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Generation of Infectious Moloney Murine Leukemia Viruses with Deletions in the U3 Portion of the Long Terminal Repeat

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Deletional analysis within the long terminal repeat (LTR) of Moloney murine leukemia virus (M-MuLV) was performed. By molecular cloning, deletions were made in the vicinity of the *XbaI* site at −150 base pairs (bp) in the U3 region, between the tandemly repeated enhancers and the TATA box. The effects of the deletions on LTR function were measured in two ways. First, deleted LTRs were fused to the bacterial chloramphenicol acetyltransferase gene and used in transient expression assays. Second, infectious M-MuLVs were generated by transfection of M-MuLV proviruses containing the deleted LTRs, and the relative infectivity of the mutant viruses was assessed by XC-syncytial assay. Most of the deleted LTRs examined showed relatively high promoter activity in the transient chloramphenicol acetyltransferase assays, with values ranging from 20 to 50% of the wild-type M-MuLV LTR. Thus, the sequences between the enhancers and the TATA box were not absolutely required for transient expression. However, infectivity of viruses carrying the same deleted LTRs showed more pronounced effects. Deletion of sequences from −195 to −174 bp reduced infectivity 20- to 100-fold. Deletion of sequences within the region from −174 to −122 bp did not affect infectivity, indicating that this region is dispensable. On the other hand, deletion of sequences from −150 to −40 bp reduced infectivity from 5 to 6 logs, although the magnitude of the reduction partly may have reflected threshold envelope protein requirements for positive XC assays. The reduced infectivity did not appear to result from a failure of proviral DNA synthesis or integration by the mutant. Thus, the infectivity measurements identified three functional domains in the region between the enhancers and the TATA box.

Retroviruses generate viral DNA with long terminal repeats (LTRs) during reverse transcription (reviewed in references 21 and 22). The LTRs are essential for viral replication and expression because they contain the signals for viral DNA integration, transcription initiation, and RNA cleavage and polyadenylation. In particular, the U3 portion of the LTR contains canonical eucaryotic promoter signals (TATA and CCAAT homologies at approximately −30 and −80 base pairs [bp], respectively) as well as transcriptional enhancers ([illegible] approximately −340 to −180 bp) (10, 20). As such, the retroviral LTR is an excellent model system for studying normal eucaryotic gene expression.

In the experiments reported here, sequences in the U3 portion of the Moloney murine leukemia virus (M-MuLV) LTR necessary for LTR function were investigated by deletional analysis. We previously demonstrated the requirement for the enhancers (12), so emphasis was placed on the sequences between the enhancers and the TATA box. The activities of the deleted LTRs in transient expression assays were assessed, similar to experiments reported by others (8, 10). In addition, we extended the analyses by generating infectious M-MuLVs containing the deleted LTRs. The infectivities of the mutant viruses were measured, and in some cases the results obtained by the two techniques were markedly different.

MATERIALS AND METHODS

Cells and viruses. NIH-3T3 mouse cells (19) were grown in Dulbucco modified Eagle medium supplemented with 10% calf serum as described previously (4). Procedures for cell transfections, viral infections, XC plaque assays, and reverse transcriptase measurements have been described previously (4, 15, 16). The XC plaque assay is an indirect assay for M-MuLV infection, based on the fact that rat XC cells fuse with cells productively infected with some strains of MuLV (including M-MuLV). Briefly, NIH-3T3 cells were infected with serial dilutions of tissue culture supernatant from M-MuLV-infected NIH-3T3 cells and allowed to grow to confluency. The infected cells were then briefly irradiated with UV light to inhibit further growth and then overlayered with XC cells. The XC cells fused to form multinucleate syncytia in regions of viral infection but grew to form a dense monolayer in uninfected areas. The areas of infection were visible as plaques, which could be seen with the naked eye or with the help of a dissecting microscope.

Molecular cloning. Molecular cloning was done by standard protocols (13). A detailed description of the generation of the proviral plasmids with altered LTRs and of the chloramphenicol acetyltransferase (CAT) expression plasmids was published in Overhauser and Fan (15).

Molecular and biochemical analyses. Viral RNA was extracted from infected cells by the method of Campos and Villarreal (1), and dot-blot hybridization on nylon membranes was performed as described previously (15). Metabolic labeling of infected cells with [^{35}S]methionine, immunoprecipitation with anti-gp70 serum, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were done as described previously (3). Southern blot hybridization of high- and low-molecular-weight DNA from Hirt pellets and supernatants of infected cell nuclei was done as described previously (15). DNA sequence analysis was done by the method of Maxam and Gilbert (14). CAT enzyme assays were performed as described previously (6, 15).

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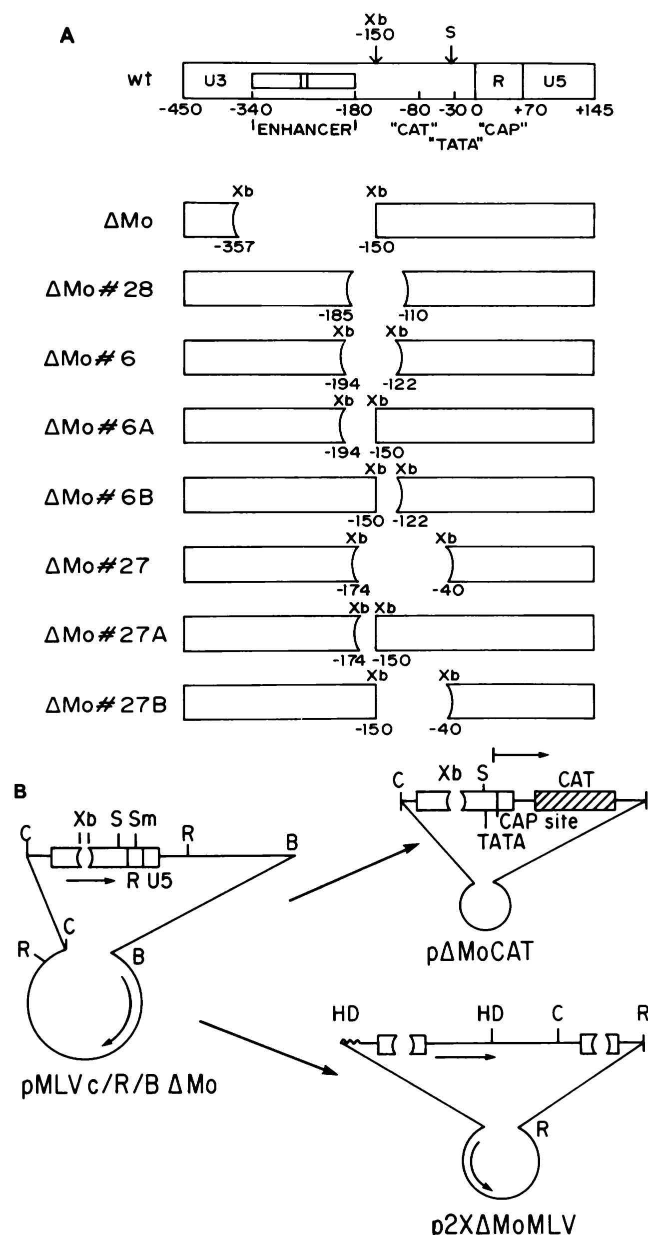

FIG. 1. M-MuLV LTR and its deletions. (A) Diagram of the wild-type M-MuLV LTR is shown at the top. Numbering of bases is from the transcriptional start site (cap) at the U3-R junction. The tandemly repeated enhancers are shown, as well as the location of the TATA and CCAAT (CAT) homologies. The locations of the restriction endonuclease sites from *Xba*I (Xb) and *Sac*I (S) are also shown. The deleted LTRs are shown below the wild-type LTR, and the extent of the deletions are indicated. All deleted LTRs contain *Xba*I sites (resulting from the addition of *Xba*I linkers) except for ΔMo 28. (B) Recombinant cloning strategy. LTR deletions were initially generated in a plasmid subclone containing a single LTR from a circularly permuted clone of M-MuLV viral DNA, pMLV C/R/B. For transient expression assays, the U3 region of the deleted LTRs was fused to the bacterial *cat* gene to give the derivative ΔMoCAT. The direction and start site of transcription from the M-MuLV LTR is indicated by the arrow. For recovery of infectious virus, the deleted LTR was also used to generate a complete M-MuLV proviral organization with deleted LTRs at both 5′ and 3′ sides, p2XΔMoMLV. A detailed description of the cloning strategies was published previously (15). Restriction enzyme abbreviations: R, *Eco*RI; C, *Cla*I; Xb, *Xba*I; S, *Sac*I; Sm, *Sma*I; B, *Bam*HI; HD, *Hin*dIII.

RESULTS

Generation of the M-MuLV LTR deletions. The deletion mutants and the strategy for their analysis is shown in Fig. 1. Deletions were generated in a subclone of circularly permuted M-MuLV DNA containing the LTR (pMLV C/R/B, Fig. 1B). Random deletions were made at the *Xba*I site at −150 bp in the LTR by cleavage with *Xba*I followed by digestion with exonuclease III and S1. *Xba*I linkers were also added during the religation to regenerate an *Xba*I site at the site of the deletions. The extent of each deletion was determined by nucleotide sequence analysis (14). The altered LTRs were then used to generate transient expression plasmids in which the region of the LTR containing the promoter was fused to the bacterial *cat* gene. The *cat* sequences were fused to the LTR at +30 bp with respect to the LTR transcription start site. The deleted LTRs were also inserted into plasmids containing the M-MuLV coding sequences to yield proviral constructs with altered 5′ and 3′ LTRs. The construction of the *cat* and proviral plasmids has been described previously (12, 15).

The deleted LTRs are shown in Fig. 1A. The ΔMo deletion (−357 to −150 bp) has been described previously (12) and shows no promoter activity by transient assay. The ΔMo LTR lacks the tandemly repeated enhancer sequences, as well as sequences between the tandem repeats and the *Xba*I site. It was not possible to generate infectious virus carrying the ΔMo LTR. The other deletions, ΔMo 28, 6, and 27, removed various amounts of sequence between the enhancers and the TATA box (−30 bp), including sequences with CCAAT homology (centered at −80 bp) and also GC-rich sequences (between the enhancers and the *Xba*I site at −150). In ΔMo 6 and 27, it was possible to generate subsets of these deletions by restoring wild-type M-MuLV sequences to either side of the *Xba*I site. These deletions are referred to as A and B (e.g., ΔMo 6A and 6B); the A derivatives lack sequences only to the 5′ side of the *Xba*I site, while the B derivatives lack sequences to the 3′ side of the *Xba*I site.

Promoter activity of the deleted LTRs. NIH-3T3 cells were transfected with LTR-*cat* plasmids, and LTR promoter activity was assessed by the transient activity of CAT enzyme (6). To quantify the relative activities of the different LTRs, it was important to establish conditions of linearity with respect to the amount of transfected *cat* DNA as well as the incubation time for the CAT reaction. A representative CAT assay is shown in Fig. 2. As shown, the amount of CAT enzyme activity was linear with respect to the amount of transfected *cat* DNA. Under these conditions, the relative amount of CAT activity at a given DNA concentration should be proportional to the relative promoter activity. Graves et al. (8) have shown that deletions within an equivalent region of the Moloney murine sarcoma virus (M-MSV) LTR affect the level of transcription in transient expression assays (S1 analysis of transcribed RNA); furthermore, they showed that these quantitative changes were not complicated by alteration in the start site for transcription.

A summary of the relative activity of the different deleted LTRs is shown in Table 1. The acetylation values resulted from several CAT enzyme assays with independent preparations of LTR-*cat* fusion plasmids. With the exception of the ΔMo LTR, all other deletions showed significant levels of promoter activity, with the least active deletions showing approximately 20% of the wild-type M-MuLV LTR activity. The most extensive deletion, ΔMo 27 (−174 to −40 bp) showed the lowest promoter activity, while ΔMo 6 and 28

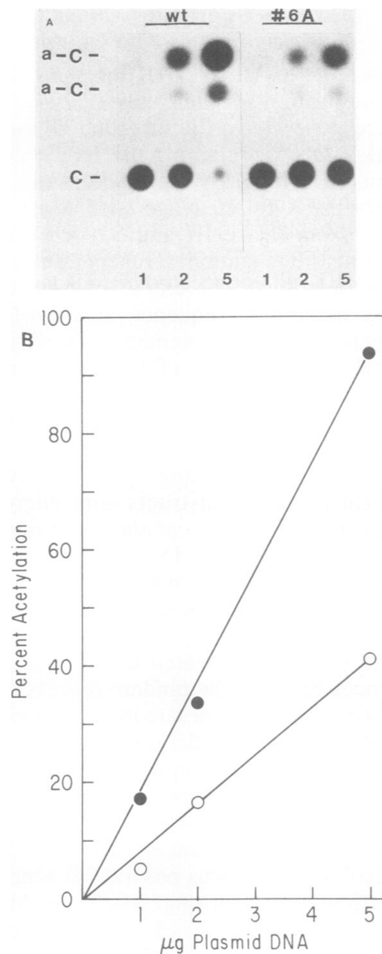

FIG. 2. Transient expression assays. (A) Representative transient expression assays for two LTR-*cat* plasmids, wild-type pMoCAT and pΔMo 6B CAT are shown. Replicate 9-cm dishes of NIH-3T3 cells were transfected with different amounts of the two plasmids. An all cases, 15 μg of calf thymus carrier DNA was also added, as was 50 μM chloroquine. After 48 h of incubation, sonic extracts of the cells were prepared, and equal amounts were assayed for CAT enzyme activity by the addition of [^{14}C]chloramphenicol. After incubation (30 min at 37°C), acetylated chloramphenicol (a-C) was separated from unacetylated chloramphenicol (C) by thin-layer chromatography on silica gel plates. An autoradiogram of the plate is shown. (B) For quantification, the regions of the silica gel plate were excised, and the amount of radioactivity was determined by scintillation counting. The percentage of acetylation achieved is shown. The amount of CAT enzyme activity was proportional to the amount of transfected plasmid DNA under these conditions. Symbols: ●, pMoCAT; ○, pΔMo 6A CAT.

showed promoter activities within 50% of wild type. Subdivision of the deletions generally gave activities between 25 and 75% of wild type. Thus, it appeared that sequences between the enhancers and TATA box were not required for maximal promoter activity in the transient assays. This is consistent with the results of Graves et al. (8). Laimins et al. (10) reported that sequences between the enhancers and the *Xba*I site are required for maximal promoter activity. However, a number of the deletions described here lacked these sequences.

Infectivity of M-MuLVs with altered LTRs. Plasmid DNAs containing M-MuLV proviral DNAs with the altered LTRs at both ends were transfected into NIH-3T3 cells to generate stocks of cells producing the mutant viruses. The transfected cells were serially transferred and assayed at each passage for evidence of XC syncytial plaques. All proviral plasmids except ΔMo 27 produced XC syncytial plaques after the first passage, and confluently infected cultures were obtained within three passages. In cells transfected with ΔMo 27 provirus, cultures remained negative for XC plaques for the first six to eight transfers. Thereafter, small areas of XC syncytial plaques became evident, and cultures became confluently positive in the next two to three transfers. However, the syncytia induced in the ΔMo 27-transfected cultures were generally much smaller than those induced by the other viruses. Southern blot analysis confirmed that these cultures were infected only with ΔMo 27 M-MuLV (not shown).

The relative infectivity of the altered M-MuLVs is shown in Table 2. For these experiments, tissue culture supernatants were harvested from transfected cultures which were XC confluent. The concentration of virus particles in the supernatants was determined by assaying a portion for reverse transcriptase activity. The amount of infectious XC-positive virus present was determined by titration on NIH-3T3 cells. The relative infectivity of the viruses was then obtained by adjusting the infectivity of each viral stock for the amount of virus particles present. Considerably larger differences in infectivity for the different viruses were observed than in the transient expression assays for LTR promoter activity (Table 2). The magnitude of the differences was also somewhat dependent on the infection protocol. A macroscopically visible XC plaque resulted from a group of cells which were originally infected by a single infectious M-MuLV particle. These cells could have arisen either by division of an initially infected cell or by spread of infectious virus to neighboring cells. The density at which the NIH-3T3 cells were seeded prior to infection influences the number of cell divisions an infected cell can undergo prior to reaching confluency, at which point further virus spread is limited. Thus, the appearance of XC plaques on NIH-3T3 cells seeded at relatively high densities is more dependent on cell-to-cell spread of virus than for assays on cells seeded at lower densities. As shown in Table 2, wild-type M-MuLV gave equivalent titers of infectivity when assayed under conditions of higher cell density (3-day assay) or lower cell density (5-day assay). However, deletion mutants with re-

TABLE 1. Activity of deleted LTRs in transient expression assays[a]

LTR	% of wild-type activity
Wild type	100 (7)
ΔMo	<0.1 (3)
ΔMo 28	60 ± 10 (4)
ΔMo 6	60 ± 13 (7)
ΔMo 6A	52 ± 22 (7)
ΔMo 6B	76 ± 14 (7)
ΔMo 27	18 ± 10 (5)
ΔMo 27A	25 ± 13 (4)
ΔMo 27B	35 ± 10 (5)

[a] LTR-*cat* plasmids for each of the deletions were tested by transfection and transient CAT enzyme assay in NIH-3T3 cells as described in the legend to Fig. 2. In each experiment, each plasmid was transfected at three DNA concentrations (1, 2, and 5 μg), and CAT activity relative to the wild-type M-MuLV LTR was determined. The values shown are the averages of several independent experiments, and in all cases more than one preparation of plasmid DNA was used. The numbers in parentheses indicate the number of independent transfections performed for each construct.

TABLE 2. Relative infectivity of M-MuLVs containing deleted LTRs

Expt no.	Virus	Titer of infectious virus[a]		No. of virus particles (cpm of dTTP incorporated)[d]	Normalized infectivity[c]	
		3-Day assay	5-Day assay		3-Day assay	5-Day assay
1	Wild type	3.0×10^6	5.0×10^6	9.7×10^5	1.0	1.0
	ΔMo 28	1.0×10^5	5.0×10^5	13.0×10^5	0.03	0.08
	ΔMo 6	1.0×10^4	6.0×10^4	3.2×10^5	0.01	0.04
	ΔMo 6A	2.0×10^4	3.0×10^5	8.9×10^5	0.006	0.06
	ΔMo 6B	4.0×10^6	4.0×10^6	10.7×10^5	1.19	0.71
2	Wild type	—[d]	1.4×10^6	6.7×10^5	—	1.0
	ΔMo 27	—	$<10^0$	5.0×10^4	—	$<10^{-5}$
	ΔMo 27A	—	1.0×10^6	8.0×10^5	—	0.6
	ΔMo 27B	—	8.8×10^2	2.4×10^5	—	0.002

[a] Tissue culture supernatant was harvested from infected cells in standard conditions (10 ml of medium incubated with 10^6 cells for 24 h) to provide the viral stocks. The concentration of infectious virus was determined by titration on NIH-3T3 cells with the UV-XC assay. For the 3-day assays, the NIH-3T3 cells were seeded at 2×10^5 per 9-cm dish and reached confluency 3 days after infection. For the 5-day assays, the cells were seeded at 10^5 per 9-cm dish. UV irradiation and XC cell overlay were done at 3 and 5 days postinfection, respectively.

[b] Portions of the same culture supernatants used for the infectivity measurements were concentrated by centrifugation, and the number of virus particles was quantified by the reverse transcriptase assay with exogenously added poly(rA):oligo(dT) template-primer (4). Values shown are for a 1-h reaction, per milliliter of supernatant. Typical incorporation values ranged from 10^4 to 10^5 cpm. Supernatant from uninfected cells contained less than 2,000 cpm.

[c] Normalized values were determined by dividing the values for infectious titer by the reverse transcriptase values for the same supernatant. The values are expressed relative to the infectivity of wild-type M-MuLV, which was assigned a value of 1.

[d] —, Not done.

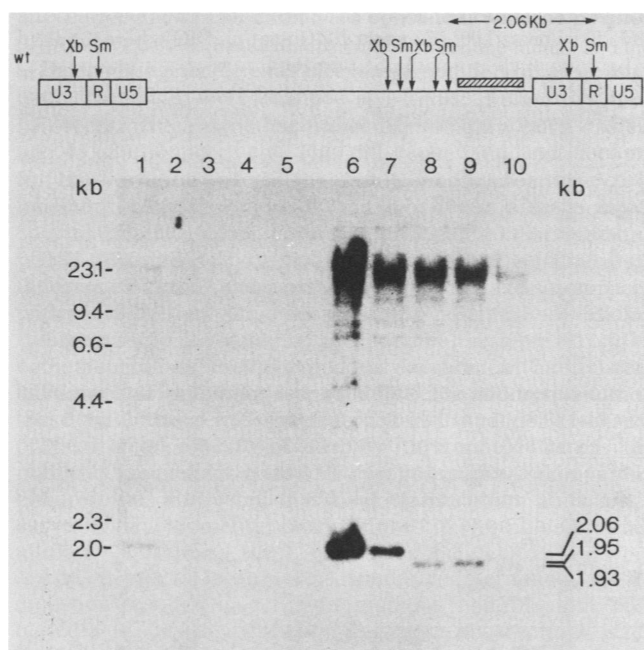

FIG. 3. Viral DNA in infected cells. NIH-3T3 cells were infected with equal amounts of wild-type (wt), ΔMo 27, or ΔMo 27B M-MuLV, as determined from reverse transcriptase activities of the stocks. For wild-type M-MuLV, the multiplicity of infection was 0.5 to 1.0 XC PFU/cell. After 48 h of incubation, nuclei were prepared and fractionated into high-molecular-weight and low-molecular-weight DNA by the Hirt procedure. The DNAs were digested with *Sma*I and analyzed by Southern blot hybridization. The probe used to detect virus-specific DNA was a ^{32}P-labeled *Hpa*I fragment, shown in the diagram above the autoradiograph as a hatched box. Xb, *Xba*I; Sm, *Sma*I. Lanes 1 to 5, Hirt supernatant (low-molecular-weight) DNA; lanes 6 to 10, Hirt pellet (high-molecular-weight) DNA. Lanes 1 and 6, DNA from cells chronically infected with wild-type M-MuLV (clone A9 [4]); lanes 2 and 7, cells freshly infected with wild-type M-MuLV; lanes 3 and 8, cells infected with ΔMo 27 M-MuLV; lanes 4 and 9, cells infected with ΔMo 27B; lanes 5 and 10, uninfected NIH-3T3 cells. M-MuLV DNA results in a 2.0- to 2.1-kilobase (kb) *Sma*I fragment which hybridizes with the labeled probe. The corresponding *Sma*I fragments from ΔMo 27 and ΔMo 27B M-MuLV DNA are 1.93 and 1.95 kilobases, respectively. The hybridization to high-molecular-weight DNA in lanes 6 to 10 results from endogenous MuLV proviruses present in all mouse cells; endogenous viral DNA is highly methylated and resistant to digestion with *Sma*I.

duced infectivity showed more pronounced effects in the 3-day assays than in the 5-day assays (e.g., ΔMo 6 M-MuLV). This result is consistent with these viruses having reduced virus production or reduced efficiency of propagating infectious virus.

The results shown in Table 2 indicated that two deletions between the tandemly repeated enhancer sequences and the *Xba*I site at −150 bp reduced viral infectivity 20-fold (100-fold in the 3-day assay; ΔMo 28 and 6A). On the other hand, infection with ΔMo 27A M-MuLV was the same as with the wild type, as was a deletion of equivalent size (approximately 30 bp) to the 3′ side of the *Xba*I site (ΔMo 6B). Thus, the sequences absent from ΔMo 27A and 6B are not essential to virus replication. Further deletion to the 3′ side of the *Xba*I site to remove the promoter-proximal (but not the TATA) sequences drastically reduced infectivity (three orders of magnitude: ΔMo 27B, −150 to −40 bp). This suggests that the sequences between −122 and −40 bp (the difference between ΔMo 6B and 27B) are very important for virus replication. The largest deletion, ΔMo 27, which removed sequences to both the 5′ and 3′ side of the *Xba*I site, had no measurable infectivity by the XC assay. However, after several passages of the infected cells, virus production could be detected by the reverse transcriptase assay. Cultures producing ΔMo 27 M-MuLV generally produced less virus than wild-type M-MuLV-infected cultures (Table 2).

Detailed analysis of infection by ΔMo 27 M-MuLV. Infection by the two most defective viruses, ΔMo 27 and 27B, was studied in more detail. Since the LTRs function in viral DNA synthesis and integration as well as in transcription, it was important to differentiate between these processes. ΔMo 27- and 27B-infected cells were examined for their ability to

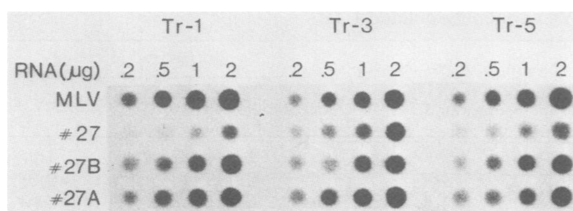

FIG. 4. Virus-specific RNA in infected cells. NIH-3T3 cells were infected with wild-type (MLV), ΔMo 27, and ΔMo 27B M-MuLVs as described in the legend to Fig. 3. Cytoplasmic RNA was extracted, and total cytoplasmic RNA was analyzed by dot-blot hybridization with the same labeled M-MuLV DNA as in Fig. 3. The infected cells were transferred (1:10) serially, and the analysis was repeated for transfers 1, 3, and 5 (Tr-1, Tr-3, and Tr-5, respectively). An autoradiogram of the dot-blot is shown. There was no visible hybridization of the labeled probe with RNA extracted from uninfected NIH-3T3 cells (not shown).

synthesize and integrate proviral DNA (Fig. 3). NIH-3T3 cells were infected with wild-type or mutant virus stocks at high multiplicity. The multiplicity of infection for wild-type M-MuLV was approximately 0.5 to 1. The mutant-infected cells were infected with equal numbers of virus particles (as measured by the reverse transcriptase assay), even though such cells would show many fewer XC plaques. High-molecular-weight and low-molecular-weight nuclear DNA from the infected cells was separated by the Hirt fractionation procedure (9). The DNA was digested with *Sma*I endonuclease, and the relative amount of high-molecular-weight nuclear (integrated) proviral DNA was determined by agarose gel electrophoresis and Southern blot hybridization.

ΔMo 27 and 27B-M-MuLV showed levels of integrated proviral DNA comparable to wild-type M-MuLV-infected cells. There was at most a threefold difference in the level of integrated proviral DNA, which contrasts with the greater than five-log difference in relative infectivity. It should also be noted that ΔMo 27 and 27B M-MuLVs, which themselves differed by two orders of magnitude in infectivity, showed the same amount of integrated proviral DNA. Unintegrated (Hirt supernatant) viral DNA was not present at significant concentrations in nuclei from either wild-type- or mutant virus-infected cells. Thus, these mutants did not appear to be markedly deficient in viral DNA synthesis or integration.

The minor difference in proviral DNA levels in mutant and wild-type virus-infected cells (Fig. 3) may have resulted from the multiplicity of infection. To perform the infections with equal numbers of virus particles for the mutant and wild-type virus stocks, the multiplicity of infection was somewhat less than 1, which could ensure that each cell was initially infected with virus. During the 2-day incubation period, secondary virus spread in the wild-type M-MuLV-infected culture could have increased the amount of proviral DNA, while it is unlikely that this could occur in cultures infected with ΔMo 27 and 27B.

ΔMo 27, 27A, and 27B M-MuLV-infected cells were also examined for steady-state levels of viral RNA (Fig. 4). ΔMo 27A and 27B M-MuLV-infected cells contained two- to fourfold less virus-specific RNA than wild-type M-MuLV-infected cells. ΔMo 27 M-MuLV-infected cells had approximately 10-fold less virus-specific RNA than wild-type M-MuLV-infected cells, but viral RNA was easily detectable. Thus, as predicted from the infectivity and CAT assays, the mutant-infected cells showed lower levels of virus-specific RNA. However, the levels detected were somewhat surprising given the extremely low infectivity of ΔMo 27 M-MuLV as measured by the XC plaque assays.

One potential concern with the viral analyses could be that virion RNA from the infecting virus might have hybridized and given an overestimate of viral RNA synthesis in the mutant-infected cells. To avoid this potential problem, steady-state viral RNA levels were measured after the infected cells were transferred one, three, and five times. The viral RNA levels in the infected cells did not change significantly with passage (Fig. 4), although input viral RNA would decrease with continued cell growth.

To further minimize the effects of input virion RNA, synthesis of envelope-specific viral protein was examined (Fig. 5). Expression of *env* glycoprotein was chosen for two reasons. First, *env* glycoprotein is expressed from spliced subgenomic mRNA (21); this RNA arises by splicing of newly synthesized viral RNA. In contrast, *gag* polyprotein is synthesized on unspliced mRNA, and infecting virion RNA can actually be directly translated during infection (5). Second, the basis of the XC syncytial assay is *env* glycoprotein. Cells infected with ΔMo 27 M-MuLV made significantly less $Pr80^{env}$, consistent with lack of XC syncytia. However, the mutant-infected cells did in fact express low levels of *env* glycoprotein—one-tenth the level of comparable wild-type M-MuLV-infected cells. This suggests a threshold level of *env* glycoprotein before infected cells can induce XC syncytia formation.

DISCUSSION

In the experiments described here, the effects of deletions within the M-MuLV LTR were measured by transient expression assays on LTR-*cat* plasmids and also by infectivity assays on infectious virus carrying the altered LTRs. It was important to use both techniques since possible artifacts exist for either method. In the transient expression assays, the tested sequences are not in standard chromatin structure. Furthermore, they are present at very high copy number in the cells, which might titrate out transcription factors (7). Thus, the quantitative effects might not accu-

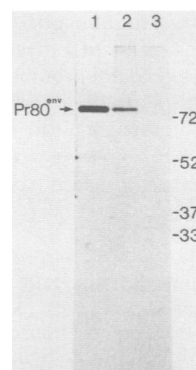

FIG. 5. Viral envelope protein in infected cells. NIH-3T3 cells were infected with wild-type and ΔMo 27 M-MuLV as described in the legend to Fig. 3. Two days after infection, cells were labeled for 1 h with [^{35}S]methionine. Cytoplasmic extracts were prepared, immunoprecipitated with an anti-M-MuLV gp70 serum (3), and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12.5% gel. An autoradiogram of the gel is shown. Lane 1, cells infected with wild-type M-MuLV; lane 2, cells infected with ΔMo 27 M-MuLV; lane 3, uninfected cells. Each lane contains the immunoprecipitate from 5×10^6 cpm of labeled cell extract. $Pr80^{env}$ is the predominant *env*-specific protein labeled under these conditions. Sizes are indicated in kilodaltons.

rately mirror the situation for the same promoter when integrated at low copy number in standard chromatin. The study of infectious M-MuLV with the deleted LTRs avoids these problems since proviral DNA is inserted into host cell DNA at low copy number, and it takes on the configuration of standard chromatin. Infectivity assays are also more sensitive, since it is possible to detect differences in infectivity over at least six orders of magnitude by standard titration methods. The major drawback to the infectivity measurements is that there may be a nonlinear relationship between infectivity and promoter activity. As described in Results, factors such as the rate of viral spread and threshold *env* requirements for a positive XC syncytial reaction may accentuate differences in infectivity which result from smaller changes in promoter activity. To some extent, these factors can be minimized by measuring viral RNA and protein levels shortly after high-multiplicity infection (Fig. 4 and 5).

In general, the differences in promoter activity as measured by the transient expression assay were relatively minor. Many of the deletions showed CAT activities within 50% of wild type, and the most defective deletions still showed 20 to 25% of wild-type activity. In contrast, when the same deletions were analyzed as infectious virus, much larger differences were seen. Moreover, some deletions which had very similar promoter activities in the transient assays showed marked differences when analyzed as infectious virus (e.g., ΔMo 27A and 27B). These results raise a general note of caution for both viral and nonviral genes. It is important to verify conclusions about promoter activities obtained from transient expression data with independent analyses, preferably on the same promoters present in the appropriate copy numbers and chromatin configurations (e.g., low copy number in standard chromatin for cellular genes).

We have also examined the infectivity of M-MuLVs containing other LTRs which show decreased activity in the transient assay. In particular, one LTR which contained an inversion of the enhancer and GC-rich sequences (Mo^-) also showed CAT activity which was 20% of the wild-type activity (4a). However, in this case relative infectivity of Mo^- M-MuLV was 5% of wild type, at least three logs higher than that of ΔMo 27 M-MuLV.

The deletion mutants described here divided the U3 region of the LTR between the tandemly repeated enhancers and the TATA box into three functional regions. Sequences around the *Xba*I site (−174 to −122 bp) are apparently nonessential, since they could be deleted without affecting viral infectivity and with little effect on promoter activity in transient assays. On the other hand, sequences between the enhancers and the *Xba*I site, and also those proximal to the TATA box, appear to be required for maximal infectivity.

Deletion of sequences between the enhancers and the *Xba*I site gave a moderate (20- to 100-fold) reduction in viral infectivity. Comparison of ΔMo 6A and 27A indicates that the critical sequences lie between −195 and −174 bp. In fact, if the reduced infectivity of ΔMo 28 (also 20- to 200-fold) results only from deletion of sequences to the 5′ side of the *Xba*I site, this would further narrow the critical sequences to between −185 and −174 bp. The region between the enhancers and the *Xba*I site has also been implicated for efficient promoter activity by Laimins et al. (10) on the basis of transient expression assays. However, our CAT assays did not show dramatic reductions for the deletions in this region. One possible explanation for the difference between these results and those of Laimins et al. could be the nature of the deletions examined. The deletions studied here were internal deletions which retained all other LTR sequences; in the deletions studied by Laimins et al., sequences to the 5′ side of the tandemly repeated enhancers were also absent. Laimins et al. also pointed out a homology in this region (−182 to −155 bp) with the GC-rich sequences in the 21-bp repeats of the simian virus 40 origin of replication. The 21-bp repeats of simian virus 40 have been shown to bind a specific cellular transcription factor, SP-1 (2). The results reported here are consistent with the role of the GC-rich sequences in efficient virus infectivity, although the portion of GC-rich sequences between −174 and −155 bp are nonessential (i.e., ΔMo 27A).

The importance of the sequences between the enhancers and the *Xba*I site in transient LTR expression is also evident in undifferentiated F9 embryonal carcinoma (EC) cells. Gorman et al. (7) recently reported that promoters lacking enhancers can function in F9 EC cells. Furthermore, they concluded that negative regulatory factors bind to the M-MSV LTR in undifferentiated F9 EC cells, which prevents transcription. Deletion of the tandem repeats (but not the GC-rich sequences) resulted in an LTR which could function in undifferentiated F9 EC cells. On the other hand, we previously reported that ΔMo LTR (deleted from −357 to −150 bp) was not functional in the same cells (12). Together, these experiments suggest an absolute requirement for the GC-rich sequences for LTR expression in undifferentiated F9 EC cells.

The sequences proximal to the promoter appeared to be the more important ones for LTR expression when integrated provirus was analyzed. The exact nature of the defect for mutants lacking these sequences remains to be completely elucidated. The results in Fig. 3 indicated that proviral DNA synthesis and integration were not affected, which suggests that viral RNA or protein synthesis was deficient. Indeed, viral protein synthesis by ΔMo 27 M-MuLV was reduced (Fig. 5). However, the residual level of protein synthesis, as well as the steady-state RNA level in infected cells, was surprising in light of the extreme defectiveness. Comparison of the ΔMo 6B and 27B deletions suggests that the important sequences lie between −122 and −40 bp, and the behavior of the ΔMo 28 (−185 to −110) deletion further suggests that the critical sequences lie between −110 and −40 bp. This region contains the CCAAT homology at −80 bp. Srinivasan et al. (17) also reported a 10-fold reduction in focus-forming efficiency during transfection of Abelson murine leukemia virus DNAs which lacked the CCAAT sequences. The difference between the transient expression and the infectivity assays was pronounced for deletions in this region. Deletion of these sequences had a relatively minor effect on promoter activity as measured by CAT assays. This is consistent with the results of Graves et al. (8), who concluded that the promoter-proximal (CCAAT) sequences are not required for transient M-MSV LTR activity when the enhancer sequences are present. Yet deletion of these sequences reduced viral infectivity 3 to 5 logs.

Another possible explanation for the low infectivity of mutants lacking the promoter-proximal sequences could be that the deletions decreased viral RNA stability. The deleted sequences would be missing from the 3′ end of viral RNA. However, such a decrease in viral RNA stability appears unlikely, since cells infected with these mutants actually showed steady-state levels of viral RNA which were higher than predicted from their relative infectivity (Fig. 4).

It seems possible that the LTR regions identified in our experiments might bind protein factors which are important

in viral transcription. In this light, chromatin studies of proviral DNA are interesting. We have mapped DNase I-hypersensitive sites in the wild-type M-MuLV LTR (18). Recent high-resolution experiments have identified the two major DNase I-hypersensitive sites in the enhancer sequences and one at the TATA box. Interestingly, no DNase I-hypersensitive site was detected in the region of CCAAT sequences, and only a very weak site was detected in the region of the GC-rich sequences (T. Thompson and H. Fan, unpublished). Thus, these sequences do not appear to organize chromatin structure even though they are important for expression.

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