JOURNAL OF VIROLOGY, July 1987, p. 2344–2348
0022-538X/87/072344-05$02.00/0

Vol. 61, No. 7

Methylation of Specific Cytosine Residues Enhances Simian Virus 40 T-Antigen Binding to Origin Region DNA

VAN G. WILSON

Department of Medical Microbiology and Immunology, College of Medicine, Texas A&M University, College Station, Texas 77843

Received 8 January 1987/Accepted 6 April 1987

Specific binding of simian virus 40 large T antigen to origin region DNA requires the interaction of T antigen with multiples of a consensus recognition pentanucleotide sequence (5′-G[T]-A[G]-G-G-C-3′). To assess the interaction of T antigen with cytosine residues in the recognition sequences, bacterial methylases were used to methylate simian virus 40 form I DNA in vitro at specific cytosine residues. Methylation of a subset of the cytosine residues in the pentanucleotide sequences resulted in enhanced binding of T antigen to origin region DNA. Enhanced binding to the methylated pentanucleotides indicates that the methyl groups introduced on this subset of pentanucleotide cytosine residues could not have sterically interfered with the interaction of T antigen with the recognition sequences. This lack of steric interference suggests that T antigen does not make close contact in the major groove with these particular cytosine residues during normal binding.

Nucleolytic protection assays have defined three adjacent but relatively independent T-antigen binding regions (I, II, and III) on the simian virus 40 (SV40) genome (3, 15–18). Within each region, bound T antigen protects a specific subset of the guanine residues from methylation by the reagent dimethylsulfate (3, 13, 18). A consensus pentanucleotide recognition sequence which accounts for all of the T antigen-protected guanines in the origin region has been defined and has the sequence 5′-G(T)-A(G)-G-G-C-3′ (3, 18). For most of the region I and II pentanucleotide guanines, prior methylation also inhibits binding by T antigen (8). The guanine protection and inhibition data support the conclusion that close contact occurs in the major groove between T-antigen amino acids and these particular guanine residues. However, similar studies to assess the importance of pentanucleotide pyrimidine residues in T-antigen contact and binding have been lacking.

The experiments discussed in this paper were undertaken to examine the interaction of SV40 T antigen with a subset of the cytosine residues present in the region I and II pentanucleotide recognition sequences. Form I SV40 DNA was methylated in vitro by using either *Hae*III methylase or *Alu*I methylase, as specified by the manufacturer. Unmethylated DNA was prepared by a mock methylation in which the *S*-adenosylmethionine was excluded from the reaction. The bacterial enzyme *Hae*III methylase methylates the internal cytosine in the recognition sequence 5′-G-G-C-C-3′. *Hae*III methylation of SV40 DNA generates a pair of methylcytosines in four of the seven region I and II pentanucleotides (Fig. 1). *Alu*I methylase methylates the cytosine in the sequence 5′-A-G-C-T-3′; however, none of the methylcytosines generated by this enzyme occurs within the T-antigen recognition pentanucleotides (Fig. 1). As with guanine methylation, the enzymatically introduced cytosine methyl groups are located in the major groove of the DNA helix (19). If T antigen normally makes close contact with these cytosine moieties, then methylation should sterically inhibit T-antigen binding in a manner analogous to inhibition by guanine methylation.

The efficiency of the in vitro methylation was demonstrated by subjecting methylated DNA to digestion with excess amounts of the cognate restriction enzyme. *Hae*III-methylated form I SV40 DNA was more than 95% resistant to digestion with a 40-fold excess of *Hae*III or *Bgl*I, indicating successful methylation of the origin region *Hae*III sites (Fig. 2A). To further ensure the completeness of methylation in the origin region, SV40 DNA was digested with *Ava*II and end labeled by T4 DNA polymerase repair of the single-stranded overhangs, and the 682-base-pair origin-containing fragment was isolated by elution from a preparative 5% acrylamide gel. After digestion with excess *Hae*III, the origin fragment was analyzed on nondenaturing (Fig. 2B) or denaturing (data not shown) acrylamide gels. Only the intact 682-base-pair fragment was observed on either the nondenaturing or denaturing gels after *Hae*III digestion of the methylated fragment, indicating that all of the *Hae*III sites in this fragment were methylated on both strands. A similar analysis confirmed the completeness of *Alu*I methylation of origin region *Alu*I sites (data not shown).

The effect of cytosine methylation on the binding of T antigen to origin region DNA was assessed with a simple filter binding assay. For the experiments shown in Fig. 3 through 5, T antigen was purified biochemically from infected CV-1 cells as described by Tegtmeyer and Andersen (14). (Immunoaffinity-purified T antigen gives identical results [unpublished observations].) Purified T antigen was incubated with SV40 DNA that had been digested with *Ava*II and end labeled as described above. Standard reaction mixtures consisted of 20 ng of DNA in a final volume of 100 μl containing 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 7.0)–75 mM NaCl–0.1 mM EDTA–5% glycerol–0.02% (wt/vol) bovine serum albumin–750 ng of sonicated salmon sperm DNA. After 1 h at 4°C, the reaction mixture was diluted with 500 μl of ice-cold STOP buffer (20 mM PIPES [pH 7.0], 75 mM NaCl, 10 mM EDTA) and the protein-DNA complexes were collected by passage through nitrocellulose filters. After the filters were washed three times with 1.0 ml of ice-cold STOP buffer and three times with 1.0 ml of ice-cold PNE (20 mM PIPES [pH 7.0], 1 mM NaCl, 0.1 mM EDTA), the DNA was extracted from the filters with 30 μl of TBE sample buffer (10 mM Tris borate [pH 8.3], 0.2 mM EDTA, 5% glycerol, 0.2% sodium dodecyl sulfate, 0.025% bromophenol blue, 0.025% xylene cyanol). The extracted DNA was analyzed on nondenaturing

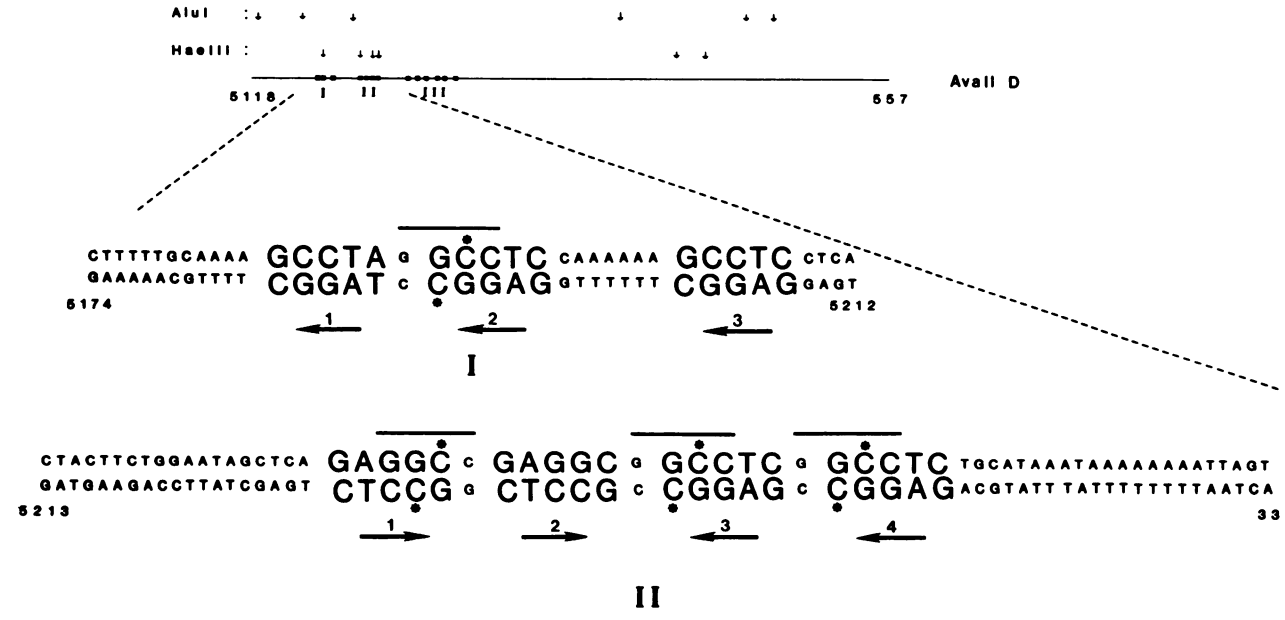

FIG. 1. T-antigen binding regions. The top line depicts the SV40 *Ava*II D restriction fragment (nucleotides 5118 to 557) which contains T-antigen binding regions I, II, and III. The closed squares represent the pentanucleotide consensus sequences. Above the *Ava*II D fragment are arrows indicating the locations of the *Hae*III and *Alu*I sites. Below the *Ava*II D fragment is an expansion of binding regions I and II. Pentanucleotide sequences are shown in enlarged type, and their orientations are indicated by arrows. The recognition sequence for *Hae*III (5′-GGCC-3′) is indicated by the solid lines. Cytosines which are methylated by *Hae*III methylase are indicated by asterisks.

acrylamide gels, as previously described (16). For quantitation of the bound fragments, the regions of the gel containing the DNA fragments were excised and the amount of radioactivity was determined by Cherenkov counting. Likewise, the relevant fragments in the marker lanes were excised and quantitated by Cherenkov counting to determine the amount of labeled input DNA fragment in the binding reaction mixtures.

At each concentration of T antigen, approximately 1.5-fold more *Hae*III-methylated *Ava*II fragment D was bound than unmethylated *Ava*II fragment D (Fig. 3). These binding studies have been performed with NaCl concentrations of 1 to 100 mM and over a 100-fold range of DNA concentrations without affecting the relative enhancement in binding to the *Hae*III-methylated origin fragment (unpublished observations). Unlike the *Hae*III-methylated DNA, the amount of *Alu*I-methylated *Ava*II fragment D bound at each T-antigen concentration was identical to that of unmethylated SV40 DNA. Since the *Alu*I-methylated and *Hae*III-methylated *Ava*II D fragments have the same total number of methylcytosines (Fig. 1), the failure to detect enhanced binding to *Alu*I-methylated origin DNA indicates that the enhancement effect was specific for certain cytosines and was not a general consequence of the presence of 5-methylcytosine in the vicinity of T-antigen binding sites. Further support for the conclusion that enhanced binding of T antigen to the *Hae*III-methylated origin fragment was related to methylation of specific cytosines comes from the observation that there was no general increase in the binding of T antigen to *Hae*III-methylated non-origin-containing restriction fragments (for example, see Fig. 4).

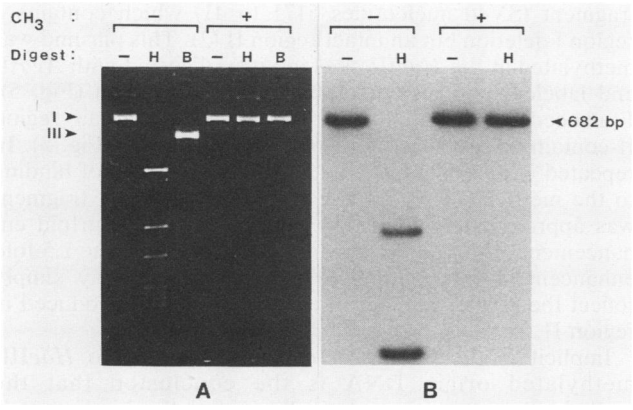

FIG. 2. Resistance of *Hae*III-methylated DNA to digestion with *Hae*III. Shown is unmethylated (−) or *Hae*III-methylated (+) SV40 DNA after mock digestion (−), digestion with *Hae*III (H), or digestion with *Bgl*I (B). Digestions were done with a 40-fold excess of enzyme. (A) Ethidium bromide-stained 1% agarose gel of form I DNA; (B) autoradiograph of the end-labeled SV40 *Ava*II D fragment. Samples were electrophoresed on a 5% nondenaturing acrylamide gel. bp, Base pairs.

To determine which of the six pairs of methylcytosines on the *Ava*II D fragment contributes to enhanced binding by T antigen, *Ava*II-digested and end-labeled SV40 DNA was further digested with *Dde*I. *Dde*I digestion divided the *Ava*II D fragment into three subfragments of 302, 270, and 110 base pairs. The 302-base-pair fragment contained binding regions II and III, including three pairs of methylcytosines. However, since this was an internal fragment, it was no longer labeled and was not detected in the binding assay. The 270-base-pair fragment was derived from the late region end of *Ava*II-D and included two pairs of methylcytosines but no T-antigen binding sites. The 110-base-pair fragment consisted of the early region end of *Ava*II-D and included binding region I, containing a single pair of methylcytosines.

The binding of T antigen to *Ava*II-digested or *Ava*II-plus-*Dde*I-digested SV40 DNA is shown in Fig. 4. No binding of

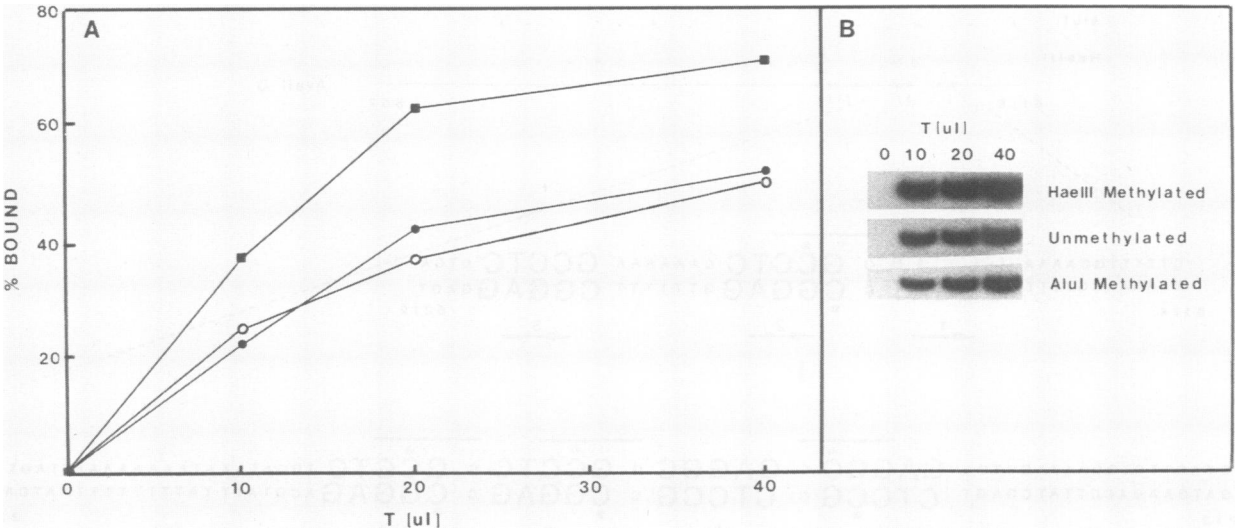

FIG. 3. T-antigen binding to unmethylated, *Hae*III-methylated, and *Alu*I-methylated SV40 DNA. SV40 form I DNA was digested with *Ava*II, end labeled, and assayed for T-antigen binding as described in the text. (A) Graph of percentage of input *Ava*II fragment D bound with increasing amounts of T antigen for *Hae*III-methylated DNA (■), *Alu*I-methylated DNA (○), and unmethylated DNA (●). Each point represents the average of at least three experimental samples. (B) Portions of an autoradiograph showing the *Ava*II D fragment region from an experiment typical of those used to derive the quantitative data shown in panel A.

T antigen to the 270-base-pair *Ava*II-*Dde*I fragment was detected for either unmethylated or *Hae*III-methylated DNA. Consequently, the two methylcytosines outside of the known T-antigen binding regions did not contribute to the enhanced binding observed with the *Ava*II D fragment. There was, however, enhanced binding to the *Hae*III-methylated 110-base-pair fragment. Approximately 1.5-fold more methylated than unmethylated 110-base-pair fragment was bound at each T-antigen concentration. Therefore, enhanced binding to the 110-base-pair fragment can be attributed to a single pair of methylcytosines in pentanucleotide 2 of region I. Furthermore, since there were approximately equimolar amounts of the *Ava*II D and 110-base-pair fragments bound at each T-antigen concentration, the enhanced binding to the *Ava*II D fragment can be accounted for almost entirely by enhanced binding to the methylated region I. This is consistent with the high-affinity binding region I being the primary contributor to fragment binding under these conditions (10, 13).

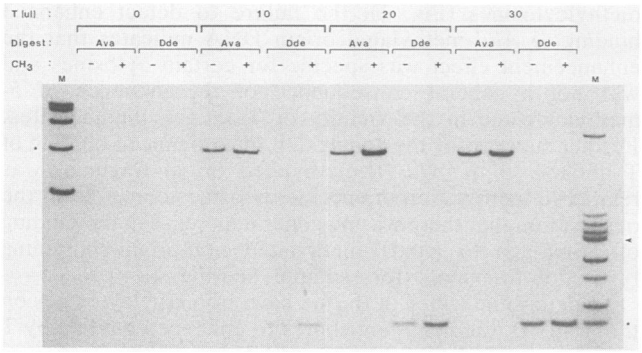

FIG. 4. T-antigen binding to isolated region I with or without *Hae*III methylation. Unmethylated (−) or *Hae*III-methylated (+) form I SV40 DNA was digested with *Ava*II and end labeled as described in the text (Ava lanes). A portion of the labeled DNA was subsequently digested with *Dde*I to subdivide the *Ava*II D fragment and isolate region I on a 110-base-pair fragment (Dde lanes). The left-hand and right-hand marker lanes (M) contained samples of the *Ava*II-digested and *Ava*II-plus-*Dde*I-digested DNAs, respectively. The *Ava*II D fragment and the *Ava*II-*Dde*I 110-base-pair fragment are marked with asterisks. The 270-base-pair fragment derived from the late region end of the *Ava*II D fragment is indicated by an arrowhead. The binding of increasing amounts of T antigen to each digested DNA sample was assayed as described in the text. Bound fragments were analyzed by electrophoresis on an 8% nondenaturing acrylamide gel and visualized by auto-radiography.

Since the region II-containing restriction fragment was not labeled in the experiment for which results are shown in Fig. 4, the effect of cytosine methylation on the binding of T antigen to region II could not be determined in this experiment. To examine whether the methylation of cytosines in three of the pentanucleotides of region II affected binding, a cloned region II sequence was tested. Plasmid pOR1 (kindly provided by A. DeLucia) contains an 84-base-pair SV40 fragment (SV40 nucleotides 5171 to 41) which contains a region I deletion but an intact region II (2). This plasmid was methylated at the *Hae*III sites in vitro, digested with *Ava*II, end labeled, and tested for binding to T antigen (Fig. 5). Enhanced binding to the methylated 892-base-pair region II-containing restriction fragment was observed (Fig. 5). In repeated experiments, the average enhancement of binding to the methylated region II-containing restriction fragment was approximately fourfold. The basis of the fourfold enhancement observed for region II binding versus the 1.5-fold enhancement for region I is not known but may simply reflect the greater number of methylcytosines introduced in region II.

Implicit in the finding of enhanced binding to *Hae*III-methylated origin DNA is the conclusion that the methylcytosines do not sterically inhibit the binding of T antigen to pentanucleotide sequences. This is in contrast to most pentanucleotide guanine residues, including those complementary to most of the origin region cytosines methylated by *Hae*III methylase, whose methylation inhibits binding by T antigen (8). Since both the guanine and cytosine methyl groups are located in the major groove (19), the failure of the cytosine methyl groups to inhibit T-antigen binding suggests

that T antigen does not normally make close contact with these cytosine residues.

Enhanced binding to a methylated 1,229-base-pair plasmid fragment also was observed in the experiment shown in Fig. 5. The methylated 1,229-base-pair fragment and the unmethylated region II-containing 892-base-pair fragment were bound to similar extents, indicating that the methylated plasmid fragment has a significant affinity for T antigen. Specific binding to the unmethylated 1,229-base-pair fragment was not observed, even at higher T-antigen concentrations, nor was there binding to the smaller 222-base-pair plasmid fragment when it was methylated or unmethylated (the 88-base-pair fragment contains no *Hae*III site and so does not become methylated). Therefore, it is likely that T-antigen binding to the 1,229-base-pair fragment was a consequence of methylation of some specific site(s) rather than simply cytosine methylation in general. It should be noted that the 1,229-base-pair fragment contains seven T-antigen consensus recognition pentanucleotides, two of which can be methylated with *Hae*III methylase. Studies are in progress to determine whether methylation of the consensus sequences is responsible for the enhanced binding of T antigen to this plasmid fragment.

There are several potential mechanisms which could explain the enhanced binding of T antigen to methylated DNA. A direct explanation could be a fortuitous hydrophobic interaction of T-antigen amino acids with the appropriately positioned cytosine methyl group. Indirect mechanisms could include an alteration in the major groove width or an overall change in the conformation of the binding region (5, 9, 11, 12). Opening the major groove could provide greater access for T-antigen amino acids to important contact nucleotides, and changes in the conformation of the binding region might favorably stabilize T antigen-DNA or T antigen-T antigen interactions (1, 12). Further studies are necessary to determine the actual mechanism by which methylation of specific cytosines enhances T-antigen binding.

Although this study was undertaken to examine biochemical features of T antigen-DNA interactions in vitro, it is nonetheless germane to consider biological implications of the findings. We showed that cytosine methylation can potentiate the binding of a viral regulatory protein to its recognition sequences in vitro. In addition, we found that methylation of a plasmid DNA sequence, which normally is not bound by T antigen, created a functional T-antigen binding site or sites de novo. Similar methylation-dependent sequence-specific binding has been observed recently for a human placenta nuclear protein (20, 21). The implication of these findings is that the methylation state of DNA may directly affect the binding of T antigen and perhaps cellular regulatory proteins as well. Although methylation is not believed to play a role in the regulation of gene expression from the SV40 genome (4, 6, 7), there is very little information about the direct interaction of T antigen with cellular genes during lytic growth or transformation. Whether the potentiation of T antigen-DNA binding by cytosine methylation is biologically significant for cellular gene expression during SV40 infection remains to be determined.

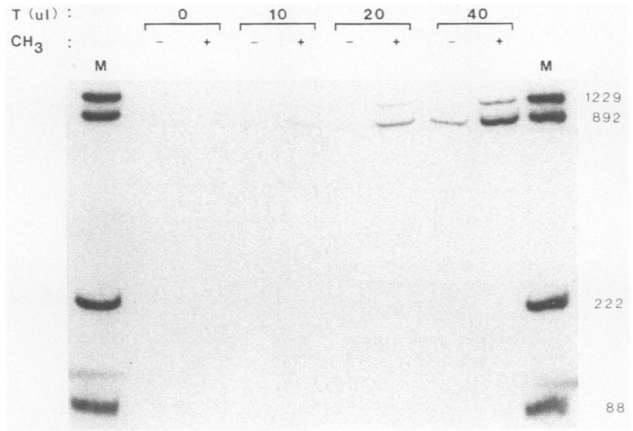

FIG. 5. T-antigen binding to methylated and unmethylated region II. Unmethylated (−) and *Hae*III-methylated (+) pOR1 form I DNA was digested with *Ava*II, end labeled, and assayed for binding to increasing amounts of T antigen, as described in the text. Bound fragments were analyzed as described in the legend to Fig. 4. The marker lanes (M) contained samples of the *Ava*II-digested plasmid DNA. The sizes of the *Ava*II fragments are indicated in the margin in base pairs.

This work was supported by grant MV-206 from the American Cancer Society and by Public Health Service grant RR05814 from the National Institutes of Health.

LITERATURE CITED

1. **Deb, S., A. L. DeLucia, A. Koff, S. Tsui, and P. Tegtmeyer.** 1986. The adenine-thymine domain of the simian virus 40 core origin directs DNA bending and coordinately regulates DNA replication. Mol. Cell. Biol. **6**:4578–4584.
2. **DeLucia, A. L., S. Deb, K. Partin, and P. Tegtmeyer.** 1986. Functional interactions of the simian virus 40 core origin of replication with flanking regulatory sequences. J. Virol. **57**:138-144.
3. **DeLucia, A. L., B. A. Lewton, R. Tjian, and P. Tegtmeyer.** 1983. Topography of simian virus 40 A protein-DNA complexes: arrangement of pentanucleotide interaction sites at the origin of replication. J. Virol. **46**:143–150.
4. **Fradin, A., J. L. Manley, and C. L. Prives.** 1982. Methylation of simian virus 40 Hpa II site affects late, but not early, viral gene expression. Proc. Natl. Acad. Sci. USA **79**:5142–5146.
5. **Fratini, A. V., M. L. Kopka, H. R. Drew, and R. E. Dickerson.** 1982. Reversible bending and helix geometry in a B-DNA dodecamer: CGCGAATT^{Br}CGCG. J. Biol. Chem. **257**:14686–14707.
6. **Graessmann, M., A. Graessmann, H. Wagner, E. Werner, and D. Simon.** 1983. Complete DNA methylation does not prevent polyoma and simian virus 40 early gene expression. Proc. Natl. Acad. Sci. USA **80**:6470–6474.
7. **Graessmann, M., C. Zeichmann, and A. Graessmann.** 1984. Methylation of the SV40 HpaII site does not affect late viral gene expression in microinjected tissue culture cells. FEBS Lett. **173**:151–154.
8. **Jones, K. A., and R. Tjian.** 1984. Essential contact residues within SV40 large T antigen binding sites I and II identified by alkylation interference. Cell **36**:155–162.
9. **Leng, M.** 1985. Left-handed Z-DNA. Biochim. Biophys. Acta **825**:339–344.
10. **McKay, R., and D. DiMaio.** 1981. Binding of an SV40 T antigen-related protein to the DNA of SV40 regulatory mutants. Nature (London) **289**:810–813.
11. **Rich, A., A. Nordheim, and A. H.-J. Wang.** 1984. The chemistry and biology of left-handed Z-DNA. Annu. Rev. Biochem. **53**:791–846.
12. **Ryder, K., S. Silver, A. L. DeLucia, E. Fanning, and P. Tegtmeyer.** 1986. An altered DNA conformation in origin region I is a determinant for the binding of SV40 large T antigen. Cell **44**:719–725.
13. **Ryder, K., E. Vakalopoulou, R. Mertz, I. Mastrangelo, P. Hough, P. Tegtmeyer, and E. Fanning.** 1985. Seventeen base pairs of region I encode a novel tripartite binding signal for SV40 T antigen. Cell **42**:539–548.
14. **Tegtmeyer, P., and B. Andersen.** 1981. Partial purification of

SV40 A protein and a related cellular protein from permissive cells. Virology **115**:67–74.
15. **Tegtmeyer, P., B. Andersen, S. B. Shaw, and V. G. Wilson.** 1981. Alternative interactions of the SV40 A protein with DNA. Virology **115**:75–87.
16. **Tegtmeyer, P., B. A. Lewton, A. L. DeLucia, V. G. Wilson, and K. Ryder.** 1983. Topography of simian virus 40 A protein-DNA complexes: arrangement of protein bound to the origin of replication. J. Virol. **46**:151–161.
17. **Tjian, R.** 1978. The binding site on SV40 DNA for a T antigen-related protein. Cell **13**:165–179.
18. **Tjian, R.** 1979. Protein-DNA interactions at the origin of simian virus 40 DNA replication. Cold Spring Harbor Symp. Quant. Biol. **3**:655–662.
19. **von Hippple, P. H., and D. G. Bear.** 1983. Location and recognition of specific DNA binding sites by proteins that regulate gene expression, p. 291–316. *In* H. Sund and C. Veeger (ed.), Mobility and recognition in cell biology. Walter de Gruyter & Co., Berlin.
20. **Wang, R. Y.-H., X.-Y. Zhang, and M. Ehrlich.** 1986. A human DNA-binding protein is methylation-specific and sequence-specific. Nucleic Acids Res. **14**:1599–1614.
21. **Zhang, X.-Y., K. C. Ehrlich, R. Y.-H. Wang, and M. Ehrlich.** 1986. Effect of site-specific DNA methylation and mutagenesis on recognition by methylated DNA binding protein from human placenta. Nucleic Acids Res. **14**:8387–8397.