Transcription of methylated eukaryotic viral genes in a soluble *in vitro* system

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ABSTRACT

SV40 and adenovirus-2 (Ad2) recombinant plasmids containing long segments of poly(dC-dG) cloned adjacent to transcription control regions were methylated *in vitro* with HhaI methylase and transcribed in a soluble *in vitro* system. The addition of up to 40 or more base pairs of poly(m^5dC-dG) immediately upstream or downstream of promoter regions was shown to have no effect on the accuracy or efficiency of specific transcription from these promoters *in vitro*. Methylation at various naturally occurring C-G sequences within or near these promoters also had no effect on transcription *in vitro*. The significance of these results with respect to possible mechanisms whereby DNA methylation might regulate eukaryotic gene expression is discussed.

INTRODUCTION

Methylation of eukaryotic DNA occurs almost exclusively as 5-methylcytosine (m^5C) in the dinucleotide sequence C-G (1). A large body of evidence has been accumulated in recent years implicating this DNA modification in the regulation of eukaryotic gene expression (1,2). These studies have established that in many cases DNA methylation is associated with transcriptional inactivity of genes. It has been shown that the extent of methylation is inversely correlated with the expression of certain cellular genes in a tissue-specific manner (3-5), and a similar correlation has been noted in some viral systems (6-8). Additionally, the cytidine analogue 5-azacytidine, which interferes with DNA methylation, can alter the pattern of gene expression in growing cells (9,10). More direct evidence suggesting a causal link between DNA methylation and the regulation of gene expression comes from DNA-mediated gene transfer experiments (11-13) and frog oocyte microinjection experiments (14,15) using *in vitro* methylated DNAs.

Double-stranded polydeoxyribonucleotides composed of alternating C-G sequences can, under appropriate conditions, assume the left-handed Z-DNA conformation (16). The finding that the Z structure in poly(dC-dG) is stabilized by methylation of cytosine residues (17) raises the intriguing possibility that modulation of DNA conformation by this modification may be involved in regulating gene

expression. While poly(m^5dC-dG) is stable in the Z conformation under near physiological salt conditions, poly(dC-dG) is not (17). Furthermore, potential Z-forming sequences have been identified in naturally occurring sequences (18,19), and there is evidence suggesting that Z-DNA may exist *in vivo* (20,21). However, what role Z-DNA might play in the regulation of gene expression is unknown.

Lacking so far is evidence for a molecular mechanism by which DNA methylation, and perhaps Z-DNA formation, might regulate the expression of genes. We have approached this problem by utilizing a soluble *in vitro* system that accurately transcribes eukaryotic genes to examine transcription from DNA templates methylated *in vitro*. For this purpose, we constructed a series of SV40 and Ad2 recombinant plasmids containing long blocks of poly(dC-dG) cloned adjacent to RNA polymerase II or III promoters. These recombinants were methylated *in vitro* using HhaI methylase, which methylates the internal cytosine in the sequence G-C-G-C (22), and then transcribed in HeLa whole-cell lysates (23). A comparison of the levels of specific transcripts synthesized from the methylated or mock methylated templates revealed that neither the accuracy nor efficiency of transcription was affected by the addition of long blocks of poly(m^5dC-dG) immediately upstream or downstream of these promoters. Methylation at a number of naturally occurring C-G sequences that are putative sites of methylation *in vivo* also was found to have no effect on transcription. We conclude that the transcription complexes in the soluble *in vitro* system are oblivious to extensive DNA methylation, and that this modification by itself is not sufficient to inhibit specific transcription *in vitro*.

MATERIALS AND METHODS

Recombinant DNA. The sources of poly(dC-dG) segments were two pBR322 plasmids containing the segments cloned as BamHI restriction fragments (24). The poly(dC-dG) inserts in pLP32 and pLP42, $(dC\text{-}dG)_{18}$ and $(dC\text{-}dG)_{21}$-dG-$(dC\text{-}dG)_2$ respectively (24), were excised from pBR322 by cleavage with BamHI and cloned into the appropriate restriction sites of the constructions described below.

The SV40 series of recombinants were constructed using the plasmid pSVRI that contains the entire SV40 genome (25) inserted at the EcoRI site in pBR322. For the construction of pSVKCG, pSVRI was cleaved with KpnI and the 3' overhangs were trimmed by digestion with *E. coli* DNA polymerase I Klenow fragment to generate blunt ends. The BamHI fragment of pLP42 was filled-in with Klenow fragment and then ligated into the trimmed KpnI site to regenerate BamHI sites. For the construction of pSVBCG1, the BglI site of pSVRI was converted to a BamHI site by digestion of the 3' overhangs with Klenow fragment and insertion of BamHI linker. The BamHI fragment of pLP42 was then ligated into this BamHI site. pSVBCG2 is identi-

cal to pSVBCG1 except that it contains two tandem inserts of the pLP42 BamHI fragment.

The Ad2 major late promoter clone pXB210 (26), which contains major late sequences extending from -66 to +190 (relative to the cap site, +1) inserted between the EcoRI and HindIII sites of pBR322, was used to construct the recombinant pXRCG. pXB210 was cleaved with EcoRI, digested with nuclease S1 to trim the 5' overhangs, and ligated to the BamHI fragment of pLP32 that had been filled-in with Klenow fragment in order to regenerate the BamHI sites. Another Ad2 major late promoter clone, pφ4 (26), which contains major late sequences extending from -400 to +33, was used in the construction of pφBCG. This recombinant contains the BamHI fragment of pLP32 inserted into the BamHI site of pφ4.

The clone pVAp, used to construct pVABCG, contains the Ad2 virus-associated (VAI and VAII) genes (25) within a BglII-HindIII fragment (Ad2 map units 24.3 to 31.5) cloned between the BamHI and HindIII sites of pBR322. The BamHI fragment of pLP42 was inserted into the BamHI site (29.2 map units) within the VAI gene in pVAp.

The structures of the above constructs are illustrated in Figure 1. The recombinant plasmids were initially screened by restriction analysis. To verify that the BamHI fragments containing the poly(dC-dG) segments were present in each clone, the DNAs were digested with BamHI, labelled at the 5' termini with [γ-^{32}P]ATP and T4 polynucleotide kinase, fractionated according to size by electrophoresis through 12.5% polyacrylamide/8M urea gels, and visualized by autoradiography. The labelled BamHI inserts of pLP32 and pLP42 were applied in parallel lanes for comparison. Standard protocols were used in the construction, analysis, amplification, and purification of the recombinant DNAs (27).

HhaI DNA Methylase. HhaI methylase was prepared from *Haemophilus haemolyticus* cells essentially as described for the preparation of HpaII methylase (11), with the following modifications. Cell cultures were grown to early stationary phase in brain-heart infusion broth containing 2 μg/ml NAD and 10 μg/ml hemin (28). The Biogel column chromatography step was omitted, as it did not yield a significant purification of HhaI methylase and resulted in substantial loss of the methylase activity. The ammonium sulfate protein precipitate was resuspended in a minimum volume of 50% glycerol, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 5 mM β-mercaptoethanol, and dialized against this same buffer for 4 hours at 4°C, followed by an additional 12 hours at -20°C using fresh dialysis buffer. The final enzyme preparation (approximately 1 to 2 ml was obtained from an 8 liter cell culture) was quick-frozen in aliquots with liquid nitrogen and stored at -80°C.

DNA methylations were carried out in alkaline pH buffer (22) consisting of 100

mM glycine-NaOH (pH 9.4), 20 mM EDTA, and 20 mM β-mercaptoethanol, in the presence of 1 mM S-adenosylmethionine. For mock methylation of DNA, 1 mM S-adenosylhomocysteine was substituted for S-adenosylmethionine. The standard reaction mixture contained typically 10 μg of supercoiled DNA and 25 μl of HhaI methylase in a final volume of 250 μl. Reaction mixtures were incubated for four hours at 16°C, after which the nucleic acids were purified by extraction with phenol and chloroform and then ethanol precipitated. The extent of DNA methylation was estimated by exhaustive digestion with HhaI restriction endonuclease followed by agarose gel electrophoresis (see Results).

In Vitro Transcription. DNA templates for *in vitro* transcription were prepared by digestion of the DNAs with the restriction endonucleases indicated in the text, followed by extraction with phenol and chloroform, and ethanol precipitation. HeLa whole-cell lysates were prepared essentially as described (23). Standard 25 μl reaction mixtures, containing 15 μl of cell extract and 1.25 μg of template DNA, were 24 mM Tris-HCl (pH 7.9), 6mM $MgCl_2$, 60 mM KCl, 0.06 mM EDTA, 1.2 mM dithiothreitol, 50 μM each ATP, CTP, UTP, and GTP, 5-10 μCi [α-^{32}P]GTP, 4 mM creatine phosphate. The RNA was purified, glyoxalated, and analyzed by agarose gel electrophoresis as previously described (29), followed by autoradiography of the dried gels without intensifying screens.

Enzymes. Restriction endonucleases, T4 DNA ligase, and *E. coli* DNA polymerase I Klenow fragment were from New England Biolabs. Nuclease S1, calf intestine alkaline phosphatase, and T4 polynucleotide kinase were from Boehringer-Mannheim.

RESULTS

Characterization of HhaI Methylase

The SV40 and Ad2 recombinants containing the cloned poly(dC-dG) segments (Figure 1) were methylated *in vitro* with HhaI methylase. This methylase modifies the internal cytosine, at the 5-position, in the sequence G-C-G-C (G-m^{5}C-G-C) (22). Every cytosine in poly(dC-dG) can be recognized as the internal cytosine (except at the ends) of a HhaI site by the methylase, since this polymer consists of repeated, overlapping HhaI sites. Furthermore, HhaI methylase is oblivious to modification at the external cytosine in the recognition sequence (G-C-G-m^{5}C), and therefore poly(dC-dG) can be methylated at every cytosine (22). Because HhaI restriction endonuclease will not cleave at G-m^{5}C-G-C (22), the extent of methylation of the polymer can be accurately estimated by assaying for protection from digestion by this enzyme. Unmodified poly(dC-dG) can be digested with HhaI restriction enzyme to tetramers and hexamers (30).

Figure 2 shows a typical restriction analysis of mock methylated (lanes B) and

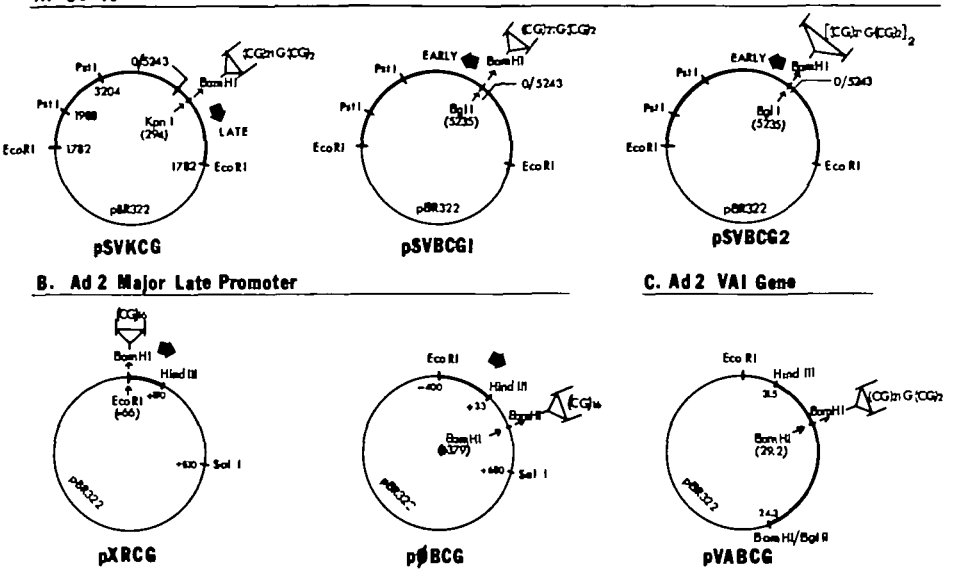

Figure 1

SV40 and Ad2 recombinant plasmids containing cloned segments of poly(dC-dG). The sequence and location of the poly(dC-dG) segments, cloned as a BamHI restriction fragment in each case, and the relevant restriction sites, are indicated. Viral sequences are represented by thick lines, pBR322 plasmid sequences by thin lines. The coordinates shown are: *Panel A*, SV nucleotide numbers (ref. 25); *Panel B*, nucleotide positions relative to the Ad2 major late cap site (position +1) (ref. 26); *Panel C*, Ad2 map units (ref. 25). The sites of the poly(dC-dG) insertions are indicated as numbers in parentheses; arrows indicate direction of transcription. See text for details on the construction as well as the significant features of these recombinants.

methylated (lanes C) DNAs following incubation in the HhaI methylase preparation (lanes A show DNAs prior to incubation with the methylase). The substrate DNA in this case was the plasmid pLP42 (24) that contains a total of 51 methylatable HhaI sites, including 31 sites in pBR322 and 20 overlapping sites in the poly(dC-dG) insert. Following exhaustive digestion with HhaI restriction enzyme, most of the methylated form I circular DNA was converted to form III linear DNA, although a detectable amount of uncleaved supercoiled DNA remained. This suggests that approximately 1 out of 50 HhaI sites per plasmid molecule was available for cleavage, and therefore that methylation of this DNA was 98% complete. However, this analysis does not distinguish a fully methylated poly(dC-dG) insert from a fully unmethylated insert. To verify that the poly(dC-dG) inserts were methylated to the same extent as the other HhaI sites in the plasmids, the following analysis was per-

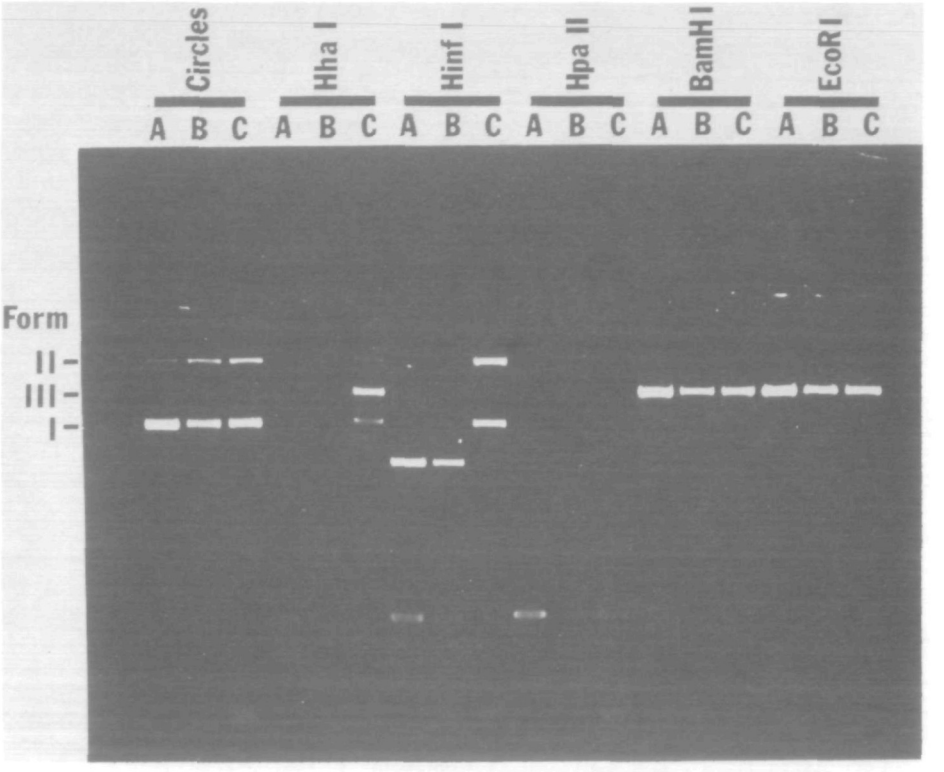

Figure 2

Characterization of HhaI DNA methylase. Plasmid DNA (pLP42) was incubated with HhaI methylase, purified, digested with the restriction endonucleases indicated, and electrophoresed through a 1.2% agarose gel containing 0.5 μg/ml ethidium bromide. Lanes (A), DNAs prior to incubation with HhaI methylase. Lanes (B), mock methylated DNAs. Lanes (C), methylated DNAs. Circles, DNAs treated as above except not digested with restriction enzymes. Form I, II, and III DNA: supercoiled, nicked circular, and linear DNA, respectively.

formed. Plasmid DNA was first digested with restriction endonucleases other than HhaI in order to generate a small restriction fragment containing the poly(dC-dG) insert and no other HhaI sites, and then digested with HhaI restriction enzyme. The results of this analysis (not shown) demonstrated that the poly(dC-dG) inserts were fully methylated (greater than 98%). Mock methylated DNA was digested to completion with HhaI restriction enzyme, demonstrating that the protection from digestion was dependent on DNA methylation.

The HhaI methylase preparation also contained HhaII methylase activity, as assayed by protection from digestion by an isoschizomer of HhaII restriction

enzyme (HinfI), which recognizes the sequence G-A-N-T-C (pBR322 contains 10 such sites). No other DNA modifications were expected, as verified by digestion of all DNAs with HpaII, BamHI, and EcoRI restriction enzymes. The extent of single-strand nicking, estimated by the conversion of form I to form II DNA, varied with the particular methylase preparation. Typically, between 25% and 50% of the supercoiled DNA was relaxed during the incubation with methylase.

In the transcription experiments described below, methylated and mock methylated DNAs to be directly compared with each other were prepared simultaneously using the same methylase preparation. The extent of DNA methylation in each case was estimated to be 98% or greater by the method described above, and all mock methylated DNAs could be digested to completion with HhaI restriction enzyme. Incubation of these DNAs in the HeLa whole-cell lysate under standard transcription conditions did not alter the state of DNA methylation (data not shown).

In Vitro Transcription of Methylated Templates

Transcription from the HhaI methylated and mock methylated recombinant plasmids shown in Figure 1 was examined in HeLa whole-cell lysates. The essential features of these recombinants and the results of the transcription experiments are described below.

SV40 Templates. The recombinants pSVBCG1 and pSVBCG2 contain, respectively, single and double inserts of a 47 base pair segment of poly(dC-dG) cloned into the SV40 BglI site (Figure 1). The BglI site lies approximately 20 base pairs downstream of the TATA box (in the early direction), and upstream of the start sites for the two major transcripts synthesized from the early region of wild-type SV40 *in vitro* (31). Figure 3 shows the two early region runoff RNAs (2.05 and 1.95 kb) that were transcribed from the wild-type template and a derivative subclone containing BamHI linker at the BglI site (lanes a and b). Two major late transcripts were also synthesized from these templates (see below). The corresponding runoff transcripts from the pSVBCG1 and pSVBCG2 templates (lanes c and d) differed from the wild-type in that the 2.05 kb transcripts were increased in size by the lengths of the respective poly(dC-dG) inserts (approximately 50 and 100 nucleotides). This indicates that the upstream transcription start site occurs either within or immediately upstream of the poly(dC-dG) inserts in these recombinants. Additionally, the levels of transcription from the upstream start site in the pSVCG1 and pSVCG2 templates were slightly reduced (less than two fold) compared to wild-type. Transcription from the downstream start site (1.95 kb runoff) in these templates remained unchanged, however, suggesting that some of the information required for accurate initiation from this site lies downstream of the poly(dC-dG) inserts.

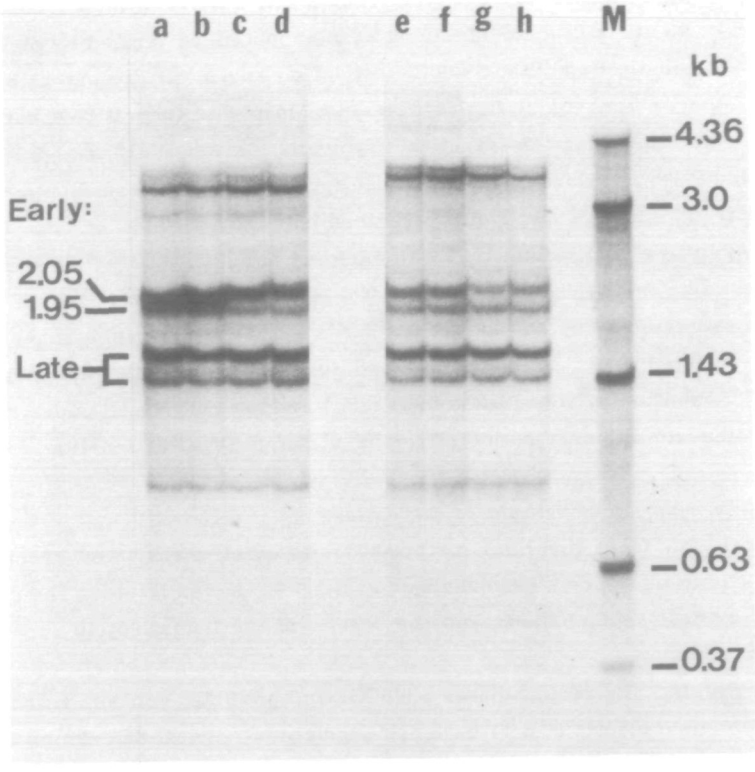

Figure 3

Transcription of SV40 early region recombinants. The templates indicated below were digested with EcoRI and PstI, transcribed in standard reaction mixtures, and the purified RNA products were glyoxalated and resolved in a 1.4% agarose gel. The templates not incubated with HhaI methylase were: lane (a), pSVRI; lane (b), pSVRI derivative containing BamHI linker inserted the BglI site; lane (c), pSVBCG1; and lane (d), pSVBCG2. The templates incubated with HhaI methylase were: lane (e), mock methylated pSVBCG1; lane (f), methylated pSVBCG1; lane (g), mock methylated pSVBCG2; and lane (h), methylated pSVBCG2. Lane (M), marker RNA prepared using an Ad2 major late promoter clone cleaved with a variety of restriction enzymes as templates.

A comparison of the levels of transcription obtained from the HhaI methylated and mock methylated pSVBCG1 and pSVBCG2 templates revealed no detectable differences in the major transcripts (Figure 3, lanes e to h). These results indicate that neither the accuracy nor efficiency of transcription from the early promoter was affected by the presence of 40 or 80 base pairs of poly(m^5dC-dG) located between the two major start sites.

The recombinant pSVKCG contains 47 base pairs of poly(dC-dG) cloned into

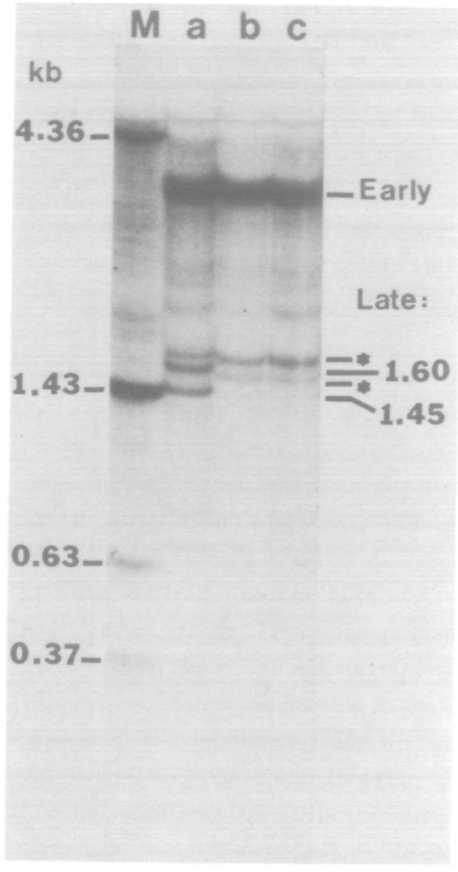

Figure 4
Transcription of SV40 late region recombinants. The templates were digested with EcoRI, transcribed in standard reaction mixtures, and the RNA was analyzed as in Figure 3. The templates were: lane (a), pSVRI; lane (b), mock methylated pSVKCG; and lane (c), HhaI methylated pSVKCG. The stars indicate the positions of the novel transcripts that were synthesized from pSVKCG. Lane (M), marker RNA.

SV40 at the KpnI site (Figure 1). The KpnI site lies between the start sites for the two major late transcripts synthesized *in vitro* from wild-type SV40 (31,32). The two runoff RNAs transcribed from the wild-type template (1.60 and 1.45 kb) are shown in Figure 4 (lane a). A third transcript (1.70 kb), which originated from the pBR322 plasmid vector (32), was also observed. Lanes b and c show the corresponding runoff RNAs that were transcribed from the HhaI methylated and mock methylated pSVKCG templates. A comparison of the transcription products obtained from the wild-type and the pSVKCG templates revealed that both major late transcripts from pSVKCG were, unexpectedly, increased in size by approximately 50 nucleotides (one transcript is not well resolved from the pBR322-derived 1.7 kb RNA in Figure 4). This indicates that the downstream start site in pSVKCG occurs near the upstream boundary of the poly(dC-dG) insert. In contrast, in

wild-type SV40, this start site occurs approximately 25 base pairs downstream of the KpnI site (32). Additionally, the levels of the major late transcripts synthesized from the pSVKCG templates were reduced approximately two fold compared to the wild-type template. Previous mutational analysis of the late promoter *in vitro* suggests that sequences overlapping with the KpnI site may constitute a transcription control region specifying the downstream start site (32). It is probable, therefore, that the insertion at the KpnI site disrupts this control region and that the novel start site is specified by an alternative control region located farther upstream. The presence of poly(m^5dC-dG) within this critical region, however, did not have any effect on late transcription.

Previous experiments have demonstrated that methylation at the unique HpaII site in SV40 specifically inhibits late gene expression in frog oocyte nuclei (15). Wild-type SV40 contains a HhaI site that overlaps with the HpaII site, located approximately 50 base pairs downstream from the KpnI site in the late direction (25). However, methylaton at this site also had no effect on *in vitro* transcription from the late region (Figures 3 and 4).

Ad2 Templates. The recombinant pXRCG (Figure 1) contains 32 base pairs of poly(dC-dG) inserted at the upstream boundary (position -66 relative to the transcription start site) of Ad2 major late promoter sequences required to obtain optimal transcription *in vitro* (26). Substitution of pBR322 sequences upstream of position -66 with the poly(dC-dG) segment had no effect on the level of transcription initiation from pXRCG compared to the parental clone, pXB210 (Figure 5A, lanes 1 and 2). However, previous studies indicate that, while specific major late sequences upstream of -66 are not required to obtain wild-type levels of transcription *in vitro* from this promoter, the presence of nonspecific template DNA upstream of -66 is required (RJ and JLM, manuscript submitted). This observation suggests that the transcription complex interacts in a nonspecific manner with template DNA upstream of -66 during transcription initiation. Despite the presence of the poly(dC-dG) segment within this region, however, HhaI methylation had no effect on either the accuracy or efficiency of transcription initiation from the pXRCG template (Figure 5A, lanes 3 and 4).

Another major late promoter clone, pφBCG, contains the same poly(dC-dG) segment inserted at a position 375 base pairs downstream from the transcription start site (Figure 1). The addition of the poly(dC-dG) insert at this site had no effect on transcription compared to the parental clone, pφ4 (data not shown). If the poly(m^5dC-dG) in pφBCG could block elongation, an RNA species approximately 375 nucleotides long, representing transcripts aborted at the site of the polymer insertion, would have been detected. However, such transcripts were not detected

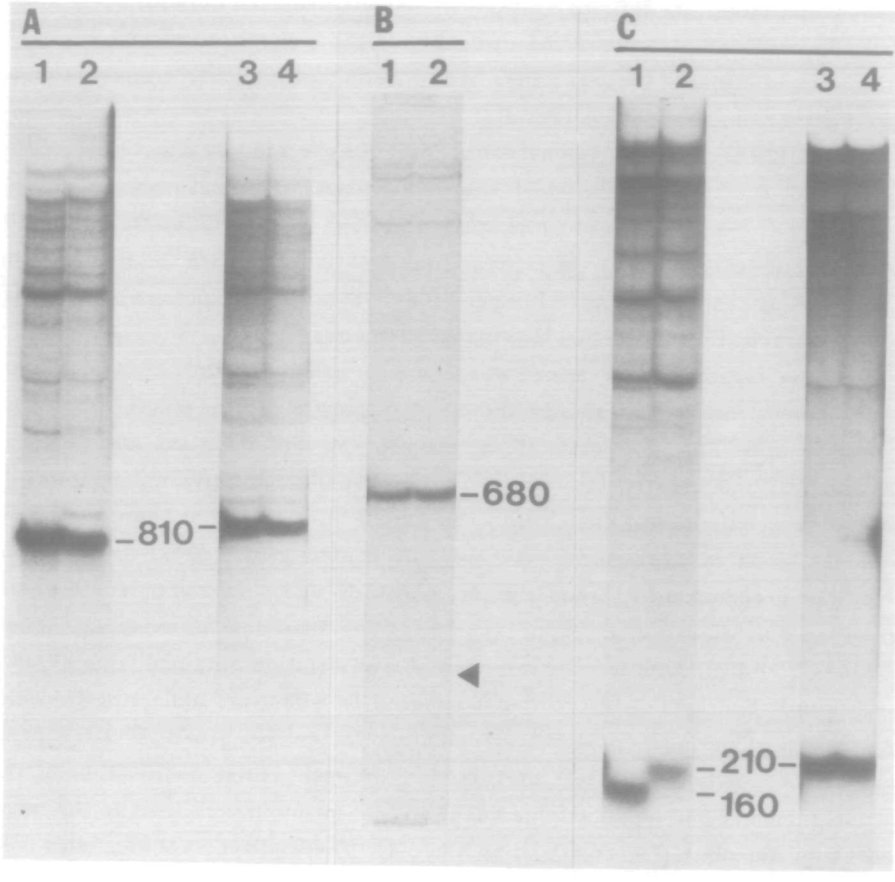

Figure 5

Transcription of Ad2 recombinants. *Panel A*, major late promoter templates, cleaved with SalI, were: lane (1), pXB210; lane (2), pXRCG; lane (3), mock methylated pXRCG; and lane (4), HhaI methylated pXRCG. *Panel B*, major late promoter templates, cleaved with SalI, were: lane (1), mock methylated pφBCG; and lane (2), HhaI methylated pφBCG. Arrowhead, position where an RNA transcript 375 nucleotides long would have migrated. *Panel C*, circular templates, containing the VA genes, were: lane (1), pVAp; lane (2), pVABCG; lane (3), mock methylated pVABCG; and lane (4) HhaI methylated pVABCG. The transcription reactions and analysis of RNA were as described in Figure 3, except that the VA transcripts were resolved in 1.8% agarose gels. The sizes of the specific transcripts are given in nucleotides.

(Figure 5B), indicating that elongation was not blocked at this site. This result is consistent with the finding that, in all cases examined where a poly(dC-dG) segment occurs downstream of a transcription start site, the levels of full-length runoff transcripts from the methylated and mock methylated templates were indistinguishable (Figures 3, 4, 5).

The major late promoter also contains a HhaI site centered at position -12 and a HpaII site centered at position -51 upstream of the transcription start site (28). Methylation at either of these two sites had no detectable effect on transcription *in vitro* (Figure 5, and unpublished results).

The Ad2 E1a promoter region contains HpaII sites located 22 and 79 base pairs upstream of the transcription start site (R.J. Roberts, personal communication). Methylation at these two sites had no effect on *in vitro* transcription from this promoter (unpublished results). In contrast, methylation at either the HpaII or the HhaI sites upstream of the Ad12 E1a gene inactivated this promoter when expression was assayed by transfection into mammalian cells (33).

All of the experiments described above involve promoters that are transcribed by RNA polymerase II. To examine the effect of poly(dC-dG) or poly(m^5dC-dG) on transcription by RNA polymerase III, a recombinant containing the Ad2 VA genes (VAI and VAII) was constructed (Figure 1). This recombinant, pVABCG, contains 47 base pairs of poly(dC-dG) cloned into the BamHI site located at the downstream boundary (position +72) of the VAI intragenic control region (34). The VAI transcript (the predominant VA transcript) from pVABCG was increased in length by the size of the poly(dC-dG) insert (approximatelty 50 nucleotides), as expected (Figure 5C, lanes 1 and 2). Additionally, the level of transcription obtained from pVABCG was reduced approximately two fold compared to wild-type, indicating that the polymerase, or perhaps an ancillary regulatory factor, was sensitive to the presence of the poly(dC-dG) insert at this site. However, HhaI methylation of the pVABCG template had no detectable effect on transcription compared to the mock methylated template (Figure 5C, lanes 3 and 4). The wild-type VAI gene also contains three HpaII sites that coincide with the intragenic control region (34). Methylation at these sites also had no effect on transcription *in vitro* (unpublished results).

DISCUSSION

We have examined the effect of extensive DNA methylation on the expression of a number of eukaryotic viral promoters transcribed by RNA polymerase II or III in a soluble *in vitro* system. These experiments demonstrate that the addition of up to 40 or 80 base pairs of poly(m^5dC-dG) in the immediate proximity of these promoter regions had no effect on either the accuracy or efficiency of transcription initiation in all cases examined. Moreover, there was no evidence that the long segments of poly(m^5dC-dG) could block transcription elongation. Finally, methylation at several naturally occurring C-G sequences located within or near promoter regions, which are putative sites of methylation *in vivo*, had no effect on the

expression of these promoters. The above results indicate that the components comprising the transcription complexes in the soluble *in vitro* system are insensitive to extensive DNA modification.

A large body of evidence has been accumulated recently that establishes an inverse correlation between the extent of DNA methylation and the expression of certain genes in eukaryotes (1,2). This suggests that DNA methylation may be utilized by eukaryotes as a mechanism for regulating gene expression. One simple model is that the state of DNA methylation alone determines the transcriptional activity of genes. In this model, methylated genes constitute poor templates for transcription as a direct consequence of altered molecular interactions between the transcription apparatus and the modified DNA. Transcription of methylated genes might then be blocked at the level of initiation or subsequent elongation, perhaps because the transcription apparatus cannot form a stable complex with methylated DNA. However, such a model appears unlikely in light of our findings that extensively methylated templates direct accurate transcription as efficiently as unmethylated templates *in vitro*.

Strong evidence in support of the hypothesis that DNA methylation is involved in the regulation of gene expression comes from experiments in which the expression of viral DNAs methylated *in vitro* was examined after microinjection of these DNAs into *Xenopus laevis* oocyte nuclei. Methylation at the unique HpaII site downstream of the SV40 late promoter was found to specifically inhibit late gene expression in frog oocytes (15). Similar results were obtained using the Ad2 E2a promoter in the oocyte system (14). However, the identical modification that inhibited SV40 late gene expression in oocytes had no effect on transcription in a soluble *in vitro* system (15). Thus, DNA methylation by itself was not sufficient to inhibit specific transcription *in vitro*, consistent with the results reported here. These results support the suggestion that the soluble system is lacking in components that confer sensitivity to DNA methylation, and that such components are distinct from those that are required for accurate transcription initiation *in vitro*.

There are notable exceptions to the inverse correlation between DNA methylation and transcriptional activity. Certain cellular genes, for example, are undermethylated despite the fact that they are not expressed *in vivo* (5,35). Conversely, complete methylation of the SV40 genome was shown to have no effect on early gene expression *in vivo* (36). Similar results were also reported with rDNA genes in the frog oocyte system (37). Somewhat surprizing, however, is the finding that the activation of a developmentally regulated gene *in vivo* is correlated with an increase in DNA methylation (38). These observations present additional arguements against simple models, such as the one discussed above, whereby the state

of DNA methylation alone determines the transcriptional activity of genes. The *in vivo* results are readily explained, however, by invoking the participation of additional factors that interact with methylated DNA and thereby regulate transcription.

A model consistent with the *in vivo* and *in vitro* data available to date is that DNA modification at specific sites regulates the accessibility of target genes to the transcription apparatus. Accessibility to the target gene could be determined by the modulation of chromatin structure, which in turn may be mediated by regulatory factors that interact with site-specific DNA modifications. Consistent with this suggestion is the observation that exogenous DNA templates are packaged into chromatin in frog oocyte nuclei (39) but not in the soluble *in vitro* system (40). If the above model is correct, this crucial difference would then explain why the soluble system does not respond to DNA modification.

The preceding discussion is concerned primarily with protein-encoding genes that are transcribed by RNA polymerase II. The effect of DNA modification on the expression of RNA polymerase III genes has not been previously studied *in vivo* or *in vitro*. Our results demonstrate that extensive methylation of the Ad2 VAI gene had no effect on transcription in the soluble *in vitro* system. Additionally, the VAI gene was transcribed as efficiently from the HhaI methylated pVABCG template as from the mock methylated template when these DNAs were microinjected into frog oocyte nuclei (A. Fradin, C. Prives, RJ, and JLM, unpublished results). Thus, the RNA polymerase III in intact frog oocytes is also oblivious to extensive modification within the VAI gene. Further study is required, however, before the question of whether DNA methylation is involved in regulating RNA polymerase III transcription *in vivo* can be adequately assessed.

It is intriguing that precisely the same methylation implicated in the regulation of eukaryotic gene expression also induces the left-handed Z-DNA conformation in poly(dC-dG) under near physiological salt conditions (17). Recent experiments have also demonstrated that cloned poly(dC-dG) segments contiguous with right-handed B-DNA can flip to the Z conformation (41,42). Thus, it is possible that in the experiments reported here the cloned poly(m^5dC-dG) segments assumed the Z conformation, although we did not determine whether this was in fact the case under any of our transcription conditions. Additionally, no effect was observed when the experiments described here were repeated in the presence of up to 100 μM spermine (RJ and JLM, unpublished results), which was previously shown to stabilize the Z structure in poly(m^5dC-dG) at much lower concentrations (17). The B-Z transition in cloned poly(dC-dG) segments is also facilitated by negative supercoiling (42); however, transcription from supercoiled templates could not be exam-

ined *in vitro* because of an endogenous relaxing activity present in HeLa cell extracts. Experiments are currently in progress to develop a soluble *in vitro* system that accurately transcribes supercoiled templates (C. Hemenway and JLM). The availability of such a system will permit further investigation into the possible role of Z-DNA in the regulation of eukaryotic gene expression.

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