Programming the Transcriptional State of Replicating Methylated DNA*

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Walter Stünkel†, Slimane Ait-Si-Ali‡, Peter L. Jones, and Alan P. Wolff‡
From the NICHD, National Institutes of Health, Bethesda, Maryland 20814

CpG methylation is maintained in daughter chromati- 

tids by the action of DNA methyltransferase at the 

replication fork. An opportunity exists for transcription 

factors at replication forks to bind their cognate se- 

quences and thereby prevent demethylation by DNA 

methyltransferase. To test this hypothesis, we injected 

a linearized, methylated, and partially single-stranded 

reporter plasmid into the nuclei of Xenopus oocytes 

and followed changes in the transcriptional activity after 

DNA replication. We find that dependent on Gal4-VP16, 

the action of DNA methyltransferase, and replication-

coupled chromatin assembly DNA replication provides a 

window of time in which regulatory factors can activate 

or repress gene activity. Demethylation in the promoter 

region near the GAL4 binding sites of the newly synthe-

sized DNA did not occur even though the GAL4 binding 

sites were occupied and transcription was activated. We 

conclude that “passive” demethylation at the replica-

tion fork is not simply dependent on the presence of 

DNA binding transcriptional activators.

CpG methylation is causal for transcriptional silencing, im-

printing, and X inactivation (1–4). CpG island hypermethyl-

ation at promoter elements of tumor suppressor genes correlates 

with their repression (5–8). The regulatory mechanisms by 

which DNA methylation interferes with gene activity have 

begun to be unraveled (2). Large repressor complexes contain-

ing methyl-CpG-binding proteins and histone deacetylases 

have been characterized (9–11), directly linking CpG methyl-

ation to transcriptional repression. To achieve the precise epi-

genetic control of gene expression, the mechanisms of mainte-

nance methylation, de novo methylation, and demethylation 

must be strictly regulated.

Several mechanisms have been proposed to keep a promoter 

in a CpG island methylation-free state (reviewed in Ref. 12).

Active demethylation occurs during early mouse development 

(13). However, the existence of proteins exerting demethylase 

activity has been controversial and remains obscure (Ref. 14 

and references therein). Alternatively, DNA replication might 

be involved in controlling the methylation state of promoters 

(15). Replicative processes might provide a window of opportu-

nity for transcription factors to bind to hemimethylated DNA 

and thereby prevent remethylation and subsequent gene re-

pression. Transcription factor binding might interfere with the 

remethylating activity of DNA methyltransferases at the replica-

tion fork (16, 17).

Transcription factor binding during the replication of DNA 

leads to subsequent demethylation concomitant with DNA rep-

lication (18). In Xenopus eggs it has been shown that replica-

tion, together with factor binding but not ongoing transcription, 

results in partial locus-specific demethylation (15). Alternatively, 

nonmethylated CpG islands may result from a transcriptionally active gene, the sequences of which also func-

tion as an origin of DNA replication at a very early develop-

mental stage (19).

All enzymatic activities and co-factors necessary for efficient 

DNA replication are present in the Xenopus oocyte. Although 

oocytes do not initiate DNA replication on double-stranded 

DNA, they can convert single-stranded DNA into double-

stranded DNA and support the concomitant assembly of chro-

matin. Previous studies using single-stranded unmethylated 

DNA showed the relief of nucleosomal repression by transcrip-

tional activators and that the assembly of transcription com-

plexes and replication-coupled chromatin assembly are highly 

competitive processes (20, 21). Here we examine whether tran-

scription factors compete with the DNA methylation mainte-

nance machinery on CpG-methylated templates directly at the 

replication fork and whether this competition can determine 

the transcriptional fate of a reporter gene. Our approach is first 
to in vitro methylate linearized template DNA and subse-
quent to use the enzyme exonuclease III to generate unidi-

rectional deletions. The modified DNA is then microinjected 

into frog oocytes. Using this partially single-stranded methyl-

ated template, we find that the stable binding of transcrip-

tional activators or repressors during the synthesis of the sec-

ond DNA strand programs the gene to remain either active or 

repressed. This programming is, under our experimental con-

ditions, independent of complete demethylation near the bind-

ing site. Hence, factor binding during the time window of sec-

ond strand synthesis might tag the sites for complete demethylation once replication is completed.

EXPERIMENTAL PROCEDURES

Plasmids—The construct pGEMH2BLuc used in this study is de-

rived from the vector PGEM-3Zf(+) (Promega). The plasmid was cut 

with EcoRI and HindIII and ligated with an EcoRI/HindIII fragment 

from the plasmid pE4 containing five Gal4 binding sites and the ade-

novirus E4 promoter (kindly provided by Mike Carey, University of 

California, Los Angeles). The minimal adenovirus E4 promoter was 

replaced with the TaqI/MluI fragment of the histone H2B promoter. 

The luciferase gene sequence ranging from +1 to +200 was polymerase 

chain reaction-amplified using specific primers containing BamHI and 

Smal restriction sites and was subsequently cloned into the Smal/

BamHI linearized vector. Expression plasmids encoding fusion proteins 
of McCP2 and Gal4-VP16 used in this study are described in Ref. 9.

Exonuclease III Digestion—Templates for second strand synthesis in

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To whom correspondence should be addressed: NICHD, National Insti-
tutes of Health, Bldg. 6, Rm. 3A17, Bethesda, MD 20814. Tel.: 301-496-
1208; Fax: 301-480-9354; E-mail: stunkelw@mail.nih.gov.
‡ Supported by a grant from the European Molecular Biology Orga-

nization. Present address: CNRS, UPR 9079, Villejuif, France.
¶ Present address: Sangamo Biosciences, Inc., Point Richmond Tech 
Center, 501 Canal Blvd., Ste. A100, Richmond, CA 94804.
vitro and in vivo were generated using exonuclease III (exo III) \(^1\) (Promega), which produces unidirectional deletions starting at the 3' end. To protect one end from digestion, the plasmid pGEMH2Bluc was cut with KpnI and SmalI. Deletions were produced starting from the SmalI end and heading into the regulatory sites of the H2B promoter and upstream Gal4 sites. 10 μg of the linear fragment were incubated with 500 units of exonuclease III at 37 °C for 10 min in a buffer containing 66 mM Tris/Cl, pH 8.0, and 0.66 mM MgCl₂. To stop the reaction, samples were heat-inactivated by incubation at 65 °C for 20 min. The lengths of the deletions were determined by the treatment of aliquots from the reactions with mung bean nuclease to cleave single-stranded DNA overhangs and by comparing them with the size of the wild-type unmethylated plasmid. After linearization of the modified DNA by standard methods, the fragments were either microinjected into oocytes or subjected to second strand synthesis in a cell-free egg extract derived from Xenopus laevis.

In Vitro Methylation of DNA—All CpG sites in the plasmid pGEMH2Bluc were methylated in vitro using Ss1 methylase following the instructions of the manufacturer (New England Biolabs). After phenol-chloroform extraction, the DNA was precipitated. The efficiency and amount of methylation were checked by restriction analysis with the methylation-sensitive restriction endonuclease HpaII.

Preparation of X. laevis Low Spin Supernatant Extract and Second Strand Synthesis—Female frogs were primed with 200 IU of human choriongonadotropin, and eggs were dejellied and crushed in a buffer containing 50 mM Tris/Cl, pH 8.0, and 0.66 mM MgCl₂. To stop the reaction, samples were mixed with 500 ng of exonuclease III-digested template, and the reaction was allowed to proceed for 2 h at room temperature. The buffer system used contained 10 mM β-glycerophosphate, 3 mM creatine phosphate, 5 mM MgCl₂, 3 mM ATP, and 10 ng creatine kinase. The reactions were stopped with proteinase K and incubation at 1 h at 37 °C. DNA fragments were further purified using the Rapid PCR Purification system according to the manufacturer's instructions (Life Technologies, Inc.).

Microinjection into X. laevis Oocytes and Primer Extension—Oocytes preparation and DNA microinjections were performed as described (22). In vitro transcribed mRNA (2–5 ng) encoding the Gal4-activator domain was microinjected into the oocyte nuclei. The efficiency of microinjection was monitored by primer extension as described (22). An oligonucleotide complementary to the luciferase gene was annealed to the mRNA and subjected to second strand synthesis in a cell-free egg extract derived from X. laevis.

RESULTS

Establishing a System Suitable for Analysis of the Transcription of Replicating Methylated DNA—To examine whether transacting factor binding interferes with the process of DNA remethylation during the time course of replicative processes, we used a single-stranded methylated H2B minimal promoter and a partial luciferase sequence. After linearization with KpnI and SmalI, one strand was deleted unidirectionally with exo III, B, the length of deleted DNA under our experimental conditions varies between 1 and 2.5 kilobase pairs (kb) as determined by mung bean nuclease treatment and subsequent religation of samples. Lanes 2-6 represent time points for exo III digestion for 3 (lanes 2 and 3), 5 (lanes 4 and 5), and 10 min (lane 6). Lane 1 represents no digestion. bp, base pairs.

![Fig. 1. Constructs used in the present study and the strategy for generating single-stranded DNA.](image)

A. plasmid DNA used in this study was generated under "Experimental Procedures." 5× Gal4 binding sites were cloned in front of a complete histone H2B promoter and a partial luciferase sequence. After linearization with KpnI and SmalI, one strand was deleted unidirectionally with exo III, B, the length of deleted DNA under our experimental conditions varies between 1 and 2.5 kilobase pairs (kb) as determined by mung bean nuclease treatment and subsequent religation of samples. Lanes 2-6 represent time points for exo III digestion for 3 (lanes 2 and 3), 5 (lanes 4 and 5), and 10 min (lane 6). Lane 1 represents no digestion. bp, base pairs.

![Fig. 2.](image)

Transcriptional State of Replicating Methylated DNA

Micrococcal Nuclease Treatment of Oocytes—Injected oocytes (20/sample) were homogenized in MNase buffer (DNase I buffer + 3 mM CaCl₂). The oocyte extract was divided into four fractions and subsequently digested with increasing amounts (1–10 units) of MNase ( Worthington) at room temperature for 15 min. Reactions were stopped with 1 volume of a buffer containing 1% SDS/20 mM EDTA. DNA was precipitated according to standard methods and analyzed by electrophoresis, blotted, and hybridized as described above.

\(^1\) The abbreviations used are: exo III, exonuclease III; DNMT, DNA methyltransferase.
methylated templates shows that there is no major change in the methylation status of all HpaII sites in the H2B promoter after DNA synthesis (Fig. 2, C and D, lanes 4-6). Aphidicolin sensitivity indicates that DNA polymerase α is responsible for the polymerizing activity (Fig. 2, C and D, lanes 4-6). We conclude that in vitro methylated and further modified templates are subjected to second strand synthesis carried out by DNA polymerase α-primase.

The Efficient Transcription of Linearized Methylated Templates Depends on a Transcriptional Activator—As described previously, DNA topology plays a significant role in transcription and template linearization and has been reported to exert a dramatic decrease in transcription efficiency (23, 24). The exact mechanism of this effect is not known and may be explained by a decreased affinity of transacting factors for their cognate sequences. Transcription of microinjected linearized...
Fig. 3. Template linearization leads to loss of transcriptional efficiency in the absence of transcriptional activators like Gal4-VP16. A, 20 oocytes for each lane were microinjected with 2 and 5 ng of RNA encoding the activator Gal4-VP16 6 h prior to DNA template injection (1 ng). After overnight incubation at 18 °C, transcription was assayed via primer extension as described under “Experimental Procedures.” H2Bluc denotes the position of the test RNA, and H4 denotes the position of the endogenous H4 RNA as an RNA recovery control. Nonmethylated was compared with methylated DNA, and supercoiled (sc) was compared with linearized (lin) DNA. B, linearized template DNA is effectively packaged into nucleosomal arrays in the oocyte. 12 h after injection half of the oocytes used for the transcription experiments were crushed in micrococcal nuclease buffer and subsequently treated with increasing amounts of micrococcal nuclease (Worthington). The reactions were stopped, and DNA was prepared according to standard procedures and blotted onto Hybond-N membranes after electrophoresis. The membranes were subsequently hybridized against a randomly labeled fragment comprising the inserted DNA used in this study.

DNA into frog oocytes is restored by co-injection of strong activators like Gal4-VP16 (Fig. 3A, lanes 8, 9, 12, 13, 16, and 17). In contrast, when we use a supercoiled circular template, the stimulation of transcription caused by the activator is less pronounced (Fig. 3A, compare lanes 2 and 3 with lanes 4 and 5). Template methylation abolishes transcription completely in the absence of the activator (Fig. 3A, lanes 6 and 7) (25). Gal4-VP16 is capable of binding to methylated DNA when present before chromatin assembly regardless of whether double-stranded, single-stranded exoIII-treated, nonmethylated, or methylated DNA is used. A, for each lane, 20 oocytes were injected with 5 ng of Gal4-VP16 RNA 6 h prior to DNA template injection (1 ng), and transcription was assayed by primer extension. A quantification of the results is shown in B. A.U., arbitrary units. C, 5 ng of RNA encoding the Gal4-DNA binding domain (Gal4DBD) alone was injected into 20 oocytes/lane 6 h prior to DNA injection, and transcription was assayed as in A. H2Bluc denotes the position of the test RNA, and H4 denotes the position of the endogenous H4 RNA as an RNA recovery control.

Fig. 4. Gal4-VP16 renders injected templates active when present before chromatin assembly regardless of whether double-stranded, single-stranded exoIII-treated, nonmethylated, or methylated DNA is used. A, for each lane, 20 oocytes were injected with 5 ng of Gal4-VP16 RNA 6 h prior to DNA template injection (1 ng), and transcription was assayed by primer extension. A quantification of the results is shown in B. A.U., arbitrary units. C, 5 ng of RNA encoding the Gal4-DNA binding domain (Gal4DBD) alone was injected into 20 oocytes/lane 6 h prior to DNA injection, and transcription was assayed as in A. H2Bluc denotes the position of the test RNA, and H4 denotes the position of the endogenous H4 RNA as an RNA recovery control.
Activator RNA is injected during or after replication-coupled chromatin assembly, the activating potential of Gal4-VP16 is decreased in the case of exo III-treated DNA (Fig. 5B, lanes 7 and 8 and lanes 11 and 12). As shown in a DNase I hypersensitivity assay (Fig. 5D), the accessibility of Gal4-VP16 to its binding sites (and therefore the transactivating potential) decreases once rapid chromatinization after the injection of exo III-treated DNA occurs. DNase I-hypersensitive sites around the H2B promoter are lost when activator RNA is injected 2 h later than the partially single-stranded template (Fig. 5D).

The Transcriptional State of a Gene Can Be Programmed for the Injection of methylated DNA—To determine whether second strand synthesis of methylated DNA provides a “window of opportunity” to assemble either an active or a repressive transcriptional state, we microinjected RNA encoding the McCP2 repression domain, fused to a Gal4-DNA binding domain, into oocytes before injection of the nuclease-modified template DNA. McCP2 has been reported to repress transcription in vivo by recruiting histone deacetylases (9, 10). Incubation of the injected oocytes with increasing amounts of trichostatin A (and therefore the transactivating potential) decreases once rapid chromatinization after the injection of exo III-treated DNA occurs. DNase I-hypersensitive sites around the H2B promoter are lost when activator RNA is injected 2 h later than the partially single-stranded template (Fig. 5D).

The Transcriptional State of a Gene Can Be Programmed for the Injection of methylated DNA—To determine whether second strand synthesis of methylated DNA provides a “window of opportunity” to assemble either an active or a repressive transcriptional state, we microinjected RNA encoding the McCP2 repression domain, fused to a Gal4-DNA binding domain, into oocytes before injection of the nuclease-modified template DNA. McCP2 has been reported to repress transcription in vivo by recruiting histone deacetylases (9, 10). Incubation of the injected oocytes with increasing amounts of trichostatin A, a potent inhibitor of histone deacetylases, relieved the transcriptional inhibition because of endogenous histone-deacetylase repressor complexes recruited to methylated DNA as compared with injection of non-trichostatin A-treated oocytes (Fig. 6, compare lane 2 with lanes 3-6). However, in the presence of the hybrid McCP2-Gal4, this alleviation of repression by trichostatin A is less pronounced (lanes 8-11). One explanation for this finding is the specific targeting of abundant protein repressor complexes to Gal4 binding sites upstream of the histone H2B promoter. The process of replication or second strand synthesis is therefore a time window for commitment of the transcriptional status toward repression.

Gal4-VP16 Expression Does Not Lead to Demethylation during Second Strand Synthesis—To determine whether binding of Gal4-VP16 precluded remethylation of newly synthesized templates, we performed restriction analyses to study the methylation status of microinjected DNA after the microinjection of either double-stranded or exonuclease III-treated single-stranded templates (Fig. 7). It is apparent that the bulk of template treated with exonuclease is size-reduced (Fig. 7A, compare lanes 1 and 3). In contrast, when injected into oocytes followed by purification and subsequent restriction, the full-length HindIII fragment appears, indicating the resynthesis of the second DNA strand (Fig. 7B, lanes 4, 10, and 12). The sensitivity of HpaII against restriction cleavage is restored in the case of the DNA that is injected in single-stranded methylated form (compare lanes 6 and 12). This indicates that no complete demethylation of both strands occurs either in the absence (Fig. 7B) or presence (Fig. 7C) of functional Gal4-VP16. On the contrary, no apparent de novo methylation is observed for the injected nonmethylated template (Fig. 7B, lanes 4-6), from which we conclude that demethylation is not induced by transcription factor binding near the HpaII sites within the H2B promoter in the frog oocyte during ongoing synthesis of the second strand under our experimental conditions.
A. The position of the endogenous H4 RNA as an RNA recovery control. H2BLuc

B. Targeting of the MeCP2 repression domain during second strand synthesis leads to transcriptional repression of injected constructs. A. After microinjection of 5 ng of repressor RNA (MeCP2 repression domain fused to the Gal4 DNA binding domain; Gal4MRD) 6 h prior to nuclear DNA injections, the oocytes were incubated in MBSH buffer containing increasing amounts of trichostatin A (lanes 3-6 and lanes 8-11). Results were quantitated in B. TSA. trichostatin A. H2BLuc denotes the position of the test RNA, and H4 denotes the position of the endogenous H4 RNA as an RNA recovery control. A.U., arbitrary units.

**FIG. 6.** Targeting of the MeCP2 repression domain during second strand synthesis leads to transcriptional repression of injected constructs. A, after microinjection of 5 ng of repressor RNA (MeCP2 repression domain fused to the Gal4 DNA binding domain; Gal4MRD) 6 h prior to nuclear DNA injections, the oocytes were incubated in MBSH buffer containing increasing amounts of trichostatin A (lanes 3-6 and lanes 8-11). Results were quantitated in B. TSA. trichostatin A. H2BLuc denotes the position of the test RNA, and H4 denotes the position of the endogenous H4 RNA as an RNA recovery control. A.U., arbitrary units.

**DISCUSSION**

There are two major findings made in this study. First, transcriptional activators or repressors are capable of programming the transcriptional state in the context of methylated CpG dinucleotides within regulatory sites during DNA synthesis. Second, upon binding of transactivators, no demethylation events were observed on newly synthesized CpG-methylated DNA, indicating that the target sites for DNMTs are accessible even in the presence of site-specific DNA-binding proteins.

**DNA Methylation, Methyl CpG-dependent Gene Silencing, and the Replication Machinery Are Associated**—Components of the DNA methylation machinery such as DNMT1 associate with replication foci (26). DNMT1 forms a complex with proliferating cell nuclear antigen in vivo (27). In addition, the inhibition of DNMT1 diminishes DNA replication, and DNMT1 interacts with histone deacetylases and the transcriptional repressor DMAP1 at replication foci (28, 29). It has been proposed that remethylation of newly synthesized CpG islands is directly coupled to the migrating replication fork. After DNA methylation and once chromatin is assembled, methyl-CpG-binding proteins could recruit histone deacetylases (9, 10). Methyl-CpG-binding proteins like methyl-CpG-binding protein-2 and 3 have been reported to bind hemimethylated DNA at the replication fork in late S phase (30). DNA methylation at specific cytosine residues might also serve as a signal recognized by the replication machinery, and this may lead to initiation of replication at more specific genomic sites. This mechanism may be used only by a subset of origins because not every origin of replication appears to have methylated CpG sequences (31).

**DNA Demethylation during Replication**—Little is known concerning factors that are involved in regulation of the cellular methylation pattern. DNA methylation at the specific dinucleotide sequence “CpG” plays a key regulatory role for the transcriptional activity for some loci and potentially for other nuclear processes like replication, DNA repair, and recombination (31, 32, 33). In addition, aberrant methylation of CpG islands in the vicinity of active promoters correlates strongly with the escape from normal cell cycle regulation and therefore oncogenesis (34). Under normal physiological conditions, these CpG islands are methylation-free except for sequences located on the inactive X chromosome where CpG methylation is part of the cellular dosage compensation mechanism in females (reviewed in Ref. 35).

Progress has been made in elucidating the underlying mechanisms for specification and maintenance of an unmethylated CpG island. Protein-DNA interactions at specific CpG dinucleotides are crucial for defining the unmethylated state. In those studies the oriP region of the Epstein-Barr virus was used to demonstrate a site-specific demethylation once the specific factor Epstein-Barr nuclear antigen-1 was bound followed by subsequent replication (18).

DNA demethylation was shown to be a two-step process requiring transacting factor association and replication of a specific sequence. Other transcription factors were shown to operate in an analogous manner in other systems. It has been demonstrated for X. laevis embryos that a massive demethylation occurs at specific regulatory sites of reporter genes after the mid-blastula transition (15). These observations lead to the hypothesis that the replicative processes concomitant with the binding of transcription factors play a role in keeping a CpG sequence free of methylation. An alternative hypothesis is that active demethylating proteins are involved in establishing the demethylated state. The existence of such demethylases has been controversial (10, 11, 36). At present, to our knowledge none of the studies described attempted to address the question of how the transcriptional state of a gene is affected once the regulatory site for a transcription factor has been methylated and DNA synthesis of the second strand is ongoing. Two possible scenarios can be envisaged. (i) Binding of the transcriptional regulator and the remethylating activity like DNMT1 would compete for their target sites, depending on the affinity toward the particular site, and the influence of remethylation may dominate over transcription factor binding, resulting in transcriptional repression. (ii) Another possibility is that the transacting factor would dominate over remethylating activities and therefore program the transcriptional state, allowing rapid initiation of transcription.

It is technically challenging to set up an experimental system that allows the investigation of newly synthesized methylated DNA and its effect on gene transcription. Because of the low activity of bacterial CpG methylases on single-stranded DNA, in vitro CpG methylation of single-stranded circular DNA is not efficient. To circumvent this problem we linearized plasmid DNA harboring regulatory sequences and then in vitro methylated the template DNA with the methylase SssI. To generate a partially single-stranded methylated template, we digested the linear DNA with exonuclease III, giving rise to unidirectional deletions of one strand as a function of time. The resulting heteroduplex DNA was used as a template in oocyte microinjection studies. From initial studies using oocyte microinjections, we concluded that linearized DNA is sufficiently stable in the oocyte after a 12-h incubation and is assembled into nucleosomes (see Fig. 3B). However, as reported by others, linearization of supercoiled plasmid DNA leads to a decrease in transcriptional efficiency (23, 24). Our studies confirmed these previous findings, but it became clear that binding of a strong transcriptional activator such as Gal4-VP16 is capable of overcom-
Fig. 7. Second strand synthesis does not lead to demethylation in either the absence or presence of Gal4-VP16. A, Southern blot showing the DNA fragment pattern before oocyte injection and after restriction digest with the enzymes indicated (nonmethylated DNA). The bulk of DNA in lane 3 depicts the population of exonuclease III-deleted DNA. The arrow denotes the 600-base pair HindIII fragment. B, second strand synthesis in the oocyte leads to an unchanged methylation status in the vicinity of the Gal4 sites and the H2B promoter in the absence of Gal4-VP16. C, Gal4-VP16 expression in the oocyte has no influence on the methylation pattern shown in B.

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