# DNA methylation directs a time-dependent repression of transcription initiation

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Background: The regulation of DNA methylation is required for differential expression of imprinted genes during vertebrate development. Earlier studies that monitored the activity of the Herpes simplex virus (HSV) thymidine kinase (*tk*) gene after injection into rodent cells have suggested that assembly of chromatin influences the methylation-dependent repression of gene activity. Here, we examine the mechanism of methylation-dependent HSV *tk* gene regulation by direct determination of nucleoprotein organization during the establishment of a transcriptionally silenced state after microinjection of templates with defined methylation states into *Xenopus* oocyte nuclei.

Results: The transcriptional silencing conferred by a methylated DNA segment was not immediate, as methylated templates were initially assembled into active transcription complexes. The eventual loss of DNase I hypersensitive sites and inhibition of transcription at the HSV *tk* promoter only occurred after several hours. Flanking methylated vector DNA silenced the adjacent unmethylated HSV *tk* promoter, indicative of a dominant transmissible repression originating from a center of methylation. The resulting repressive nucleoprotein structure silenced transcription in the presence of activators that are able to overcome repression of transcription by nucleosomes.

Conclusions: Silencing of transcription by DNA methylation is achieved at the level of transcription initiation and involves the removal of transcriptional machinery from active templates. This transcriptional repression can occur by indirect mechanisms involving the time-dependent assembly of repressive nucleoprotein complexes, which are able to inhibit transcription more effectively than nucleosomes alone.

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## Background

DNA methylation is proposed to have an active role in gene regulation [1,2]. Normal mammalian development requires both paternal and maternal genomes [3,4]. In the case of imprinted genes, paternal and maternal alleles are differentially expressed during embryogenesis in a methylation-dependent manner. Inappropriate methylation caused by targeted mutation of the DNA methyltransferase gene in mice leads to embryonic lethality [1,2]. Regions of DNA within imprinted genes differ in methylation state depending on the state of the parental allele [5–7]. These regions have been suggested to act as imprinting marks and are often found several kilobases from the promoter that is differentially regulated in response to imprinting [5–7]. Methylation states are continually changing in the early embryo [8] and the exact sites of methylation also show considerable variation [9]. Although DNA methylation is generally correlated with the inactivity of imprinted genes [1,2,10,11], a decrease in DNA methylation leads to the transcriptional repression of the *Igf2* and *Igf2R* genes [1,2]. Molecular mechanisms to explain these diverse results have proven elusive.

Available transient transfection or *in vitro* transcription assays demonstrate invariably that the repression of transcription is dependent on DNA methylation [12,13]. Certain transcription factors fail to associate stably with methylated recognition elements [14]. Both histones [15,16] and proteins recognizing methylated CpG sequences [17,18] can bind selectively to methylated DNA and potentially block transcription factor access. Specialized chromatin structures have also been implicated in maintaining the transcriptionally silenced state [19–25].

Nuclease-resistant chromatin is enriched in histone H1 and 5-methylcytosine [16], and a preference of histone H1 for methylated DNA has been reported [17]. Graessmann and colleagues [20,21] have suggested that chromatin assembly might be critical for DNA methylation to repress gene expression. In these early studies, transcriptional activity itself was not investigated; instead, the Herpes simplex virus (HSV) thymidine kinase (*tk*) gene was injected into rodent cells and thymidine kinase activity was monitored at various times after injection. Activity was detected soon after injection (< 48 hours) of both methylated and

unmethylated genes; however, at later times (> 48 hours), the enzymatic activity disappeared selectively when a methylated gene was injected. Nucleosome assembly on these templates was not investigated in detail, although chromatin was implicated in the repression of gene activity because prior histone association with the DNA template prevented the detection of thymidine kinase activity when the methylated gene, but not the unmethylated gene, was injected [20,21].

These results suggested that the repression of gene expression as a result of DNA methylation might be mediated by indirect mechanisms requiring time to achieve their effects. However, as assays for nucleoprotein structure and transcription itself were not carried out in these studies, a molecular dissection of the mechanism of repression was not possible. Consistent with the view that DNA methylation inhibits transcription indirectly, two separate studies [22,25] have suggested that transcriptional repression has a non-linear dependence on DNA methylation within a chromatin environment [25], and that, in certain circumstances, transcriptional repression is transmissible in *cis* to unmethylated promoter DNA [22]. Drugs that inhibit histone deacetylase alleviated methylation-dependent transcriptional repression [25], indicating a role for chromatin structure in this repression. We have attempted to integrate these various issues by microinjecting templates with defined methylation states into *Xenopus* oocyte nuclei, in which the transcription and nucleoprotein organization can be assayed directly under controlled conditions.

Here, we report the unexpected finding that methylated and unmethylated HSV *tk* promoters have equivalent transcriptional activity and competitive strength immediately following injection into *Xenopus* oocyte nuclei. Nucleoprotein complexes detected by DNase I hypersensitive sites are also assembled on the HSV *tk* promoter in a methylation-independent manner after short incubations. But longer incubation times lead to the selective loss of DNase I hypersensitivity and removal of engaged RNA polymerase from the methylated template. This dominant repression of transcription directed by methylation results in the selective assembly of the inactivated promoter into a nucleosomal array. The repressive nucleoprotein structure assembled on methylated DNA silences transcription in the presence of activators that can overcome transcriptional repression by canonical nucleosomes. Thus, methylation augments significantly the effects of conventional nucleosomes in driving the establishment of a repressive state.

## Results

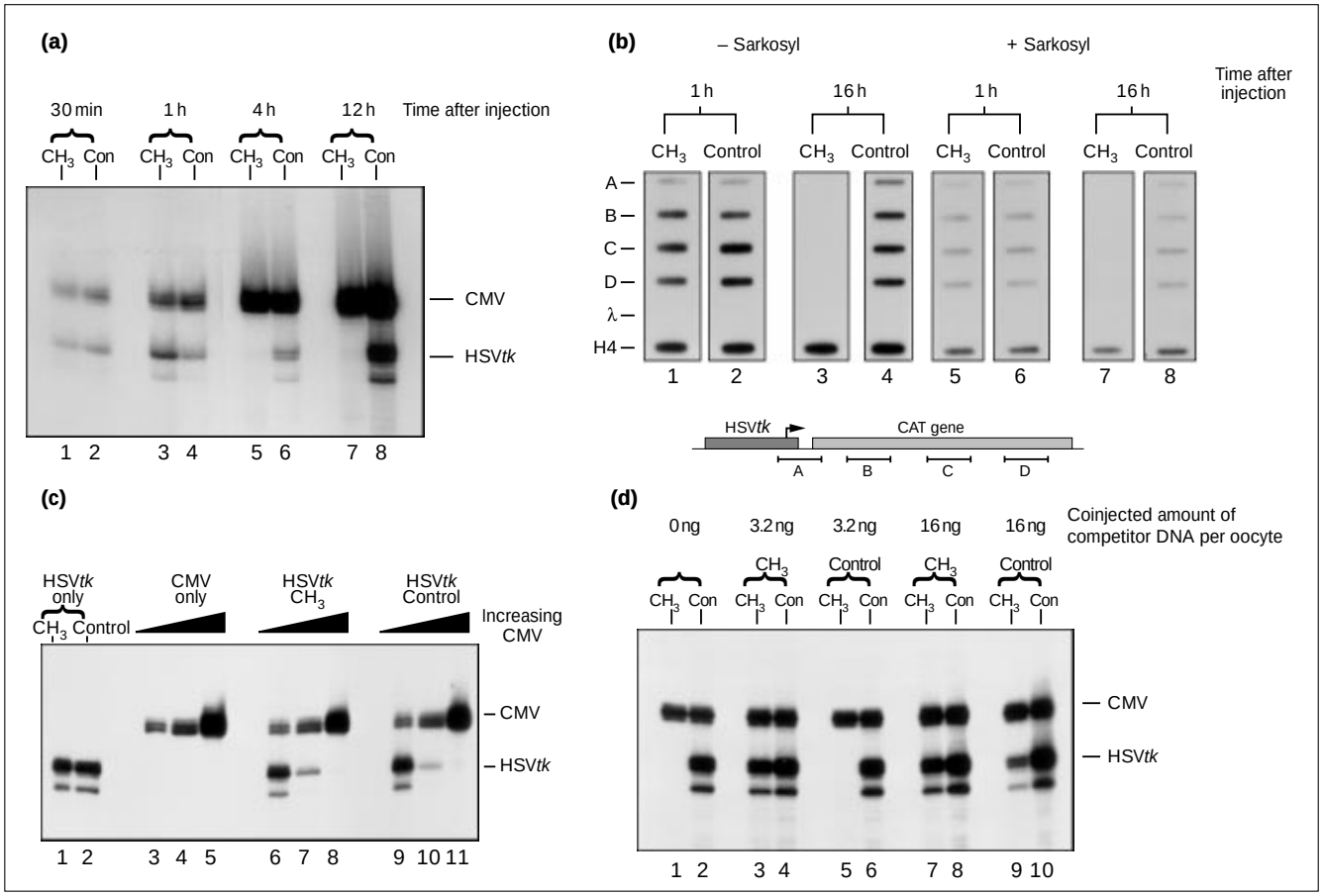
### Methylated and unmethylated HSV *tk* promoters are transcribed identically soon after microinjection, but not later

We used microinjection into *Xenopus* oocyte nuclei of a plasmid containing the HSV *tk* promoter driving the expression of a chloramphenicol acetyltransferase (CAT) reporter gene to determine how methylation might influence transcription. Methylated and unmethylated HSV *tk* templates were mixed with a plasmid containing an unmethylated cytomegalovirus (CMV) promoter driving the expression of a CAT reporter gene as a control, and transcription was assayed by primer extension at various times after injection. Methylated and unmethylated HSV *tk* constructs were transcribed equally early after injection (Fig. 1a, lanes 1–4), indicating that methylation alone was not sufficient to cause transcriptional inactivation. At later times, the methylated HSV *tk* construct was transcriptionally silenced, whereas the unmethylated control continued to be active (Fig. 1a, lanes 5–8).

We examined the distribution of RNA polymerase molecules using the detergent sarkosyl, which strips the DNA template of non-transcribing RNA polymerases and the vast majority of other chromatin-associated proteins, including histones [26]. Transcriptionally engaged RNA polymerases remain on the template in the presence of sarkosyl and can continue to elongate along the template in the presence of RNA precursors. The relative distribution of RNA polymerase along the transcription unit was very similar in the presence or absence of sarkosyl (Fig. 1b, compare lanes 1–4 with 5–8). Transcription run-on experiments indicated that there was an equal distribution of RNA polymerase along the transcription unit early after microinjection of the template, which was independent of the methylation state of the template (Fig. 1b, lanes 1,2). However, at later times, DNA methylation clearly affected transcription at the level of transcription initiation (Fig. 1b, compare lanes 1,3 with 5,7). Therefore, although RNA polymerase is initially engaged on the methylated template, the transcriptional machinery is removed from the template as time progresses.

The equivalent transcriptional activity of methylated and unmethylated HSV *tk* promoters at early times after microinjection was surprising as it was consistent neither with models for methylation-dependent transcriptional repression, whereby methylation-specific repressor proteins bind to the promoter and prevent transcriptional activators from binding, nor with a failure of transcription factors to bind methylated promoter sequences [14,17,18]. In order to investigate further the equivalent transcriptional activity of methylated and unmethylated HSV *tk* promoters at early times after microinjection into oocytes, we then examined their competitive strength for the sequestration of transcription factors. We used template competition assays in which a fixed mass of methylated or unmethylated HSV *tk* promoter constructs were transcribed in the presence of increasing masses of competitor unmethylated CMV promoter template (Fig. 1c). This assay showed that the competitor template inhibited transcription from both HSV *tk* constructs with equivalent efficiency (Fig. 1c, compare

**Figure 1**

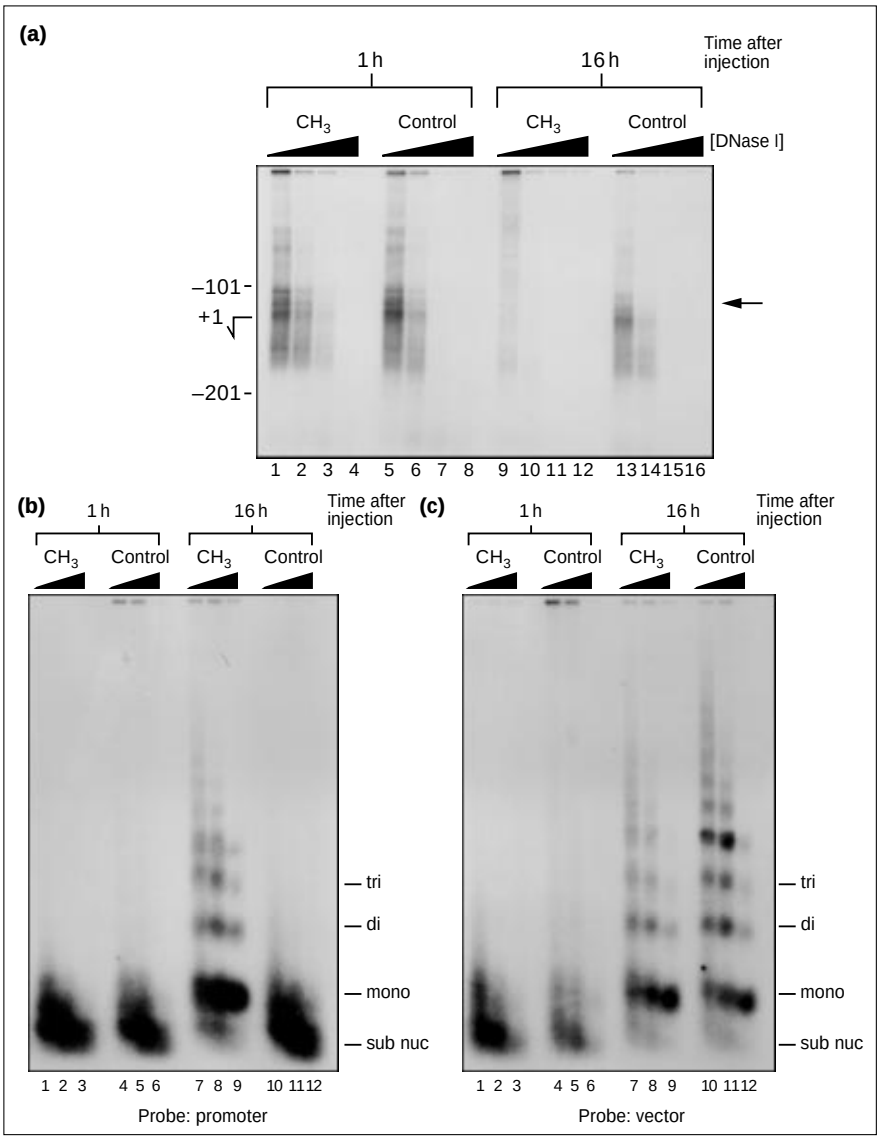



(a) Time-dependence of transcriptional repression by DNA methylation. Methylated ($CH_3$) or mock-methylated (Con) plasmid pBS-HSV*tk* was injected into oocyte nuclei and transcription from the HSV*tk* promoter assayed by primer extension at 30 min, 1 h, 4 h and 12 h after injection, as indicated. Coinjection of pCMVCAT (0.25 ng per oocyte) serves as an internal standard. (b) The methylated HSV*tk* promoter is devoid of transcriptional complexes after chromatin assembly. Methylated ($CH_3$) and unmethylated (Control) plasmid pBS-HSV*tk* was injected into oocytes and the germinal vesicles isolated 1 h or 16 h after injection. Run-on transcription assays were performed in the presence (panels 5–8) or absence (panels 1–4) of sarkosyl as indicated. Run-on transcription products were visualized by hybridization to 100 bp PCR amplified probes covering parts of the promoter and the CAT gene. The position of the probes relative to the transcription start site and the number of labeled U residues in the sequence, complementary to each probe is for probe A: –21 to +80, 13 U; for probe B: +160 to +260, 28 U; for probe C: +360 to +460, 33 U; for probe D: +560 to +660, 27 U. A DNA fragment coding for the cDNA of the histone H4 gene was loaded as a control for transcriptional efficiency (H4), and phage lambda DNA (λ) was loaded as a hybridization control. (c) Oocytes were injected with 3 ng pBS-HSV*tk* and after 1 h with 3, 5 or 15 ng pCMVCAT in a second injection. Lanes 1 and 2: pBS-HSV*tk* only, 1 h after injection; lanes 3–5: pCMVCAT only, 1 h after injection; lanes 6–8: pBS-HSV*tk* methylated, 2 h after initial injection; lanes 9–11: pBS-HSV*tk* mock-methylated, 2 h after initial injection. (d) Methylated ($CH_3$) or mock-methylated (Con) plasmid pBS-HSV*tk* was coinjected with increasing amounts of either methylated or mock-methylated pBluescript DNA as indicated. Transcriptional activity was assayed 16 h after injection. Coinjection of pCMVCAT (0.25 ng per oocyte) serves as an internal standard.

lanes 6–8 with 9–11). Therefore, transcription complexes assembled on methylated and unmethylated HSV *tk* DNA have equivalent stability and the promoters have equivalent competitive strength. This indicates that promoter strength does not contribute to the selective repression of transcription from methylated templates.

To elucidate further the repression mechanisms involved in DNA methylation, we analyzed transcriptional activity of the HSV *tk* promoter constructs in the presence of competitor DNA, which titrates stored chromatin factors in oocytes (Fig. 1d). We wished to test for the existence of both methylation-specific repressors, using methylated competitor DNA, and general DNA-binding repressor proteins, using unmethylated competitor DNA. Methylated (Fig. 1d, lane 3), but not unmethylated (lane 5), prokaryotic plasmid DNA (pBluescript) coinjected at a concentration of 3.2 ng per oocyte was able to relieve methylation-mediated repression of the HSV *tk* promoter. Injection of a high mass of competitor DNA (16 ng),

**Figure 2**




(**a**) Loss of DNase I hypersensitivity over the methylated HSV*tk* promoter. Methylated ($CH_3$) or mock-methylated (Control) plasmid pBS-HSV*tk* was injected into oocyte nuclei and assayed for the presence of DNase I hypersensitive sites 1 h and 16 h after injection, as described in Materials and methods. The plasmid was linearized with *Nco*I and the Southern blot was probed with a *Nco*I–*Eco*RI fragment (+313 to +616 relative to the start site of transcription). The scheme at the left indicates the start site of transcription (+1) for the HSV*tk* promoter. All known regulatory elements are in the region –101 to +1. The arrow at the right indicates the major site of hypersensitivity. (**b**) Analysis of chromatin structure of the HSV*tk* promoter in *Xenopus* oocytes. pBS-HSV*tk*, either methylated to completion ($CH_3$, lanes 1–3 and 7–9) or mock-methylated (Control, lanes 4–6 and 10–12) was injected into oocytes at a concentration of 1.2 ng DNA per oocyte. Groups of 30 oocytes were collected 1 h and 16 h after injection, treated with micrococcal nuclease and further processed as described in Materials and methods. The resulting Southern blot was probed with a 125 bp *Bam*HI–*Pst*I fragment from pBS-HSV*tk*, containing the HSV*tk* promoter from –105 to +30. (**c**) The Southern blot from (b) was rehybridized with a 1045 bp *Pvu*I fragment from pBS-HSV*tk*, containing vector sequences only. Positions of subnucleosomal digestion products (sub nuc) as well as mononucleosomes (mono), dinucleosomes (di) and trinucleosomes (tri) are indicated.

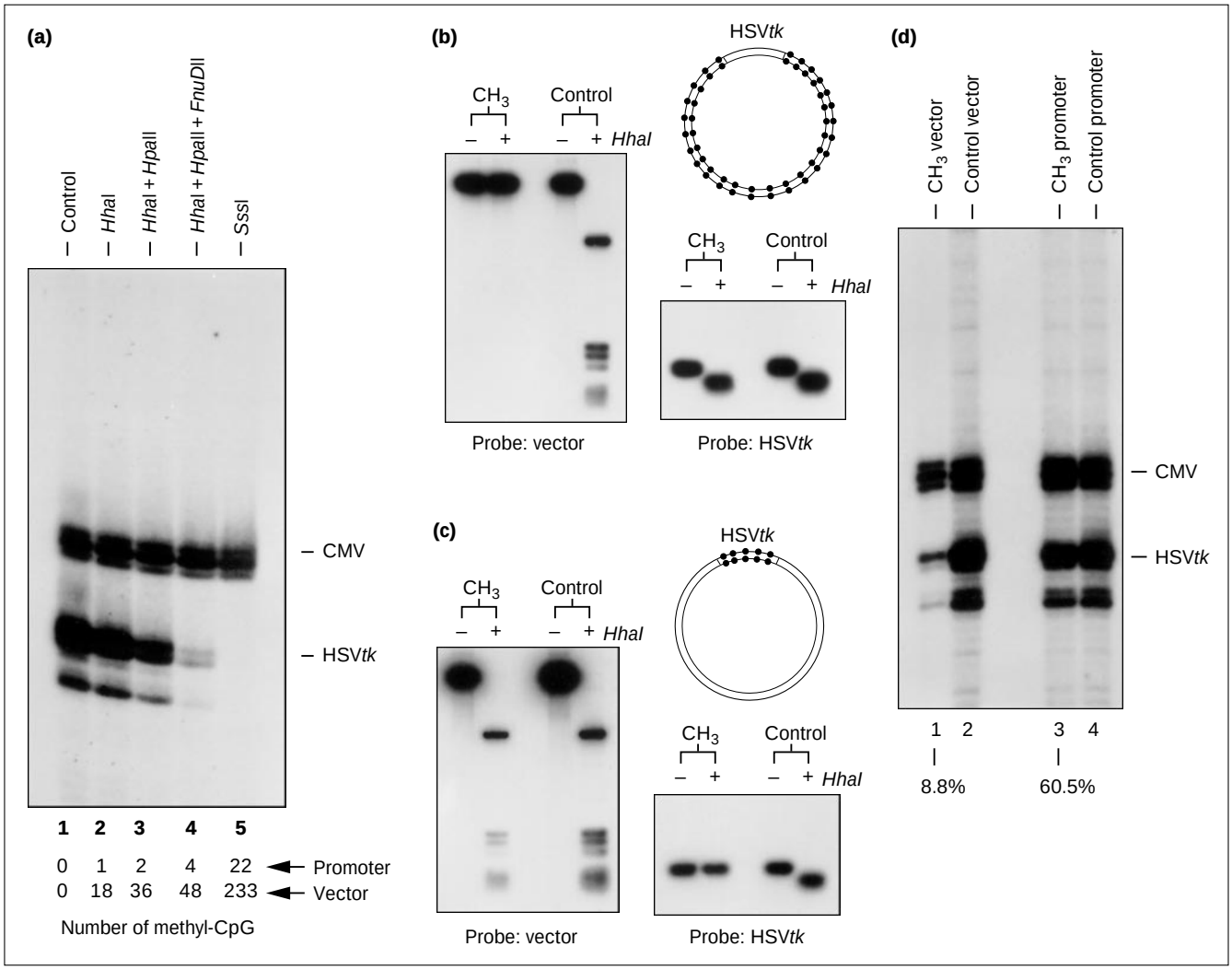
which is sufficient to titrate nucleosome assembly [27], allowed unmethylated competitor also to relieve repression (lane 9). This result indicates that transcriptional repression of methylated HSV *tk* is mediated by a factor that binds preferentially to methylated DNA and that might be required for the formation of a specialized chromatin structure.

### Time-dependent remodeling of nucleoprotein structures

The time-dependent inhibition of transcription on methylated DNA (Fig. 1a,b) implies that a dramatic restructuring of chromatin might occur. The active HSV *tk* promoter is rapidly assembled into a DNase I hypersensitive site [18] 1 hour after microinjection in a manner independent of its methylation state (Fig. 2a, lanes 1–8). The DNase I hypersensitivity of the promoter in the methylated templated is lost, however, 16 hours after injection (Fig. 2a, compare lanes 9–12 with 13–16). DNase I hypersensitive sites are generally interpreted as regions of regulatory DNA elements to which transcription factors are bound and from which nucleosomes are excluded. At early times following microinjection of methylated or unmethylated DNA into oocyte nuclei, neither promoter was assembled into a canonical nucleosomal array (Fig. 2b, lanes 1–6). However, 16 hours after injection, the methylated HSV *tk* promoter was assembled into a nucleosomal array, whereas the unmethylated promoter was not (Fig. 2b, compare lanes 7–9 with 10–12).

Selectivity was demonstrated by rehybridization of the same filter with vector DNA: at early times, the vector DNA was not assembled in a regular nucleosomal array

**Figure 3**




**(a)** Effects of DNA methylation are transmissable in *cis*. In order to achieve increasing methylation levels, plasmid pBS-HSV*tk* was either left unmethylated (Control) or methylated with a combination of prokaryotic methylases (M. *Hha*I, M. *Hpa*II, M. *Fnu*DII and M. *Sss*I), as indicated. The plasmids were injected at 1.2 ng DNA per oocyte. Transcriptional activity was assayed by primer extension 16 h after injection. Coinjection of pCMVCAT (0.25 ng per oocyte) serves as an internal standard. **(b,c)** Plasmid pBS-HSV*tk* was digested with *Bam*HI–*Xho*I and fragments containing the HSV*tk* promoter sequence (161 bp, (c)) or the remaining vector DNA (4 561 bp, (b)) were used to generate regionally methylated constructs as described. Mock-methylated control constructs were generated in parallel, by omitting the methylation step. The sites and extent of DNA methylation are depicted by the dotted region on the circular representations of the plasmid DNA molecules. Total DNA was isolated from oocytes 16 h after injection and digested with *Bam*HI and *Xho*I to release the methylated region from the plasmid. The methylation pattern was analysed by digestion with the methylation-sensitive restriction enzyme *Hha*I (one site in the promoter; 18 sites in the vector DNA) and subsequent Southern blot analysis, using either the vector fragment (left panel) or the promoter fragment (right panel) as a probe. No digestion with *Hha*I is indicated by minus (–). The appearance of shorter DNA fragments than in this minus lane indicates that the DNA was digested. **(d)** Transcriptional activity of regionally methylated constructs was assayed by primer extension 16 h after injection. Coinjection of pCMVCAT (0.25 ng per oocyte) serves as an internal standard. The relative transcriptional activity of methylated constructs was quantified using a phosphorimager and was standardized against the internal control.

(Fig. 2c, lanes 1–6), whereas at later times it was found in an array (Fig. 2c, lanes 7–12). This result was independent of DNA methylation. The assembly of the promoter of the methylated HSV *tk* construct into a regular nucleosomal array occurred concomitantly with the loss of DNase I hypersensitivity. Therefore, this remodeling of regulatory nucleoprotein complexes is not an immediate process but takes several hours, and transcriptional repression of methylated DNA is maintained within a nucleosomal environment.

### Transcriptional repression does not require promoter DNA methylation and is transmissible in *cis*

The equivalence of the promoter strength of methylated and unmethylated templates (Fig. 1c), their transcriptional activity (Fig. 1a,b) and their assembly into DNase I hypersensitive sites (Fig. 2a) soon after injection argue against mechanisms in which the transcriptional machinery is immediately prevented from association with methylated promoter elements. To test this concept independently, we used two distinct strategies for regional methylation of plasmid DNA [13]. The first approach used the sequence-specific methylases M. *Hha*I, M. *Hpa*II, M. *Fnu*DII and M. *Sss*I to target methylation to specific sites in the plasmid DNA (Fig. 3a). Transcription from the HSV *tk* plasmid was reduced only two-fold using M. *Hha*I and M. *Hpa*II with a total of 36 sites methylated in the vector DNA and two sites methylated in the promoter (Fig. 3a, lanes 2,3). Transcription from the promoter was repressed nine-fold with additional methylation to 48 vector and four promoter sites by M. *Fnu*DII (lane 4), and repression was almost complete with M. *Sss*I (223 vector and 22 promoter sites methylated; lane 5). This non-linear relationship between methylation and repression suggests that a threshold of methylation density determines transcriptional repression, although it still remains possible that repression might follow from the modification of key sites.

The second approach to examine the significance of promoter methylation in the association of the transcriptional machinery was to methylate vector DNA, but to leave promoter DNA unmethylated and *vice versa* [22]. The stability of this regional methylation *in vivo* over a 16 hour period was assessed by microinjection of DNA into *Xenopus* oocytes, followed by isolation of the DNA, its digestion with methylation-sensitive restriction enzymes and subsequent Southern blot analysis (Fig. 3b,c). Methylation of vector DNA exerted a strong repressive effect (8.8% of the activity of the unmethylated control) on transcription from the HSV *tk* promoter (Fig. 3d, lane 1), whereas methylation of the promoter alone resulted in a much weaker repression (60% of unmethylated control; Fig. 3d, lane 3). Therefore, transcriptional repression does not require promoter methylation and is transmissible in *cis*. Both of these findings indicate that a direct immediate inhibition of transcription factor binding at a promoter is not necessary in order to confer methylation-dependent transcriptional repression.

### The stability of transcriptional repression conferred by DNA methylation exceeds that conferred by nucleosomes alone

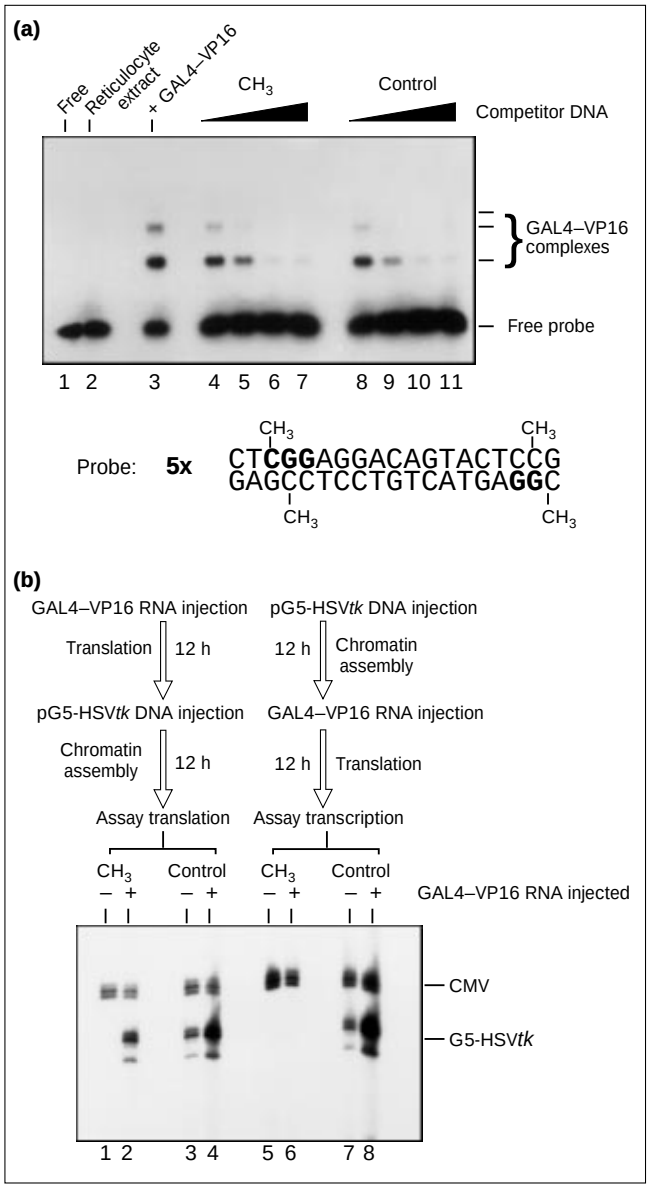
The stability of epigenetic states is in marked contrast to the plasticity of inducible gene regulation, a process known to involve nucleosome disruption. It was important to determine whether DNA methylation imposed additional repressive effects in addition to that imposed by nucleosomes alone. It has been reported previously that the prior assembly of core histones onto regulatory DNA does not prevent the function of GAL4–VP16, a strong transactivator comprising a fusion of the DNA-binding domain from the yeast protein GAL4 and the transactivation domain of the HSV protein VP16 [28]. We examined whether GAL4–VP16 could activate transcription from a methylated nucleosomal template containing five GAL4 binding sites fused to the HSV *tk* promoter. GAL4–VP16 bound with equivalent affinity to methylated and unmethylated recognition elements (Fig. 4a). Synthesis of GAL4–VP16 prior to chromatin assembly (Fig. 4b, lanes 1–4) led to transcription from both methylated and unmethylated templates. Transcription was not eliminated in the presence of GAL4–VP16 (compare lanes 2 and 4), as was observed in the absence of activator (compare lanes 1 and 3). This demonstrates that the presence of a strong activator can reduce the silencing effect of methylated DNA. In contrast, if the templates were assembled into chromatin prior to the synthesis of GAL4–VP16 (Fig. 4b, lanes 5–8), then GAL4–VP16 could not activate transcription from the methylated template. This result indicates that bound GAL4–VP16 can prevent the assembly of a repressive chromatin structure on methylated DNA; however, once such a structure is assembled, the activator cannot function.

## Discussion

The major new conclusions from this work are, first, that the process of silencing transcription by DNA methylation involves both the inhibition of transcription initiation and the removal of engaged transcriptional machinery from active templates (Figs 1,2); and second, that methylation-dependent silencing of transcription directs the time-dependent assembly of a repressive structure that includes a higher-order nucleosomal DNA structure, but that represses transcription more effectively than the nucleosomal structure alone (Figs 3,4). Our results confirm and extend existing data consistent with indirect mechanisms of transcriptional repression, such as the time-dependent elimination of HSV *tk* activity following the microinjection of methylated templates into mammalian cells [20,21].

Our results establish that HSV *tk* gene expression is regulated by DNA methylation at the level of transcription (Fig. 1a), and more specifically at the initiation step (Fig. 1b), in a time-dependent manner. This differential gene expression is established with methylated and unmethylated HSV *tk* promoters of equivalent competitive strength (Fig. 1c). We also demonstrate that transcriptional activity states correlate with the remodeling of nucleoprotein structures, such as the time-dependent loss of DNase I hypersensitivity and the assembly of nucleosomal arrays on the methylated promoter (Fig. 2). These results extend significantly the earlier work that assayed enzymatic activity without corresponding temporal analysis of nucleoprotein organization [20,21]. We also confirm results obtained from

**Figure 4**




GAL4–VP16 fails to activate transcription from the methylated and chromatinized HSV *tk* promoter. (a) Electrophoretic mobility shift assay of GAL4–VP16. The unmethylated restriction fragment containing five GAL4-binding sites was used as a radiolabelled probe. A monomer of the sequence with major GAL4–DNA contacts (in bold), as well as positions of CpG-methylation, is indicated. Lane 1, free probe; lane 2, probe incubated with reticulocyte extract; lanes 3–11, probe incubated with *in vitro* translated GAL4–VP16; lanes 4–7, in the presence of 2×, 5×, 10× and 50× molar excess of unlabelled, methylated GAL4-binding fragment; lanes 8–11, in the presence of 2×, 5×, 10× and 50× molar excess of unlabelled, unmethylated GAL4-binding fragment. (b) The effect of overexpressed GAL4–VP16 on transcription was assayed as indicated. RNA encoding GAL4–VP16 was injected either before (lanes 1–4) or after (lanes 5–8) chromatin assembly of methylated (lanes 1, 2 and 5, 6) or unmethylated (lanes 3,4 and 7,8) plasmid pG5-HSVtk, as indicated in the figure. Transcriptional activity was assayed by primer extension as before. Coinjection of pCMVCAT (0.25 ng per oocyte) serves as an internal standard.

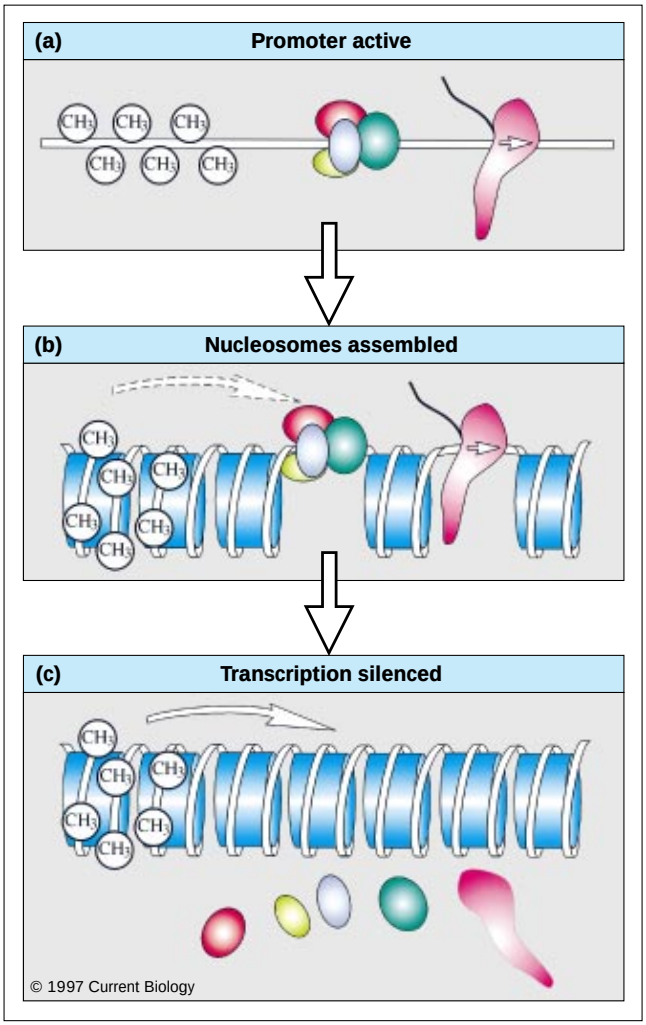
earlier work using a distinct system in mammalian cells that demonstrated that methylation density is important for transcriptional repression in a chromatin environment [25] and that the effects of DNA methylation on gene activity are transmissible in *cis* [22]. Importantly, we demonstrate that the repressive effects of chromatin assembled on methylated DNA exceed those of the conventional nucleosomal structure (Fig. 4). This result, together with the selective titration of transcriptional repression by methylated DNA (Fig. 1d), suggests that additional methylation-specific transcriptional repressors assist the establishment of a repressive nucleoprotein architecture.

Our results contribute to understanding the complex association between DNA methylation and the regulation of imprinted genes. Consistent with genetic evidence [1,2], DNA methylation has a decisive role in establishing the repression of actively transcribed genes (Figs 1,2). In contrast to existing models [29,30], DNA methylation does not interfere with the immediate access of transcription factors to promoters. Instead, the repression of transcription directed by methylated DNA requires considerable time to be established (Figs 1,2). This might allow the transient expression of methylated genes during development [1,2]. The access of transcriptional regulators to methylated DNA (Figs 1,2) would also allow the regulatory elements associated with genes to influence the establishment of differential gene activity, even though these elements and genes are methylated [31].

A strong transcriptional activator (GAL4–VP16) can resist silencing only if it gains immediate access to methylated templates before the time-dependent assembly of a repressed state (Fig. 4, lane 2). In contrast, once such a repressed state has been assembled, GAL4–VP16 can no longer activate transcription (Fig. 4, lane 6). This demonstrates that DNA methylation is a component of a process that removes the interactions between regulatory regions of DNA and the transcriptional machinery. This is an important requirement in the compartmentalization of the genome into active and inactive domains [32].

The density-dependent transmissible effects of DNA methylation on gene activity (Fig. 3) can explain the variable distance of segments of DNA at which methylation is regulated and the promoters that are affected by the methylation [5–7], in addition to the considerable variation in the exact sites of methylation within such regulatory DNA [9]. A useful model for considering the influence of DNA methylation on gene activity is the assembly of heterochromatin structures in *Drosophila* and *Saccharomyces cerevisiae* [33,34]. The nucleation of a specialized chromatin structure at a methylated DNA segment through association with a methylation-selective repressor [17,18] might lead to a spreading of the interactions between repressor proteins and DNA, and therefore

**Figure 5**




A model for methylation-dependent silencing. **(a)** At times immediately following microinjection, the promoter is active, transcription factors (colored ellipsoids) and RNA polymerase (pink) are engaged on the template, independent of the methylation status of naked DNA. **(b)** Nucleosomes (blue) are assembled onto the transcription unit and flanking DNA. At early times, this association is revealed through the generation of a DNase I hypersensitive site. However, concomitant with the assembly of nucleosomes, methylation-specific repressive effects begin to be exerted (broken arrow). **(c)** Transcription is silenced, the transcriptional machinery erased from DNA and the promoter assembled into nucleosomes. The methylation center directs a continual silencing function in *cis* (arrow).

to the transmissible influence on transcription (Fig. 5). The time-dependence of methylation dependent silencing may also reflect the staged assembly of such a repressive chromatin structure.

## Materials and methods

### *Microinjection of* Xenopus *oocytes*

DNA solution (30 nl containing 1.2 ng per oocyte unless stated otherwise) was injected into *Xenopus* oocyte nuclei as described [27].

### *Methylation of templates*

Plasmids were methylated *in vitro* using methylases *Hha*I, *Hpa*II, *FnuD*II and *Sss*I under conditions recommended by the manufacturer (New England Biolabs). Regional methylation was achieved using phagemid single-stranded (ss) DNA. This was annealed to gel-purified restriction fragments at a molar ratio of 3:1 (fragment DNA: ssDNA). To prevent the methylase *Sss*I from binding to and methylating the ssDNA region, T4 gene 32 protein (Boehringer Mannheim) was added (10 $\mu g\ \mu g^{-1}$ ssDNA) prior to the methylation reaction. After methylation, the DNA was purified, filled in and ligated by standard procedures. Regionally methylated constructs were gel-purified prior to microinjection.

### *Nucleic acid extraction and primer extension*

Oocytes (30–40) were collected and homogenized in 20 mM Tris-HCl (pH 8.0), at 10 $\mu$l per oocyte. This homogenate was used for both RNA and DNA analysis. RNA was isolated using RNAzol (Cinna Scientific). A 30 mer oligonucleotide (3′–TACCTCTTTTTTTAGT-GACCTATATG-GTGG–5′) complementary to the CAT gene mRNA was used for primer extension. Extension products were separated on 6% sequencing gels and visualized by autoradiography. DNA was purified by incubation at 45°C for 2 h in 15 mM EDTA, 20 mM Tris-HCl (pH 8.0), 0.5% SDS and 500 $\mu g\ ml^{-1}$ proteinase K followed by phenol/chloroform extraction and ethanol precipitation. After RNase treatment, the DNA was separated by agarose gel electrophoresis and Southern blot analysis was carried out.

### *Transcription run-on experiments*

Injected oocytes were placed in isolation buffer (25 mM Tris-HCl, pH 8.0, 10% glycerol, 5 mM $MgCl_2$ and 2 mM DTT) and nuclei isolated as described [26]. Samples of 40 germinal vesicles were collected by centrifugation at 1500 × *g* and resuspended in 440 $\mu$l nuclear freezing buffer (40% glycerol, 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 0.1 mM EDTA and 0.5 mM DTT). To 220 $\mu$l nuclei suspension was added: 60 $\mu$l of 5× transcription buffer (25 mM Tris-HCl, pH 7.8, 12.5 mM $MgCl_2$, 375 mM KCl, 1 mM each of ATP, CTP and GTP), 2 $\mu$l RNasin (Promega), 16 $\mu$l (160 $\mu$Ci) of $\alpha$-[$^{32}$P]UTP (3,000 Ci $mmol^{-1}$). Sarkosyl was included in the 5× transcription buffer at 3% (wt $vol^{-1}$) where appropriate. The transcription reaction was carried out for 5 min at room temperature and was stopped by the addition of SDS and EDTA to 0.5% and 5 mM respectively. RNA was purified using RNazol as described above. RNA was resuspended in 50 $\mu$l $H_2O$ and digested with 1 U RNase-free DNase for 10 min at 37°C. Nuclear transcripts were separated from unincorporated nucleotides on a Sephadex G50 column. Prior to hybridization, the RNA was heated for 10 min at 80°C.

Four probes covering part of the HSV-tk promoter and the CAT gene were generated by PCR and 1 $\mu$g of each was loaded onto a nylon membrane (Hybond $N^+$, Amersham) using a Schleicher and Schuell slot blot apparatus. 1 $\mu$g of a 600 bp fragment, containing the histone H4 gene and 1 $\mu$g of phage lambda DNA were loaded as transcription and hybridization controls, respectively. Hybridization was carried out in 800 $\mu$l of a 50% formamide buffer (Hybrisol I, Oncor) for 48 h at 42°C. Filters were washed twice in 0.2× SSC, 1% SDS for 20 min at 60°C, once in 2× SSC, 5 $\mu g\ ml^{-1}$ RNase A for 5 min at room temperature and finally three times in 0.2× SSC, 1% SDS at room temperature for 5 min.

### *Digestion with nucleases*

Injected oocytes (35 per sample) were homogenized in buffer E (70 mM KCl, 20 mM HEPES pH 7.5, 1 mM DTT and 5% sucrose), using 20 $\mu$l per oocyte; $MgCl_2$ was added to a final concentration of 5 mM and the homogenate divided into four aliquots. Either DNase I or micrococcal nuclease (10, 15, 20 and 30 U) were added and the samples were incubated for 5 min at room temperature. The reaction was stopped by addition of an equal volume of buffer containing SDS, EDTA and proteinase K, to final concentrations of 0.5%, 15 mM and 500 $\mu g\ ml^{-1}$, respectively. Purification and concentration of DNA samples was carried out as before. The DNA was then linearized with *Nco*I and separated on a 1.6 % agarose gel in TAE buffer. Southern blot analysis was performed using an *Nco*I–*Eco*RI fragment from the

plasmid, adjacent to the promoter sequence for DNase I, or with the promoter or vector sequences indicated in the text for micrococcal nuclease.

### *Expression of GAL4–VP16 and construction of the GAL4-responsive transcription plasmid*

A 152 bp fragment containing five GAL4-binding sites was amplified by PCR from pG5E4T [35] and cloned in front of the HSV *tk* gene from –105 to +51 fused to the CAT reporter gene. Construction of pGAL4–VP16 pSP64 was performed by cloning a PCR-amplified fragment containing the coding region of the GAL4–VP16 protein [34] into pSP64. A 30 nl sample of *in vitro* transcribed mRNA encoding GAL4–VP16 was injected into the oocyte cytoplasm. Synthesis of over-expressed proteins was monitored by incubation of injected oocytes in [$^{35}$S]–methionine and subsequent analysis of newly translated proteins by SDS-PAGE and autoradiography.

### *Electrophoretic mobilty shift assay*

The electrophoretic mobility shift assay was performed as previously described [14].

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