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Methylation of the enhancer region of avian sarcoma virus long terminal repeat suppresses transcription

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The effect of methylation of an enhancer on transcription was studied. A 245 bp enhancer-containing a fragment of the LTR of the avian sarcoma virus was methylated in vitro and ligated back into a vector which lacked the upstream enhancer sequence. The transient expression in QT6 cells indicated that methylation of the enhancer-containing sequence severely reduced the extent of transcription.

Methylation; Enhancer; Long terminal repeat; Transfection; Transcription

1. INTRODUCTION

In many eukaryotic systems, it has been demonstrated that methylation of a gene markedly reduces its ability to support transcription [1–4]. It appears that methylation of the main body of the gene does not inhibit transcription but methylation of the 5′-upstream regulatory domains can severely curtail the extent of transcription [5–11]. At least in some cases, the inactivated genes can be reactivated by inhibitors of DNA methylation, suggesting that methylation may play a critical role in gene silencing [12–14].

Several groups recently demonstrated that in viral systems, in vitro methylation of 5′-regulatory sequences results in marked reduction in the levels of RNA synthesis. Recently, several groups have presented evidence for the presence of an enhancer domain in the sequence between nucleotides −208 and −119 of the U3 region of RSV [15–19]. By using restriction enzymes which are methyl-sensitive we provided evidence that methylation of the *PvuI* site (−113) in the LTR of the provirus in nonpermissive cells inhibits viral RNA synthesis [7]. Here we present evidence which indicates that in vitro methylation of the sequence between −299 and −54 of the U3 region of LTR markedly reduces the level of transcription of neomycin-specific RNA that is promoted by the ASV LTR.

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2. MATERIALS AND METHODS

2.1. *Cells*

QT6 cells were grown in medium 199 as described [20].

2.2. *Construction of vectors*

pATV-6D3A, containing a neomycin (*neo*) gene from transposon 5 (Tn5) which is under the control of the avian sarcoma virus LTR, has been described [21]. pATV-6D3AΔ250 and pATV-6D3AΔ250ΔRI were constructed in the following way.

DNA from pATV-6D3A was digested with *Eco*RI and the resulting fragments were ligated and used to transform *E. coli* HB101. Transfor-

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mants were selected on *amp* and *neo* plates and DNA was prepared from isolated colonies and characterized. Clones in which the 250 bp *Eco*RI fragment was deleted were identified and from one (pATV-6D3AΔ250) (fig.1), supercoiled DNA was prepared by CsCl-ethidium bromide gradient centrifugation. The DNA was linearized with *Eco*RI by partial digestion, the ends were filled in with Klenow fragment of DNA polymerase I, ligated and used to transform *E. coli* HB101. DNA was isolated from several transformants and characterized with restriction enzymes. One of them, in which the *Eco*RI site in the downstream LTR is removed (pATV-6D3AΔ250ΔRI) (fig.1), was used in all subsequent experiments.

2.3. *Methylation of the U3 region of LTR*

The 250 bp *Eco*RI fragment containing sequences −299 to −54 was isolated from pATV-6D3A by gel elution. About 1 μg of this fragment was methylated in vitro with rat liver DNA methylase essentially as in [22]. Briefly, the 250 bp *Eco*RI fragment (1 μg) was methylated for 40 h at 30°C in a total volume of 500 μl containing 20 μM Tris, pH 7.8, 40 mM KCl, 5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 0.05% Triton X-100, 200 μg/ml *S*-adenosyl-L-methionine (SAM) and 1000 U DNA methylase from rat liver. To determine the level of methylation, 15 μl of the above mixture were incubated with 5 μM [^{3}H]SAM instead of unlabeled SAM. After deproteinization with pronase and phenol, the methylated DNA was concentrated 10-fold with butanol and dialysed twice. A control DNA was treated in the same way except that SAM was omitted from the incubation. Incorporation of radioactivity indicated that at least 70% of the available CpG doublets were methylated.

2.4. *Ligation*

pATV-6D3AΔ250ΔRI was linearized by *Eco*RI, deproteinized by phenol and precipitated with ethanol at −20°C. The DNA was collected and about 10 μg linearized DNA was mixed with 1 μg methylated or unmethylated 250 bp fragment and ligated with 50 U T_4 ligase in 50 μl at 15°C. Linearized pATV-6D3AΔ250ΔRI was also ligated under identical conditions. Following ligation, the DNA was phenol extracted and precipitated with ethanol at −20°C.

2.5. *Transfection*

Ligated DNAs were resuspended in transfection buffer (Hepes-buffered saline) and used to transfect QT6 cells. Transfections were as described in [16]. Briefly, 1 day before transfection, the cells were seeded at about 2×10^6 cells per 100 mm plate and the following day the medium was replaced with 5 ml fresh DME medium containing 10% fetal bovine serum. 2 h later, 5 μg/plate of the test DNA along with 15 μg calf thymus DNA in calcium phosphate [23] was mixed with the medium. In some experiments, about 200000 cpm ^{3}H-labeled pPL-21 plasmid DNA was mixed with test DNA. 5–5.5 h later, the cells were subjected to glycerol treatment followed by feeding with fresh DME or medium 199 containing 10% fetal bovine serum. The cells were incubated for an additional 36–40 h, harvested and RNA was isolated by the CsCl method [24,25].

2.6. *Quantitation of* neo*-specific RNA by dot-blot analysis*

RNA samples were serially diluted and spotted onto a nylon or cellulose nitrate paper using a dot-blot apparatus. The filters were baked in vacuo and hybridized with ^{32}P-labeled 0.7 kb *neo*-specific probe [16] or 18 S rRNA (pXC-1) probe. The probe from the neo filter was removed and rehybridized with a ^{32}P-labeled actin probe.

3. RESULTS

The effect of methylation of avian sarcoma virus LTR on transcription was studied. The methylated and unmethylated 250 bp fragments were ligated separately with pATV-6D3AΔ250ΔRI DNA that had been linearized by *Eco*RI. Gel analysis of an aliquot from each sample indicated that all of the 250 bp fragment was ligated and that methylation of DNA appeared to have no effect on ligation as identical results were obtained with methylated and unmethylated DNA (not shown).

The ligated DNAs were introduced into QT6 cells by the calcium phosphate technique as in [16]. In some experiments a radiolabeled plasmid marker DNA was mixed with test DNA, and the amount of ^{3}H radioactivity in each DNA sample was determined. These results indicated that in all transfections the uptake of plasmid DNA was approximately the same. About 1.0–1.7% of the

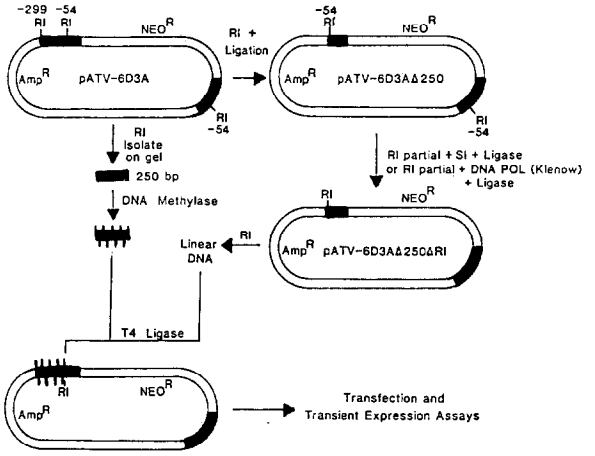

Fig.1. Scheme illustrating vectors and the fragments used. Construction of the vectors, methylation of the 250 bp *Eco*RI fragment, digestion and transfection are as described in sections 2 and 3.

plasmid is taken up by the cells (fig.2, lower right). Serial dilutions ranging from 13.5 to 0.6 μg RNA were spotted onto a cellulose nitrate paper, baked and hybridized with *neo*-specific DNA probe. The extent of hybridization, as determined by the intensity of each spot in the autoradiogram (fig.2, NEO), indicated at least 6–10-fold reduction upon methylation suggesting that methylation of the 250 bp enhancer sequence affects the levels of transcription. Unmethylated 250 bp fragment which is ligated to pATV-6D3Δ250ΔRI (fig.2,NEO) showed only moderately decreased levels compared with the unmethylated pATV-6D3A indicating that ligation per se has not markedly affected the expression levels. This result was expected because enhancers act in an orientation-independent way [26] and therefore ligation of the 250 bp fragment in either orientation should enhance transcription [15]. As expected, deletion of the 250 bp fragment drastically reduced transcription (fig.2) confirming previous results [16] that the 250 bp fragment contains the enhancer element. Direct quantitation of the radioactivity in each spot indicated that methylation of the enhancer reduced the extent of transcription by more than 80%. Digestions of the RNA samples with pancreatic deoxyribonuclease before hybridization did not alter these results indicating that the RNA was not contaminated by plasmid DNA.

In order to show that suppression of transcription of neomycin RNA was due to methylation and not to some other artefact of transfection or hybridization, the probe was removed from the same filter and rehybridized with actin probe. There was no difference in the amount of actin-specific RNA between the methylated and unmethylated samples (fig.2, actin), strongly suggesting that methylation affected transcription of only the LTR-directed neomycin sequences. Additional experiments with ribosomal DNA probe supported these results (fig.2, rDNA). From these data we conclude that methylation of the enhancer region alone is sufficient to reduce transcription.

4. DISCUSSION

The present results here provide strong evidence for a loss of transcriptional activity upon methylation of the enhancer region. The vector used contains a *neo* gene flanked by LTRs. We have observed, like others, that the enhancer sequences are localized in the U3 region at -119 to -299 [15–19]. In these experiments, the 3′-end LTR is not modified. The TATA box in the 5′-end LTR is also intact. Since only the 245 bp *Eco*RI fragment of the upstream LTR is methylated and since only the sequences between -119 and -208 are important for enhancer activity, we conclude that methylation of the enhancer markedly reduces transcription promoted by the upstream LTR. Another noteworthy feature of this study is that the unmethylated LTR that is 3′ to the *neo* gene cannot overcome the inhibitory effect of the methylated 5′ LTR enhancer, suggesting that the enhancer and promoter sequences immediately upstream of the *neo* gene are important.

By examining a large number of genes, it has been concluded that gene expression is inversely correlated with methylation of the upstream regulatory sequences [1,2,4]. The same appears to be valid for other retroviral genes. For example, methylation of the LTRs of Moloney sarcoma virus inhibits the transforming ability of the *src* gene [27]. In a similar study, it has been shown that methylation of M-MuLV genes by the mammalian DNA methylase but not by the bacterial *Hpa*II DNA methylase inhibits gene expression, suggesting that methylation of specific regions of the gene is important in gene regulation [6]. The over-

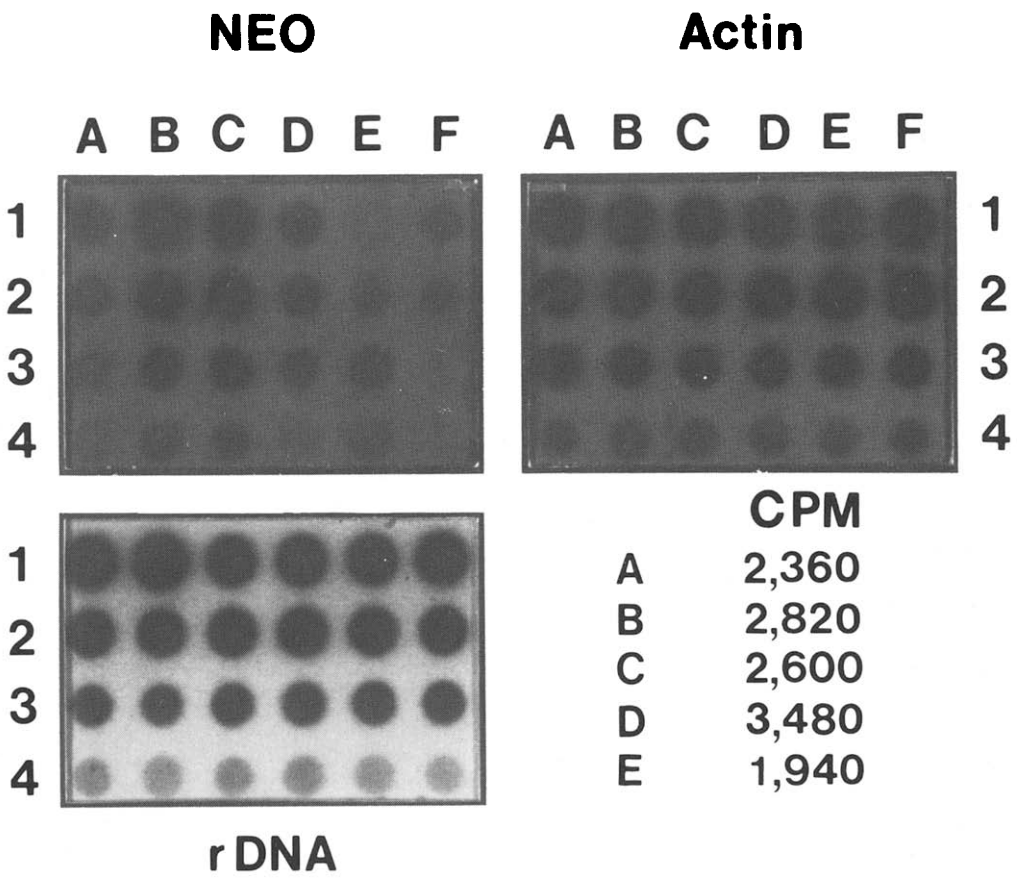

Fig.2. Analysis of RNA for *neo*-specific transcripts: Total cellular RNA was isolated by the CsCl method [24,25]. Serial 3-fold dilutions (1–4) ranging from 13.5 to 0.6 μg (NEO and Actin panels) or 3–0.1 μg (rDNA) were applied on a nylon membrane and the blots were hybridized as described [16] with a neomycin-specific DNA probe. The filters were exposed to X-ray film for 72 h for *neo*, 54 h for actin and 8 h for rDNA. (A) DNA from pATV-6D3AΔ250ΔRI; (B) pATV-6D3A DNA; (C) unmethylated 250 bp *Eco*RI fragment ligated to pATV-6D3AΔ250ΔRI; (D) methylated 250 bp *Eco*RI fragment ligated to pATV-6D3AΔ250ΔRI; (E) mock transfection with calf thymus DNA; (F) normal QT6 cellular RNA. (Lower right panel) The amount of ^{3}H-labeled plasmid DNA taken up by the cells (given as cpm) in each of the transfection experiments.

whelming evidence suggests that tissue-specific factors control gene expression by binding to the upstream regulatory sequences [26]. An elegant study by Cedar and co-workers [11] indicated that in vitro methylation of the α-actin gene resulted in reduced levels of expression in fibroblasts but normal levels in myoblasts. In the latter, normal expression appears to result in demethylation of the α-actin gene. These results suggest that cell-specific factors are important in recognizing the methylated or unmethylated DNAs.

We suggest that methylation of the enhancer sequences precludes binding of the positively acting cellular regulatory factors which results in decreased levels of transcription. In vitro experiments with purified transcription factors will directly address these issues.

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REFERENCES

[1] Doerfler, W. (1983) Annu. Rev. Biochem. 52, 93–124.
[2] Cedar, H. (1984) in: DNA Methylation (Razin, A. et al. eds) pp.147–164, Springer, New York.
[3] Jaenisch, R. and Jahner, D. (1984) Biochim. Biophys. Acta 782, 1–9.
[4] Bird, A. (1986) Nature 321, 209–213.
[5] Kruczek, I. and Doerfler, W. (1983) Proc. Natl. Acad. Sci. USA 80, 7586–7590.
[6] Simon, D., Stuhlmann, H., Jahner, D., Wagner, H., Werner, E. and Jaenisch, R. (1983) Nature 304, 275–277.
[7] Katz, R.A., Mitsialis, S.A. and Guntaka, R.V. (1983) J. Gen. Virol. 64, 429–435.
[8] Yen, P.H., Patel, P., Chinnault, A.C., Mohandas, T. and Shapiro, L.J. (1984) Proc. Natl. Acad. Sci. USA 81, 1759–1763.
[9] Toniolo, D., D'Urso, M., Martini, G., Persico, M., Tufano, V., Battistuzzi, G. and Luzzatto, L. (1984) EMBO J. 3, 1987–1995.
[10] Keshet, I., Yisraeli, J. and Cedar, H. (1985) Proc. Natl. Acad. Sci. USA 82, 2560–2564.
[11] Yistraeli, J., Adelstein, R.S., Melloul, D., Nudel, U., Yaffe, D. and Cedar, H. (1986) Cell 46, 409–416.
[12] Groudine, M., Eisenman, R. and Weintraub, H. (1981) Nature 292, 311–317.
[13] Wolf, S.F., Jolly, D.J., Lunmen, K.D., Friedman, T. and Migeon, B.R. (1984) Proc. Natl. Acad. Sci. USA 81, 2806–2810.
[14] Jones, P. (1984) in: DNA Methylation (Riggs, A. et al. eds) pp.165–187, Springer, New York.
[15] Luciw, P., Bishop, J.M., Varmus, H.E. and Capecchi, M.R. (1983) Cell 33, 705–716.
[16] Mitsialis, S.A., Caplan, S. and Guntaka, R.V. (1983) Mol. Cell. Biol. 3, 1975–1984.
[17] Laimins, L.A., Tsichlis, P. and Khoury, G. (1984) Nucleic Acids Res. 12, 6427–6442.
[18] Cullen, B.R., Raymond, K. and Ju, G. (1985) Mol. Cell. Biol. 5, 438–447.
[19] Weber, F. and Schaffner, W. (1985) EMBO J. 4, 949–956.
[20] Guntaka, R.V. and Weiner, A.J. (1978) Nature 274, 274–276.
[21] Mitsialis, S.A., Young, J.F., Palese, P. and Guntaka, R.V. (1981) Gene 16, 217–225.
[22] Graessmann, M., Graessman, A., Wagner, H., Wagner, E. and Simon, D. (1983) Proc. Natl. Acad. Sci. USA 80, 6470–6474.
[23] Graham, F.L. and Van der Eb, A.J. (1973) Virology 52, 456–467.
[24] Guntaka, R.V. and Weiner, A.J. (1978) Nature 274, 274–276.
[25] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[26] Gluzman, Y. (1985) Eukaryotic Transcription, Cold Spring Harbor Press, Cold Spring Harbor, NY.
[27] McGeady, M.L., Jhappan, C., Ascione, R. and Vande Woude, G.F. (1983) Mol. Cell. Biol. 3, 305–314.