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**Cytosine methylation in CTF and Sp1 recognition sites of an HSV *tk* promoter: effects on transcription *in vivo* and on factor binding *in vitro***

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**ABSTRACT**

We methylated specific cytosine residues within or immediately around the CTF and Sp1 binding sites of the Herpes simplex virus thymidine kinase promoter. The efficiency of transcription *in vivo* was reduced at least 50-fold compared with transcription from the unmethylated promoter. However, methylation within the CTF recognition site had no effect on the affinity of CTF for this site *in vitro*. Methylation of the Sp1 site resulted in only a small decrease in the affinity of this factor for its recognition site. *In vivo* studies showed that the same gene inserted in different vector DNAs was regulated differently by methylation in the promoter. These results show that cytosine methylation can inhibit transcription by a mechanism other than directly blocking the binding of transcription factors.

**INTRODUCTION**

Gene expression in mammalian cells is regulated by several mechanisms, one of which is DNA methylation. A considerable number of reports show an inverse correlation between CpG methylation and gene activity. Actively transcribed genes are almost invariably found to be undermethylated, while heavily methylated genes are mostly inactive (1–3). Transfection or microinjection of cloned genes has shown that DNA methylated *in vitro* is most often not transcribed *in vivo*. Methylation of the promoter regions alone can bring about gene inactivation (4–6).

How does methylation affect promoter function? This could operate at a numbers of levels. Cytosine methylation increases helix stability (7–8) and thus regional base-stacking could be modified. Unusual DNA secondary structures, such as left-handed Z-DNA, may be induced by DNA methylation (9–10) then, in part, regulate gene expression. The interaction of specific proteins with specific DNA sequence elements in the promoter regions of genes plays a major role in the regulation of transcription (11). Thus, it is reasonable to expect that promoter methylation might affect the interaction of regulatory proteins with DNA. For example, interaction of restriction enzymes with DNA can be affected by DNA methylation (12–13).

The second distal element (dsII) of the Herpes simplex virus thymidine kinase (HSV *tk*) promoter (see Fig. 1a and ref. 14) was shown by DNaseI footprinting to interact with the transcription factors CTF and Sp1 (15–16). We have shown that the presence of a single methylated CpG in the promoter of the HSV *tk* gene is sufficient to cause a 98% reduction in transcription when the DNA is microinjected into frog oocytes (6). The aim of the work we report here was to find whether DNA methylation inhibits transcription of the HSV *tk* gene by interfering directly with the binding of these transcriptional activator proteins to their respective binding sites or whether other events are necessary for this inhibitory effect.



## MATERIALS AND METHODS

### *Preparation of cell extracts*

The HeLa whole cell extract was prepared as previously described (19–20). Whole oocyte extracts were prepared by a modification of the Laskey et al. (21) and Glikin et al. (22) procedures. Defolliculated and dispersed *Xenopus Laevis* oocytes were washed and centrifuged for 30 min at 250,000 × g in an equal volume of extraction buffer (30 mM Tris-HCl, pH 7.9; 90 mM KCl; 10 mM Na β-glycerophosphate; 1 mM EGTA and 1 mM DTT). After a rapid homogenization (in the same tube) and recentrifugation, the clear supernatant was carefully removed and stored at –70°C. With this modification it was possible to extract three times more protein (8–9 mg/ml) than previously described.

### *Sp1 gel retardation assays and competition studies*

The synthetic 32-mer oligonucleotide 5'-ACAAGACGCTGGGCGGGCCGGAT-CCGGTTCG-3' was 5'-labeled with <sup>32</sup>P and annealed with its complementary strand (23). 20–40 μg of extract proteins (2 μl of HeLa whole cell extract or 4 μl of whole oocyte extract) were added to a reaction mixture containing 25 mM Tris-HCl (pH 8), 0.1 mM ZnCl<sub>2</sub>, 0.5 mM DTT, 0.5 mM EDTA, 50 mM KCl, 0.5 μg BSA, 0.5 μg poly d(I.C) and 10% (vol/vol) glycerol in a total volume of 16 μl. After 5 min. at room temperature, the end-labeled duplex **Sp1-0** (2 μl, 20 fmol) and various quantities (2 μl, 0–500 fmol) of unlabeled competitor oligonucleotide (**Sp1-0**, **Sp1-C**, **Sp1-D** or **Sp1-E**) were added. Incubation was continued for a further 20 min. 6 μl aliquots were then loaded on a 6% nondenaturing polyacrylamide gel (24). Electrophoresis was carried out at 10V/cm. The gel was dried, and the amount of bound oligonucleotide duplex was quantitated by densitometry of the autoradiograph.

### *CTF gel retardation assays and competition studies*

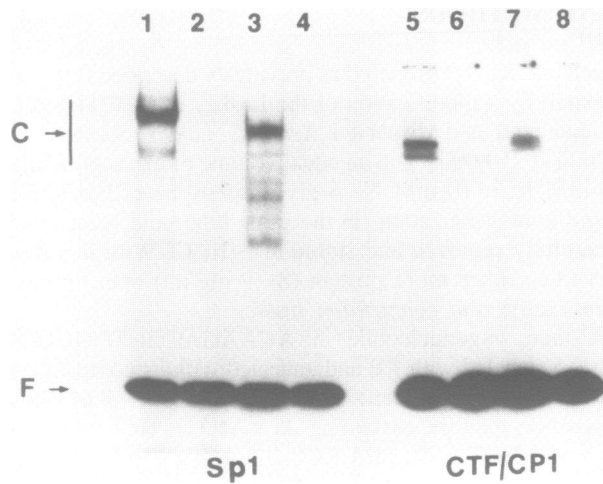
The synthetic 27-mer oligonucleotide 5'-CAGTGAATTCGCCAATGACAAGACGCT-3' was 5'-labeled and annealed with its complementary strand. The binding reactions were carried out as described above, except that the reaction buffer was that of Superti-Ferga et al. (25) and the unlabeled competitor oligonucleotides were **CTF-0** or **CTF-A**.

### *Methylation interference assay*

The duplex **CTF-0**, <sup>32</sup>P-labeled at one of its 5'-ends, was treated with dimethyl sulfate for 3 min as described by Maxam and Gilbert (26), purified by ethanol precipitation (twice) and used in a band-shift assay. The mixture was then loaded on a 6% non-denaturing polyacrylamide gel, the bands containing the bound and unbound duplexes were excised from the gel and the DNAs were electroeluted, phenol extracted and ethanol precipitated. G >> A cleavage of the eluted methylated duplexes with NaOH and electrophoresis (20% acrylamide –7 M urea) were carried out following published protocols (27).

### *Construction of pUC/dsIIio vectors*

The plasmids M13/dsIIio and M13/ψ-dsIIio were prepared as described (6). The cloned *tk* fragment of M13/dsIIio was excised by *EcoRI* and *HindIII* digestion, and this fragment was subcloned either in pUC18 to give pJJio or in pUC19 to give pJJio-inv. The M13/ψ-dsIIio DNA was digested with *BamHI*, blunt-ended with Klenow polymerase and religated. The *tk* fragment of this mutated DNA was excised by *EcoRI* and *HindIII* digestion, blunt-ended with Klenow polymerase and subcloned into the blunt-ended *HindIII* site of either pJJio to give pJPS, or pJJio-inv to give pJPS-inv. The pJPS and the pJPS-inv constructs contain the *tk* and the pseudo-*tk* genes in the same orientation, where the *EcoRI* and the *BamHI* sites of the pseudo-*tk* gene, which contains a 20 bp deletion in the 5'-noncoding region, have been mutated out.



**Figure 2.** Detection of Sp1 and CTF/CP1 binding activities in crude extracts by band-shift assay. The binding reactions were carried out using the unmethylated duplexes **Sp1-0** (lanes 1–4) or **CTF-0** (lanes 5–8) and either HeLa (lanes 1, 2, 5 and 6) or oocyte (lanes 3, 4, 7 and 8) whole cell extracts. Lanes 2 and 4: competition with 25 equivalents of unlabeled duplex **Sp1-0**; lanes 6 and 8: competition with 25 equivalents of unlabeled duplex **CTF-0**. F and C denote the positions of the free and complexed forms of the oligonucleotides.

#### *In vivo transcription assays*

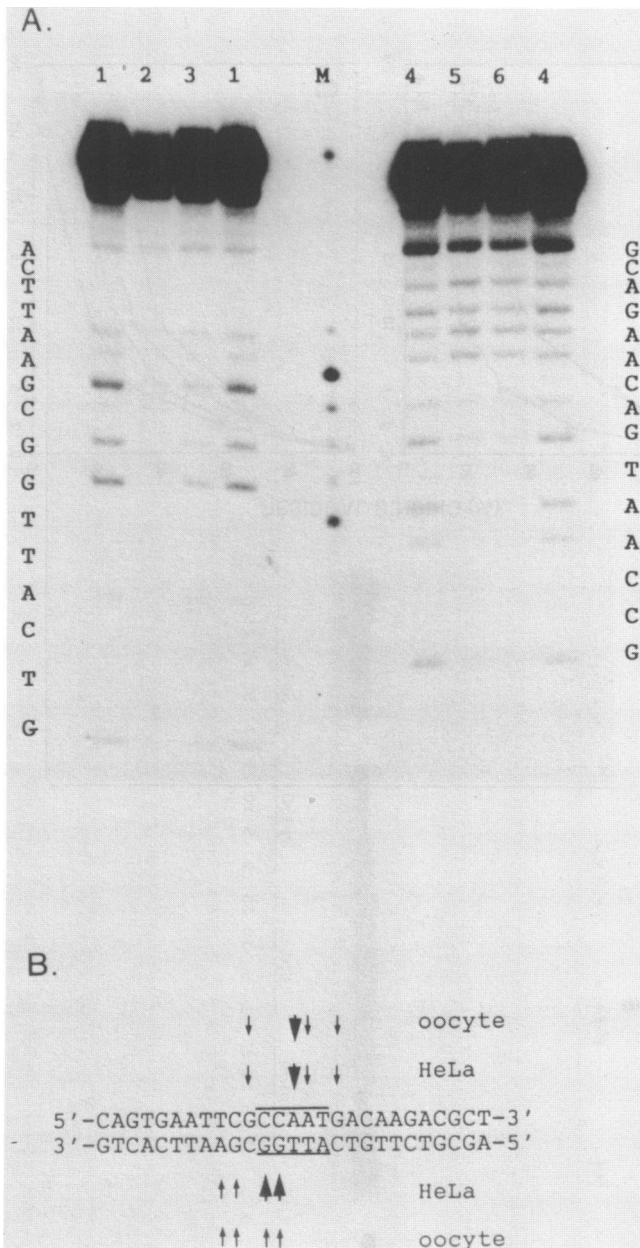
Methylated and unmethylated DNAs were microinjected into the nuclei of *Xenopus Laevis* oocytes. Total oocyte RNA was purified 16–20 hrs after injection and assayed by primer extension for accumulated HSV *tk* transcripts (6).

## RESULTS

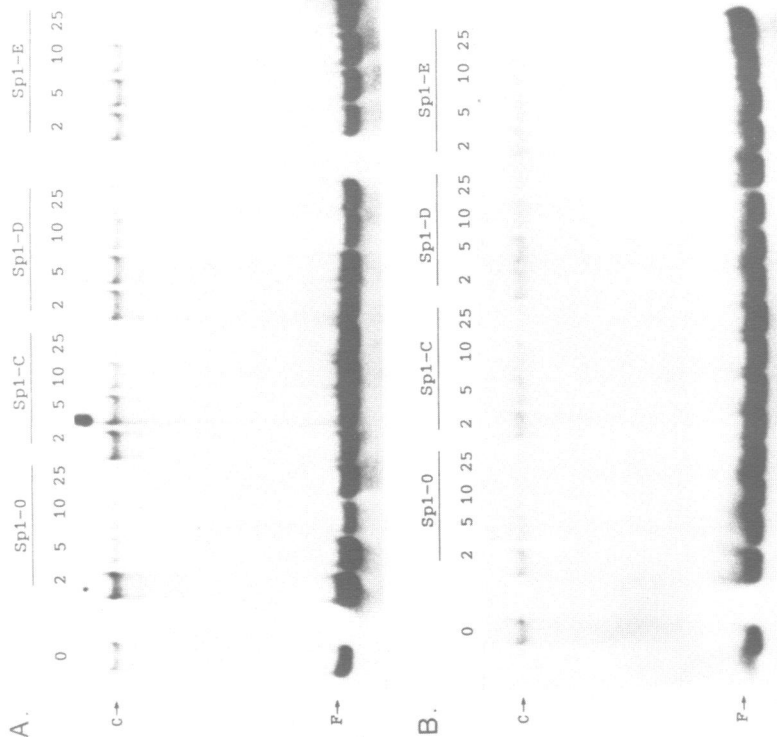
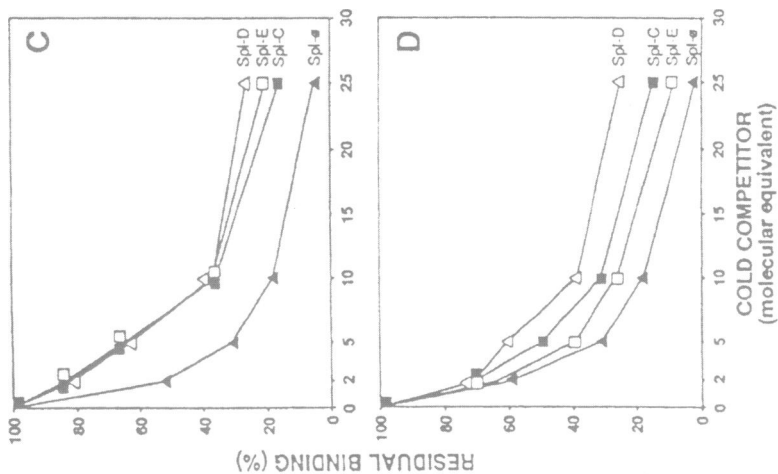
### *Frog oocyte and HeLa cell proteins specifically interact with the GC-box and the CCAAT-box elements of the HSV tk promoter*

Labeled oligonucleotide duplexes containing the Sp1 or the CTF binding sites of the HSV *tk* promoter (Fig. 1b, oligos **Sp1-0** and **CTF-0** resp.) were incubated with the crude HeLa or oocyte cell extracts in the presence of a large excess of nonspecific competitor DNA and then analyzed by nondenaturing gel electrophoresis. The bands of retarded mobility indicate the formation of GC-box (Fig. 2, lanes 1, 3) and CCAAT-box specific (Fig. 2, lanes 5, 7) protein-DNA complexes. The binding could be abolished by the addition of 25 molar equivalents of unlabeled duplexes **Sp1-0** (Fig. 2, lanes 2, 4) or **CTF-0** (Fig. 2, lanes 6, 8) to the assay. It is worth noting that the retarded complexes, formed with HeLa or frog Sp1 protein (17,28), had different electrophoretic mobilities (Fig. 2, lanes 1 and 3). This suggests that the Sp1 factors from the two species are most likely differently glycosylated (29).

As the CCAAT-box of the HSV *tk* gene is a low affinity binding site (30), we decided to carry out methylation interference experiments (31–32) in order to confirm that the duplex **CTF-0** was indeed bound by the CTF/CP1 factor. Labeled DNA probes were partially methylated by reaction with dimethylsulfate (DMS) and used as substrates in protein-DNA binding experiments. The complexed (C) and free (F) forms of DNA were recovered from the native gels, cleaved with NaOH and analysed on denaturing



**Figure 3.** Analysis of the protein binding pattern at the HSV *tk* CCAAT region by methylation interference. (A) Lanes 1 and 4: free form of CTF-0 duplex; lanes 2 and 5: complexed forms using whole oocyte extract; lanes 3 and 6: complexed forms using HeLa whole cell extract. (B) Summary of the methylation interference analysis. A and G residues that interfere with protein binding are indicated by arrows. Solid arrows represent a greater degree of interference than weak arrows. The CTF/CP1 consensus sequence is bracketed.



polyacrylamide gels. The retarded band (C) contained only those duplexes where methylation of guanine or adenine residues did not interfere with the protein-DNA binding, whereas the free (F) band contained the entire range of modified duplexes. The methylation interference analysis (Fig. 3) clearly demonstrated that the contacts made by the proteins bound to the CTF/CP1 consensus sequence are the same for the HeLa and the oocyte extracts. A similar methylation interference pattern was previously described for the binding of partially purified CP1 factor (30,33). We thus conclude that the protein binding to the CTF-0 oligonucleotide duplex was CTF/CP1.

*Competition-binding experiments : influence of a single methylated CpG site*

In our previous work (6), we observed that the methylation of single CpG sites within a modified *tk* promoter was sufficient to suppress transcription in the nuclei of *Xenopus* oocytes. In order to find out whether the downregulating effect observed *in vivo* was caused by the interference of the methylated sites with the binding of the transcription factors Sp1 and CTF to their respective recognition sequences, we carried out a series of band-shift competition experiments.

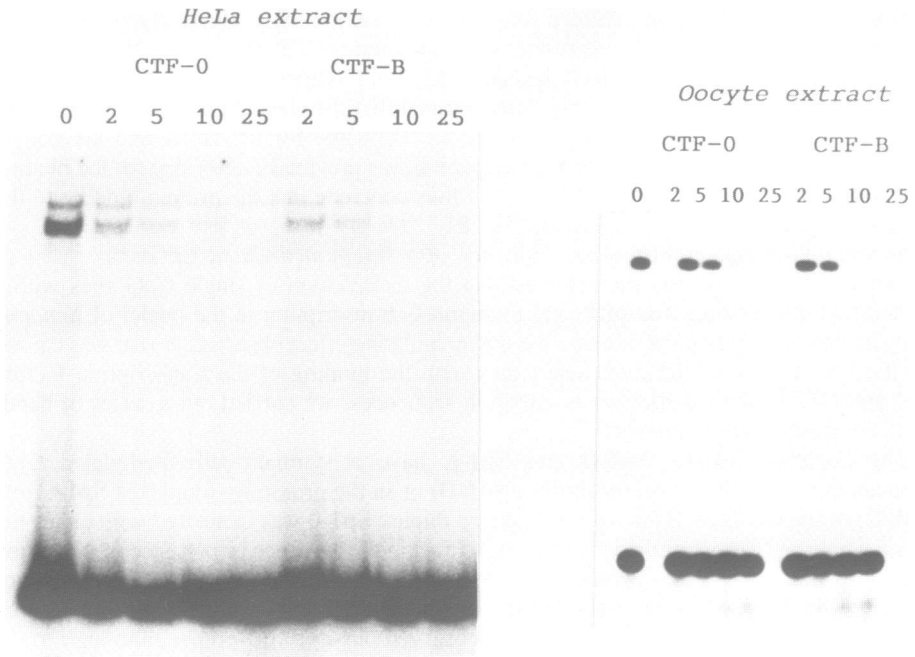
The duplexes Sp1-C, Sp1-D and Sp1-E have a symmetrically-methylated CpG dinucleotide either in the center (duplex Sp1-D) or in the proximity (duplexes Sp1-C and Sp1-E) of the GC-box. Radioactively-labeled duplex Sp1-0 was incubated with either the HeLa whole cell extract or the whole oocyte extract in the absence or presence of increasing quantities of unlabeled competitor DNA (Sp1-0, Sp1-C, Sp1-D or Sp1-E). The result of this experiment (Fig. 4) indicates that the methylated duplexes were poorer competitors than the unmethylated ones. However, the observed one-third difference, although found with either HeLa or oocyte extract (Fig. 4A and 4B respectively), was between one and two orders of magnitude smaller than the *in vivo* effect on the specific *tk* transcription (6) (Fig. 6, lane 1–3).

A similar experiment was carried out with the duplexes containing the CCAAT-box of the HSV *tk* promoter. As shown in Figure 5, no difference was observed between the methylated CTF-B and the unmethylated CTF-0 duplex in a competition experiment with the labeled duplex CTF-0. Cytosine methylation of the CpG site, situated immediately adjacent to the CCAAT-box, therefore does not affect the binding of the transcription factor CTF/CP1 *in vitro*.

*Cytosine methylation of the tk promoter and downregulation of transcription : the role of vector DNA*

In order to find out whether the nature of the vector influenced the extent of the downregulation, we carried out the point methylation (methylation of a single CpG site) and complete *Hpa*II methylation experiments with three different constructs. M13/dsIIio, a 9.2 kb construct, contains the *tk* gene cloned in the polylinker of M13mp9 (6). pJJio (4.3 kb) contains the *tk* gene in a pUC vector. pJPS is derived from pJJio and contains, in addition to the *tk* gene also the reference pseudo-*tk* gene (Fig. 7A). The advantage of

**Figure 4.** Evaluation of relative affinities of Sp1 for methylated and unmethylated duplexes. A and B: Various quantities of unlabeled duplexes Sp1-0, Sp1-C, Sp1-D or Sp1-E were used to compete with a fixed amount of the labeled Sp1-0 duplex for the binding of the transcription factor Sp1. The numbers at the top of the lanes represent the molar excess of the competing unlabeled duplex. The experiments used either the HeLa (panels A and C) or the oocyte (panels B and D) whole cell extracts. C and D: Competition curves. The values obtained in the absence of competitor were taken as the 100% reference.



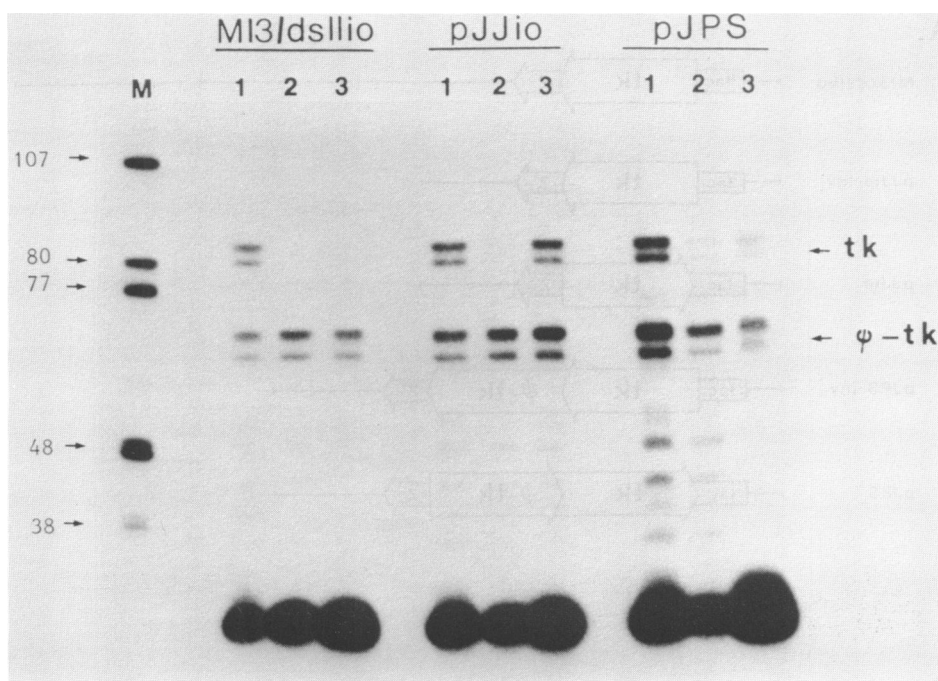
**Figure 5.** Evaluation of the relative affinities of CTF/CP1 factor for methylated and unmethylated duplexes. Various quantities of unlabeled duplex (CTF-0 or CTF-b) competed with a fixed amount of labeled CTF-0 duplex for the binding of the transcription factor CTF/CP1. All the other details are described in the legend to Figure 4.

this latter construct is that the pseudo-*tk* gene serves as an internal control for the transcription experiments. Interestingly, the pseudo-*tk* promoter of pJPS, in which the *Bam*HI and *Eco*RI restriction sites were eliminated (see Materials and Methods), is 1.7 times more active than the wild-type *tk* promoter.

After microinjection of these constructs into *Xenopus* oocytes, the levels of *tk* transcription were studied by primer extension. With the clones containing the M13 vector, the downregulation of transcription of the methylated DNAs was almost complete (6)(Fig. 6). In contrast, the methylated clones containing pUC sequences were transcribed relatively efficiently. With the point methylated constructs pJJio and pJPS specific *tk* transcription was reduced by only slightly more than 50% (Fig. 6, lanes 3). It thus appears that the extent of transcriptional downregulation of the *tk* gene *in vivo* is influenced by the nature of the vector DNA.

The vector sequence upstream from the *tk* promoter of M13/dsIIio differs from those of pJJio and pJPS (see Fig. 7A). To eliminate the possibility that these sequences play a role in the inhibition of *tk* transcription by methylation, we constructed two additional plasmids, where the pUC vector was inverted relative to the *tk* gene. The new constructs, pJJio-inv and pJPS-inv, and M13/dsIIio have the same 165 bp fragment immediately upstream from the *Eco*RI site of the promoter. The results of the primer extension experiments, following microinjection of these vectors into oocytes, showed (Fig. 7B) that the orientation of the insert did not significantly influence the extent of downregulation of specific *tk* transcription. Although the results were often variable (Fig.6, cf Fig.7), we





**Figure 6.** Primer extension analysis of *tk* mRNA isolated from microinjected oocytes. Equimolar amounts of M13/ $\psi$ -dsIIio and either the M13/dsIIio or pJJio vectors were microinjected into the nuclei of *Xenopus* oocytes. With the exception of the *Hpa*II-methylated pJPS DNA, the pJPS constructs were injected alone, as they contain a copy of the reference gene. Lane M: marker DNA; lane 1: unmethylated DNA; lane 2: *Hpa*II-methylated DNA; lane 3: methylation of the internal cytosine residue of the GC-box (site D in figure 1). The reverse transcripts obtained by primer extension of the test *tk* mRNA and the reference  $\psi$ -*tk* mRNA are indicated by arrows.

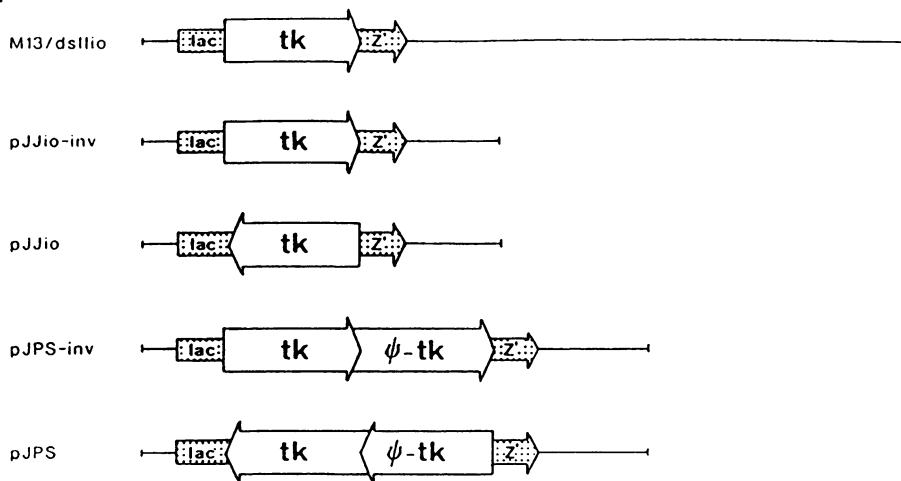
never observed inhibition of *tk* transcription comparable to that seen with the methylated M13 constructs.

## DISCUSSION

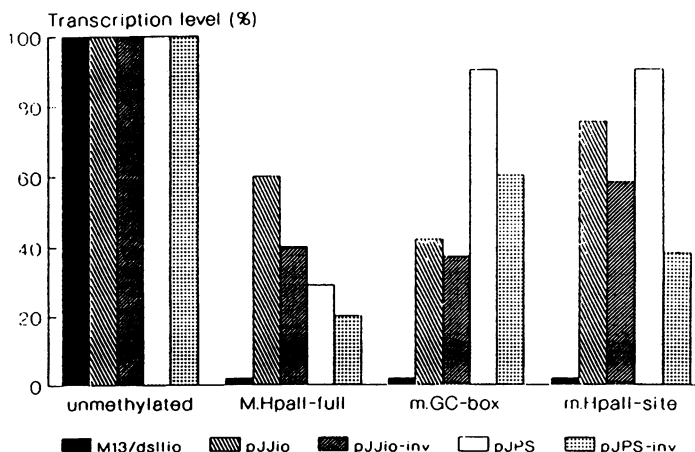
We have shown that methylation of single CpG sites within the modified HSV *tk* promoter strongly inhibits the transcription of the *tk* gene in the nuclei of *Xenopus Laevis* oocytes (6, and this work). How is this inhibition achieved? The four methylated CpG sites investigated are situated in, or immediately adjacent to, the binding sites of the transcription factors CTF and Sp1. We tested whether the observed downregulation was mediated by altered binding of one or both of these factors to DNA by performing quantitative band-shift experiments with methylated and unmethylated oligonucleotide duplexes containing the *tk* promoter binding sites for these proteins.

Our clearest results were with the CTF/CP1 recognition site. Methylation of the CpG situated immediately adjacent to the HSV *tk* CCAAT-box reduced transcription by at least 50-fold when compared to the unmethylated promoter. However, this methylation had no effect on the affinity of CTF/CP1 for its recognition site *in vitro*. Therefore we conclude that DNA methylation does not inhibit transcription by a simple direct effect on the binding of CTF to its recognition sequence.

A.



B.



**Figure 7.** (A) Schematic diagram of the *tk* constructs used in the microinjection experiments. The circular DNAs are shown in linear form for ease of comparison. (B) Influence of vector DNA on *tk* transcription in oocytes. Autoradiographic exposures were scanned by densitometry to quantitate the relative transcription efficiencies. The first set of columns represents the transcriptional activity of the unmethylated DNA constructs, adjusted to 100%. The set designated M.HpaII-full represents the transcription of M.HpaII methylated constructs; the set designated m.GC-box represents the transcription of the constructs methylated at the internal cytosine residue of the GC-box (site D in figure 1) and the last one (m.HpaII-site) the methylation of the internal cytosine residue of the HpaII site situated immediately to the vicinity of the GC-box (site E in figure 1).

Methylation of a CpG site situated either in or immediately adjacent to the HSV *tk* GC-box also blocked transcription, but reduced the binding of the transcription factor Sp1 by only approximately one-third. Therefore, the effect of methylation on transcription factor binding to the HSV *tk* promoter cannot account for the magnitude of the reduction in transcription.

This finding does not imply that cytosine methylation never represses transcription by directly preventing factor binding (34–36). Methylation of a CpG in the middle of the major late transcription factor of the adenovirus major late promoter inhibits both binding and transcription (36). But in view of our results this mechanism cannot be general. DNA methylation seems to regulate transcription in more than one way.

Other observations support the idea that repression of the HSV *tk* promoter by methylation is indirect (37–38). Should the inhibition of transcription be mediated simply by differences in transcription factor affinity for the methylated versus unmethylated binding sites, the magnitude of repression would be similar in the plasmids M13/dsIIio, pJJio-inv and pJPS-inv. The binding sites and flanking sequences in these plasmids are identical, but the vector differs. The effect of methylation was found to be different for the three constructs. DNA sequences within the pUC vector interfere with the process of regulation by methylation of the *tk* promoter. The plasmid pBR322 (pUC is a derivative of pBR322) when coinjected with SV40, can inhibit transcription of the SV40 DNA (39). However, coinjection of equivalent amounts of pJJio and the internal control construct M13/ $\psi$ -dsIIio, showed that both DNAs were transcribed with similar efficiency. The pUC sequences thus appear to have no effect on the activity of the unmethylated *tk* promoter, though they do affect the response to methylation.

Even though CpG methylation of the CTF and Sp1 binding site has little effect on factor binding *in vitro* in gel mobility shift assays, DNA methylation can repress transcription *in vitro* (unpublished result, see also 40). To detect the effect of methylation *in vitro*, it was reported that the template DNA must be circular and the concentration of added nuclear proteins high (40). These results imply that the effect of methylation may depend on competition between transcription factors and other nuclear proteins (perhaps histones and HMG proteins) for binding to promoter DNAs in a structure which requires circular DNA—chromatin would be one possibility (41). According to this competition model, methylation would favour formation of a repressed structure, while in the absence of methylation transcriptional activator proteins would favour the formation of an active transcription complex. This could explain how promoters normally repressed by methylation can be reactivated by a strong enhancer (42) or a transactivator protein (43), and perhaps why methylation of an Sp1 site did not reduce transcription when the site was linked to the  $\beta$ -globin TATA box and the SV40 enhancer (17).

If transcriptional repression by methylation is mediated by the binding of inhibitory chromatin proteins in the place of transcription factors, these factors should be absent from the repressed promoter. Alternatively methylation may alter the nucleoprotein structure of a gene independently of transcription factor binding. *In vivo* footprinting experiments with injected methylated or unmethylated DNA, currently in progress in our laboratory, may allow these two possibilities to be distinguished.

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