Two Blocks in Moloney Murine Leukemia Virus Expression in Undifferentiated F9 Embryonal Carcinoma Cells as Determined by Transient Expression Assays

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Transient expression assays were used to investigate the restriction of Moloney murine leukemia virus (MoMuLV) expression in undifferentiated mouse F9 embryonal carcinoma (EC) cells. We previously reported that the MoMuLV long terminal repeat (LTR) is inactive in undifferentiated F9EC cells due to inactivity of the tandemly repeated MoMuLV transcriptional enhancers. Others suggested that the inactivity was due to the presence of negative regulatory elements that interact with the MoMuLV tandem repeats. Two heterologous enhancer sequences that are active in undifferentiated F9 EC cells were inserted into the MoMuLV LTR: the B enhancers from the F101 variant of polyomavirus and a cellular enhancer sequence isolated from EC cells that we previously identified. The chimeric LTRs were then fused to the bacterial chloramphenicol acetyltransferase gene and tested for expression by transfection into F9 EC or NIH 3T3 cells. Insertion of these enhancers either upstream or downstream of the MoMuLV tandem repeats resulted in transcriptionally active LTRs in undifferentiated EC cells, which did not support the existence of negative regulatory elements interacting with the tandem repeats. In our previous MoMuLV enhancer deletion constructs, the GC-rich sequences downstream from the tandem repeats were also deleted, which might have contributed to the inactivity in EC cells. However, restoration of the GC-rich sequences did not yield an active LTR. The experiments also suggested that the EC cellular enhancer was preferentially active in undifferentiated EC cells and inactive in NIH 3T3 cells. The possibility of negative regulatory sequences in the vicinity of the MoMuLV primer-binding site was tested by inserting MoMuLV sequences from +30 to +419 base pairs into the LTR-chloramphenicol acetyltransferase gene constructs downstream of the transcriptional start site. Transient expression assays confirmed that these sequences reduced expression from functional LTRs in undifferentiated F9 EC cells but reduced expression significantly less in NIH 3T3 cells. Moreover, equivalent sequences from myeloproliferative sarcoma virus did not exhibit this effect. These results supported restriction of MoMuLV expression in undifferentiated F9 EC cells at two levels, inactivity of the MoMuLV enhancers and interaction of negative regulatory factors in the vicinity of the primer-binding site.

Murine embryonal carcinoma (EC) cells are derived from malignant stem cell carcinomas and share many properties with normal embryonic stem cells. These cells have been used as experimental systems to investigate regulation of gene expression in undifferentiated cells. Undifferentiated cells express a different, restricted set of genes in comparison with differentiated cells, which presumably reflects differences in regulatory and transcriptional factors.

Viruses have been useful tools in investigating expression in undifferentiated EC cells. In particular, a number of viruses are unable to productively infect undifferentiated EC cells. In the case of the DNA tumor virus polyomavirus, the restriction has been attributed to the inability of polyomavirus transcriptional enhancer elements to function in these cells (8, 9, 19, 24, 29).

Retroviruses are also restricted for infection of undifferentiated EC cells (28, 30, 33). In the case of Moloney murine leukemia virus (MoMuLV), restriction may occur at several different levels. Stewart et al. (30) provided evidence for de novo methylation of integrated MoMuLV proviral DNA. We and others demonstrated that the transcriptional elements in the MoMuLV long terminal repeat (LTR) are not functional in undifferentiated F9 EC cells (12, 23). Others have suggested that this may be due to interaction of negative

regulatory factors in F9 EC cells with the tandemly repeated enhancers in the MoMuLV LTR (12). Finally, work with MoMuLV-based retroviral vectors has suggested a negative regulatory region of the MoMuLV genome in the vicinity of the tRNA primer-binding site (1, 25).

In the work reported here, we investigated the restriction of MoMuLV expression in undifferentiated F9 EC cells by transient expression assays of modified MoMuLV LTRs. Insertion of sequences from other viral and cellular genes that increased expression was particularly useful and allowed conclusions about the mechanisms involved in restriction to be drawn.

MATERIALS AND METHODS

Cell lines. NIH 3T3 cells were grown in monolayers in Dulbecco modified Eagle medium supplemented with 10% calf serum. F9 EC cells were grown in the same medium containing 10% fetal calf serum and 0.1 mM (final concentration) β -mercaptoethanol.

Recombinant DNA cloning. Molecular cloning procedures were performed by standard protocols (26). Construction of LTR-chloramphenicol acetyltransferase (CAT) gene fusion plasmids has been described previously (6, 14, 23). Isolation of the F9 EC cellular enhancer element has also been described previously (32).

Construction of Mo/PB⁺-CAT involved first restoring a

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*Sma*I site located at +30 base pairs (bp) in the MoMuLV LTR that had been converted to a *Hind*III site in pMo-CAT. The primer-binding site region was a MoMuLV *Sma*I-to-*Pvu*I fragment spanning from +28 to +422 taken from a molecular clone of MoMuLV DNA. After end filling of the *Pvu*I site with a Klenow fragment of DNA polymerase I, this fragment was inserted into pMo-CAT at +30 bp by blunt-end ligation. This resulted in restoration of the remainder of the R and U5 regions, as well as downstream sequences, to the MoMuLV LTR. As a result of these manipulations, a 2-nucleotide insertion within the R region (at +28 bp) was present in all vectors containing viral primer-binding sequences. The myeloproliferative sarcoma virus (MPSV) primer-binding site was isolated as a corresponding *Sma*I fragment from an MPSV plasmid (pUC LTR-5' [no. 3], a gift from Eric Barklis) and inserted into pMo-CAT in an analogous manner.

DNA transfections. DNA transfections were performed by calcium phosphate precipitation essentially as described by Graham and van der Eb (13), as modified by Chen and Okayama (3). Briefly, cells were plated 1.5 to 2 h prior to transfection. The DNA-calcium phosphate precipitate was formed in a buffer containing BES (*N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid) instead of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and added to cells that were then incubated in a 3% CO₂ atmosphere for 18 h. Cells were then shocked with a 15% glycerol solution (1 min for F9 EC cells and 2 min for NIH 3T3 cells), washed, and fed with fresh medium. Cell extracts were prepared 40 h after transfection, and standardized reactions for bacterial CAT enzyme activity were performed as previously described (11).

T2 RNase analysis. Transcription from LTR-CAT fusion constructs was mapped by T2 RNase digestion of hybrids between a radioactive antisense RNA probe and cytoplasmic RNA from transfected cells (4, 27). The antisense RNA probe used in the analysis was synthesized *in vitro* by using SP6 RNA polymerase from a plasmid containing the MoMuLV LTR-CAT gene junction and spanned from the *Xba*I site in the LTR (−150) to an *Eco*RI site (+250) in the CAT gene. Labeled RNA probe was synthesized by incubating linearized SP6 expression plasmid DNA (1 µg) in a 20-µl reaction mixture containing 40 mM Tris (pH 7.5), 6 mM MgCl₂, 2 mM spermidine, 50 mM NaCl, 20 mM dithiothreitol, 100 µg of bovine serum albumin per ml, 500 µM CTP, GTP, and ATP, 12 µM UTP, 100 µCi of [α -³²P]UTP, 18 U of RNasin, and 15 U of SP6 RNA polymerase (Promega Biotec). Components were incubated at 37°C for 1 h, after which 1 U of RNase-free DNase I (Promega Biotec) was added. The reaction was terminated by phenol-chloroform extraction, and RNA was precipitated with the addition of 2 volumes of ethanol in the presence of 300 mM sodium acetate. After an additional round of ethanol precipitation, the probe was stored in ethanol at −20°C.

RNA was isolated from cells transfected with the LTR-CAT plasmids by using the Nonidet P-40 lysis method as described by Campos and Villarreal (2). For mapping, 30 µg of cytoplasmic RNA was hybridized with 250,000 cpm of antisense RNA probe in 30 µl of hybridization buffer (80% deionized formamide, 40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)], pH 6.4, 400 mM NaCl, 1 mM EDTA) at 60°C for 14 to 16 h. The resulting hybrids were digested by the addition of 300 µl of 50 mM sodium acetate (pH 5.0)–100 mM NaCl–2 mM EDTA containing 60 U of T2 RNase (Bethesda Research Laboratories, Inc.) per ml and were incubated at 30°C for 2 h. The digestion was terminated by

phenol-chloroform extraction, and the resulting hybrids were ethanol precipitated in the presence of 10 µg of carrier tRNA. The T2 RNase-resistant products were analyzed by electrophoresis in an 8% polyacrylamide gel containing 8 M urea and then by autoradiography.

RESULTS

Altered LTRs. The altered MoMuLV LTRs used in this study are diagrammed in Fig. 1. Each altered LTR was fused to the bacterial CAT gene; the resulting expression plasmids were transfected into undifferentiated F9 EC cells. Transfection into NIH 3T3 cells was used to compare behavior in a differentiated mouse cell. The Δ Mo deletion (−150 to −357 bp) lacked the 75-bp tandem repeats, which have been shown to have enhancer activity, as well as GC-rich sequences to the 3' side of the tandem repeats (21, 23). The Mo⁺ LTR was very similar to the wild-type MoMuLV LTR except that it contained a 4-bp deletion and insertion of an *Xba*I restriction site to the 5' side of the tandem repeats as well as removal of the original *Xba*I site at −150 bp (6). This facilitated insertion of sequences upstream of the tandem repeats. The Δ Mo+G/C LTR was derived from the Δ Mo LTR by restoration of the GC-rich sequences downstream from the tandem repeats (−180 to −150 bp). Polyomavirus F101 (PyF101) enhancer sequences inserted into the wild-type, Δ Mo, and Mo⁺ LTRs have been described previously (5, 6, 23).

Expression of PyF101-containing LTRs. Gorman et al. (12) previously suggested that the inability of the Moloney murine sarcoma virus LTR (closely related to the MoMuLV LTR) to function in undifferentiated F9 EC cells may be due to the presence of dominant negative regulatory factors in F9 EC cells that interact with the tandem repeats. One possible implication of this was that the MoMuLV tandem repeats might affect the activity of adjacent enhancers that are functional in these cells. Moreover, the relative position of these enhancers might be important. In order to test this, we studied LTRs containing insertions of the enhancers from the F101 variant of polyomavirus. The B enhancers from PyF101 show alterations that result in expression in undifferentiated F9 EC cells (24). LTRs containing PyF101 B enhancers are also shown in Fig. 1 (Mo+PyF101, PyF101+Mo, and Δ Mo+PyF101).

The PyF101-containing LTRs were tested for expression in undifferentiated F9 EC cells by transfection of the CAT expression plasmids. Cytoplasmic extracts of the transfected cells were assayed for CAT enzyme activity, and the results are shown in Table 1. For comparison, the same plasmids were transfected into NIH 3T3 cells. The wild-type MoMuLV LTR and the Δ Mo LTRs were both inactive in undifferentiated F9 EC cells, as described previously (23). As expected, the Δ Mo LTR was inactive in NIH 3T3 cells, indicating the requirement of enhancers for efficient LTR function (21, 23). Interestingly, all PyF101-containing LTRs were active in undifferentiated F9 EC cells, and insertions upstream (PyF101+Mo) or downstream (Mo+PyF101) of the inactive MoMuLV tandem repeats resulted in comparable activity. Thus, the MoMuLV tandem repeats did not appear to exert a repressive effect on adjacent active enhancers regardless of relative position. In NIH 3T3 cells, all three PyF101-containing LTRs showed expression levels essentially equivalent to that of the wild-type MoMuLV LTR.

Addition of GC-rich sequences to the Δ Mo LTR. We previously reported that the Δ Mo LTR is inactive in undifferentiated F9 EC cells (23). This result contrasted with the

FIG. 1. LTR-CAT gene plasmids. The construction of the LTR-CAT plasmids Mo-CAT, Δ Mo-CAT, and Mo⁺-CAT have been previously described (6, 23). These constructs contained the entire U3 regions ($\square$) and half of R regions from the various MoMuLV LTRs. The 75-bp enhancer elements in the U3 region are indicated. Nucleotides are numbered relative to the MoMuLV transcription start site at 0. The standard promoter elements (TATA and CCAAT homologies) are located at -30 and -80 bp, respectively, in the U3 region. Δ Mo+G/C was constructed by inserting a synthetic oligonucleotide containing the MoMuLV sequences between -180 and -150 into Δ Mo-CAT. The Mo+PyF101 LTR represents insertion of the B enhancer elements from the F101 mutant of polyomavirus into the wild-type MoMuLV LTR via *Xba*I linkers at -150 bp (5). The Δ Mo+PyF101 and PyF101+Mo LTRs represent insertion of the same polyomavirus fragment into the Δ Mo and the Mo⁺ LTRs, respectively.

TABLE 1. Relative activity of chimeric LTRs in transient expression assays^a

Expt no.	LTR ^b	% Acetylation in:	
		F9 EC cells	NIH 3T3 cells
1	Mo	0.3	13.8
	ΔMo	0	0
	PyF101+Mo	22.8	20.8
	Mo+PyF101	30.8	13.8
	ΔMo+PyF101	64.8	14.8
2	Mo	1.0	11.8
	ΔMo	0.8	0.3
	ΔMo+G/C	3.1	0.1
	Mo+PyF101	>99	16.8
	Mo+	2.0	17.8

^a LTR-CAT expression plasmids were transfected into cells, and CAT enzyme activity was measured 48 h later. In all cases, 10 μ g of plasmid DNA was precipitated in the presence of 10 μ g of calf thymus carrier DNA per 10-cm culture dish. Amounts of cell extracts were adjusted for equal A₂₈₀, and reactions were carried out for the same lengths of time. Assays were repeated at least three times with extracts from independent transfections. The amount of acetylation was determined by thin-layer chromatography on silica gel plates and autoradiography. Percentages of acetylation were determined by scintillation counting of excised radioactive spots.

^b The nature of the LTRs fused to the bacterial CAT gene is shown in Fig. 1.

finding of Gorman et al. (12) that deletion of the tandem repeats from the Moloney murine sarcoma virus LTR restored activity of the LTR in undifferentiated F9 EC cells. Since the Δ Mo LTR lacked both the tandem repeats and also adjacent downstream GC-rich sequences, it seemed possible that the inactivity of the Δ Mo LTR might result from lack of the GC-rich sequences. Therefore, the GC-rich sequences were restored to the Δ Mo LTR by insertion of a synthetic oligonucleotide, giving a construct analogous to the enhancer-negative Moloney murine sarcoma virus LTR of Gorman et al. (12). Assay of the Δ Mo+G/C in undifferentiated F9 EC cells showed little if any activity (Table 1). Thus, the experiments represented in Table 1 did not support the interaction of negative regulatory factors with the MoMuLV tandem repeats in F9 EC cells.

Insertion of cellular enhancers active in F9 EC cells. The previous experiments utilized enhancers from PyF101 as the active enhancers in undifferentiated EC cells. One potential drawback to using the PyF101 enhancers was that like many viral enhancers, the inserted sequences contained several enhancer core elements (15, 17, 34), which might complicate the interpretation. We wished to test a different, preferably cellular enhancer that was also active in these cells. We recently described isolation and characterization of sequences from PCC4 EC cells that showed enhancer activity in undifferentiated PCC4 cells and which apparently activated transcription of a retroviral vector driven by the

FIG. 2. MoMuLV LTRs containing the EC cellular enhancer. A 320-bp F9 EC cellular enhancer sequence (32) was inserted as an *Xba*I fragment into the *Xba*I sites of Mo-CAT, Δ Mo-CAT, and Mo⁺-CAT (Fig. 1). Clones containing the cellular enhancer in both orientations were selected in each case.

MoMuLV LTR (32). The EC enhancer sequences were inserted as a 320-bp fragment into the MoMuLV LTRs to give the constructs shown in Fig. 2. These sequences were inserted in both orientations (+ and -) with respect to the MoMuLV LTR. The EC enhancer was able to potentiate transcription of the MoMuLV LTR in all positions within the MoMuLV LTR and regardless of orientation (Fig. 3). The ability of the EC enhancer to potentiate expression of the inactive MoMuLV LTR in either orientation and at different positions within the MoMuLV LTR supported the notion that the constructs were predominantly functioning as enhancers. The highest transcriptional activity was consistently observed with Mo+EC⁺ or Mo+EC⁻ CAT-containing LTRs with the EC sequences inserted downstream of the MoMuLV enhancers. This suggests that the MoMuLV enhancers cooperated with the EC enhancers in the undifferentiated EC cells, even in a condition in which they were not active by themselves.

Another noteworthy feature of the EC enhancer-containing LTRs was observed in the assays of NIH 3T3 cells. The Δ Mo+EC⁻ LTR was inactive in NIH 3T3 cells, while it was active in the undifferentiated F9 EC cells. Thus, the EC enhancer may only be active in undifferentiated F9 EC cells and not in differentiated cells such as NIH 3T3. Two other results were consistent with this; the Δ Mo+EC⁺ LTR was also inactive in NIH 3T3 cells, and the Δ Mo+EC⁻ LTR was also inactive in a line of differentiated rat hepatoma cells (not shown).

It was important to ensure that the CAT enzyme activity measured indicated the amount of transcription initiated from the MoMuLV promoters in these chimeric constructs. Therefore, RNase protection experiments were carried out. A radioactive antisense RNA probe spanning the MoMuLV cap site of pMoCAT (-150 to +250 bp) was first prepared by *in vitro* transcription from a plasmid containing the appropriate pMoCAT sequences inserted downstream from a

FIG. 3. CAT activities of chimeric LTRs containing the EC enhancer. CAT activities were measured as described in Table 1. The locations of the substrate [¹⁴C]chloramphenicol (C) and the two acetylated forms of chloramphenicol (aC) are indicated. Percentages of acetylation (%Ac) were determined by scintillation counting of excised radioactive spots and are indicated above the lanes. Chimeric MoMuLV LTR insertions containing the EC cellular enhancer are designated by + or - to indicate the orientation of the enhancer with respect to the direction of transcription from the MoMuLV promoter.

bacterial SP6 promoter. F9 EC cells were transfected with CAT expression plasmids driven by the EC or PyF101 enhancer-containing LTRs. Total cellular RNA was extracted from a portion of each transfected culture, and the remainders of the cultures were assayed for CAT enzyme activity. Equal amounts of cellular RNA from each transfected culture were hybridized with the radioactive antisense RNA probe and then digested with T2 RNase. The digested samples were then analyzed by electrophoresis on a denaturing polyacrylamide gel followed by autoradiography. As shown in Fig. 4, transfection with CAT constructs containing the three EC and PyF101 enhancer-containing LTRs yielded a protected 280-nucleotide fragment in all cases, characteristic of transcription from the MoMuLV cap site. (Full-length antisense fragment was present in all samples, including RNA of control untransfected F9 EC cells [-]; the only labeled fragment associated with plasmid transfection was the 280-nucleotide fragment.) No other transcriptional start sites within the vicinity of the MoMuLV cap site were detected. It was important that the relative intensities of the protected 280-nucleotide fragments paralleled the amount of CAT enzyme measured for each transfected culture (Fig. 4). This supported the notion that the CAT activities accurately

FIG. 4. Quantitative T2 RNase protection analysis of transcripts in F9 EC cells. Duplicate cultures of F9 EC cells were transfected with each chimeric LTR-CAT plasmid shown. Total cytoplasmic RNA was prepared 48 h after transfection from one culture for each plasmid, and 30 μ g was hybridized with a uniformly labeled RNA probe spanning the LTR-CAT gene junction sequence. The sizes of T2 RNase-resistant fragments were determined on an 8% polyacrylamide gel containing 8 M urea. Correctly initiated CAT gene transcripts gave a protected fragment of 280 bp (arrow). The pBR-*Hinf*I lane represents pBR322 DNA digested with *Hinf*I and end labeled with [α - 32 P]ATP. Untransfected F9 EC cytoplasmic RNA (lane -) was also subjected to the same hybridization, and analysis showed nonspecific RNase protection of the original probe band (430 bp). The remaining cultures were harvested and tested for CAT activities as shown on the right. C, Chloramphenicol; aC, acetylated form(s) of chloramphenicol.

reflected the amount of transcription from the MoMuLV promoters. As expected, transfection of pMoCAT into NIH 3T3 cells yielded the 280-nucleotide protected fragment (Fig. 5), and transfection of this plasmid into F9 EC cells resulted in no protected fragment (not shown).

Effect of the MoMuLV primer-binding region on expression in F9 EC cells. We previously generated infectious MoMuLVs containing the Mo+PyF101 and Δ Mo+PyF101 LTRs by transfection into NIH 3T3 cells. While the virus could productively infect NIH 3T3 and other differentiated cells, it could not productively infect undifferentiated F9 EC cells (H. Fan, unpublished data). This indicated that an additional block to MoMuLV infection may exist in undifferentiated F9 EC cells. On the basis of MoMuLV-based retrovirus vectors, others have suggested the importance of sequences downstream from the mRNA cap site at the 3' end of the LTR, near the tRNA primer-binding region (1, 25). Deletion of sequences from this region increased the effi-

FIG. 5. T2 RNase protection in NIH 3T3 cells. Expression of Mo-CAT and Δ Mo-CAT plasmids was tested in NIH 3T3 cells as described for Fig. 4. Undigested probe appeared at 430 bp. As expected, Mo-CAT showed correct initiation at 280 bp, and Δ Mo-CAT did not.

ciency of these vectors, suggesting the binding of negative regulatory factors in undifferentiated F9 EC cells. We wished to test this possibility directly in transient expression systems. Therefore, CAT expression vectors were constructed. These were related to the vectors used for Fig. 1 to 5 but additionally contained sequences from +30 to +419 bp of the MoMuLV genome which included the tRNA primer-binding region in either orientation (Fig. 6A). The added sequences represented 5' untranslated sequences from genome-length MoMuLV RNA and would not be expected to introduce additional translational start sites. CAT expression plasmids containing the primer-binding region as well as the wild-type, Mo+PyF101, and Δ Mo+PyF101 U3 regions were constructed.

Expression of these plasmids was first tested in NIH 3T3 cells. As shown in Fig. 6B, insertion of the primer-binding region sequences in either orientation downstream of the wild-type MoMuLV U3 region (Mo/PB⁺, Mo/PB⁻) had little effect on expression. Insertion of these sequences downstream of the Mo+PyF101 or Δ Mo+PyF101 U3 regions reduced expression approximately twofold.

In contrast, all plasmids containing the primer-binding region showed a proportionally larger decrease (5- to 10-fold) in expression in undifferentiated F9 EC cells as shown in Fig. 6B. (Constructs with the wild-type MoMuLV U3 region were not tested since the wild-type MoMuLV enhancers are inactive in undifferentiated F9 EC cells.) Both Mo+PyF101/

FIG. 6. Effect of primer-binding site sequences from MoMuLV on expression. (A) Schematic representation of LTR-CAT constructs containing the MoMuLV tRNA primer-binding site. The primer-binding site region from MoMuLV was inserted into pMo-CAT to give pMo/PB-CAT as described in Materials and Methods. Similar plasmids containing the PyF101 enhancer sequences were also constructed (Mo+PyF101/PB-CAT and ΔMo+PyF101/PB-CAT). (B) Transient expression of constructs containing MoMuLV primer-binding site sequences. Orientation of the primer-binding site fragment in the plasmids is designated as either 5' to 3' (+) or 3' to 5' (-). Constructs were transfected into F9 EC cells and NIH 3T3 cells, and levels of CAT enzyme expression were measured. C, Chloramphenicol; aC, acetylated form(s) of chloramphenicol; %Ac, percentage of acetylation.

PB⁻ and ΔMo+PyF101/PB⁻ constructs showed a reproducibly larger inhibition than the equivalent constructs with the primer-binding region inserted in the positive orientation, suggesting a secondary directionality effect. These results were therefore consistent with the presence of factors in undifferentiated F9 EC cells that interacted in a negative fashion with sequences in the MoMuLV primer-binding region.

Effect of the primer-binding region from MPSV. Weiher et al. (35) have suggested on the basis of retroviral vectors that primer-binding region sequences from MPSV (a v-mos-containing murine retrovirus [7, 20]) may be less subject to repressive effects in F9 EC cells than MoMuLV primer-binding sequences. We tested this by generating CAT

FIG. 7. Effects of the MPSV primer-binding site in F9 EC cells. The corresponding primer-binding site fragment of MPSV was inserted into ΔMo+PyF101-CAT and Mo+PyF101-CAT (Fig. 1). Constructs containing either the MoMuLV primer-binding site sequences (PB⁺) or the MPSV primer-binding site (MP⁺) were transfected into F9 EC cells, and expression of CAT enzyme was compared. C, Chloramphenicol; aC, acetylated form(s) of chloramphenicol; %Ac, percentage of acetylation.

expression plasmids equivalent to those of Fig. 6 but which contained primer-binding sequences from MPSV. Plasmids containing MPSV primer-binding sequences consistently showed significantly less inhibition in F9 EC cells compared with the MoMuLV-containing constructs (Fig. 7). These results provided more direct evidence that MPSV primer-binding sequences are less inhibitory to expression in F9 EC cells.

DISCUSSION

In these experiments, factors that restrict expression of MoMuLV in undifferentiated EC cells were investigated. The results supported findings from previous experiments by ourselves and others (12, 23) that inactivity of the MoMuLV enhancers was a major factor. Several lines of evidence did not support the existence of negative regulatory elements in F9 EC cells that interacted with the MoMuLV tandem repeats. First, insertion of PyF101 enhancers into the MoMuLV LTR yielded LTRs that were functional in F9 EC cells regardless of whether they were inserted upstream or downstream of the MoMuLV tandem repeats. Second, the ΔMo+G/C LTR, which was very similar to the enhancer-deletion construct of Gorman et al. (12), was also inactive in F9 EC cells. Third, for chimeric LTRs containing the EC cellular enhancers, the presence of the MoMuLV enhancers upstream actually potentiated transcriptional activity in F9 EC cells. Loh et al. (25) also recently reported lack of evidence for negative regulatory factors in F9 EC cells.

On the other hand, the ΔMo+PyF101 LTR, which lacks the MoMuLV enhancers, was consistently somewhat more active in F9 EC cells than LTRs containing both PyF101 and MoMuLV enhancers (Mo+PyF101 and PyF101+Mo) (Fig. 2). This could be consistent with minor negative regulatory factors. However, equivalent constructs containing the EC cellular enhancers (ΔMo+EC⁺ or ΔMo+EC⁻) did not show the same effect.

The EC cellular enhancers also yielded LTRs that were active in F9 EC cells. The original experiments (32) sug-

gested that these enhancers activated transcription from the LTR of an MoMuLV-based vector in PCC4 EC cells from an upstream position. The results reported here are consistent with this and further indicate that these EC enhancers can activate an MoMuLV LTR when inserted at different positions and orientations within the LTR itself. It was noteworthy that the EC cellular enhancers were nonfunctional in differentiated NIH 3T3 or rat hepatoma cells, as judged from the inactivity of $\Delta\text{Mo}+\text{EC}^+$ or $\Delta\text{Mo}+\text{EC}^-$ in those cell types. The EC cellular enhancers may only be active in undifferentiated stem cells. We previously generated several other chimeric MoMuLV LTRs driven by heterologous viral enhancers, including those from polyomavirus, simian virus 40, and human T-cell leukemia virus types I and II. All of these LTRs showed some level of activity in NIH 3T3 cells. The $\Delta\text{Mo}+\text{EC}$ LTRs are the first chimeric LTRs that are active only in EC cells but not in differentiated cells such as NIH 3T3 cells or hepatoma cells.

In the experiments reported here, a 320-bp fragment containing the EC cell enhancer core region was used. In other experiments where a larger 2.3-kilobase fragment containing the EC cell was used, chimeric LTRs showed activity in NIH 3T3 cells as well as in undifferentiated EC cells (M. Taketo, unpublished data). This suggests that while the EC cell enhancer core region is not enough to be functional in differentiated cells, sequences that are normally adjacent to the core region of the enhancer may confer activity in differentiated cells.

We recently generated infectious MoMuLV driven by the chimeric $\text{EC}^- + \text{Mo}$ LTR. This virus replicates in NIH 3T3 cells, presumably due to the presence of the MoMuLV enhancers. However, the virus cannot productively infect undifferentiated F9 EC cells, presumably due to other blocks in infection such as those at the primer-binding site. Attempts to generate infectious MoMuLVs driven by the $\Delta\text{Mo}+\text{EC}$ LTRs have been unsuccessful so far.

Previous experiments using retroviral vectors indicated that MoMuLV 5' untranslated sequences near the tRNA primer-binding site may be involved in blockage of expression in undifferentiated F9 EC cells. The results in the previous work were quantified by colony formation efficiencies, a somewhat indirect assay. The transient expression assays reported here provided a more direct assay and confirmed that MoMuLV sequences from +30 to +419 bp reduced the expression of LTRs with functional enhancers in F9 EC cells. Moreover, the reduction was less pronounced or absent in NIH 3T3 cells. Thus, the reduction of expression in F9 EC cells did not result simply from insertion into the mRNA transcript of ~400 nucleotides between the mRNA cap and the initiation codon for CAT protein. Insertion of the equivalent primer-binding sequences from the MPSV genome did not show as pronounced inhibition of CAT gene expression in the F9 EC cells (Fig. 7), consistent with experiments using retroviral vectors.

The results shown in Fig. 6 and 7 confirm that a second block to MoMuLV replication in undifferentiated F9 EC cells results from the 5' untranslated sequences near the tRNA primer-binding site. The most likely interpretation is that F9 EC cells contain negative regulatory factors that can interact with MoMuLV DNA or RNA from this region and prevent viral gene expression. This situation is reminiscent of regulation for human immunodeficiency virus (HIV), a human retrovirus. DNase footprint assays have identified host cell factors that bind to HIV LTR sequences downstream from the cap site (10, 36). Moreover, this general region of the HIV LTR is also necessary for response to *tat*,

the HIV transactivating protein. Recent experiments by Kao et al. have suggested that *tat* transactivation of HIV may result from antiattenuation; i.e., in the absence of *tat*, transcription does not proceed outside of the LTR. Perhaps F9 EC cells have a protein factor that interacts with MoMuLV sequences near the tRNA primer-binding site to prevent further transcription. It should be noted however, that insertion of the MoMuLV primer-binding sequences into a functional LTR in either orientation resulted in inhibition of transient CAT gene expression. This suggests that the putative F9 EC inhibitory factor interacts with MoMuLV DNA. In contrast, HIV LTR sequences confer *tat* responsiveness only when in the plus orientation. It should also be noted that these experiments do not exclude the physical binding of NIH 3T3 factors to MoMuLV primer-binding sequences, particularly if these factors do not markedly reduce expression.

The behavior of the primer-binding-site-containing LTR constructions in NIH 3T3 cells also suggested interaction among factors bound to different regions of the DNA. In particular, Mo-CAT and Mo/PB-CAT showed indistinguishable levels of CAT gene expression. In contrast, Mo+PyF101/PB-CAT consistently showed twofold-less activity than Mo+PyF101-CAT (Fig. 6) in these cells. This could be explained by a negative interaction between factors bound to the MoMuLV primer-binding site and factors bound to the PyF101 enhancers. This is consistent with the biological infectivities of MoMuLVs driven by the wild-type and Mo+PyF101 LTRs (5). Mo+PyF101 MoMuLV has an infectivity-to-particle ratio fivefold lower than that of wild-type MoMuLV in NIH 3T3 cells. On the other hand, the two LTRs show the equivalent transcriptional activities in CAT expression plasmids lacking the primer-binding sequences.

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