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Suppression of the Hypomethylated Moloney Leukemia Virus Genome in Undifferentiated Teratocarcinoma Cells and Inefficiency of Transformation by a Bacterial Gene under Control of the Long Terminal Repeat

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The Moloney leukemia virus (M-MuLV) genome was introduced into undifferentiated teratocarcinoma cells by transfection of a plasmid with the virus genome linked to pSV2neo, which carries a bacterial drug resistance gene, *neo*, or by cotransfection with pSV2neo. In the resulting cells, the M-MuLV genome remained hypomethylated, but its expression was suppressed in cells in an undifferentiated state. The pattern of DNA methylation of the viral genome remained unchanged when the cells were induced to differentiate into epithelial tissues. However, spontaneous M-MuLV expression was detected with differentiation of the cells. To determine to what extent the viral long terminal repeat (LTR) was responsible for this suppression in undifferentiated cells, I constructed plasmids in which *neo* was placed under the control of the promoter sequence of the dihydrofolate reductase gene or the M-MuLV LTR, and compared the biological activities of the plasmids in Ltk$^-$ cells and in undifferentiated teratocarcinoma cells. In Ltk$^-$ cells, these plasmids were highly efficient in making the cells resistant to selection by G418. However, in undifferentiated teratocarcinoma cells, the M-MuLV LTR promoted *neo* gene expression at only 10% of the expected efficiency, as compared with the expression of the *neo* gene under the control of the simian virus to or dihydrofolate reductase promoter. Thus, the mechanisms of gene regulation are not the same in undifferentiated and differentiated teratocarcinoma cells.

DNA methylation plays an important role in gene regulation in endogenous type C viruses. Endogenous avian and murine leukemia viruses can be activated by the treatment of cells with 5-azacytidine, which strongly inhibits DNA methylation (7, 10, 16). It has also been shown that the transcriptionally inactive Moloney leukemia virus (M-MuLV) genome acquired through germ line integration of mice or by exogenous infection of rat cells is heavily methylated (10, 25). Hypomethylated M-MuLV provirus molecularly cloned from BALB/cMo mice becomes biologically active upon transfection of fibroblast cells (8). Also, methylation of the molecularly cloned M-MuLV genome by mammalian methylase inactivates its biological activity in transfection of mouse fibroblast cells (19). Thus, the murine retrovirus genome seems to be repressed by DNA methylation in these cells.

Infection of undifferentiated murine teratocarcinoma cells with M-MuLV suppresses the viral genome (4, 22). The M-MuLV genome infecting nullipotent F9 cells becomes methylated immediately after integration into host cellular DNA, and this is thought to cause the suppression (24). However, from the study of M-MuLV infection of a pluripotent cell line derived from PCC4 cells, it was shown that the freshly integrated M-MuLV genome is devoid of detectable DNA methylation but that the expression is suppressed (5, 17). Therefore, it was proposed that DNA methylation may not play a role in gene regulation in undifferentiated cells and that the repression of the M-MuLV genome in undifferentiated cells may involve discrimination against transcription initiation from the promoter sequence of the virus. Recently, it was reported that the expression of a bacterial drug resistance gene, CAT, under the control of the M-MuLV long terminal repeat (LTR), is blocked in undifferentiated teratocarcinoma cells (13). This observation is consistent with the hypothesis presented above. It was also proposed that the hypomethylated state is a requirement for gene expression in differentiated cells, whereas it is irrelevant for gene regulation in undifferentiated cells (17). In this communication, evidence for this hypothesis is provided.

MATERIALS AND METHODS

Cell cultures. EC-A1 cells, a subline of PCC4 Aza1 cells, were undifferentiated pluripotent teratocarcinoma stem cells (5). SC-1 cells were a mouse embryo fibroblast line derived from a feral mouse (9). Ltk$^-$ cells, thymidine kinase-deficient derivatives of L cells, were kindly provided by K. Yamanishi (Osaka University). Culturing of all cells was carried out in MEM alpha medium (GIBCO Laboratories) supplemented with 10% heat-inactivated calf serum.

Differentiated EC-A1 cells were obtained by treating the culture with medium containing 0.1 μM retinoic acid (Sigma Chemical Co.) for 1 week. After completion of the treatment, cultures consisted entirely of cells with an epithelial morphology.

Transfection and biological assays. EC-A1 cells and Ltk$^-$ cells were transfected by the procedure of Graham and Van der Eb (6). Briefly, 24 h after seeding of 5×10^5 cells on 6-cm plates, the preparations were transfected with 1 μg of plasmid DNA together with 10 μg of carrier calf thymus DNA. After 24 h of incubation, the cells were trypsinized and seeded onto 10-cm plates in medium containing 400 μg of G418 (GIBCO) per ml. Colonies were scored ca. 12 days

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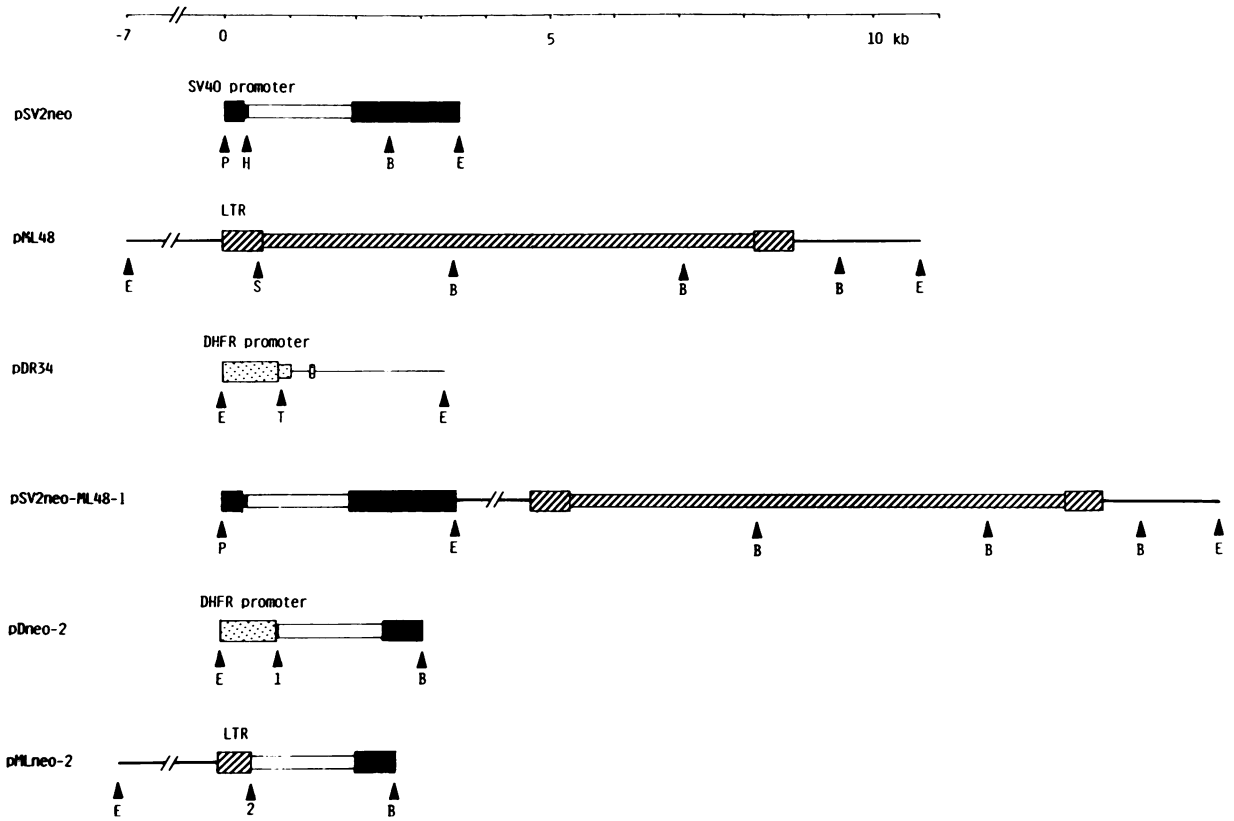

FIG. 1. Plasmids used. The black boxes represent sequences derived from SV40. The open boxes represent the fragment derived from Tn*5*, which carries the coding sequence of the *neo* gene. The shaded boxes represent the M-MuLV genome, and the lines on both sides of pML48 are cellular flanking sequences. The dotted boxes represent the tentative promoter sequence and the coding regions of the DHFR gene. The thin lines of pDR34 are the first and second introns. The blunt end of the *Taq*I site of pDR34 was ligated to the blunt end of the *Hin*dIII site of pSV2neo. The junction is indicated as site 1 in pDneo-2. The *Sma*I site of the 5′ LTR of pML48 was changed to the *Hin*dIII site and ligated to the *Hin*dIII site of pSV2neo. The junction is indicated as site 2 in pMLneo-2. Restriction enzyme cleavage sites are indicated as follows: B, *Bam*HI, E, *Eco*RI; H, *Hin*dIII; P, *Pvu*II; S, *Sma*I; and T, *Taq*I.

after transfection. If necessary, the colonies were ring-cloned and propagated for further analysis.

The expression of virus from EC-A1 cells was assayed by cocultivation with SC-1 cells and the subsequent reverse XC test of SC-1 cells (15). Cells to be tested were first treated with 25 μg of mitomycin C per ml for 30 min at 37°C. After trypsinization, 10^5 test cells were cocultivated with 2×10^5 SC-1 cells for 3 days. SC-1 cells were then trypsinized and counted, and 10^5 cells were mixed with 5×10^5 XC cells. Plaques appearing in 2 days were scored.

Extraction of cellular RNA and DNA. Total cellular RNA was isolated by sedimentation through cesium chloride (2). High-molecular-weight DNAs were isolated from cultured cells as described previously (14).

Plasmid DNAs were isolated as described previously (14). pSV2neo was kindly provided by P. J. Southern. pML48 was obtained from H. Fan through J. W. Gautsch. pDR34 was a kind gift from R. T. Schimke. p8.2 was provided by R. A. Weinberg and was the recombinant plasmid carrying the unintegrated 8.2-kilobase (kb) M-MuLV genome at the *Hin*dIII site. Other plasmids were constructed as described below.

Hybridization procedure. High-molecular-weight DNAs (5 μg) were digested with a three- to fourfold excess of restriction endonucleases, size separated through electrophoresis on a 0.7% agarose gel, and transferred onto a nitrocellulose sheet as described previously (20). Dot blot hybridization of total cellular RNA was carried out as described previously (27). Plasmid DNAs were labeled with ^{32}P by the nick translation procedure (14). Specific activities of the probes were 1×10^8 to 4×10^8 cpm/μg.

RESULTS

Construction of plasmids. The plasmids used are shown in Fig. 1. pSV2neo is a plasmid carrying *neo*, the bacterial drug resistance gene, and transcription of this gene is promoted by the simian virus 40 (SV40) early gene promoter (21). pML48 carries an 18-kb insert at the *Eco*RI site, and this insert consists of the integrated full-length M-MuLV genome and flanking cellular sequences (1). pDR34 carries part of the dihydrofolate reductase (DHFR) gene and includes the 5′ promoter sequence, the first exon, the first intron, the second exon, and part of the second intron (3). pSV2neo-ML48-1 was constructed so that the 18-kb fragment of pML48, including the M-MuLV genome and the flanking cellular sequences, was inserted into the *Eco*RI site of plasmid pSV2neo in the same transcriptional orientation. The DHFR gene has a *Taq*I site 6 base pairs downstream of the initiation codon, and this site was cut and made to form a blunt end. The sequence that spanned from the *Hin*dIII site to the *Bam*HI site of pSV2neo and contained the entire *neo* gene and part of the SV40 sequence, including the polyadenylic acid attachment signal, was isolated. The *Hin*dIII site of this fragment was made to form a blunt end and was then

ligated to the blunt-end *Taq*I site, downstream of the DHFR promoter sequence. In the resulting construction, pDneo-2, the *neo* gene was efficiently transcribed from the DHFR promoter upon introduction into mouse cells. pMLneo-2 was constructed so that the *Sma*I site of the M-MuLV 5′ LTR from pML48 was converted to the *Hin*dIII site by linker insertion, and part of the pSV2neo sequence spanning from the *Hin*dIII site to the *Bam*HI site was ligated downstream of the LTR. With this construction, the M-MuLV LTR efficiently promoted transcription of the *neo* gene.

Introduction of the hypomethylated M-MuLV genome into undifferentiated EC-A1 cells by transfection with pSV2neo-ML48-1. The M-MuLV genome was introduced into undifferentiated EC-A1 cells or into SC-1 cells by transfection with pSV2neo-ML48-1. G418-resistant colonies were isolated and assayed for virus production. Five G418-resistant SC-1 clones were isolated and designated SC-1(NM)p1 to SC-1(NM)p5. All the clones produced M-MuLV, indicating that the M-MuLV in this construction was biologically active (data not shown). Five G418-resistant EC-A1 clones, designated EC-A1(NM)p1 to EC-A1(NM)p5, were randomly isolated and tested for M-MuLV expression. They were found to be virus negative, as confirmed by cocultivation with SC-1 cells and the subsequent reverse XC cell assay of SC-1 cells (data not shown). Total cellular RNA was isolated from EC-A1(NM)p clones and analyzed by dot blot hybridization. None of the undifferentiated EC-A1(NM)p clones expressed RNA hybridizable to M-MuLV (Fig. 2, spots b to f). Therefore, it seems that the lack of virus production is due to the block of transcription of the viral genome. If this were so, it is interesting to note that I could not detect biological activity of M-MuLV in undifferentiated EC-A1(NM)p cells even though the SV40 promoter nearby actively promoted transcription of the *neo* gene. Strong hybridization to M-MuLV was noted for RNA isolated from SC-1(NM)p1 cells (Fig. 2, spot a).

As a probe to detect M-MuLV, the 329-base *Sma*I fragment of M-MuLV was isolated from p8.2, and this fragment hybridized specifically to M-MuLV. The 2.2-kb *Hin*dIII-*Bam*HI fragment from pSV2neo was used to detect the *neo* gene, and the whole pBR322 plasmid was used as a probe to detect the pBR322-related sequence. DNA was isolated from one of the clones, EC-A1(NM)p5, and analyzed by Southern blotting (20). When the M-MuLV-specific probe was applied, the *Bam*HI-digested DNA revealed the 3.0-kb internal fragment of M-MuLV (Fig. 3, lane a). The 6.8-kb band was

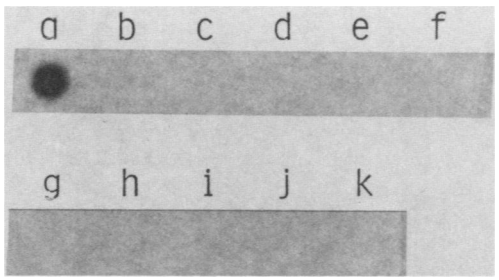

FIG. 2. Dot blot analysis of RNA from EC-A1 clones carrying the hypomethylated M-MuLV genome. Total cellular RNA (5 μg) was spotted onto a nitrocellulose sheet. The 8.2-kb M-MuLV fragment was used as a probe. The spots represent RNA from SC-1(NM)p1 cells (spot a), EC-A1(NM)p1 to EC-A1(NM)p5 cells (spots b to f), respectively, and EC-A1(NM)c1 to EC-A1(NM)c5 cells (spots g to k, respectively).

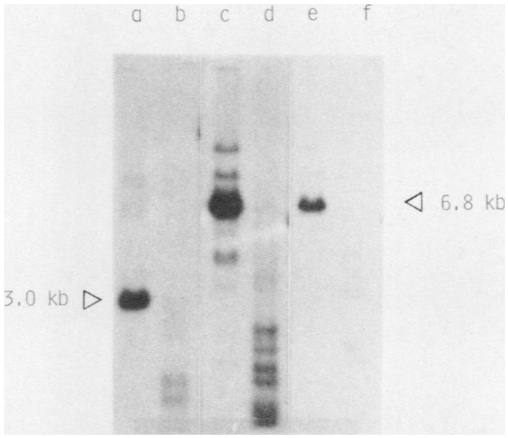

FIG. 3. *Hpa*II digestion pattern of transfected sequences in EC-A1(NM)p5 cells. DNA was isolated from undifferentiated EC-A1(NM)p5 cells. DNA (5 μg) was digested with *Bam*HI (lanes a, c, and e) or *Bam*HI plus *Hpa*II (lanes b, d, and f). Probes used were the M-MuLV-specific 329-base *Sma*I fragment (lanes a and b), the 2.2-kb *Hin*dIII-*Bam*HI fragment from pSV2neo (lanes c and d), and pBR322 (lanes e and f).

detected by two probes, the 2.2-kb *Hin*dIII-*Bam*HI fragment from pSV2neo and pBR322 (Fig. 3, lanes c and e). This 6.8-kb fragment corresponds to a region of pSV2neo-ML48-1 from the *Bam*HI site near the polyadenylic acid signal of the SV40 sequence, via the coding region of the *neo* gene and a portion of pBR322, to the *Bam*HI site of the cellular flanking sequence 3′ to the M-MuLV genome of the pML48 insert. The M-MuLV genome contained 33 *Hpa*II sites, 15 of which were located within the 3.0-kb *Bam*HI fragment. The pSV2neo sequence within the 6.8-kb fragment contained 25 *Hpa*II sites. The bands for M-MuLV, *neo*, and pBR322 disappeared upon digestion with *Hpa*II, indicating that these sequences were hypomethylated at *Hpa*II sites (Fig. 3, lanes b, d, and f).

The M-MuLV genome remained hypomethylated in undifferentiated EC-A1(NM)p5 cells for at least 6 months in cultures. This is in contrast to the M-MuLV genome introduced via infection with virions into undifferentiated EC-A1 cells, in which the transcriptionally inactive viral genome was methylated. DNA from one of the virion-infected clones, EC-A1(M)28, was analyzed (Fig. 4). As is clear, the 3.0-kb *Bam*HI fragment was heavily methylated. One explanation for the lack of methylation of the M-MuLV genome in EC-A1(NM)p5 cells is that active transcription of the *neo* gene nearby may interfere with methylation of the transcriptionally inactive M-MuLV genome. During the construction of pSV2neo-ML48-1, the *neo* gene and the M-MuLV genome are separated by the 7-kb cellular flanking sequence at one end and the 4-kb fragment of the cellular flanking sequence plus part of pBR322 at the other end.

Introduction of the hypomethylated M-MuLV genome into undifferentiated EC-A1 cells by cotransfection. Cotransfection of mammalian cells was shown to result in nonspecific ligation of foreign DNA fragments (29). Attempts were made to introduce the M-MuLV genome into undifferentiated EC-A1 cells by cotransfection with pML48 and pSV2neo, together with an excess amount of carrier calf thymus DNA. With this method one can expect to obtain EC-A1 cells carrying the M-MuLV genome separated from the *neo* gene by fragments of carrier DNA. Nineteen G418-resistant undifferentiated EC-A1 clones were isolated from the cotrans-

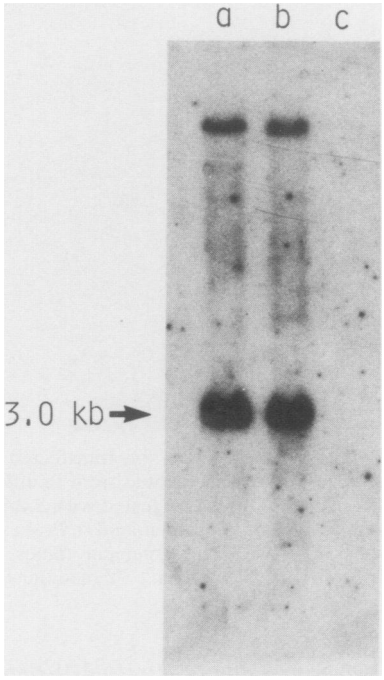

FIG. 4. *Hpa*II digestion pattern of the M-MuLV genome in virion-infected EC-A1(M)28 cells. DNA was isolated from undifferentiated EC-A1(M)28 cells, which were cloned from EC-A1 cells infected with M-MuLV. DNA (5 μg) was digested with *Bam*HI (lane a), *Bam*HI plus *Hpa*II (lane b), or *Bam*HI plus *Msp*I (lane c).

fected culture, and their DNAs were analyzed for the presence of the M-MuLV genome. Five clones were found to be positive for the M-MuLV genome, and these were designated EC-A1(NM)c clones. As in the case of EC-A1(NM)p clones, none of the EC-A1(NM)c clones expressed M-MuLV-related RNA, as determined by dot blot hybridization (Fig. 2, spots g to f).

DNA from one such clone, EC-A1(NM)c3, was digested with *Bam*HI or doubly digested with *Bam*HI and *Hpa*II. As is clear from Fig. 5, M-MuLV (Fig. 5A), *neo* (Fig. 5B), and pBR322 (Fig. 5C) were again hypomethylated at *Hpa*II sites. The M-MuLV sequence in the EC-A1(NM)c3 cells was also hypomethylated at *Hha*I sites (data not shown).

Lack of change in the methylation pattern of M-MuLV during cell differentiation. DNA was isolated from undifferentiated and differentiated EC-A1(NM)c3 cells and digested with *Bam*HI, *Eco*RI, or *Hin*dIII or with each of these enzymes plus *Hpa*II. When probed with the M-MuLV-specific *Sma*I fragment, *Bam*HI produced a single 3.0-kb band, *Eco*RI produced an 18-kb major band and two other minor bands, and *Hin*dIII produced a 9.7-kb major band and two other minor bands (Fig. 6A). All of these bands were sensitive to digestion with *Hpa*II in undifferentiated cells. Changes in the patterns of the bands were not evident with differentiation of the cells, nor was there any change in the sensitivity to *Hpa*II (Fig. 6B).

Spontaneous expression of the hypomethylated M-MuLV genome after cell differentiation. The M-MuLV genome in undifferentiated EC-A1 cells is suppressed, regardless of the state of DNA methylation (5, 17). On the other hand, in mouse fibroblasts the newly integrated M-MuLV genome hypomethylated at the *Hpa*II sites is transcriptionally active. As shown above, EC-A1(NM)p5 cells and EC-A1(NM)c3 cells carried the M-MuLV genome hypomethylated at the *Hpa*II sites. In contrast, a virion-infected clone, EC-A1(M)8, carried the methylated M-MuLV genome. The expression of M-MuLV in these cells in the differentiated stage was studied.

EC-A1(NM)p5, EC-A1(NM)c3, and EC-A1(M)28 cells were induced to differentiate by treatment with retinoic acid. The differentiated EC-A1(NM)p5, EC-A1(NM)c3, and EC-A1(M)28 cells were passaged, and M-MuLV expression was monitored at each passage by cocultivation with SC-1 cells. Differentiated EC-A1(NM)p5 cells and EC-A1(NM)c3 cells started to produce a low level of M-MuLV spontaneously at passages 2 and 3, respectively (Table 1). As determined from the number of cells used and the number of virus-positive dishes, the spontaneous induction frequency was calculated to be 2×10^{-6} for passage-2 EC-A1(NM)p5 cells and 6×10^{-6} for passage-3 EC-A1(NM)c3 cells. Virus expression was not detected in differentiated EC-A1(M)28 cells up to passage 5 (Table 1).

Expression of the *neo* gene in Ltk$^-$ cells. The data obtained indicate that the expression of the hypomethylated M-MuLV genome in undifferentiated cells is suppressed, probably at the transcriptional level. To estimate the extent of involvement of the LTR in the suppression of transcription of the viral genome in undifferentiated cells, I constructed plasmid

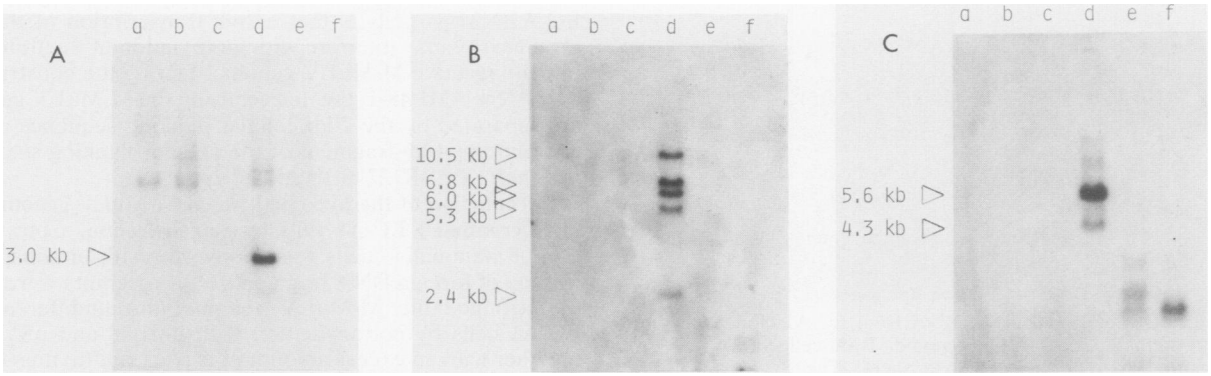

FIG. 5. *Hpa*II digestion pattern of transfected sequences in EC-A1(NM)c3 cells. DNA was isolated from undifferentiated EC-A1 cells (lanes a, b, and c) and from EC-A1(NM)c3 cells (lanes d, e, and f). DNA (5 μg) was digested with *Bam*HI (lanes a and d), *Bam*HI plus *Hpa*II (lanes b and e), or *Bam*HI plus *Msp*I (lanes c and f). Probes used were the M-MuLV-specific fragment (A), the *Hin*dIII-*Bam*HI fragment from pSV2neo (B), and pBR322 (C).

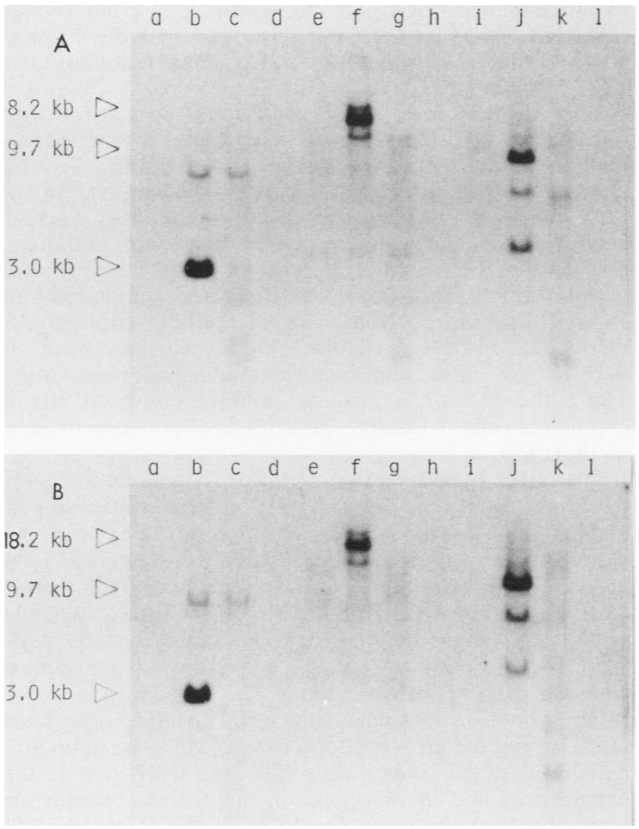

FIG. 6. Restriction enzyme digestion pattern of the M-MuLV genome in undifferentiated and differentiated EC-A1(NM)c3 cells. DNA was isolated from undifferentiated (A) and differentiated (B) EC-A1(NM)c3 cells. DNA from EC-A1 cells was used as a control (lanes a, e, and i). The other lanes represent DNA from EC-A1(NM)c3 cells. The probe used was the M-MuLV-specific *Sma*I fragment. Enzymes used were *Bam*HI (lanes a and b), *Bam*HI plus *Hpa*II (lane c), *Bam*HI plus *Msp*I (lane d), *Eco*RI (lanes e and f), *Eco*RI plus *Hpa*II (lane g), *Eco*RI plus *Msp*I (lane h), *Hin*dIII (lanes i and j), *Hin*dIII plus *Hpa*II (lane k), and *Hin*dIII plus *Msp*I (lane l).

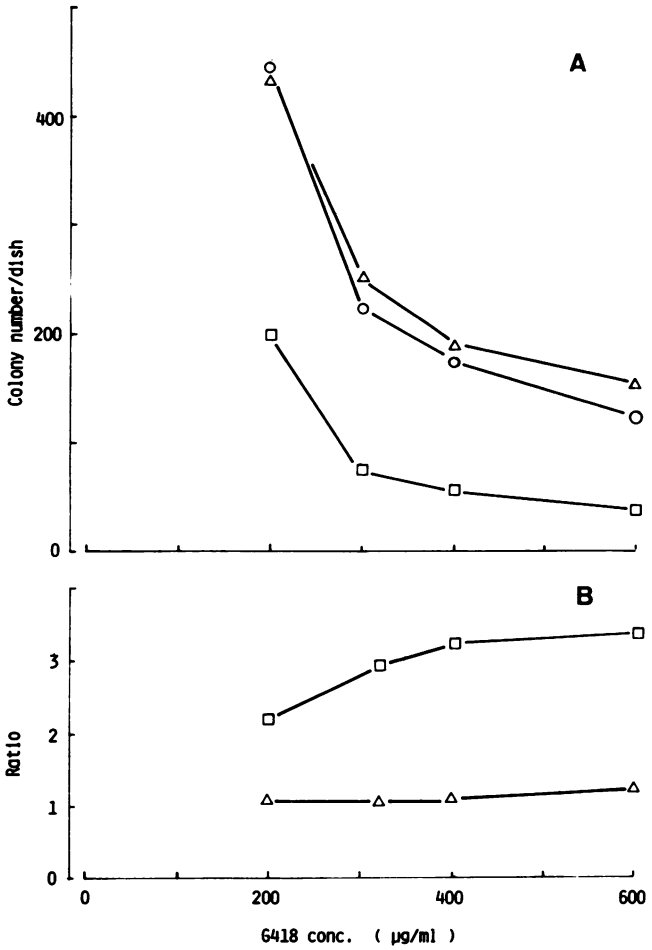

FIG. 7. Strength of the controlling sequences in the three constructions of the *neo* gene. (A) Ltk$^-$ cells were transfected with pSV2neo (○), pDneo-2 (□), or pMLneo-2 (△), and the colonies which developed with various concentrations (conc.) of G418 were scored. (B) Colony-number ratios. Symbols: □, pSV2neo/pDneo-2 colony-number ratio; △, pMLneo-2/pSV2neo, colony-number ratio.

pMLneo-2, in which the *neo* gene is placed under the control of the M-MuLV LTR (Fig. 1). The DHFR gene is a housekeeping gene, the function of which is required in any cell type. The *neo* gene placed under the control of the DHFR promoter might be as active in differentiated as in undifferentiated cells. Therefore, pDneo-2 was used as a standard plasmid, together with pSV2neo. The actual number of colonies obtained in the transfection experiment could be influenced by a variety of factors. However, I considered that the number of colonies obtained reflected the relative strength of the promoter sequence in each construction. An experiment was performed in which these plasmids were transfected in Ltk$^-$ cells, which were then challenged with various concentrations of G418 (Fig. 7). In Ltk$^-$ cells, pMLneo-2 was as active as pSV2neo, and pDneo-2 was the least active of the three. The number of colonies increased with decreasing concentrations of G418 (Fig. 7A). The pSV2neo/pDneo-2 colony-number ratio and the pMLneo-2/pSV2neo colony-number ratio were plotted against concentrations of G418. The pSV2neo/pMLneo-2 colony-number ratio remained close to unity with various concentrations of G418 (Fig. 7B). The pSV2neo/pDneo-2 colony-number ratio was ca. 3 at 600 μg of G418 per ml, and this ratio decreased when the concentration of the drug was decreased (Fig. 7B). By extrapolation of the slope, this ratio decreased to 1 in the absence of the drug. Thus, pDneo-2 transformed Ltk$^-$ cells at a relatively higher efficiency with a lower concentration of the drug. This indicates that at a low concentration of a selective drug, cells expressing small amounts of the *neo* gene transcript from the DHFR promoter are as viable as cells expressing a high copy number of the SV40-promoted

TABLE 1. Virus expression in differentiated EC-A1(NM)p5, EC-A1(NM)c3, and EC-A1(M)28 cells

Cell line[a]	No. of virus-positive dishes/total no. of dishes at passage[b]:				
	1	2	3	4	5
EC-A1(NM)p5	0/5	1/5	5/5	NT[c]	NT
EC-A1(NM)c3	0/5	0/5	2/5	NT	NT
EC-A1(M)28	0/5	0/5	0/5	0/5	0/5

[a] Retinoic acid-induced differentiated cells were used.
[b] Differentiated cells were passaged weekly by seeding 2×10^5 cells per 6-cm dish. At each passage, 10^5 cells were mixed with 2×10^5 SC-1 cells and cultivated for three days. The cells were then assayed by the reverse XC test.
[c] NT, Not tested.

TABLE 2. Comparison of transforming activity of pSV2neo, pDneo-2, and pMLneo-2

Plasmid	No. of G418-resistant colonies/μg of DNA on indicated cells:			EC-A1/Ltk⁻ ratio[a]	Normalized ratio
	Ltk⁻	SC-1	EC-A1		
pSV2neo	321	121	58	0.18	1.0
pDneo-2	122		21	0.17	0.94
pMLneo-2	283	98	6	0.02	0.11

[a] The number of colonies on EC-A1 cells was divided by that on Ltk⁻ cells.
[b] The value obtained for pSV2neo was normalized to unity.

neo gene transcript. Thus, assuming that the average copy numbers of pSV2neo and pDneo are the same in the transfected cells, the number of colonies obtained on Ltk⁻ cells reflects the strength of the promoter sequences.

Activity of the LTR-promoted *neo* gene in undifferentiated cells. Three cell lines were used for the transfection assay of the plasmids. pSV2neo and pMLneo-2 exhibited similar levels of biological activity in Ltk⁻ and SC-1 cells. The lowest number of transformants was obtained for all three plasmids when EC-A1 cells were used as the recipients (Table 2). This may have been due to the inefficiency of EC-A1 cells as hosts for the transfection assay. Nevertheless, pSV2neo yielded the highest number of colonies on EC-A1 cells. pMLneo-2, in contrast, yielded the lowest number of the three. Ratios of the number of transformants on EC-A1 and Ltk⁻ cells were calculated for each plasmid. The ratio obtained for pSV2neo was normalized to unity, whereas the ratios obtained for pDneo-2 and pMLneo-2 were normalized to that for pSV2neo. From the values obtained, it became clear that the SV40 and DHFR promoters functioned efficiently in undifferentiated EC-A1 cells, whereas the LTR functioned at only 10% of the expected efficiency in undifferentiated EC-A1 cells (Table 2).

DISCUSSION

In a previous study on gene regulation of the M-MuLV genome in teratocarcinoma cells, it was proposed that two independent mechanisms are involved in the suppression of the viral genome (17). The first mechanism operates in undifferentiated cells and is independent of the state of DNA methylation of the M-MuLV genome. Repression of M-MuLV in undifferentiated cells was suggested to involve discrimination against LTR-promoted transcription of the genome. The second mechanism operates in differentiated cells and may involve condensation of methylated DNA by a specific DNA-binding protein, making the DNA refractory to transcription by RNA polymerase II.

The present data indicate that the transfectionally introduced M-MuLV genome hypomethylated at *Hpa*II and *Hha*I sites remains inactive in undifferentiated cells. As to why the viral genome in the transfected cells was not methylated, one possibility is that the de novo methylase of undifferentiated teratocarcinoma cells does not methylate an actively transcribed sequence. If so, the M-MuLV and pBR322 sequences that are near the *neo* sequence that should be transcribed actively for cells to survive would not be methylated under selective conditions. Another possibility is that methylation continues to play an important role in the suppression of genes in undifferentiated cells. In this case, only cells in which *neo*, M-MuLV, and pBR322 escaped methylation would be selected. The latter situation is difficult to envisage because sequences with the same hypomethylated state, *neo* and M-MuLV, are differentially regulated. Although *neo* is transcribed actively from the SV40 promoter, M-MuLV nearby is silent in undifferentiated EC-A1(NM) clones.

The suppression of M-MuLV in undifferentiated teratocarcinoma cells seems to be absolute. I screened more than 10^6 undifferentiated cells for each of five EC-A1(NM)p clones and five EC-A1(NM)c clones by cocultivation with SC-1 cells and found no expression of the virus when the cells were in the undifferentiated stage. In addition, I cocultivated EC-A1 and F9 cells for more than 6 months with M-MuLV-producing SC-1 cells. The resulting cell lines, EC-A1(M)m and F9(M)m, carried multiple copies of the methylated M-MuLV genome. These cell lines were also negative for virus expression (unpublished observation). Thus, the M-MuLV genome in undifferentiated teratocarcinoma cells is inactive, regardless of the state of *Hpa*II methylation.

The absolute suppression of M-MuLV can be accounted for by the inefficiency of the LTR in undifferentiated cells. M-MuLV LTR-promoted transcription of the *neo* gene was suppressed by 90% in undifferentiated EC-A1 cells. For the absolute suppression of M-MuLV, sequences other than the LTR may be required. Another possibility is that splicing of viral mRNA may be inefficient in undifferentiated cells. Incomplete splicing was thought to be the cause of suppression of SV40 in teratocarcinoma stem cells (18).

Other investigators have shown the absolute suppression of LTR-promoted transcription of the CAT gene in undifferentiated teratocarcinoma cells (13). The CAT gene in their experiments and the *neo* gene in the present investigation were placed at the same *Sma*I site of the M-MuLV LTR. Nevertheless, suppression of the former was absolute, whereas that of the latter was partial. The discrepancy in the results may be due to the different assays used. CAT gene expression was detected by a short-term biochemical assay without selective pressure on the cells, and *neo* gene expression was assayed by selecting transfected cells for drug resistance.

It was shown that the hypomethylated M-MuLV genome was highly active upon transfection of mouse fibroblasts, whereas the same sequence methylated by mammalian DNA methylase was inactive (19). The M-MuLV genome in EC-A1(NM)p5 cells and EC-A1(NM)c3 cells was hypomethylated in both undifferentiated and differentiated cells. The same hypomethylated M-MuLV sequence became activated in differentiated cells. Therefore, this shows that in differentiated cells, the hypomethylated sequence can be transcribed as long as it has a functional promoter sequence.

The conformation of chromatin, detected by DNase I sensitivity, correlates with the transcriptional activity of the genes (23, 28). The working hypothesis is that the methylated sequence is condensed only in differentiated cells, making the sequence refractory to transcription. A nuclear protein isolated from human tissue specifically binds to methylated DNA (11), and such a protein may be involved in the condensation of chromatin. In undifferentiated cells, this condensation of chromatin does not take place, regardless of the state of methylation of the sequences. Thus, one can expect that the conformation of chromatin regions with methylated sequences changes upon differentiation. It was reported that chromatin in undifferentiated cells is generally sensitive to DNase I digestion (26). The same workers also studied the DNase I sensitivity of transfectionally introduced genes in teratocarcinoma cells and found that these genes are sensitive to the enzyme (12). I tested the DNase I

sensitivity of methylated M-MuLV in EC-A1 cells and found that the viral sequence in undifferentiated cells was sensitive to DNase I digestion but became resistant to the enzyme in differentiated cells (K. Iwasaki and O. Niwa, manuscript in preparation).

All of this evidence indicates that the mechanism of gene regulation in undifferentiated teratocarcinoma cells differs from that in differentiated teratocarcinoma cells.

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