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Inactivation of the Moloney Murine Leukemia Virus Long Terminal Repeat in Murine Fibroblast Cell Lines Is Associated with Methylation and Dependent on Its Chromosomal Position

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The expression of a retroviral vector with the Moloney murine leukemia virus (Mo-MuLV) long terminal repeat (LTR) promoter after integration into the genome of murine fibroblast cell lines was monitored with the *Escherichia coli*-derived β-galactosidase (β-gal) gene as the reporter. Monoclonal cell lines derived after retroviral infection exhibited a marked heterogeneity in their expression of the reporter gene. We studied two monoclonal cell lines with a single unrearranged copy of the vector provirus integrated into their genome. The first, BB10, expressed the marker enzyme in only 8% of its cell population, whereas in the second, BB16, β-gal expression could be detected in over 98% of the cells. Treatment of BB10 with the DNA-demethylating agent 5-azacytidine raised the number of β-gal-positive cells to over 60%. Transfection experiments showed that the Mo-MuLV LTR promoter-enhancer is potentially fully functional in both the BB10 and BB16 cell lines. The inactivated provirus from BB10 cells was cloned and subsequently used to generate retrovirus stocks. The promoter-enhancer activity of its LTR after infection with these BB10-derived viruses showed a variation similar to that of the original virus stocks. Our data showed that (1) inactivation of the Mo-MuLV LTR is a frequent event in murine fibroblast cell lines, (2) inactivation is associated with de novo methylation of cytidine residues, (3) the frequency of inactivation of the provirus must be determined by its chromosomal position, (4) the process of methylation of sequences within the LTR is not necessarily the same as the transcription-repression mechanism that is operating in undifferentiated embryonal carcinoma cells.

Retroviral vectors offer several advantages for gene transfer experiments. First, the highly efficient integration process results in the insertion of a DNA copy of the virus vector with a predictable structure into the host cell genome. Second, they can be used to infect a wide variety of host cells and may carry, apart from the gene of interest, a dominant selectable marker. Third, high-titer helper-free stocks of recombinant retroviruses can be obtained with relative ease. Therefore, retroviral vectors are widely used for experiments aimed at the development of genetic therapies for human genetic disorders (11).

So far, applications have been seriously hampered by the fact that the retroviral regulatory elements are repressed in certain cell types. It has been amply documented (2, 9, 27, 28, 35, 36, 52, 57) that the Moloney murine leukemia virus (Mo-MuLV) long terminal repeat (LTR) is expressed inefficiently in undifferentiated embryonal carcinoma (EC) cell lines and in preimplantation embryos. The restriction of expression occurs at several levels but is caused at least partially at the level of transcription initiation. Within the LTR, the enhancer sequences are targets for repressing factors (10, 14, 20, 26, 52). It has been shown that de novo methylation of CpG doublets is associated with this suppression (6, 12, 31, 50; reviewed in reference 55). Methylated LTRs are inefficient as promoters in murine fibroblast cell lines, whereas unmethylated LTRs are fully functional (19, 46).

An analogous or identical suppression mechanism has been suggested to operate after retrovirus-mediated gene transfer into hematopoietic stem cells (18, 29, 53). As a consequence, vectors that are functional in mature hematopoietic cells often are not expressed in blood cells of animals transplanted with infected stem cells (29, 54, 58). As hematopoietic stem cells are considered to be a potential target tissue for gene therapy, much effort has been put into the improvement of vectors to overcome the suppression phenomenon. Modification of the enhancer within the LTR has been exploited to prevent the suppression (53). The introduction of additional promoters into the viral transcription unit was partially successful, but expression levels in blood cells in vivo still remain variable in most cases (3, 54, 58). However, recent experiments suggest that failure to reconstitute the bone marrow of the recipient or failure to infect the donor stem cells may have contributed to the variable expression levels observed in vivo, thereby possibly overemphasizing the involvement of the transcription-suppression phenomenon (4, 24, 32, 59).

In an effort to gain more insight in the suppression mechanism, we used the retroviral vector BAG (37) (Fig. 1A), which contains the *Escherichia coli* β-galactosidase gene (β-gal) as a reporter to monitor the expression of the Mo-MuLV LTR promoter. The activity of β-gal can be visualized in individual cells by a sensitive in situ assay. The BAG vector also carries the Tn*5*-derived neomycin phosphotransferase gene (Neo^r), which is driven by the simian virus 40 early promoter and is therefore independent of the Mo-MuLV LTR activity. Here we report on the stability of the Mo-MuLV LTR-driven expression in murine fibroblast cell lines. We demonstrate that there is a marked heterogeneity in β-gal expression within monoclonal BALB/c 3T3 cell lines containing a single integrated copy of the BAG

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vector provirus. We show that inactivation of the Mo-MuLV LTR is directly associated with de novo methylation of cytidine residues. In addition, we demonstrate that the frequency of inactivation depends on the chromosomal position of the provirus. These results help to provide insight into the mechanisms involved in the down-regulation of transcription of the Mo-MuLV LTR.

MATERIALS AND METHODS

Cell lines and virus preparation. All sera and tissue culture media were purchased from GIBCO Laboratories (Grand Island, N.Y.). All culture plastics were obtained from Greiner (Nürtingen, Federal Republic of Germany). The hypoxanthine phosphoribosyltransferase-negative ($HPRT^-$) BALB/c 3T3 cell line B77 and the EC cell line P19 have been described before (30, 34). The psi-2-BAG cell line (37) (Fig. 1A), which produces helper-free ecotropic BAG viruses, was obtained from the American Type Culture Collection (ATCC 9560). This vector contains β-gal from *E. coli* driven by the Mo-MuLV LTR, together with the neomycin phosphotransferase gene from transposon Tn*5* that renders bacteria and eucaryotic cells resistant to kanamycin and G418, respectively. Both cell lines were grown in high-glucose (4.5 g/liter) Dulbecco modified Eagle medium supplemented with 8% fetal calf serum in a 5% CO_2 atmosphere at 37°C. To isolate virus stocks, near-confluent cultures were grown in fresh medium for 16 h, after which the medium was isolated and filtered through a 0.45-μm-pore-size filter (Gelman Sciences, Inc., Ann Arbor, Mich.) and stored at −80°C until further use. Virus infections and titrations were performed as described previously (21); titers of the stocks ranged from 1×10^5 to 3×10^5 Neo^r CFU/ml when tested on B77 cells.

Cell lines containing a single proviral copy of the BAG virus were generated by infection of subconfluent B77 cell cultures with a low multiplicity of infection (MOI < 0.001) in the presence of 8 μg of Polybrene (Sigma) per ml. Twenty-four hours after infection, the cultures were split 1 in 10 in medium containing 400 μg of G418 (GIBCO) per ml. Twelve days postinfection, individual G418-resistant colonies were isolated, expanded, and assayed for β-gal expression. Individual cell lines derived in this manner were designated BB1 to BB19. These cell lines were routinely grown in medium containing 400 μg of G418 per ml. The demethylating agent 5-azacytidine (azaC) (Boehringer GmbH, Mannheim, Federal Republic of Germany) was used at 4 μM concentration unless stated otherwise.

Southern blot analysis. High-molecular-weight cellular DNA was isolated, and 10-μg samples were digested for 16 h in 400 μl with 40 units of *Hin*dIII or *Sma*I (Pharmacia, Uppsala, Sweden). After 60 min, a 20-μl sample was removed from the reaction mixture and added to 0.5 μg of adenovirus type 2 DNA and incubated for a further 15 h. The restriction fragments of the latter incubation were resolved by electrophoresis on a 1% agarose gel. Complete digestion of the adenovirus DNA indicated complete digestion of the chromosomal DNA. The digested chromosomal DNA was size fractionated on a 1% agarose gel and transferred to a GeneScreen Plus membrane (Dupont, NEN Research Products) by standard procedures (40). Hybridization was performed to either a Neo^r-specific probe (the 1,321-bp *Hin*dIII-*Sma*I fragment of pRSVneo [13]) or a β-gal-specific probe (the 154-bp *Bam*HI-*Bgl*I fragment of plasmid pBAG, encoding the amino terminus of the β-gal protein). Probes were labeled to high specific activity with [α-^{32}P]dCTP by the random primer method (8).

RNA analysis. Cytoplasmic RNA was isolated from near-confluent cell cultures as described previously (43). Quantitation of specific transcripts was performed after transfer of serial dilutions of RNA onto nitrocellulose membrane via the slot-blot procedure (Schleicher & Schuell, Dassel, Federal Republic of Germany) and hybridization with specific probes. Apart from the probes mentioned above, a nearly full-length cDNA clone of human elongation factor 1 (5) was also used as a positive control.

β-Gal cytochemistry. To reveal the bacterial β-gal activity in situ, cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Research Organics Inc., Cleveland, Ohio) essentially as described by Sanes et al. (41). Cells were incubated in the staining solution for 14 to 20 h before enumeration of the fraction of β-gal-positive cells. With the use of a standard bright-field microscope, a minimum of 500 cells were scored in all cell lines tested.

Recombinant DNA techniques. All recombinant DNA techniques followed standard protocols (40). To clone the inactivated provirus from the BB10.c37 cells, genomic DNA was isolated and 10 μg was digested to completion with *Nhe*I. Subsequently, the digested DNA was recovered and circularized with T4 DNA ligase (Boehringer) in a volume of 1 ml. The ligated DNA (3 μg) was used for transformation into the transformation-competent *E. coli* MC1061 (40) and plated onto agar plates containing 50 μg of kanamycin per ml. A clone containing a plasmid (pBB10c37.1) with the desired structure was used for further study.

CAT plasmids and assay. A 10-μg sample of the plasmid to be tested was transfected into 2×10^5 cells by the calcium phosphate precipitation procedure (15) in a 10-cm tissue culture dish. Plasmid pMuLV-CAT was constructed by inserting the *Nhe*I-*Sma*I fragment from the plasmid pBAG (kindly provided by C. Cepko, Harvard Medical School, Boston, Mass.), containing almost the complete U3 region and part of the R region of the MuLV LTR (nucleotides 7846 to 8292; numbered according to reference 45) into plasmid pBL-CAT5 (48) (kindly supplied by R. Offringa). This plasmid was digested with *Bgl*II, and the sticky ends were filled in with DNA polymerase (Klenow fragment) and digested with *Xba*I prior to ligation with the BAG *Nhe*I-*Sma*I fragment. Plasmids pBL-CAT5 and pRSV-CAT (14) were included as negative and positive controls, respectively. Forty hours after transfection, the cells were lysed and 50 μg of the protein lysate (100 μg for P19 cells) was used for the chloramphenicol acetyltransferase (CAT) activity assays. These were performed as described previously (13). The incubation time of the assays was 60 min (120 min for P19 cells).

RESULTS

β-Gal is expressed heterogeneously within BAG virus-infected clones. We studied the Mo-MuLV LTR promoter activity in murine fibroblast cell lines infected with a recombinant retroviral vector by exploiting several features of the BAG vector (Fig. 1A). In a first series of experiments, the percentage of β-gal-expressing cells was determined in 135 independent BAG-infected $G418^r$ BALB/c 3T3 (B77) colonies. After staining, most of the colonies showed a mosaic phenotype with respect to β-gal expression (Fig. 1B). A large portion of the colonies contained considerable numbers of β-gal-negative cells. The results are summarized in Fig. 1C. A similar heterogeneity was observed in a number of other BAG-infected murine fibroblast cell lines, including Swiss 3T3 and PA317 (20a). In a control experiment, colonies

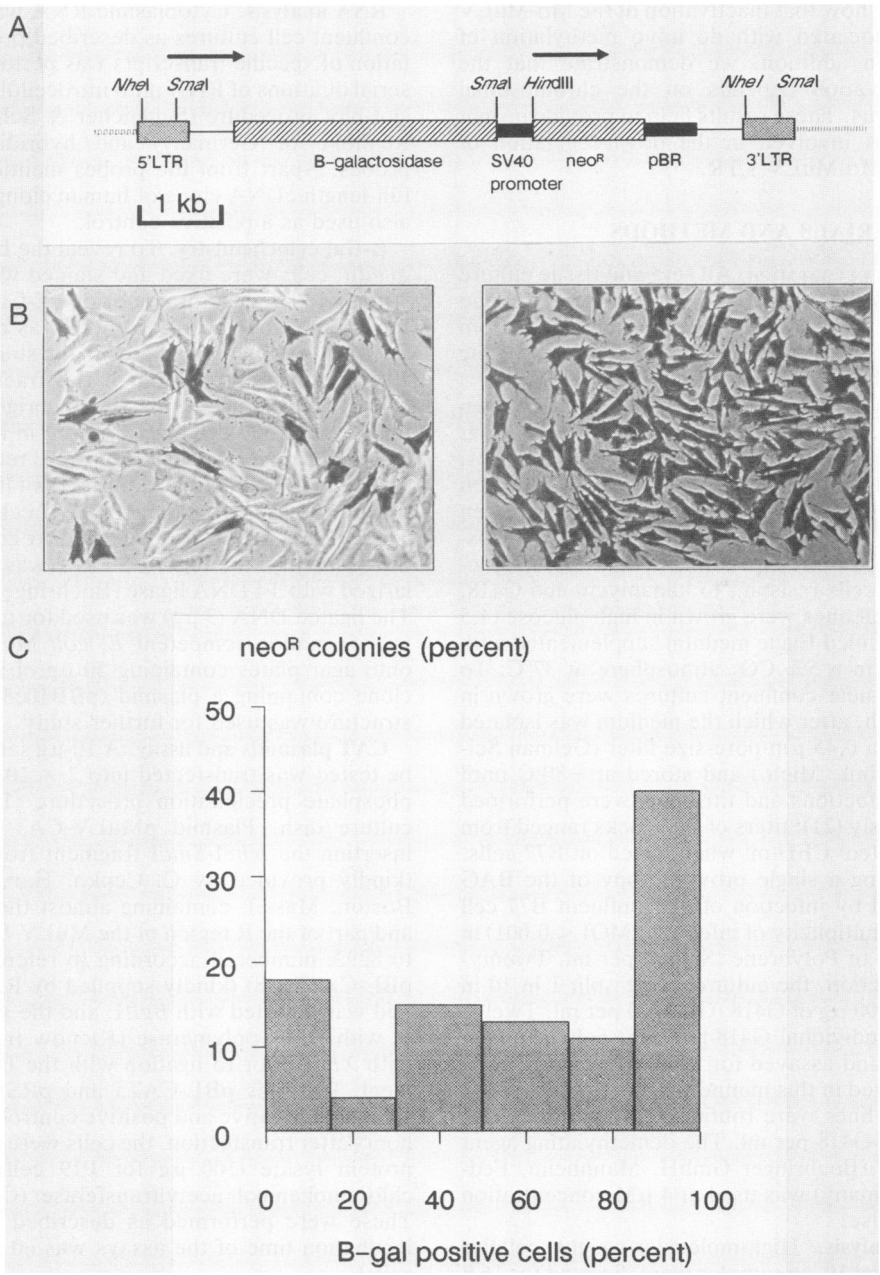

FIG. 1. (A) Schematic representation of the BAG vector used in this study. The 5′ ends of the transcripts encoded by this vector are indicated by horizontal arrows. Both transcripts end at the polyadenylation site in the 3′ LTR. Relevant restriction sites are indicated. A detailed description of this vector is given by Price et al. (37). pBR indicates the pBR322-derived origin of replication. (B) Photomicrographs of two BAG virus-infected and G418-resistant B77 clones. B77 cells were infected with BAG virus at low MOI and grown in medium containing 400 μg of G418 per ml for 12 days to select for infected cells. After selection, the cells were fixed and stained for the bacterial β-gal as described previously (41). Phase-contrast photomicrographs of two lines representing extreme phenotypes are shown (left, BB10; right, BB16; see text). (C) Histogram representing the fraction of β-gal-positive cells in 135 monoclonal BAG-infected B77 cell clones. B77 cells were infected at low MOI with BAG virus. Following selection with 400 μg of G418 per ml for 12 days, the resulting colonies were stained for the bacterial β-gal activity. The fraction of β-gal-positive cells was determined microscopically. On the ordinate, the number of colonies is shown as the percentage of the total number of colonies analyzed.

derived from the psi-2-BAG virus-producing cell line were assayed. In the latter experiment, all colonies consisted exclusively of β-gal-positive cells, excluding the possibility that the observed heterogeneity is due to improper fixation or staining.

To study the heterogeneity within the mosaic cell lines in more detail, we isolated 20 independent BAG-infected B77 cell lines. In these cell lines, the percentage of β-gal-expressing cells ranged between 8 and >98%. Two clones with the extreme phenotypes, BB10 (containing 8% β-gal-positive cells) and BB16 (>98% of the cells β-gal positive) were used for further study. Southern analysis established

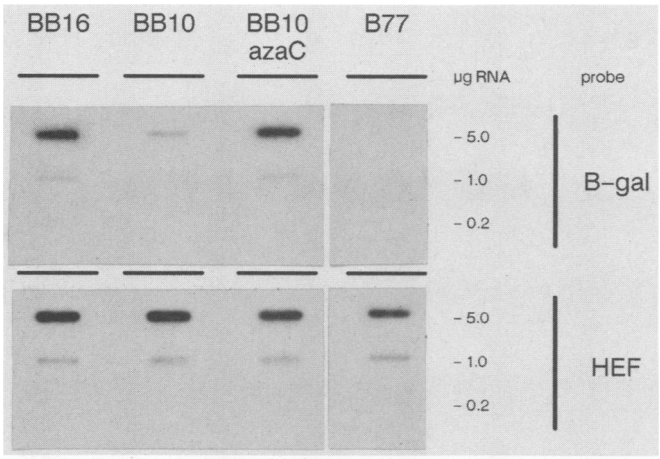

FIG. 2. Slot-blot quantitation of the β-gal-specific transcripts in B77, BB16, and BB10 cells and in BB10 cells treated with azaC. Cytoplasmic RNA was isolated from near-confluent cultures of B77, BB10, and BB16 cells and in addition from BB10 cells that had been treated with 4 μM azaC for 72 h. Serial dilutions of cytoplasmic RNA (5, 1, and 0.2 μg) were applied onto a nitrocellulose membrane with a slot-blot apparatus and hybridized with a β-gal-specific probe. As a control, a parallel filter was hybridized with a human elongation factor 1-specific (HEF) probe.

that both BB10 and B16 carried a single unrearranged copy of the vector provirus in their genomes (data not shown). A screening of the cytoplasmic RNA isolated from BB10, BB16, and the parental B77 cells for β-gal-specific sequences showed that the hybridization signal correlates with the relative abundance of the number of β-gal-positive cells (Fig. 2). The high frequency of inactivation suggests that the shutdown of β-gal expression is not the result of mutation of vector sequences but of some epigenetic mechanism.

Inactivation of LTR is reversed by addition of azaC. It is now well documented that in undifferentiated EC cells the block in transcription initiation is associated with the methylation of cytidine residues in CpG dinucleotides in the Mo-MuLV LTR (reviewed in reference 55). To study whether the Mo-MuLV LTR promoter could be reactivated by demethylation in the BB10 cells, we grew this cell line in various concentrations of the DNA-demethylating agent azaC for 3 days and determined the percentage of β-gal-positive cells. The fraction of β-gal-positive BB10 cells increased in a dose-dependent manner (Fig. 3A), whereas azaC did not change β-gal expression in B77 (0% β-gal positive) and in BB16 (>98% β-gal positive). Maximum stimulation in BB10 cells was achieved at 4 μM azaC (Fig. 3A); higher concentrations severely reduced the viability of the cells. These results indicate that the heterogeneity in the BB10 cell line is associated with de novo methylation of cytidine residues.

In a parallel experiment, a polyclonal cell culture of BAG virus-infected and G418-selected B77 cells containing 33% β-gal-positive cells was grown in 4 μM azaC for 72 h. This increased the number of β-gal-positive cells to 80%, indicating that the methylation-associated shutdown is a general phenomenon in BAG-infected B77 cells and not a peculiarity of the BB10 cell line. Thus, BB10 is a useful model for studying LTR inactivation in murine fibroblast cell lines.

Apart from the effect on the methylation of DNA, azaC also reduces DNA synthesis (25). To verify that the observed effect results from demethylation rather than reduction of DNA synthesis, we treated BB10 cells for 3 days with various concentrations of 1-β-D-arabinofuranosylcytosine-5′-triphosphate, a known inhibitor of DNA polymerases α and β (61). No alterations were seen in the fraction of β-gal-positive cells, even at concentrations which reduced [^{3}H]thymidine incorporation to less than 10% (data not shown).

To study whether the azaC-induced expression was stable in time, we grew BB10 cells with various concentrations of azaC for 72 h. After this treatment, the cell population was cultured in G418-containing culture medium. At regular intervals, subcultures were analyzed for β-gal expression. The results are shown in Fig. 3B. After the initial increase caused by azaC, the fraction of β-gal-expressing cells in the

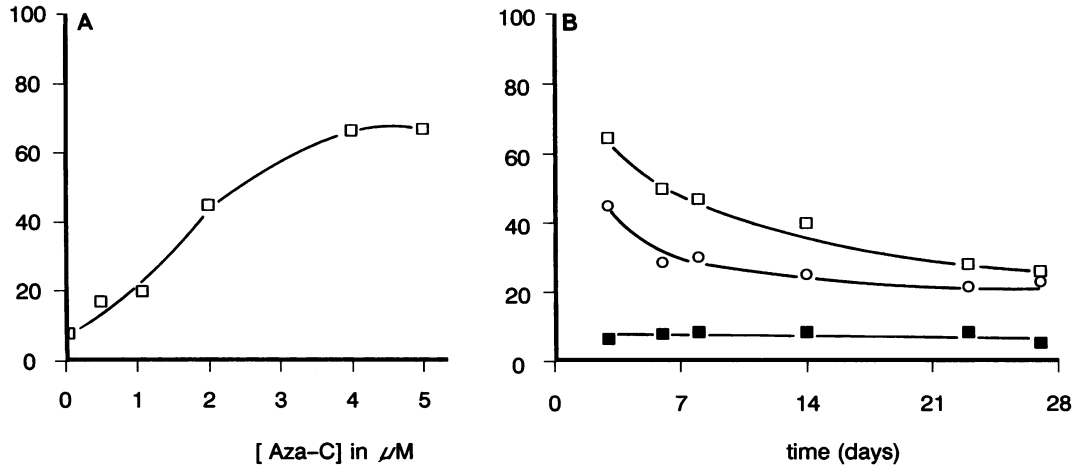

FIG. 3. (A) azaC-dependent increase in the relative number of β-gal-expressing cells. Confluent BB10 cultures were split 1 in 10 and subsequently grown in the presence of various concentrations of azaC for 72 h. After fixation, the cultures were stained for β-gal. The percentage of β-gal-positive cells was determined by microscopically scoring at least 500 cells. (B) Decrease in the fraction of β-gal-positive cells after azaC treatment of BB10 cells. Confluent BB10 cultures were split 1 in 10 and grown for 72 h in the presence of 4 μM (□) or 2 μM (○) azaC or in the absence of azaC (■) to induce β-gal expression. Subsequently, the cells were fed fresh medium without azaC, and at regular intervals subcultures were fixed and stained for β-gal activity.

culture gradually decreased. These results show that the vector provirus LTR again becomes inactivated after having been induced by azaC. Thus, inactivation of the LTR is independent of the integration process of the virus. A similar decrease was seen in parallel cultures grown without G418, excluding the possibility that the decrease was induced by the selection (data not shown). Experiments in which BB16 and B77 cells were grown in mixed cultures did not reveal significant differences in growth rates or survival after azaC treatment between β-gal-positive and -negative cells (data not shown).

Helper viruses are not involved in azaC-induced reactivation. The murine genome contains many copies of defective endogenous retroviruses. The majority of these are heavily methylated and thus transcriptionally inactive (reviewed in reference 7). There is evidence that in BALB/c mice, as the result of recombination between defective endogenous retroviruses, replication-competent viruses can be generated (7). Conceivably, the same could occur in the azaC-treated cell lines in which the BAG vector might be rescued from the initially β-gal-positive cells. Subsequent reinfection of the β-gal-negative cells might then contribute substantially to the increase of the fraction of β-gal-expressing cells. Several types of experiments were performed to test this hypothesis. First, no reverse transcriptase activity could be detected in the conditioned medium from BB10 and BB16 cells after azaC treatment of the cells. Second, culture medium from azaC-treated cells could not confer G418 resistance to B77 cells (<0.2 $G418^r$ CFU/ml), nor could it induce β-gal expression after infection of B77 cells (<0.2 $\beta\text{-gal}^+$ induction units per ml). And third, no additional integration sites could be detected in a Southern analysis of *Hin*dIII-digested genomic DNA from nine subclones isolated after azaC treatment of BB10 cells (Fig. 4). These results clearly demonstrate that rescue of the BAG virus is not responsible for the increase of the fraction of β-gal-positive cells resulting from azaC treatment.

Sequences within the 5′ LTR of BB10 cells but not in that of BB16 cells are methylated. To determine whether sequences within the 5′ LTR of the BAG vector provirus are methylated, we exploited the fact that the LTR contains a site for the restriction endonuclease *Sma*I. This enzyme is inhibited when the inner cytidine residue of its recognition sequence, 5′-CCCGGG-3′, is methylated (38). *Hin*dIII-digested chromosomal DNA from BB10 (8% β-gal-positive cells), BB16 (>98% β-gal-positive cells), and B77 (parent) cells was digested for 16 h with excess *Sma*I. After transfer of the restriction fragments to a nylon membrane, the blot was hybridized with a β-gal-specific probe (Fig. 5). As expected, no signal could be detected in the lanes containing B77 DNA. In the BAG virus-infected cell lines BB10 and BB16, a single hybridizing fragment was detected in the *Hin*dIII-digested samples. Since the vector contains only a single *Hin*dIII site, the size of this fragment depends on the location of the nearest *Hin*dIII site in the bordering chromosomal DNA and thus differs between the BB10 and BB16 cell lines. In BB16, a single hybridizing fragment was detected in the *Hin*dIII-*Sma*I-digested sample, comigrating with the corresponding fragment of *Sma*I-digested pBAG DNA. This indicates that in BB16 neither *Sma*I site flanking the β-gal gene is methylated. In BB10 DNA, however, an additional fragment is seen in the *Hin*dIII-*Sma*I-digested DNA. Since the size of this fragment approximates the size of the corresponding *Hin*dIII fragment, we conclude that the *Sma*I site in the 5′ LTR is refractory to cleavage in a part of the DNA molecules. On the basis of the intensity of the signals, we estimate that in approximately 30% of the DNA molecules the *Sma*I site is protected by methylation. This is considerably lower than the fraction of β-gal-negative cells in the BB10 cell line, suggesting that the methylation of the *Sma*I site in the 5′ LTR is not the direct cause of the transcriptional shutdown but rather a delayed event.

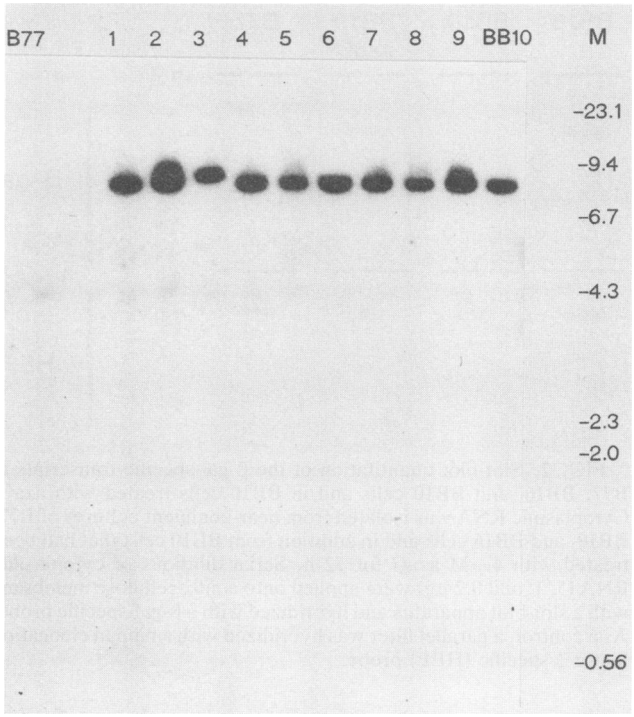

FIG. 4. Southern analysis of BB10 subclones isolated after azaC treatment. BB10 cells were treated with 4 μM azaC for 72 h. Following this treatment, subclones were isolated after limiting dilution of the cell population. From uninfected B77 cells, untreated BB10 cells, and the nine subclones (lanes 1 to 9), genomic DNA was isolated and 10-μg samples were digested with *Hin*dIII and subsequently fractionated on a 1% agarose gel. The Southern blot was hybridized with a Neo^r-specific probe. The positions of the size markers (M) are indicated (sizes in kilobases).

Similar results have been reported in EC cell lines, in which the inactivation precedes the general methylation of proviruses by many days (12, 55). However, our observation that addition of azaC to the culture medium results in a rapid increase in expression of β-gal, as shown above, suggests that methylation of specific sequences within the LTR is somehow involved in the repression.

LTR enhancer-promoter sequences are potentially fully functional in B77 and derived cell lines. Our results suggest that, in contrast to the situation in EC cells, inactivation of the LTR can occur in cells in which the enhancer is potentially fully functional. To illustrate this difference, we determined the LTR promoter activity in both cell systems. The Mo-MuLV LTR was cloned in front of the CAT gene and introduced into B77, BB16, BB10, and P19 EC cells. The results are summarized in Table 1. As expected, the MuLV LTR was equally active in B77 and its BAG-infected derivatives BB10 and BB16, whereas it was repressed in P19 cells. Transfection of the pRSV-CAT plasmid induced a significant conversion of the substrate (see reference 14), showing that the lack of expression after transfection of

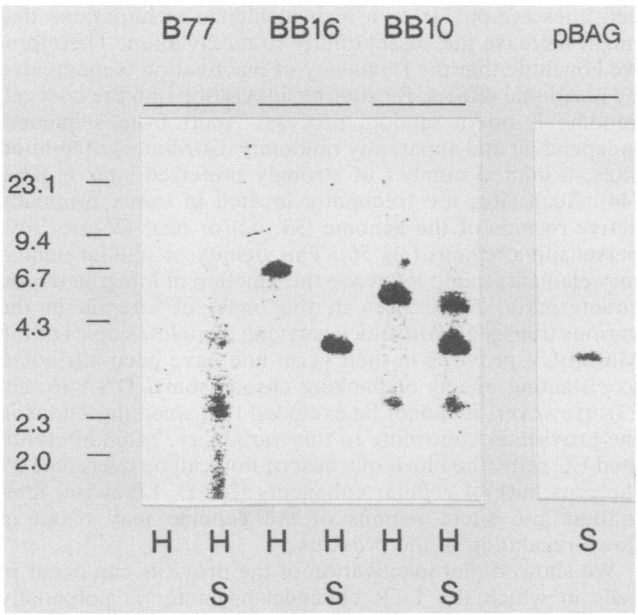

FIG. 5. Southern analysis of B77 clones infected with the BAG virus. Genomic DNA (10 μg) of BB10, BB16, and uninfected B77 cells was digested with *Hin*dIII (lanes H) or with *Hin*dIII and *Sma*I (lanes H and S) and fractionated on a 1% agarose gel. As a control, *Sma*I-digested plasmid pBAG DNA (10 pg) was used. The Southern blot was hybridized with a β-gal gene-specific probe. The positions of the size markers are indicated (sizes in kilobases).

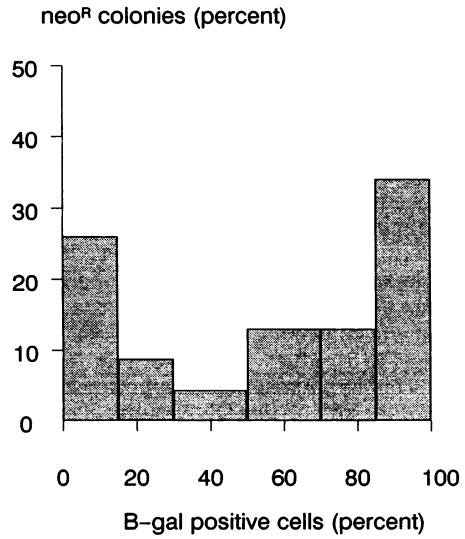

FIG. 6. Histogram representing the fraction of β-gal-positive cells in 23 monoclonal BB10c37 virus-infected B77 cell clones. B77 cells were infected at low MOI with BB10c37 virus. Following selection with 400 μg of G418 per ml for 12 days, the resulting colonies were stained for the bacterial β-gal activity. The fraction of β-gal-positive cells was determined microscopically. On the ordinate, the number of colonies is shown as the percentage of the total number of colonies analyzed.

pMuLV-CAT in P19 cells is not just caused by inefficient transfection. These observations indicate that the MuLV LTR promoter-enhancer is actively repressed in the undifferentiated EC cells, whereas in the B77 cells it is fully functional. Apparently, inactivation of the MuLV LTR after integration can occur in cell types in which the LTR is potentially fully functional.

Frequency of inactivation is chromosome-position dependent. As shown above, an epigenetic mechanism is responsible for the shutdown of expression in murine fibroblast cell lines. The heterogeneity within the cell lines is generated by temporal variations in the occurrence of the inactivating event in individual cells within a certain cell line. Apart from this heterogeneity within monoclonal cell lines, a large variation is seen between different cell lines with respect to the overall frequency of inactivation. This variation, illustrated in Fig. 1C, might be caused by the vicinity of regulatory elements in the flanking host cell genome, viz., a chromosome position effect. Alternatively, retroviruses are known to have a high mutation rate (47), and mutations might increase the susceptibility to methylation. To differentiate between these two hypotheses, we decided to clone the inactivated provirus from BB10 cells. To eliminate the β-gal-positive cells from the BB10 population, we derived several subclones which contained less than 1 β-gal-positive cell per 10^4. Genomic DNA of one of these subclones, BB10.c37, was used to clone the provirus. This is made possible by the fact that the provirus contains a pBR322-derived origin of replication and the Neo^r gene which confers resistance to kanamycin in bacteria. Chromosomal DNA was restricted with *Nhe*I and subsequently circularized. After transformation to *E. coli*, a clone which had the expected restriction pattern was isolated and designated pBB10c37.1. As the *Nhe*I site is located in the extreme 5′ part of the LTR, a plasmid is generated which contains virtually the complete 5′ LTR (Fig. 1A). After transfection into psi-2 cells, this LTR drives transcription and provides the polyadenylation signal as well. BB10 virus-containing medium was isolated 3 days posttransfection and used to infect B77 cells. A total of 23 G418^r clones were isolated and stained for β-gal. After staining, most colonies were mosaic with respect to β-gal expression. The spectrum of phenotypes in this group of clones was equivalent to that of the colonies derived after infection with the original stocks (Fig. 6). In addition, DNA sequence analysis of the LTR-containing *Nhe*I-*Sma*I fragment from plasmid pBB10c37.1 revealed a sequence identical to that of parental clone (data not shown). From these results, we conclude that the BAG provirus in BB10 is not inactivated as the result of mutation of vector sequences. Therefore, we favor the hypothesis that the shutdown of expression is governed by regulatory elements in the flanking host cell genome.

TABLE 1. Activity of Mo-MuLV LTR promoter linked to the CAT gene in B77 and its derivative cell lines and in the EC cell line P19

Cell line	% Acetylation			Ratio (MuLV/RSV)
	pBL-CAT5	pRSV-CAT	pMuLV-CAT	
B77	0.06	13.9	34.1	2.45
BB10	0.04	12.1	26.2	2.17
BB16	0.06	4.2	10.0	2.38
P19	0.06	1.9	0.2	0.11

DISCUSSION

We studied the expression from the Mo-MuLV LTR promoter in murine fibroblast cell line B77 infected with the

retroviral vector BAG (37). We showed that cell lines containing a single integrated copy of the vector provirus are heterogeneous in their expression of the reporter, *E. coli*-derived β-gal. In 60% of the G418^r colonies, more than 15% of the cells were β-gal negative (Fig. 1). This frequency is too high to be explained by mutational events but rather suggests an epigenetic mechanism. Treatment of β-gal-negative cells with the DNA-demethylating agent azaC resulted in resurrection of β-gal activity (Fig. 3A). However this azaC-induced reactivation was temporary (Fig. 3B); removal of azaC from the culture medium resulted in a gradual decline of the fraction of β-gal-expressing cells. From these data, we inferred that an epigenetic mechanism, viz., the de novo methylation of C residues, is responsible for shutdown of expression. This hypothesis was supported by the fact that the *Sma*I site in part of the DNA molecules isolated from BB10 cells, but not from BB16 cells, was refractory to digestion with *Sma*I, which is sensitive to methylation of its recognition site (38). The involvement of methylation of C residues in transcription repression is well established. Methylated LTRs are inefficient as promoters in murine fibroblast cell lines, whereas unmethylated LTRs are fully functional (19, 31, 46, 50; reviewed in reference 55).

Several other groups have studied the stability of Mo-MuLV-driven gene expression in fibroblast cell lines. It has been shown that such a loss of proviral gene expression can result from chromosome instability and subsequent loss of the vector provirus (49). However, in our case chromosome loss is not involved in the shutdown of expression as the provirus is obviously retained. In an extensive study, Xu and co-workers (60) assessed the stability of retroviral vectors after integration and observed, as in our study, shutdown of provirus expression without apparent alterations of vector sequences. In contrast to our results, these researchers were unable to restore expression by azaC treatment in their cells. However, in their study, the cells were grown in azaC-containing medium for only 24 h. In our hands, a 24-h treatment with azaC resulted only in a moderate reactivation of expression, whereas at least 3 days are required for a maximum effect (data not shown).

The mechanism by which the shutdown is accomplished is still obscure. In our study, we showed that demethylation alone is sufficient to reactivate transcription of the provirus in murine fibroblast cell lines. This suggests that the presence of methylated C residues in the 5′ LTR of the provirus constitutes the only block to transcription. Transfection experiments in which the MuLV LTR was linked to the CAT gene (Table 1) demonstrated that the MuLV LTR enhancer-promoter is potentially fully functional in these cells. In contrast, provirus expression in EC cells can only be restored by azaC treatment after differentiation of the cells (36), and extensive methylation of inactive proviruses can only be observed after a lag period of many days (12; reviewed in reference 55). This suggests that the extensive methylation in these EC cells is a consequence of the inactivation rather than an initial step. Our data suggest that the inactivation by way of methylation of the Mo-MuLV LTR is not necessarily the same as the transcription repression mechanism that is operating in undifferentiated EC cells. The proteins that are involved in maintaining the methylation patterns have been identified, but little is known about the factors responsible for de novo methylation (reviewed in reference 1).

The frequency of the epigenetic shutdown of expression varies between different cell clones. We demonstrated that the inactivation of the Mo-MuLV LTR in murine fibroblast cell lines is not due to a high incidence of mutations that might increase the susceptibility to methylation. Therefore, we conclude that the frequency of inactivation is modulated by positional effects. Retrovirus integration into the host cell genome is not a random process. Apart from sequence-independent and apparently randomly distributed integration sites, a limited number of strongly preferred sites is used (44). Such sites are frequently located in transcriptionally active regions of the genome (33, 42) or near DNase I-hypersensitive regions (39, 56). The vicinity of cellular regulatory elements might influence the function of integrated viral counterparts. Differences in the onset of viremia in the various transgenic *Mov* mice carrying complete copies of the Mo-MuLV provirus in their germ line have been attributed to *cis*-acting effects of flanking chromosomal DNA (6, 22, 23). However, it cannot be excluded that small mutations in the proviruses contribute to this variability. In undifferentiated EC cells, the block of transcription can be overcome by the proximity of cellular enhancers (2, 51). Likewise, integration into silent regions of the genome may result in down-regulation of the provirus.

We showed that inactivation of the provirus can occur in cells in which the LTR enhancer-promoter is potentially fully functional. This implies that the shutdown mechanism is dominant over the viral regulatory elements, i.e., that the regulatory elements in the provirus LTR are not sufficient for sustained expression of the provirus in a position-independent manner. Recently, regulatory elements (termed dominant control regions) have been identified that can confer cell-type specificity and position independence to linked genes after transfer into the mouse germ line (16, 17). We conclude that the Mo-MuLV LTR does not contain sequences that can act as a dominant control region in murine fibroblast cell lines. As a consequence, the Mo-MuLV LTR enhancer-promoter might not be sufficient to ensure sustained high-level expression in the absence of selective pressure, even in cells in which the enhancer-promoter is potentially fully functional. It could be the position-dependent inactivation that is responsible for the clonal variation in expression often observed, e.g., in the progeny of individual stem cells after retrovirus-mediated gene transfer (3, 4, 24, 32, 54, 59).

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