA [pH 7.4] buffer followed by one wash in a 1M NaCl containing buffer. The beads were resuspended in 30 μl of 10mM Tris-HCl EDTA [pH 7.4] 0.1% SDS solution and boiled for 5 minutes. The supernatant (fraction S, corresponding to unbound proteins) and boiled fraction (fraction B, corresponding to bound proteins) were loaded onto an SDS-PAGE gel, dried and exposed to autoradiography. The autoradiograms were scanned, the amount of proteins in each fraction was quantified and the percentage of protein stably bound to the beads was calculated as boiled/ (boiled +supernatant)*100.

Cell culture and transient transfections- HEK 293 cells, a human adenovirus type 5 transformed human embryonal kidney cell line (Graham et al., 1977) (ATCC: CRL 1573), were grown in Dulbecco's modified Eagle's medium (high glucose)

supplemented with 10% fetal calf serum and 2 mM glutamine. For transient transfection experiments, HEK 293 cells were plated 18 h prior to transfection at a density of 7×10^5 cells/100-mm tissue culture dish. The cells were transfected with 5 µg of Xpress-FTR plasmid using the calcium phosphate protocol (1988). The medium was replaced 24 h after transfection and the cells were harvested 48 h after transfection.

Immunoprecipitations and Western Blot Analysis- Nuclear extracts were isolated as previously described (Szyf et al., 1991). For the immunoprecipitation assay, 400 µg of nuclear extracts were incubated with 10 µl of the agarose-conjugated PCNA antibody (PC10 Santa Cruz) at 4°C overnight. For the competition experiments the reactions were pre-incubated with 100 nM final concentration of competitor oligonucleotides (Sc and H1) for 1 h before addition of the PCNA antibody. After the overnight incubation, the beads were spun and washed three times with PBS. The immune complexes were resolved on a 7.5 % SDS-polyacrylamide gel electrophoresis. After transferring to a polyvinylidene difluoride membrane and blocking the nonspecific binding with 5% milk, Xpress-FTR protein was detected using mouse anti-Xpress antibody (Invitrogen) at a 1:5000 dilution, followed by peroxidase conjugated anti-mouse secondary antibody (Jackson ImmunoResearch) at a 1:20000 dilution, and enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

Nuclear Extract Binding assay - Nuclear extracts were isolated as previously described (Szyf et al., 1991) and binding was performed utilizing 500 µg of hairpin bound beads (mix 1/1 of oligonucleotides B1 and B2 coated beads) for 5 min. The beads and supernatant were separated by a magnet and the beads were washed twice with 50 µl of 10mM Tris-HCl EDTA [pH 7.4] buffer followed by one wash in a 1M NaCl containing buffer. The beads were resuspended in 30 µl of 10mM Tris-HCl EDTA [pH 7.4] 0.1% SDS solution and boiled for 5 minutes. The supernatant (fraction S, corresponding to unbound proteins) and boiled fraction (fraction B, corresponding to

bound proteins) were loaded onto an SDS-PAGE gel and western blot analysis was performed as above.

Results

Coupled in vitro transcription/translation of recombinant human DNMT1 deletion mutants - In order to map the DNMT1 target recognition domain, DNMT1 deletion mutant peptides were created using a coupled *in vitro* transcription/translation rabbit reticulocyte system (Fig. 1A, B). The constructs were designed as follows: DNMT1 bears the entire coding sequence of the enzyme starting at the upstream ATG initiation site (Yoder et al., 1996), construct M bears the coding sequence of the DNMT1 starting at the downstream ATG initiation site (Yen et al., 1992), construct PS bears a single base mutation converting the previously proposed catalytic site proline-cysteine dipeptide to proline-serine (Kumar et al., 1994), construct N-FTR bears the N-terminal fork targeting region and the central domain region contained within amino-acids 122-1112, construct N-CAT bears the N-terminal and catalytic domains with a deletion of the central domain from amino-acid 652 to 1113, construct CAT encodes the entire catalytic domain as proposed by homology to bacterial cytosine methyltransferases plus the N-terminal portion responsible for nuclear localization (NLS) (Bestor et al., 1988; Bestor, 1992; Kumar et al., 1994), construct FTR bears the N-terminal region between amino-acids 122 and 652, construct 5'-FTR is a deletion of FTR containing the region between amino-acids 122 and 417, and construct 3'-FTR contains the region between amino-acids 417 and 731 (Fig. 1A). We utilized a luciferase construct as a control (Fig. 1A). Following coupled *in vitro* transcription/translation in the presence of [^{35}-S] methionine, the peptides were size fractionated by SDS-PAGE and visualized by autoradiography (Fig. 1B). All the peptides migrated according to their expected sizes.

Oligonucleotide design- Hemimethylated and nonmethylated CG-containing phosporothioate hairpin oligonucleotides were designed as probes and competitors for

our binding assays (Table 1). Since oligonucleotides B1 and B2 are biotinylated at the 5' arm of the hairpin, they can be conjugated to streptavidin-coated magnetic beads and used as solid-state probes for peptide binding experiments. The remaining oligonucleotides (Sc, H1 and N1) were utilized as competitors. The 5' arm of the hairpins containing methylated cytosines behaves as the methylation guiding parental strand and the 3' arm behaves as the methyl-acceptor nascent strand of replicating DNA. The hairpin substrates we utilized contained an inosine (I) instead of the methyl acceptor cytosine. These substrates have been previously shown to form a high affinity complex with DNMT1 that could be dissociated only by boiling (Bigey et al., 1999). Results from these experiments have also indicated that the inosine (I) group substitution, as well as synthesis of the backbone with a phosphorothioate modification, results in increased hairpin binding affinity to DNMT1 (Bigey et al., 1999). Moreover, the CG sites present in the hairpins are separated by 4 bases to avoid tandem CGs, which have been demonstrated to be poor substrates for DNA methyltransferases (Bigey et al., 1999).

The DNMT1 target recognition domain resides in the N-terminal region. Previous studies have shown that DNMT1 forms a low affinity-complex with both hemimethylated and nonmethylated DNA (Flynn et al., 1996). It has been proposed that the initial binding of DNMT1 with DNA does not discriminate between hemimethylated and nonmethylated DNA (Flynn et al., 1996), and that discrimination between the two substrates occurs during catalysis (Flynn et al., 1996). In our binding assays (Figs. 2-5), the ability of a polypeptide to form a stable complex with the magnetic bead conjugated substrate is measured by comparing the abundance of [^{35}-S] labeled polypeptide in the supernatant fraction (indicated as S in Figs. 2-5) versus the abundance in the fraction eluted by boiling the hairpin bound beads (indicated as B in Figs. 2-5). As observed (Fig. 2A), in the absence of any competitor, peptide M is present exclusively in the bound fraction (B), whereas a luciferase peptide, used as a negative control, is unable to form a

complex with the hemimethylated solid-state substrate, and is therefore present exclusively in the supernatant fraction (S).

To test the binding specificity of the M peptide for the hemimethylated solid-state substrate, we incubated [^{35}-S]-methionine labeled *in vitro* translated M peptide with the substrate in the presence of increasing concentrations of either a hemimethylated (H1) or a nonspecific (Sc) oligonucleotide. As observed (Fig. 2B), in the absence of any competitors (N), M is able to form a stable complex with the hemimethylated hairpin. This is indicated by the presence of M exclusively in fraction B (Fig. 2B). Complex formation is not challenged by an excess of 100 μM of nonspecific oligonucleotide (Sc), since M remains exclusively in fraction B. Conversely, complex formation is partly abolished by a challenge with 10 μM of cold hemimethylated competitor (H1), and is completely abolished by a challenge with 100 μM of cold competitor, as indicated by the disappearance of M from B, and its presence in S.

To determine which domain of DNMT1 interacts specifically with the hemimethylated substrate, we incubated the different [^{35}-S]-methionine labeled recombinant polypeptides with the solid-state hemimethylated substrate in the presence 100 μM of either hemimethylated (H1), nonmethylated (N1) CG bearing hairpin oligonucleotides or a nonspecific competitor (Sc) (Table 1). As observed in the autoradiograms (Fig. 3A) and in the graphical representations (Fig. 3B), all the polypeptides form a stable complex with the solid-state substrate in the absence of any competitors. This is indicated by their presence in fraction B, suggesting that both C-terminal and N-terminal domains can recognize and form a stable complex with the substrate. However, the binding of the catalytic domain peptide (CAT) to the hemimethylated substrate is completely abolished by nonspecific (Sc) as well as specific competitors (H1, N1). This is evident from the presence of the peptide exclusively in fraction S and its complete absence from fraction B. The binding of peptides bearing

either the N-terminal domain (N-FTR, N-CAT, FTR, and 5'-FTR) or the complete protein (M and DNMT1) is not competed by the nonspecific competitor (Sc) (>80% remain bound) but is abolished (<40% remain bound) by the hemimethylated competitor (H1). The nonmethylated CG bearing hairpin (N1) is a weaker competitor as indicated by the distribution of the polypeptide in both the bound and unbound fractions. In all of these cases the hemimethylated hairpin is 2-4 fold more effective in competing out binding to the solid-state substrate than the nonmethylated homolog (Fig. 3B). The pro-ser mutant (PS) also shows a preference for the hemimethylated substrate, but interestingly it is competed less efficiently by the H1/N1 competitor pair. The fact that the competition profiles of DNMT1 and N-CAT are similar indicates that the central region of the enzyme (amino-acids 652-1113) is not required for target specificity. This is confirmed by the similarity of competition profile of these two peptides with the FTR peptide competition profile. Moreover, results indicating that binding of F-CAT to the hemimethylated substrate is competed as efficiently by the nonspecific competitor (Sc) as it is by the hemimethylated competitor (H1) further confirms that the central region is not essential for target recognition. The 5'-FTR peptide still retains hemimethylated binding specificity, which is indicated by the fact that H1 competes for binding more efficiently than N1 and Sc. Interestingly, the binding specificity of 3'-FTR peptide for the hemimethylated substrate is weaker than the more inclusive FTR peptides, since all competitors have a similar effect on binding. Taken together, these results support the hypothesis that the target recognition domain of DNMT1 resides in the N-terminal segment (amino-acids 122-417) of the protein, overlapping with the fork targeting region.

We then tested whether the FTR peptide still binds specifically to hemimethylated DNA when both CAT and FTR peptides are present in the same reaction, as is the case with the natural product. We tested their affinity to hemimethylated DNA under two conditions, in the absence or in the presence of competitor DNA. The presence of

competitor DNA mimics the *in vivo* scenario, where DNMT1 has to discriminate between its specific target and the bulk of non-CG DNA. The results (Fig. 4A) demonstrate that in the absence of any competitors both CAT and FTR peptides can bind the target, in agreement with results from Fig. 3 showing that both peptides have high affinity to DNA. However, in the presence of a nonspecific competitor (Fig. 4B), as is the case when DNMT1 interacts with the genome *in vivo*, only FTR is bound to hemimethylated DNA. Having both CAT and FTR peptides concurrently also verifies that the differences observed between distinct peptides in the competition experiments depicted in Fig. 3 are not the result of impurities in the *in vitro* translation reaction of one peptide versus another. For clarity only the bound fractions were loaded.

PCNA/FTR complex can be disrupted by a hairpin oligonucleotide- Previous reports have shown that modified hairpin oligonucleotides can work as *bona fide* antagonists of DNMT1 and inhibit DNA replication (Bigey et al., 1999; Knox et al., 2000). Since the target recognition of DNMT1 may overlap with its PCNA binding site, we tested the hypothesis that the DNMT1/PCNA complex could be disrupted by FTR binding to the modified hairpin oligonucleotides. We transfected HEK 293 cells with an Xpress-tagged FTR expression vector followed by PCNA immunoprecipitations in the presence of either a nonspecific oligonucleotide (Sc), a hemimethylated hairpin (H1), or no competitor (N). The results (Fig. 5A, top panel) indicate that the PCNA/DNMT1 complex can be disrupted by H1, but not by Sc. The presence of IgG and PCNA in all the immunoprecipitations was also tested (Fig. 5A, middle and bottom panels). We also immunoblotted the supernatants of these immunoprecipitations to confirm FTR expression (Fig. 5A, bottom panel). Since the previous experiments had been performed using *in vitro* translated peptides, we tested the ability of the FTR peptide to bind the hairpin solid-state substrate in HEK 293 nuclear extracts. The results (Fig. 5B) demonstrate that the FTR peptide does indeed bind the solid substrate under these

conditions, as indicated by the FTR presence in the bound fraction (B). These results suggest that the disruption of the PCNA/DNMT1 complex might be a possible mechanism by which these modified hairpin oligonucleotides inhibit DNA replication.

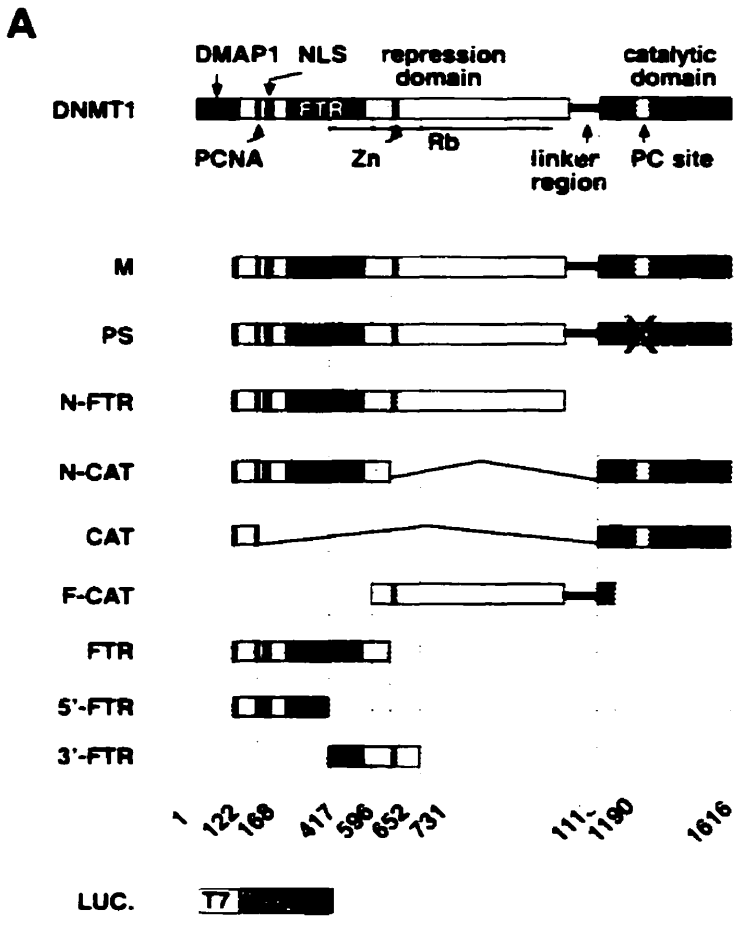

A
DMAP1
NLS
repression domain
catalytic domain
DNMT1
FTR
PCNA
Zn
Rb
linker region
PC site
M
PS
N-FTR
N-CAT
CAT
F-CAT
FTR
5'-FTR
3'-FTR
1
122
168
417
596
652
731
1111-1190
1616
LUC.
T7
B
N-FTR
N-CAT
CAT
FTR
M
F-CAT
5'-FTR
3'-FTR
PS
DNMT1
LUC.
220 kD
97 kD
66 kD

Fig 1. ***In vitro*** **translated human DNMT1 deletion mutant peptides.** **A,** Physical maps of constructs of the different deletion mutants of human DNMT1 are shown relative to a schematic representation of the full length human DNMT1 (construct DNMT1). The previously proposed structural motifs and functional domains are illustrated DMAP1 – DNMT1 associated protein binding region (aa 1-126), PCNA - PCNA binding motif (aa 162-174), NLS- Nuclear Localization Signal (aa 194-213), FTR- Fork Targeting Region (aa 320-567), Zn-Zinc Binding peptide, containing the CXXC region (aa 653-691), Rb - Rb binding region (aa 416-913), repression domain (653-812), Linker region- linker introduced in the catalytic domain containing constructs (10 aa between aa 1112-1113), PC site, Pro-Cys dipeptide site (aa 1225-1226). The following peptides were translated: DNMT1, full length peptide (aa 1-1616); M, DNMT1 protein initiated at the downstream ATG (aa 122-1616); PS, as construct M but the cysteine at position 1226 was converted to a serine by site-directed mutagenesis; N-FTR, includes aa 122-1112; N-CAT, includes aa 122-652 and 1113-1616; CAT, includes aa 122-168 and 1113-1616; F-CAT, includes aa 596-1190; FTR, includes aa 122-652; 5'-FTR, includes aa 122-417; 3'-FTR, includes aa 417-731; LUC., luciferase control construct. **B,** The different constructs were incubated in a coupled *in vitro* transcription-translation reaction mix (Promega) in the presence of [^{35}S]-methionine as described in materials and methods. The radiolabeled peptides were fractionated on SDS-PAGE and exposed to autoradiography. The positions of molecular weight markers are indicated.

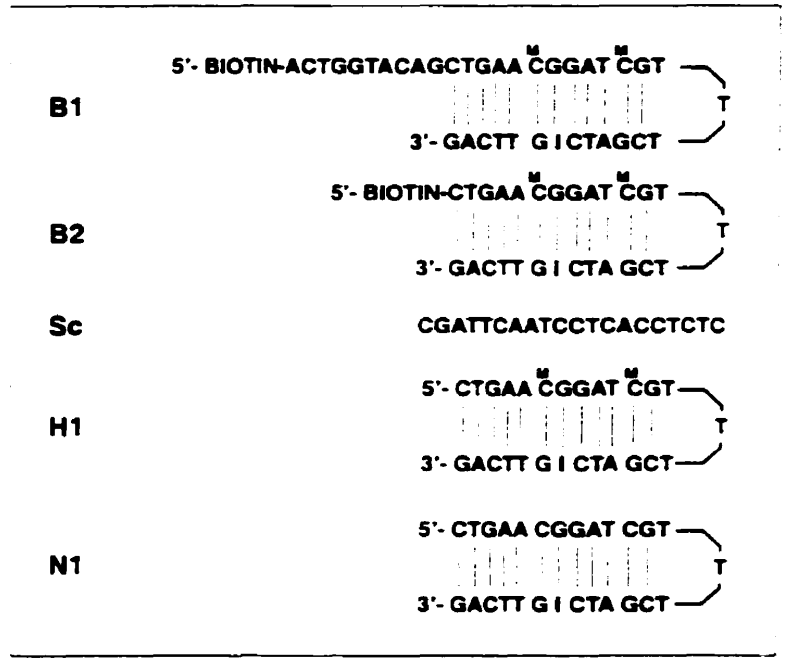

B1
5'- BIOTIN-ACTGGTACAGCTGAA CGGAT CGT
T
3'- GACTT G I CTAGCT
B2
5'- BIOTIN-CTGAA CGGAT CGT
T
3'- GACTT G I CTA GCT
Sc
CGATTCAATCCTCACCTCTC
H1
5'- CTGAA CGGAT CGT
T
3'- GACTT G I CTA GCT
N1
5'- CTGAA CGGAT CGT
T
3'- GACTT G I CTA GCT

Table 1. Hairpin oligonucleotide sequences and modifications. B1 and B2 are solid-state biotinylated hemimethylated substrates that were conjugated to avidin-coated magnetic beads. The remaining oligonucleotides (Sc, H1, N1) served as competitors for the binding assays. BIOTIN indicates biotin-modified oligonucleotides. The shaded M indicates the position of methylated cytosines and the shaded I indicates inosine substitution. All oligonucleotides contain phosphorothioate modified backbones.

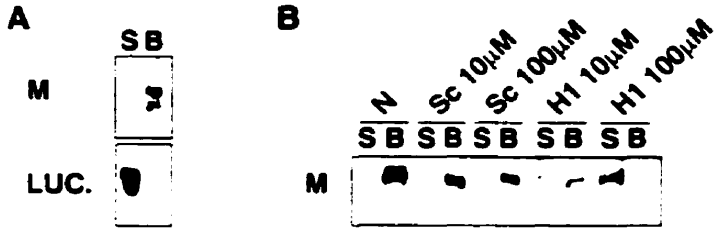

A
S B
M
LUC.
B
N
Sc 10μM
Sc 100μM
H1 10μM
H1 100μM
S B S B S B S B S B
M

Fig 2. Specificity of the stable complex formed between *in vitro* translated human DNMT1 and hemimethylated hairpin. **A,** [^{35}S]-methionine-labeled constructs M and LUC. were *in vitro* transcribed and translated, as described in materials and methods, and incubated at room temperature with avidin-coated magnetic beads, bound to biotinylated hemimethylated hairpins B1 and B2. The hairpin-bound (B) and unbound (S) fractions were separated on SDS-PAGE and exposed to autoradiography. **B,** Construct M was *in vitro* transcribed and translated and pre-incubated with either no competitor (N), increasing concentrations of a nonspecific phosphorothioate oligonucleotide (Sc) or hemimethylated hairpin (H1). Following 30 min. of pre-incubation, avidin-coated magnetic beads bound to biotinylated hemimethylated hairpins B1 and B2 were added to the mix and the fractions were analyzed as in A.

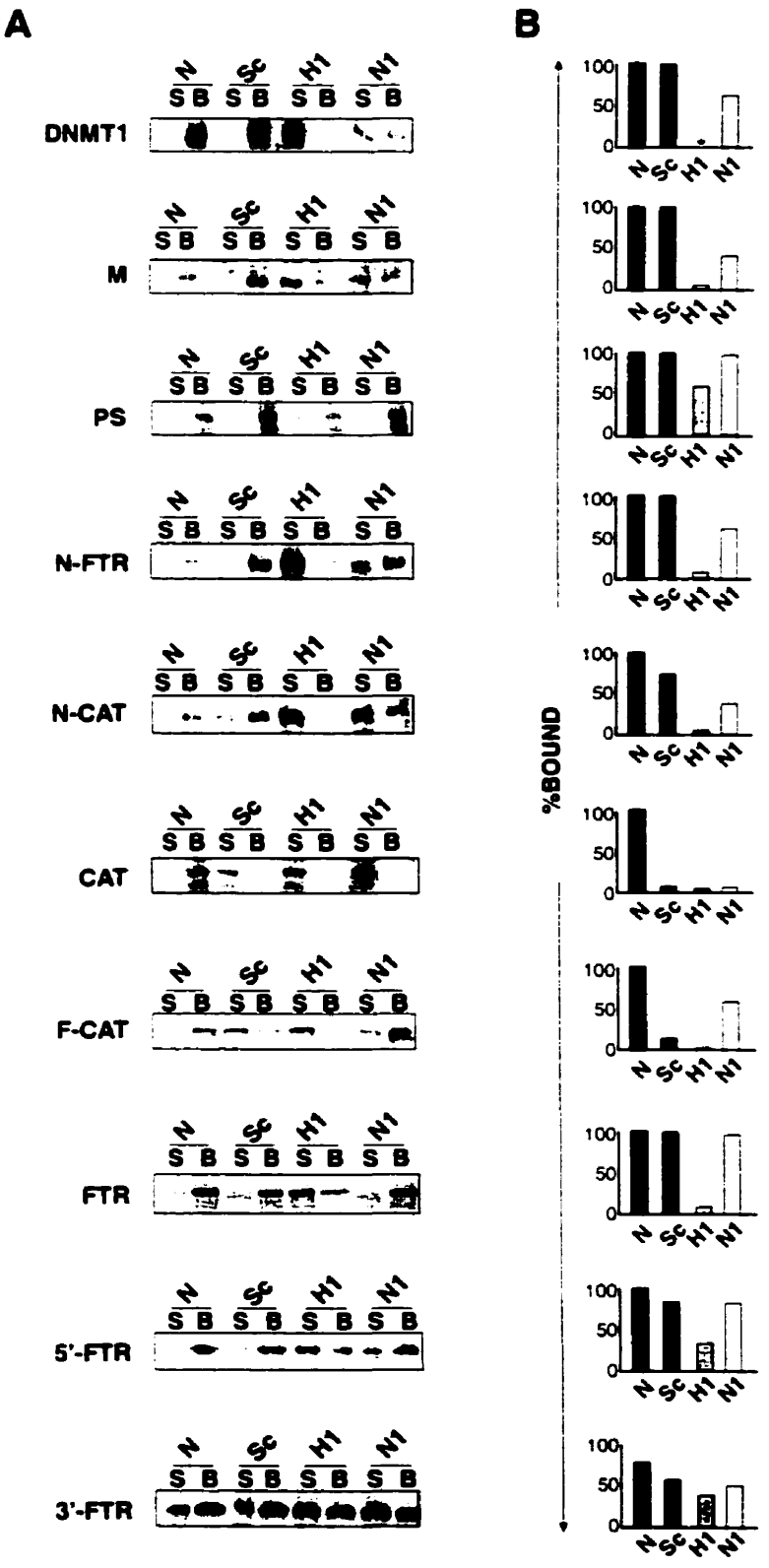

A
B
N
Sc
H1
N1
S B
DNMT1
M
PS
N-FTR
N-CAT
CAT
F-CAT
FTR
5'-FTR
3'-FTR
%BOUND
100
50
0

Fig 3. Binding Specificity of various DNMT1 deletion mutant peptides to the hemimethylated substrate. **A,** [^{35}S]-methionine-labeled DNMT1 deletion mutant peptides were *in vitro* transcribed and translated, as described in materials and methods, and preincubated with either no competitor (N), 100 μM of a nonspecific phosphorothioate oligonucleotide (Sc), hemimethylated hairpin (H1), or nonmethylated CG bearing hairpin (N1). Following 30 min. of pre-incubation at room temperature, avidin-coated magnetic beads bound to biotinylated hemimethylated hairpins (B1 and B2) were added to the mix as described in the materials and methods. The hairpin-bound (B) and unbound (S) fractions were separated on SDS-PAGE and exposed to autoradiography. **B,** Bound (B) and unbound (S) fractions were and quantified by densitometry and the results were plotted as percentage bound.

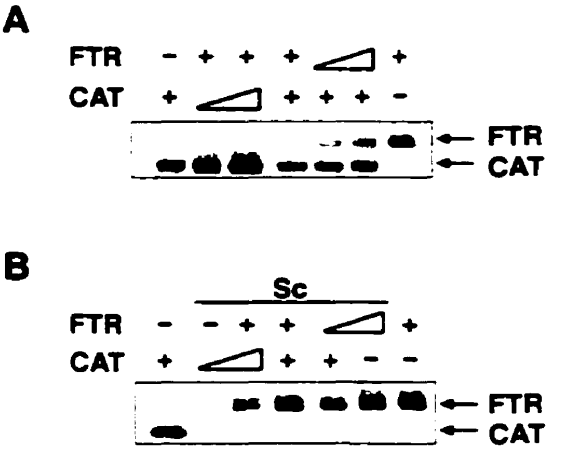
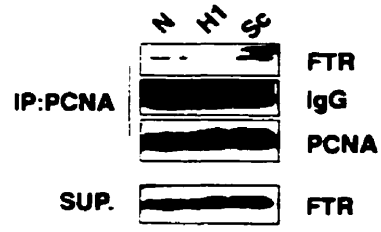

A
FTR
CAT
FTR
CAT
B
Sc
FTR
CAT
FTR
CAT

Fig 4. Differential interaction of the catalytic and N-terminus regions of the DNMT1 with the hemimethylated substrate when present concurrently. A, FTR and CAT peptides were *in vitro* transcribed and translated and different ratios of both peptides were incubated at room temperature with avidin-coated magnetic beads bound to biotinylated hemimethylated hairpins (Bl and B2) in the absence of any competitors, as described in the materials and methods. The hairpin-bound (B) and unbound (S) fractions were separated on SDS-PAGE and exposed to autoradiography. **B,** A similar experiment was performed, but with the addition of nonspecific competitor (Sc) where indicated. The bound and the unbound fractions were separated as above. The arrows indicate the expected positions of FTR and CAT peptides. Plus sign (+) indicates presence of indicated peptide, minus sign (-) indicates absence of indicated peptide, and the triangle indicates increasing concentrations of the indicated peptides.

A



B

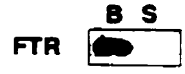


Fig 5. The FTR-PCNA complex can be disrupted by hairpin oligonucleotides. A, An Xpress-tagged FTR construct was transiently transfected into HEK 293 cells and immunoprecipitated utilizing a PCNA antibody in the presence of either no competitor (N), a hemimethylated competitor (H1), or a nonspecific competitor (Sc). Western blot analysis of FTR, IgG and PCNA of immunoprecipitated fractions was performed as indicated. Western blot analysis of the FTR present in supernatant fractions was also performed. **B,** Nuclear extracts from Xpress-tagged FTR transfected HEK 293 cells were incubated with avidin-coated magnetic beads and bound to biotinylated hemimethylated hairpins B1 and B2. The hairpin-bound (B) and unbound (S) fractions were separated on SDS-PAGE and analyzed by western blot with an anti-Xpress antibody.

Discussion

Recognition of hemimethylated CGs, the DNMT1 target sequence, is a critical step in the replication of the DNA methylation pattern and the epigenetic information that it encodes. Although the first mammalian *dnmt* has been cloned and its cDNA sequenced more than decade ago (Bestor et al., 1988; Bestor, 1988), the domain responsible for its target recognition has not yet been clearly defined. Based on sequence homology between mammalian and bacterial cytosine methyltransferases the entire catalytic domain, including the target recognition domain, has been proposed to reside in the C-terminus of the protein (Kumar et al., 1994). The additional N-terminal and central domains of the protein were proposed to perform regulatory roles (Bestor, 1992; Chuang et al., 1996). This hypothesis is based on the assumption that the structure of the mammalian DNMT1 follows the rules laid out in bacteria, and that DNMT1 is an evolutionary hybrid of a primordial DNMT plus at least two additional regulatory protein modules (Bestor, 1992). Our results are in agreement with recent target specificity studies utilizing a mouse-prokaryotic hybrid DNMT, DNMT1-HhaI, containing the intact N-terminus of DNMT1 and most of the coding sequence of HhaI, demonstrating that it has a 2.5-fold preference for hemimethylated DNA, whereas HhaI by itself has preference for unmethylated DNA (Pradhan and Roberts, 2000). Moreover, structural analysis of *de novo* DNMTs, DNMT3a and DNMT3b (Bird and Wolffe, 1999; Okano et al., 1999), which lack the DNMT1 N-terminal region and recognize nonmethylated CGs, further supports the hypothesis that the hemimethylated target recognition domain resides within the DNMT1 N-terminus.

To delineate the DNMT1 target recognition domain, we performed experiments utilizing a solid-state hemimethylated substrate and *in vitro* translated deletion mutant peptides. Competition assays with hemimethylated and nonmethylated substrates revealed that the N-terminal domain specifically recognizes a hemimethylated CG

substrate. The previously described catalytic domain, which has been proposed to contain all the elements required for catalytic activity including target recognition (Kumar et al., 1994), binds DNA but does not exhibit specificity to hemimethylated CG duplex DNA as determined by competition assays. Moreover, when the catalytic domain and the N-terminal domain are concurrently present in the reaction with nonspecific DNA, the substrate binds to the N-terminal domain suggesting that it bears the target recognition domain. Interestingly, the PS mutant is competed less efficiently by the H1/N1 competitor pair, indicating the formation of a tighter complex. This result is supported by a previous study indicating that EcoRII DNMT mutants with substitution of the conserved cysteine for serine bind tightly to DNA (Wyszynski et al., 1993). These mutants resemble the wild-type enzyme in that their binding to substrate is not eliminated by the presence of nonspecific DNA in the reaction (Wyszynski et al., 1993).

Deciphering whether the catalytic components of DNMT1 reside entirely in the C-terminal domain or whether they reside elsewhere is critical for both structure function and crystal structure analysis of the protein, as well as for drug design. Identifying the location of the different components of the catalytic domains of DNMTs is also critical for interpretation of knockout experiments by homologous recombination which target putative catalytic domains (Li et al., 1993). If these alleles, which are inactivated at the C-terminus still maintain the sequence-specific target recognition domain, some of the previously described biological effects of this knockout could be attributed to the DNA binding effects of the truncated protein rather than inhibition of methylation.

The catalytic domain should, by definition, include the substrate recognition domain, otherwise there is no catalysis. Our results shed doubt on the previous designation of the DNMT1 C-terminus as the exclusive catalytic domain. The data presented in this paper suggests that this conclusion might be revisited. In contrast to many bacterial DNMTs (Malone et al., 1995), the target recognition domain of the

mammalian DNMT1 resides at the N-terminus, at a significant distance from the conserved catalytic and AdoMet binding domains. Our data is consistent with the hypothesis that the N-terminal is not just a regulatory domain, as has been previously suggested (Bestor, 1992; Zimmermann et al., 1997), but it is the substrate recognition module of the enzyme. Therefore, the catalytic component of the vertebrate enzyme is composed of two modular domains, a target recognition domain in the N-terminus in addition to the previously described AdoMet binding and methyl-transfer domains in the C-terminus of the enzyme. The fact that the target recognition domain and methyl-transfer domain are located at such a distance on the linear amino acid sequence must not necessarily translate to a similar distance in the tertiary structure of the enzyme. The results presented here might explain the failure of previous attempts to demonstrate DNA methylation activity in the previously postulated catalytic domain of the enzyme (Zimmermann et al., 1997). In spite of the striking structural homology of the C-terminus of the vertebrate to bacterial DNMTs, its expression in bacterial or mammalian cells does not result in DNA methylation activity (Margot et al., 2000). These results illustrate the risk in predicting biochemical functions exclusively from sequence homology.

We had previously demonstrated that the hairpin oligonucleotides utilized here can form a stable complex with DNMT1 (Bigey et al., 1999), inhibit its activity, and inhibit DNA replication in parallel with a transcriptional upregulation of the tumor supressor p21 (Knox et al., 2000; Milutinovic et al., 2000). With the knowledge that the PCNA interaction domain of DNMT1 and its target recognition domain are overlapping, we were able to show that the PCNA-DNMT1 complex could be disrupted by the hemimethylated hairpins. These results might provide an alternative mechanism by which DNMT1 inhibition can supress oncogenic transformation (Szyf et al., 2000). Since DNMT1 is a multifunctional protein, identifying the domains important for

transformation is critical for DNMT1 anti-oncogenesis drug design. Moreover, the identification of the target recognition domain allows a more complete structure-function analysis of DNMT1, and it will help the development of novel direct inhibitors of this enzyme (Szyf, 1998a).

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Chapter VI

General discussion

The research described in this thesis focuses on questions concerning the complex relationship between methylation, methyltransferases, and different aspects of DNA replication. In this regard, the study of methylation and replication has recently seen great advancements. Three novel mammalian DNA methyltransferases (DNMTs) as well as four novel methyl-CG-binding protein (Bird and Wolffe, 1999), one of which with demethylase activity (Bhattacharya et al., 1999; Cervoni et al., 1999; Ramchandani et al., 1999), have been characterized in the last three years. Moreover, DNMT1 and methyl-CG-binding proteins have been shown to associate with histone deacetylases and chromatin remodeling complexes, providing a possible connection between DNA methylation, chromatin structure, transcriptional regulation, and DNA replication (Bird and Wolffe, 1999; Fuks et al., 2000). DNA methylation is fundamental for mammalian development and functions in modulating gene expression, genomic imprinting and X-chromosome inactivation (Bird and Wolffe, 1999; Szyf et al., 2000). Moreover, imbalances in DNA methylation and DNA methyltransferase 1 (DNMT1) activity occur in innumerable human cancers (Szyf, 1998a; Szyf et al., 2000).

Initiation of DNA replication, where control over the frequency and timing of replication takes place, is poorly understood in eukaryotes in general, and in mammalian cells in particular (Zannis-Hadjopoulos and Price, 1999). The study of DNA replication in prokaryotic systems and lower eukaryotes has advanced our understanding of the first principles of replication. Initiation of DNA replication in these systems generally starts from specific sequences; thus, it seems logical to propose that higher eukaryotes might have conserved this specificity of initiation. However, autonomous replication assays demonstrating that replication could initiate at multiple locations on plasmids carrying human sequences challenged this hypothesis, and it was suggested, due to the complexity of higher eukaryotic genomes, that any large region could support replication (Krysan and Calos, 1991). Nevertheless, later site-specific recombination experiments have

clearly demonstrated the need of specific sequences for DNA replication in mammalian cells (Aladjem et al., 1998; Malott and Leffak, 1999). These experiments presented demonstrated that sequences formerly characterized as *bona-fide* chromosomal initiation sites of DNA replication can initiate replication when transferred to new chromosomal locations (Aladjem et al., 1998; Malott and Leffak, 1999).

Recently, technical advancements have facilitated the characterization of new mammalian origins of replication and of proteins involved in this function (Zannis-Hadjopoulos and Price, 1999). The identification and characterization of several novel initiation sites of mammalian DNA replication in our laboratory has allowed for a greater understanding of the general principles governing origin function in mammalian cells (Araujo et al., 1999; Pelletier et al., 1999; Tao et al., 2000; Wu et al., 1993b). For instance, results presented in Chapters II and III demonstrate that certain origins are associated with methylated CG regions, such as the initiation sites contained within the human *dnmt1* locus, whereas other origins, such as those within the *c-myc* locus, are unmethylated. The functional significance of DNA methylation within initiation sites of DNA replication is still unclear; nevertheless, as discussed below, it is possible that methylation may be important for establishing proper timing of replication of different loci within the S-phase of the cell cycle.

Recent results indicate that HDAC2 associates with DNMT1 at replication foci during late S-phase (Rountree et al., 2000). This result suggests the possibility that during late S-phase, HDAC2 joins DNMT1 to replicate hypoacetylated and methylated regions of the genome, wheres the unmethylated regions, associated with hyperacetylated histones, replicate early, without the need for HDAC2. A logical assumption then would be that early replicating, unmethylated regions, do not need the presence of DNMT1. Interestingly however, results presented in Chapter IV demonstrate that even unmethylated initiation sites of DNA replication are inhibited when DNMT1 is inhibited.

This suggests that DNMT1 is important for DNA replication regardless of the methylation state of the genome. The identification of *Drosophila* DNMTs, where DNA methylation is absent, and the finding that one of these DNMTs, DmMTR1, associates with PCNA supports the hypothesis that DNMT1 is important for replication regardless of DNA methylation (Hung et al., 1999). The recent identification of a number of novel mammalian DNMTs and MBDs, now point to the question of whether these proteins also function in DNA replication. To date, the relationship between the activity of these different DNMTs and MBDs, the patterns of DNA methylation, and the control of DNA replication in mammalian cells remains largely unknown.

In *E. coli*, timing and frequency of initiation of DNA replication is controlled by the methylation status of the eleven GATC sites contained within the bacterial origin of replication, *oriC* (Boye and Lobner-Olesen, 1990; Campbell and Kleckner, 1988; Campbell and Kleckner, 1990; Landoulsi et al., 1990; Lu et al., 1994; Russell and Zinder, 1987; Slater et al., 1995). Following initiation of replication at *oriC* the bacterial DNA becomes transiently hemimethylated, and this hemimethylated state at *oriC* is maintained until DNA replication is complete. The hemimethylated state of the bacterial origin inhibits initiation of replication from occuring until the previous round of replication is complete (Boye and Lobner-Olesen, 1990; Campbell and Kleckner, 1988; Campbell and Kleckner, 1990; Landoulsi et al., 1990; Lu et al., 1994; Russell and Zinder, 1987; Slater et al., 1995). A similar control mechanism does not seem to operate in mammalian cells, since results presented in Chapter III demonstrate that there is no lag between DNA replication and methylation. These experiments verified that there is no lag between replication and methylation by measuring the methylation status of short nascent DNA (~ 1kb in length) both at specific origins of replication as well as at a population of orgins and comparing it to the methylation status of genomic DNA. Thus, hemimethylation of the initiation sites of replication does not seem to have the inhibitory effect on replication

that is observed in *E. coli*. However, mammalian DNA methylation does appear to affect the relative time within the cell cycle that a particular locus is replicated. Experiments performed with imprinted genes and X-linked genes have revealed that the methylated genes on the transcriptionally inactive allele replicate later than the active, unmethylated, counterpart (Torchia et al., 1994). This finding is supported, first, by the observations mentioned above, that DNMT1 at replication foci associates during late S-phase of the cell cycle with HDAC2 (Rountree et al., 2000), and second, by the fact that housekeeping genes replicate at the early part of the S-phase of the cell cycle (Zannis-Hadjopoulos and Price, 1999).

Inhibitors of DNMT1 have been shown to act as inhibitors of tumorigenesis, but the mechanism by which these inhibitors work remains unknown (MacLeod and Szyf, 1995; Ramchandani et al., 1997). Data presented in Chapters IV and V indicate that inhibition of DNMT1 leads to inhibition of DNA replication, potentially by a mechanism that involves disruption of protein-protein interactions that may be needed for replication (Knox et al., 2000). The results presented in these Chapters raise the possibility that the interaction between PCNA and DNMT1 may be necessary for initiation of replication (Chuang et al., 1997). DNMT1 binds to PCNA's p21 binding domain and this interaction may be a signal for cells to shift from G1 to the S-phase of the cell cycle. This hypothesis is supported by the observation that when p21 is bound to PCNA, pol-δ dependent replication is inhibited (Flores-Rozas et al., 1994). Inhibition of DNMT1 leads to transcriptional upregulation of p21 (Milutinovic et al., 2000), and recent results indicate that DNMT1 participates in a transcriptional repressor complex with Rb and E2-F to repress E2-F target genes (Robertson et al., 2000). Therefore, one possibility is that DNMT1 inhibition might disrupt this repressor complex, and since the p21 gene contains E2-F binding sites, it may provide an explanation as to how DNMT1 inhibition results in a transcriptional upregulation of p21(Milutinovic et al., 2000). Importantly, the p21

promoter is not methylated, therefore, the transcriptional upregulation of p21 observed following DNMT1 inhibition cannot be the result of promoter demethylation (Milutinovic et al., 2000). These results are important because, as mentioned previously, the accepted dogma is that DNMT1 inhibitors may be effective in cancer treatment only because they could promote DNA demethylation of the tumor suppressors that become methylated in neoplastic cells. These recent results point towards a new understanding in the potential use of DNMT1 as an anticancer agent. In order to design good DNMT1 inhibitors it is critical to understand the function of the different domains of the enzyme. Results presented in Chapter V demonstrate that the amino terminal region of DNMT1 is critical for its ability to recognize hemimethylated CGs, before it can catalyze the methylation reaction. This result is important since it provides novel targets for DNMT1 inhibitor design, such as dominant-negative DNMT1 forms that could be active for replication but negative for methylation and vice-versa.

Knowledge of the basic science behind how the epigenetic program is maintained, altered, and regulated will guide us in the development of pharmacological and biomedical therapies not only for cancer, but also for many other diseases.

Claims to originality

The following results presented in this thesis are original:

1) The identification and characterization of *in vivo* initiation sites of DNA replication within the human *dnmt1* locus.

2) The identification of OBA/Ku binding sites within the human *dnmt1* locus.

3) The demonstration that DNA replication and methylation are concurrent events in mammalian cells.

4) The demonstration that mammalian origins of DNA replication are differentially methylated.

5) The demonstration that DNMT1 inhibition represses DNA replication.

6) The demonstration that the amino-terminal region of DNMT1 is responsible for hemimethylated DNA recognition.

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