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# Long Terminal Repeat Enhancement of v-*mos* Transforming Activity: Identification of Essential Regions

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The transforming efficiency of recombinant DNA clones containing the Moloney sarcoma virus v-*mos* sequence was enhanced by introducing the Moloney sarcoma virus long terminal repeat (LTR) in either the 5′ or 3′ position relative to v-*mos*. We analyzed the polyadenylated RNA expressed in cells transformed by these recombinant DNA clones and examined the structural integrity of integrated copies of the DNA. In each case, we demonstrated the presence of v-*mos* containing RNA transcripts in the polyadenylated RNA and showed that these RNA transcripts are consistent with the structure of the transfected DNA. The analysis of DNA from these transformed cells showed that the relative positions of the v-*mos* and LTR sequences within the transfected DNA were conserved in the integrated DNA copies. These results demonstrate that a single LTR can successfully enhance the transforming activity of v-*mos* from either a 5′ or a 3′ relative position. The results from the transfection analysis of recombinant clones containing only portions of the LTR introduced 3′ to v-*mos* demonstrate that the essential region of the LTR responsible for the enhancement of transformation is a region within the unique 3′ sequences of the LTR containing the 73-base-pair tandem repeat sequence.

The essential components of the Moloney murine sarcoma virus (MSV) proviral genome responsible for cell transformation are the acquired v-*mos* sequence and the proviral long terminal repeat (LTR) (7, 18). The retroviral LTR has been shown to contain transcriptional control elements that function to ensure viral RNA transcription of the provirus (9, 15, 29, 30, 33). The results from transfection assays using subgenomic MSV proviral DNA clones have shown that the LTR enhances the transforming activity of v-*mos* with equivalent efficiency from either a 5′ or a 3′ position relative to v-*mos* (7). Although these results suggest that a single LTR can enhance the transforming activity of v-*mos*, these assays do not provide evidence that will exclude the possibility that tandem integrations or rearrangements of the transfected recombinant DNA are responsible for the enhancement of the transforming activity. In this report, we present the analysis of polyadenylated RNA expressed in cells transformed by the transfection of recombinant DNA clones containing v-*mos* and a single LTR and examine the structure of the integrated form of the transfected DNA in these cells. Furthermore, to determine the essential region of the LTR responsible for enhancement, we constructed a series of recombinant DNA clones containing only portions of the LTR introduced 3′ to v-*mos* and tested these recombinant DNAs in transfection assays.

## MATERIALS AND METHODS

**DNA transfections and development of transformed cell lines.** Maps of the restriction endonuclease sites present in recombinant DNA clones of HT1 and m1 strains of MSV proviral DNA have been published elsewhere (19, 34). Descriptions of the subgenomic proviral recombinant DNA clones used in this study have been reported elsewhere (7) or are described below. DNA transfection of NIH3T3 cells was performed by modifications of established procedures (11) as previously described (7). Morphologically transformed cells were selected from individual foci by single-cell cloning either in agar or by serial dilution in microwell tissue culture dishes. The cells were maintained in Dulbecco modified minimal essential medium (GIBCO Laboratories) supplemented with 10% (vol/vol) calf serum and antibiotics.

**DNA analysis.** The cells were lysed with a buffer containing 0.6% (wt/vol) sodium dodecyl sulfate, 10 mM EDTA, 10 mM Tris-hydrochloride (pH 7.5), and 100 μg of pancreatic RNase A per ml which had been incubated at 100°C for 5 min. The lysate was incubated at 37°C for 1 h. Proteinase K (Boehringer Mannheim) was added to a final concentration of 250 μg/ml, and the incubation was continued for 2 h at 37°C. The mixture was then extracted once with an equal volume

of phenol saturated with 1 M Tris-hydrochloride (pH 8.0), twice with phenol-$CHCl_3$ (1:1), and once with $CHCl_3$. DNA was precipitated with 2 volumes of ethanol and dissolved in sterile $H_2O$.

Restriction endonuclease (New England Biolabs) digests were performed under the conditions recommended by the manufacturer. DNA samples (12 to 15 μg) were applied to 0.75% (wt/vol) agarose gels containing 0.5 μg of ethidium bromide per ml, and DNA fragments were separated by electrophoresis as reported by McDonell et al. (20). *Hin*dIII digests of lambda DNA were included in each analysis for estimating DNA fragment size. The transfer of DNA to nitrocellulose membranes (Schleicher & Schuell Co.) was performed as described by Southern (31). The blots were dried for 2 h at 80°C and pretreated with a solution containing 50% (vol/vol) formamide (Fluka), 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.2% (wt/vol) Ficoll (Pharmacia Fine Chemicals), 0.2% (wt/vol) polyvinylpyrrolidone, 50 mM sodium phosphate (pH 6.5), 1% (wt/vol) glycine, 0.1% (wt/vol) sodium dodecyl sulfate, and 250 μg of sheared salmon sperm DNA (Sigma Chemical Co.) per ml for 16 h at 43°C. After hybridization, the blots were washed three times in 2× SSC at room temperature and three times in 0.1× SSC for 15 min at 50°C. The blots were then air dried overnight at room temperature. Autoradiography was performed with Kodak SB-5 film and an intensifying screen at −70°C.

**RNA analysis.** The cells (0.3 to 0.5 ml of packed cells) were lysed by homogenization in buffer containing 25 mM Tris-hydrochloride (pH 7.5), 25 mM NaCl, 5 mM $MgCl_2$, 1 mg of heparin (Sigma) per ml, and 2% Triton X-100 (Bio-Rad Laboratories). After centrifugation of the lysate at 15,000 × *g* for 10 min at 4°C, the supernatant was mixed with an equal volume of the above buffer containing 200 mM $MgCl_2$. This mixture was incubated for 2 h at 0°C and then layered over a solution of 1 M sucrose, 25 mM Tris-hydrochloride (pH 7.5), 25 mM NaCl, and 100 mM $MgCl_2$. $Mg^{2+}$-complexed polysomes were pelleted by centrifugation at 15,000 × *g* for 20 min at 4°C. The pellet was suspended in 200 μl of 100 mM EDTA containing 1 mg of proteinase K per ml and then mixed with 5 ml of a buffer containing 25 mM Tris acetate (pH 7.5), 600 mM sodium acetate, 2 mM EDTA, and 0.5% sodium dodecyl sulfate. The mixture was homogenized and incubated at 45°C for 3 min. Polyadenylated RNA was selected on oligodeoxythymidylate-cellulose (P-L Biochemicals, Inc.) as previously described (2). RNA samples were stored under ethanol at −20°C. RNA was collected by centrifugation at 12,000 × *g* for 5 min, and the RNA pellets were dried under vacuum. RNA samples were denatured in 15 mM methyl mercury hydroxide (Alfa) for 10 min at room temperature and separated by electrophoresis on 1.2% (wt/vol) agarose gels containing 5 mM methyl mercury hydroxide (1, 3). The transfer of RNA to diazobenzyloxymethyl paper (Schleicher & Schuell) and the subsequent treatment of the blots for hybridization and autoradiography were performed as described by Alwine et al. (1).

**DNA probes.** The restriction endonuclease sites used in isolating specific DNA fragments are shown in Fig. 1. p-*mos*, a plasmid containing the entire c-*mos* sequence and 1.9 kilobases (kb) of normal mouse DNA 5′ to c-*mos* cloned into pBRSc7 (see the legend to Fig. 5) at the *Sac*I and *Hin*dIII sties, was used as the source of the *mos*-specific DNA fragment. pm1sp, a plasmid containing one copy of the m1 MSV proviral LTR plus the 5′ and 3′ cellular sequences flanking the m1 MSV

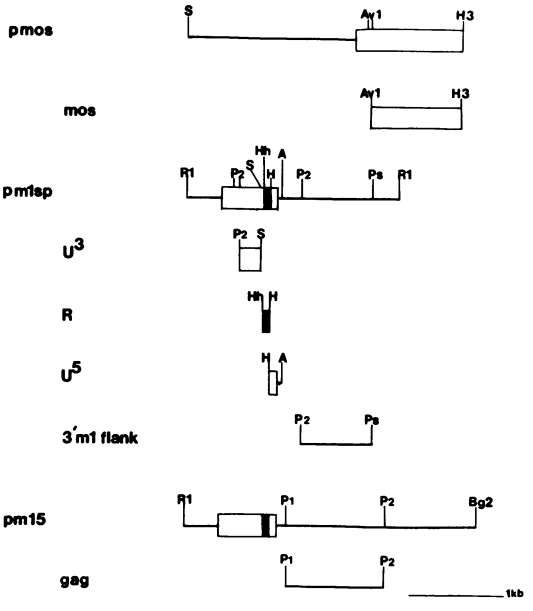


FIG. 1. DNA fragments used to prepare nick-translated DNA probes. Shown are restriction endonuclease sites present in three cloned DNA inserts (p-*mos*, pm1sp, pm15) that have been cloned into pBR322 (7, 24). These restriction sites were used in isolating DNA fragments (see the text) that represent specific DNA sequences used in the preparation of nick-translated probes. The size of the DNA fragments was estimated by comparison with the electrophoretic mobility of known DNA size markers in either agarose (20) or polyacrylamide gels (17). p-*mos*, a plasmid that contains the endogenous c-*mos* sequence (24), was used in isolating a 930-bp *Ava*I-*Hin*dIII *mos*-specific DNA fragment. pm1sp is a plasmid containing one copy of the m1 MSV proviral LTR plus the 5′ and 3′ cellular sequences flanking the m1 MSV provirus (19). A 215-bp DNA fragment that represents the U3 region of the LTR was isolated from *Pvu*II-*Sac*I digests of pm1sp DNA. A 70-bp *Hha*I-*Hin*fI DNA fragment containing all of the R sequence and a *Hin*fI-*Alu*I DNA fragment that contains the entire unique 5′ region of the LTR and 34 bp of 3′ cellular flanking sequence were also isolated from this plasmid. A 700-bp DNA fragment representing the 3′ cellular flanking sequences was isolated from *Pvu*II-*Pst*I digests of pm1sp. pm15 is a plasmid containing the 5′ LTR and 2.0 kb of adjacent virus-specific *gag* sequences cloned from m1 MSV proviral DNA (7). A 1.1-kb *Pvu*I-*Pvu*II DNA fragment representing part of the *gag* coding region of m1 MSV was isolated from this plasmid. Restriction enzymes: A, *Alu*I; Av1, *Ava*I; Bg2, *Bgl*II; H, *Hin*fI; H3, *Hin*dIII; Hh, *Hha*I; P1, *Pvu*I; P2, *Pvu*II; Ps, *Pst*I; R1, *Eco*RI; S, *Sac*I.

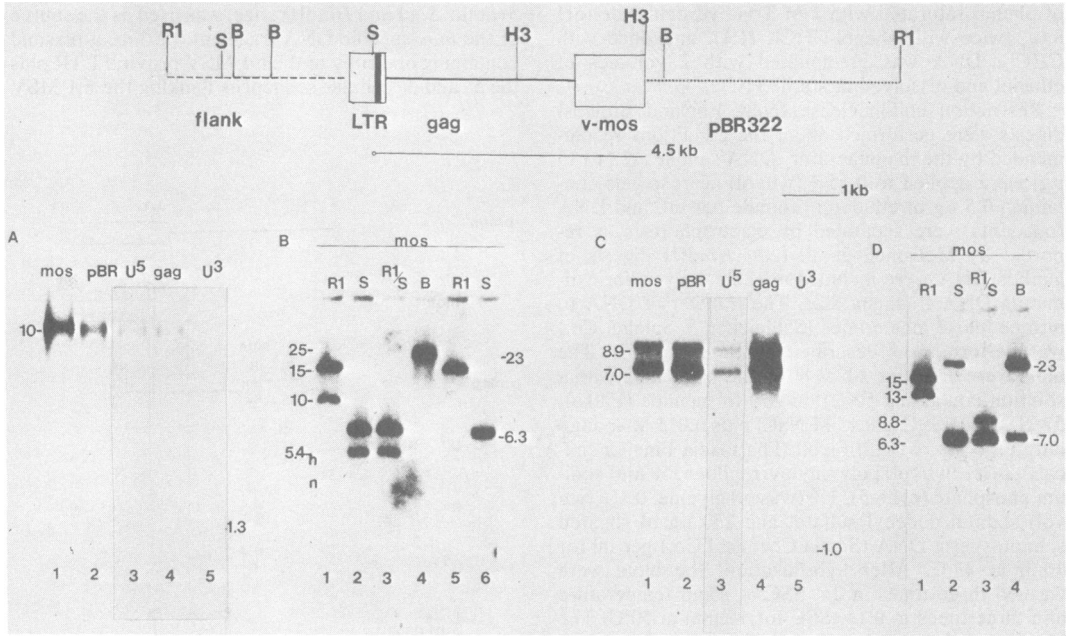


FIG. 2. Analysis of RNA and DNA from pHT25 transfectants. A schematic diagram of the structure of the 12.2-kb pHT25 plasmid DNA is shown, with various proviral, vector, and cellular flanking DNA sequences labeled. Restriction endonuclease sites utilized in analyzing the structure of the integrated DNA are indicated. pHT25 DNA was linearized by *Eco*RI digestion before transfection. The results from the analysis of two independent pHT25 transfectants are shown. (A and C) Results from hybridization analysis of polyadenylated RNA (2.5 μg/lane) isolated from these transfectants. The probes used in analyzing the RNA blots are indicated at the top of each lane, and their preparation is described in the text. The results from hybridization analysis with a *mos* probe of restriction endonuclease digests of cellular DNAs isolated from nontransformed NIH3T3 cells (B, lanes 4 and 5) and the two pHT25 transfectants (B, lanes 1 to 4, and D) are shown. The restriction endonucleases used in the various digests are indicated at the top of each lane. Restriction enzymes: B, *Bam*HI; H3, *Hin*dIII; S, *Sac*I; Rl, *Eco*RI.

provirus (19), was used as a source of DNA fragments representing the unique 3′ region (U3), the repeat sequence (R), and the unique 5′ region (U5) of the LTR, as well as that of a DNA fragment representing the 3′ flanking cellular sequences. pm15, a plasmid containing the 5′ end of m1 MSV proviral DNA, including the LTR and 2.0 kb of virus-specific *gag* sequences (7), was used to isolate a *gag*-specific DNA fragment and the U5 DNA fragment (*Hin*fI-*Pvu*I), which was used as a probe in the hybridization analysis of the pm13 transfectants. A 5.6-kb *Eco*RI DNA fragment that represents the mink cellular DNA target site of HT1 MSV provirus integration (34) was used as a probe for the HT1 MSV cellular flanking sequences (data not shown).

For restriction endonuclease digestion, plasmid DNA fragments were purified by electrophoresis on 5% (wt/vol) polyacrylamide gels (bis:acrylamide, 1:25) (17). The nick translation of purified DNA fragments ($1 \times 10^8$ to $2 \times 10^8$ cpm/μg) was performed as reported by Rigby et al. (28), and all hybridizations of RNA and DNA blots contained $5 \times 10^6$ to $10 \times 10^6$ cpm of the respective DNA probe. Each DNA probe was tested for sequence specificity before use in hybridization assays.

## RESULTS

**Analysis of cells transformed by DNA containing an LTR 5′ to v-*mos*.** The results from DNA transfection assays using cloned recombinant DNAs containing v-*mos* and a single LTR have previously been reported (7, 18). One of these clones, pHT25, was derived from HT1 MSV proviral DNA and contains the 5′ LTR and adjacent proviral sequences through v-*mos* cloned into pBR322 (Fig. 2).

The analysis of RNA and DNA from two independent pHT25 transfectants is shown in Fig. 2. If the LTR provides transcriptional control elements that insure the expression of v-*mos*, then transcription should be initiated at the repeat (R) sequence within the 5′ LTR, and the resulting RNA transcripts would contain U5, *gag*, and v-*mos* sequences. In one pHT25 transfectant, a single 10-kb RNA was detected with *mos*, U5, and *gag* probes (Fig. 2A, lanes 1, 3, and 4). The second pHT25 transfectant contained two RNA transcripts (8.9 and 7.0 kb) that

hybridized with *mos*, *gag*, and U5 probes (Fig. 2C, lanes 1, 3, and 4). The predicted primary transcript for an RNA initiated at the R sequence within the LTR and containing all of the viral sequences encoded in pHT25 is 4.5 kb, but all of the *mos*-containing RNA transcripts observed in these cells were in excess of this size. Some of this additional information can be attributed to the exression of pBR322 vector sequences since a probe to this DNA detected the same RNA transcripts as the *mos*, *gag*, and U5 probes (Fig. 2A and C, lane 2). However, the 10-kb RNA transcript (Fig. 2A) would still require additional sequences derived from host or carrier DNA even if we assume that a complete pBR322 vector sequence was expressed. A probe representing the U3 region of the LTR did not hybridize to any of the *mos*-containing RNA transcripts (Fig. 2A and C, lane 5). This demonstrates that the discrete RNA transcripts observed are not derived from tandem integrations of the transfected DNA and suggests that the termination and polyadenylation signals utilized by these RNA transcripts are acquired from either vector, host, or carrier DNA sequences. A U3 probe did detect a 1.3- and a 1.0-kb RNA transcript (Fig. 2A and C, lane 5) in the pHT25 transfectants analyzed; however, these transcripts did not hybridize with *mos*-specific probes.

To determine the number of integrated copies of pHT25 DNA and to assess the structural integrity of the integrated DNA sequences in the two pHT25 transfectants, we analyzed the cellular DNA by digestion with restriction endonucleases and by Southern analysis. *Eco*RI-digested DNA from nontransformed NIH3T3 cells contained a 15-kb DNA fragment which hybridized with a *mos* probe (Fig. 2B, lane 5). This DNA fragment corresponds to the endogenous *Eco*RI c-*mos* DNA fragment found in normal mouse cellular DNA (14, 24). The *mos* probe detected the 15-kb c-*mos* DNA fragment and a new 10-kb DNA fragment (Fig. 2B, lane 1). The size of the latter *Eco*RI DNA fragment suggests that *Eco*RI sites at the ends of the transfected pHT25 DNA have not been conserved in the integrated copy and that a portion of the transfected DNA was deleted. The extent of this deletion could be estimated from *Sac*I and *Bam*HI DNA digests (Fig. 2B, lanes 2 and 4). *Sac*I digests contained a 6.3-kb c-*mos* DNA fragment and a 5.4-kb DNA fragment derived from the transfected pHT25 DNA (Fig. 2B, lanes 2 and 4). A single *Sac*I site was present in the LTR, whereas no *Sac*I sites existed in the adjacent viral or vector sequences of the transfected pHT25 DNA (Fig. 2, schema). The 5.4-kb *mos*-containing DNA fragment is, therefore, generated from a second *Sac*I site acquired from either host or carrier DNA sequences, and its size implies that a minimum of 3 kb of pBR322 vector sequences has been deleted from the integrated DNA. pHT25 DNA also contained a 7.0-kb *Bam*HI fragment (Fig. 2). This DNA fragment was not conserved in the integrated DNA, indicating that potentially all but 340 base pairs (bp) of the pBR322 sequences have been deleted from the integrated copy. A comparison of the abundance of the endogenous c-*mos* DNA fragment relative to that of the new *mos*-containing DNA fragments observed in this pHT25 transfectant suggests that a single copy of the pHT25 DNA is present in these transformed cells (Fig. 2B, lanes 1 through 4), which would imply that the 10-kb *mos*-containing RNA (Fig. 2A) detected in these cells results from transcription from this single copy of pHT25 DNA (Fig. 2B).

The 8.9- and 7.0-kb *mos*-containing RNA transcripts (Fig. 2C) expressed in the second pHT25 transfectant also appear to be transcribed from a single integrated copy of pHT25 DNA (Fig. 2D, lanes 1 through 4). *Eco*RI digests of cellular DNA from this transfectant contained the 15-kb c-*mos* DNA fragment and a new 13-kb DNA fragment that hybridized with the *mos* probe (Fig. 2D, lane 1). Probes representing pBR322 or the cellular flanking sequences present in plasmid pHT25 DNA also anneal with a 13-kb *Eco*RI DNA fragment (data not shown). The *mos* probe hybridized to a 7.0-kb *Bam*HI DNA fragment (Fig. 2D, lane 4) and an 8.8-kb DNA fragment in *Eco*RI-*Sac*I digests (Fig. 2D, lane 3). Both of these DNA fragments correspond to the predicted size of their respective *mos*-containing DNA fragments in pHT25 plasmid DNA. These results imply that essentially all of the transfected pHT25 DNA has been conserved in this transfectant. *Sac*I digestion of DNA from these cells produced a 23-kb *mos*-containing DNA fragment (Fig. 2D, lane 2), demonstrating that the pHT25 DNA sequences are integrated in the cellular DNA. The results from the analysis of DNA from both of these pHT25 transfectants show that a single LTR introduced 5′ to the v-*mos* sequence can effect the expression of *mos*-containing RNA transcripts.

**Analysis of cells transformed by DNA containing an LTR 3′ to v-*mos*.** pHT21 is a recombinant DNA plasmid derived from HT1 MSV proviral DNA containing a single LTR 3′ to the v-*mos* sequence and cloned into pBR322 (Fig. 3). Five *mos*-specific RNA transcripts ranging in size from 6.3 to 3.2 kb were detected in the pHT21 transfectant (Fig. 3A, lane 1). An identical RNA pattern was obtained by hybridizations with a U3 probe (Fig. 3A, lane 2), whereas none of the *mos*-containing RNA transcripts annealed to the U5 probe (Fig. 3A, lane 4). This result shows

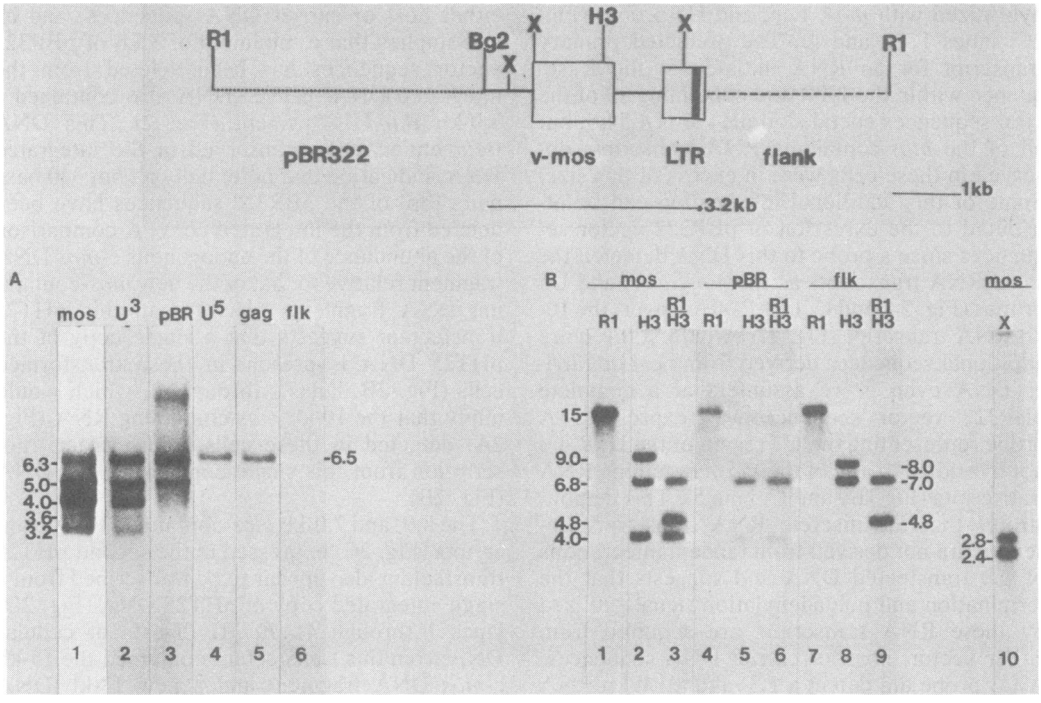


FIG. 3. Analysis of RNA and DNA from a pHT21 transfectant. A schematic diagram of the structure of pHT21 plasmid DNA is shown. The 9.9-kb plasmid DNA was linearized by *Eco*RI digestion before transfection. (A) Results from hybridization analysis of polyadenylated RNA (5 μg/lane) isolated from the pHT21 transfectant. (B) Results from hybridization analysis of DNA from these transformed cells digested with either *Eco*RI, *Hin*dIII, *Eco*RI-*Hin*dIII, or *Xba*I restriction endonucleases. The probes used in the hybridization assays are indicated at the top of the lanes and are described in the text.

that the enhancement of the transforming efficiency of v-*mos* by a 3′ LTR does not result from the tandem integration of the transfected pHT21 DNA. The *mos*-containing RNA transcripts expressed in the pHT21 transfectants presumably terminate in the 3′ LTR, utilizing the LTR polyadenylation signals. The promoter signals and transcription initiation sites for these RNA transcripts are not derived from the transfected LTR and presumably are either present in the Moloney leukemia virus sequences preceding v-*mos* or are acquired from vector, host, or carrier DNA sequences. The size of the *mos*-containing RNA transcripts expressed in this transfectant limit the use of possible promoter signals encoded in the proviral DNA to the expression of only the 3.2-kb transcript. Two of the five *mos*-containing RNA transcripts expressed in the pHT21 transfectant contained sequences that annealed to the pBR322 probe (Fig. 3A, lane 3).

The U5 probe annealed to a 6.5-kb RNA transcript in the pHT21 transfectant (Fig. 3A, lane 4). However, the expression of this RNA transcript is apparently not directly related to the expression of pHT21-encoded sequences. A probe made to the cellular flanking sequence downstream from the 3′ pHT21 LTR did not hybridize to this transcript (Fig. 3A, lane 6), but a probe specific for the *gag* region of MSV, a region not present in pHT21 plasmid DNA, did anneal to the 6.5-kb RNA transcript (Fig. 3A, lane 5). These results are consistent with this transcript representing the expression of an endogenous murine viral RNA. A similar RNA transcript is sometimes observed in polyadenylated RNA from normal NIH3T3 cells, but the level of the expression of this RNA is increased in this pHT21 transfectant and in several other MSV-transformed cells that have been examined (T. Wood, unpublished data). However, all MSV transfectants do not express this RNA transcript, as is shown for the two pHT25 transfectants analyzed in Fig. 2.

To determine the number of integrated copies of pHT21 DNA and to assess the structural integrity of the integrated DNA sequences present in these transformed cells, we analyzed the cellular DNA from the pHT21 transfectant (Fig. 3B). Hybridization with a *mos* probe detected only a 15-kb DNA fragment in *Eco*RI digests of cellular DNA from the pHT21 transfectant (Fig.

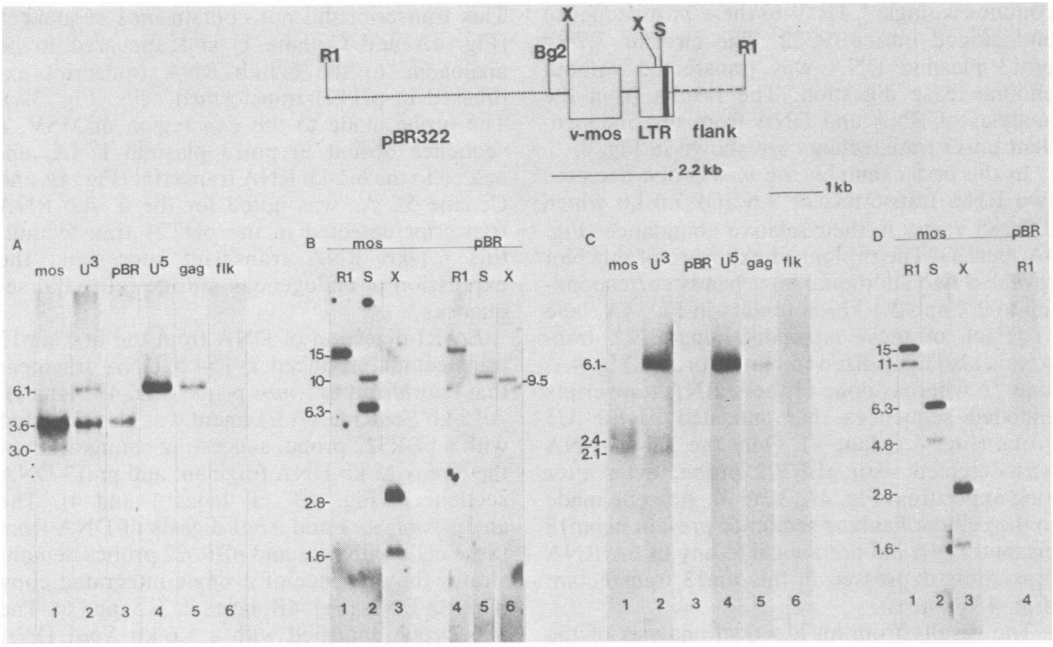


FIG. 4. Analysis of RNA and DNA from pm13 transfectants. A schematic diagram of the structure of pm13 plasmid DNA is shown. The results from the hybridization analysis of polyadenylated RNA isolated from two pm13 transfectants are shown in (A) (4 μg/lane) and (C) (5.5 μg/lane). The results from the hybridization analysis of cellular DNA isolated from these transformed cells are shown in (B and D). The restriction endonucleases used in the DNA digests are indicated at the top of each lane. Restriction enzymes: R1, *Eco*RI; S, *Sac*I; X, *Xba*I. The probes (see the text used in analyzing the RNA and DNA blots) are indicated at the top of the lanes in each panel.

3B, lane 1). A probe representing pBR322 or the cellular flanking sequence in pHT21 plasmid DNA also detected a 15-kb DNA fragment (Fig. 3B, lanes 4 and 6). These probes did not hybridize to the endogenous c-*mos* 15-kb DNA fragment (data not shown), indicating that a DNA fragment representing an integrated copy of the pHT21 DNA comigrates with the c-*mos* 15-kb DNA fragment. This interpretation was confirmed by analysis with other restriction endonucleases. *Hin*dIII digests contained the predicted 9.0-kb c-*mos* DNA fragment and two additional *mos*-containing DNA fragments of 6.8 and 4.0 kb (Fig. 3B, lane 2). The latter two DNA fragments were not affected by additional digestion with *Eco*RI, whereas the c-*mos* DNA fragment was cleaved (Fig. 3B, lane 3). The *Xba*I site immediately 5′ to v-*mos* was 2.4 kb from the single *Xba*I site present in the 3′ LTR in pHT21 plasmid DNA (Fig. 3, schema). Hybridization with a *mos* probe detected two DNA fragments in *Xba*I digests of DNA from the pHT21 transfectant, a 2.8-kb c-*mos* *Xba*I DNA fragment and a 2.4-kb *Xba*I DNA fragment derived from the integrated pHT21 DNA (Fig. 3B, lane 10). These results demonstrate that there are two integrated copies of pHT21 DNA in this transfectant, neither of which has undergone rearrangement of the relative positions of the v-*mos* and LTR sequences present in the transfected DNA. Although the size of the *Eco*RI DNA fragment indicated the loss of *Eco*RI sites from both pHT21 DNA copies present in this transfectant, pBR322 and cellular flanking sequences were retained in both of the integrated DNAs (Fig. 3B, lanes 4 through 9), offering further evidence that a major rearrangement of the transfected DNA has not occurred. Both the pBR322 and *mos* probes detected the same DNA fragments in *Eco*RI, *Hin*dIII, and *Hin*dIII-*Eco*RI digests (Fig. 3B, lanes 1 through 6). However, the relative intensity of pBR322 hybridization to the *Hin*dIII DNA fragments suggests that different amounts of pBR322 sequences are retained in each of the integrated copies. It should be noted that these two integrated copies of pHT21 DNA are apparently responsible for the expression of five *mos*-containing RNA transcripts.

A second example of the enhancement of the transforming activity of v-*mos* by a single LTR introduced in a 3′ relative position is presented by the analysis of pm13-transformed cells (Fig. 4). pm13 is derived from m1 MSV proviral DNA and, like pHT21, this recombinant DNA plasmid

contains a single LTR 3′ to the v-*mos* sequence and cloned into pBR322. The circular 7.7-kb pm13 plasmid DNA was transfected without endonuclease digestion. The results from the analysis of RNA and DNA from two independent pm13 transfectants are shown in Fig. 4.

In the first example, the *mos* probe detected two RNA transcripts of 3.6 and 3.0 kb which differed vastly in their relative abundance (Fig. 4A, lane 1). The prolonged exposure of this blot revealed two additional faint bands corresponding to 2.4 and 2.1 kb (note dots in Fig. 4A, lane 1). Each of these *mos*-containing RNA transcripts also hybridized to the U3 probe (Fig. 4A, lane 2), whereas none of these RNA transcripts encoded sequences that annealed to the U5 probe (Fig. 4A, lane 4). Only the 3.6-kb RNA was detected with pBR322 probe, even after long exposures (Fig. 4A, lane 3). A probe made to the cellular flanking sequence present in pm13 plasmid DNA did not anneal to any of the RNA transcripts expressed in this pm13 transfectant (Fig. 4A, lane 6).

The results from an identical analysis of the RNA transcripts expressed in a second independent pm13 transfectant are shown in Fig. 4C. After prolonged exposure (10 days), the *mos* probe detected two RNA transcripts of 2.4 and 2.1 kb in these transformed cells (Fig. 4C, lane 1). Both of these RNA transcripts contained sequences that hybridized to the U3 probe, whereas neither transcript annealed with the U5, flank, or pBR322 probes (Fig. 4C, lanes 2 through 4 and 6). These results are consistent with the results from the previous analysis of the pHT21 transfectant but, in addition, show that the level of the expression of *mos*-containing RNA transcripts can vary considerably in cells transformed with subgenomic v-*mos*-containing proviral DNAs. Note that the sizes of the *mos*-containing RNAs shown in Fig. 4C are identical to those of the smaller RNA transcripts detected only after prolonged exposure in the previous example (Fig. 4A). We estimate the level of *mos* RNA in the second example of pm13-transformed cells to be between 1 and 10 copies per cell (T. Wood, unpublished data). This implies that only a few copies of *mos*-containing RNA are required to induce cell transformation. Furthermore, these results suggest that the enhancement of the transforming efficiency of v-*mos* does not predispose the expression of the *mos* gene to transcribe high levels of *mos* RNA. However, we cannot exclude the possibility that high levels of *mos* RNA are transcribed in pm13-transformed cells but not processed to the cytoplasm.

A 6.1-kb RNA transcript was detected with the U5 and U3 probes in both examples of pm13-transformed cells (Fig. 4A and C, lanes 2 and 4). This transcript did not contain *mos* sequences (Fig. 4A and C, lane 1) and appeared to be analogous to the 6.5-kb RNA transcript expressed in pHT21-transformed cells (Fig. 3A). The probe made to the *gag* region of MSV, a sequence absent in pm13 plasmid DNA, annealed to the 6.1-kb RNA transcript (Fig. 4A and C, lane 5). As was noted for the 6.5-kb RNA transcript detected in the pHT21 transfectant, this 6.1-kb RNA transcript represents the expression of endogenous murine retroviral sequences.

*Eco*RI digestion of DNA from the first pm13 transfectant produced a 15-kb DNA fragment that hybridized to a *mos* probe (Fig. 4B, lane 1). A 15-kb *Eco*RI DNA fragment was also detected with a pBR322 probe, suggesting comigration of the c-*mos* 15-kb DNA fragment and pm13 DNA sequences (Fig. 4B, cf. lanes 1 and 4). The analysis of *Sac*I and *Xba*I digests of DNA from these cells with *mos* and pBR322 probes demonstrates the presence of a single integrated copy of pm13 DNA (Fig. 4B, lanes 2, 3, 5, and 6). The *mos* probe annealed with a 1.6-kb *Xba*I DNA fragment from this pm13 transfectant (Fig. 4B, lane 3). This corresponds to the predicted size of the *Xba*I DNA fragment in pm13 plasmid DNA (Fig. 4, schema) and indicates that the v-*mos* and LTR sequences in this integrated pm13 DNA have not been rearranged. The *mos* and pBR322 probes both detected a 10-kb *Sac*I DNA fragment, suggesting that the vector sequence is oriented in a 5′ position relative to v-*mos* (Fig. 4B, lane 5). This result is consistent with the expression of pBR322 sequences in the 3.6-kb RNA transcript (Fig. 4A, lanes 1 and 3).

The *mos* probe detected a 15-kb c-*mos* DNA fragment and a new 11-kb DNA fragment in *Eco*RI digests of cellular DNA from the second pm13 transfectant (Fig. 4D, lane 1). The analysis of *Sac*I digests of this DNA with the *mos* probe also demonstrates a single integrated copy of pm13 DNA (Fig. 4D, lane 2). As noted with the previous pm13 transfectant (Fig. 4B, lane 3), a 1.6-kb *Xba*I DNA fragment was detected with the *mos* probe, indicating that the relative positions of the v-*mos* and LTR sequences in the integrated copy of pm13 DNA have been conserved (Fig. 4D, lane 3). The pBR322 probe did not anneal to DNA from this pm13 transfectant, suggesting that the vector sequences have been deleted (Fig. 4D, lane 4).

The results from the analysis of RNA and DNA isolated from cells transformed by the transfection of cloned subgenomic proviral DNA (Fig. 2 through 4) have provided evidence that a single LTR can efficiently enhance the transforming activity of v-*mos* from either a 5′ or a 3′ relative position. The transfection of recombinant DNA containing an LTR 5′ to v-*mos* (i.e.,

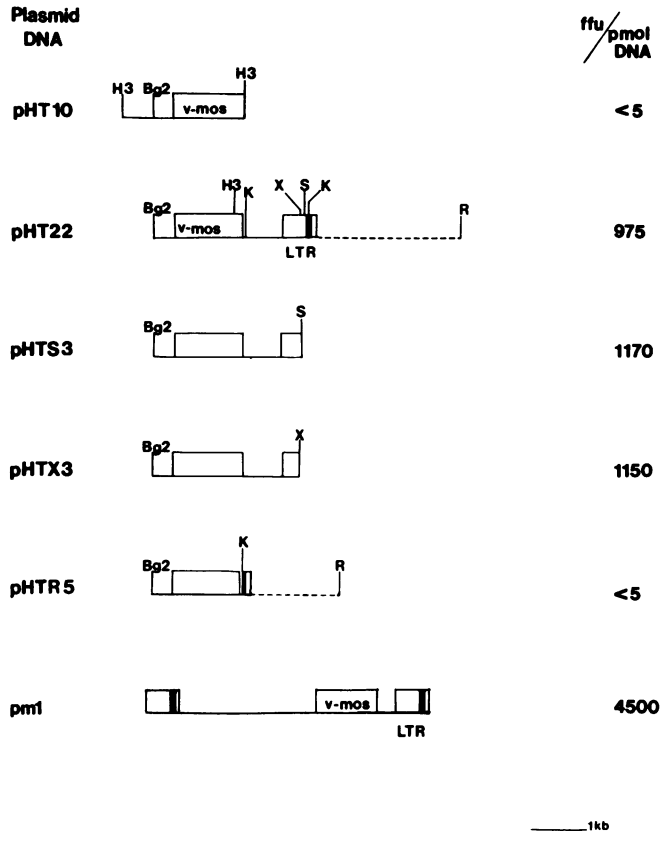


FIG. 5. Physical maps and transforming efficiency of recombinant DNA clones containing various LTR sequences introduced 3′ to v-*mos*. Descriptions of pHT10, pHT21, pHT22, and pm1sp plasmid DNAs have been previously published (7, 19). The recombinant DNA clones and the essential restriction endonuclease sites used in the construction of the recombinant DNA clones are shown. The DNA fragments used in these constructions were purified by electrophoresis in agarose gels (20), and the DNA was recovered by electroelution. The DNA fragments were extracted once with phenol-$CHCl_3$ (1:1) and precipitated with ethanol. The DNA fragments were ligated (10), and the sample was transfected into competent LE392 (32). Colonies were selected and tested for confirmation of structure by restriction endonuclease digestion. pHTS3 was constructed from the *Hin*dIII-*Sac*I DNA fragment of pHT22 by ligation to a *Bgl*II-*Hin*dIII DNA fragment from pHT10 and cloned into the *Bam*HI-*Sac*I sites of pBRSc7 (a plasmid vector with *Sac*I linkers introduced at the *Pvu*II site in pBR322). pHTX3 was constructed in a similar manner with the *Hin*dIII-*Xba*I DNA fragment of pHT22 ligated to a *Bgl*II-*Hin*dIII DNA fragment from pHT10 and cloned into the *Bam*Hi-*Xba*I sites in pBRX19 (a plasmid vector with *Xba*I linkers introduced at the *Pvu*II site in pBR322). pHTR5 was constructed with a *Bgl*II-*Kpn*I DNA fragment purified from a partial digest of pHT21 DNA. This DNA fragment was ligated to a *Kpn*I-*Eco*RI DNA fragment derived from pm1sp and cloned into the *Bam*HI-*Eco*RI sites of pBR322. Sequences representing v-*mos* and the LTR are indicated. The dashed line represents cellular flanking sequences. The cloned DNAs were linearized by digestion with either *Eco*RI (pHTS3, pHTX3), *Sal*I (pHT22, pHTR5), or *Bam*HI (pHT10, pm1) before DNA transfection (see the text). The specific activities represent the number of foci induced per $2.5 \times 10^5$ cells per pmol of DNA transfected and are expressed as focus-forming units (ffu). They were calculated from at least four replicate determinations.

pHT25) requires the acquisition of termination and polyadenylation signals for RNA expression; alternatively, an LTR 3′ to v-*mos* (i.e., pHT21, pm13) imposes the requirement of obtaining signals for the initiation of transcription. Although the LTR does encode transcriptional control signals for directing the initiation and polyadenylation of RNA transcripts (9, 15, 29, 30, 33), the enhancement of the transforming activity of v-*mos* does not apparently result from these transcriptional elements, and this implies that the LTR contains other sequences that influence the expression of v-*mos*. If this is true, then eliminating the polyadenylation signals

from an LTR introduced 3′ to v-*mos* should not influence its transforming efficiency and could serve to identify the regions of the LTR responsible for enhancement.

**Determination of the sequences responsible for enhancement by an LTR introduced 3′ to v-*mos*.** To identify the essential sequences required for enhancement by an LTR 3′ to v-*mos*, we tested the transforming activity of a series of recombinant DNA clones constructed to contain various sequences derived from the LTR (Fig. 5). As previously shown, pHT10, a plasmid containing v-*mos* and 900 bp of Moloney leukemia virus sequences 5′ to v-*mos*, but no LTR sequences, exhibits a very low transforming efficiency (<5 focus-forming units per pmol of DNA) (7), whereas a plasmid containing the entire proviral DNA of ml MSV (pml) produced 4,500 focus-forming units per pmol of DNA (Fig. 5). The insertion of a single LTR 3′ to the v-*mos* sequence (pHT22) resulted in a 200-fold stimulation of the transforming efficiency observed with pHT10 (Fig. 5). An equivalent enhancement of the pHT10 transforming efficiency was observed when DNA fragments containing only sequences derived from the U3 region of the LTR were introduced 3′ to v-*mos* (Fig. 5, pHTS3 and pHTX3). However, when sequences derived from the R and U5 region of the LTR were present 3′ to v-*mos* (pHTR5), the transforming efficiency was equivalent to the level observed with the transfection of pHT10 DNA (Fig. 5). These results demonstrate that the region of the LTR responsible for the enhancement of the transforming activity of v-*mos* consists of sequences present in the U3 region and that sequences in the R and U5 region (i.e., polyadenylation signals) do not enhance the transforming activity of v-*mos*.

## DISCUSSION

The results from DNA transfection assays have shown that the transforming activity of v-*mos* is enhanced by introducing a single LTR in either a 5′ or a 3′ position relative to v-*mos* (7, 19). In this report, we have presented results from the analysis of RNA and DNA isolated from cells transformed by the transfection of recombinant DNA clones containing v-*mos* and a single LTR. In each of the transfectants, we demonstrated the presence of additional copies of v-*mos* within the cellular DNA in contrast to a single haploid DNA copy observed in normal NIH3T3 cells (14, 24). The analysis of restriction endonuclease digests of cellular DNA from these transfectants by hybridization with various probes specific for sequences present in the transfected DNA confirms the observation that a single LTR is sufficient for the enhancement of the transforming activity of v-*mos*. Tandem integrations of the transfected DNA, recreating provirus-like DNA structures, are not necessary for the expression of v-*mos* RNA transcripts or for the induction of cellular transformation. It should be noted that in these experiments we did not distinguish between the introduction of transfected DNA into the host chromosome and its insertion into high-molecular-weight carrier DNA in a "pekelasome" structure (27).

The rearrangement of the v-*mos* and LTR sequences is not required for the expression of v-*mos* RNA transcripts, and the relative positions of the v-*mos* and LTR sequences within the transfected DNAs are conserved in the integrated DNA copies. The fate of the vector and cellular flanking sequences present in the transfected plasmid DNAs varied for each of the transfectants that we examined. In one case (i.e., pm13), the entire pBR322 vector sequence present in the transfected plasmid DNA was deleted from the integrated DNA copy. Our results demonstrate that the LTR provides transcriptional control elements that insure the expression of v-*mos* RNA transcripts.

The cells transformed by the transfection of DNA containing an LTR 5′ to v-*mos* express RNA transcripts that hybridize with *mos* and U5 probes, but not with probes representing the U3 region of the LTR. The termination and polyadenylation signals utilized by the v-*mos* RNA transcripts in these transfectants are not obtained from the transfected LTR. These signals are acquired from either vector, host, or carrier DNA. Although downstream promotion provides one mechanism for the activation of an oncogene, the induction of transformation by the insertion of an LTR in a 3′ position relative to v-*mos* is inconsistent with a promoter activation model. Blair et al. first observed that these constructs induced transformation as efficiently as the 5′ LTR v-*mos* constructs (7). We have shown here that cells transformed by the transfection of DNA containing an LTR 3′ to v-*mos* express RNA transcripts that hybridize with *mos* and U3 probes. The U5 probe does not anneal to the v-*mos* RNA transcripts expressed in these cells, demonstrating that transcript initiation sites present in the transfected LTR are not utilized in the expression of these v-*mos* RNA transcripts. Again, these signals must be acquired from either vector, host, or carrier DNA sequences and, because of the high transformation frequency in their absence, must be easily acquired during transfection. Because of the size of the 3.2-kb *mos*-containing RNA transcript in the pHT21 transfectant (Fig. 3) and the size of the 2.1-kb *mos*-containing transcript in pm13 transfectants (Fig. 4), we cannot exclude the possible use of proviral sequences preceding v-*mos* to provide the initiation sites. By intro-

ducing an LTR 5′ to v-*mos*, we provide transcriptional control elements for the expression of v-*mos*-containing RNA transcripts analogous to the promoter insertion model suggested for avian leukosis virus (ALV)-induced bursal lymphomas (13, 22, 23, 25, 26). These studies show evidence for an increased expression of the chicken c-*myc* gene resulting from the insertion of an ALV LTR 5′ to c-*myc*. Payne et al. have shown that in one example of ALV-induced bursal lymphomas, the ALV LTR can also increase the expression of c-*myc* from a 3′ position (26).

The results obtained with a number of simian virus 40 (SV40) deletion mutants have suggested that important elements controlling the expression of SV40 RNA transcripts are contained in the 72-bp tandem repeats located near the SV40 origin of replication (5, 6, 12). Moreau et al. have shown that the 72-bp tandem repeat from SV40 can stimulate T-antigen expression in chimeric plasmids where the SV40 TATA box region has been substituted with conalbumin or adenovirus 2 major late promoter sequences (21). Furthermore, the level of T-antigen expression is not altered by reversing the orientation of the 72-bp tandem repeat (21). The enhancement of gene expression for genes other than those present in SV40 genomic DNA has also been demonstrated. Capecchi reported that the insertion of an SV40 DNA fragment containing the 72-bp tandem repeat into a plasmid containing the thymidine kinase gene of herpes simplex virus increases the transformation frequency of mouse LMTK$^-$ cells (8). The expression of the rabbit β-globin gene is enhanced in HeLa cells when this gene is inserted into a recombinant vector that contains SV40 DNA sequences, including the 72-bp repeat element (4).

MSV proviral DNA has been shown to contain a 73-bp tandem repeat within the U3 region of the LTR (9). Levinson et al. have demonstrated that the 73-bp repeat from m1 MSV can replace the SV40 72-bp repeat as an activator for the expression of viral sequences located downstream from the 73-bp repeat (16). We tested the ability of various sequences, derived from the LTR and introduced 3′ to v-*mos*, to enhance the transforming activity of v-*mos* (Fig. 5). The results from this analysis demonstrate that sequences within the first 300 bases of the LTR can yield the same level of enhancement of the transforming activity of v-*mos* as can a complete LTR. Sequences present in the R and U5 regions of the LTR (i.e., polyadenylation signals) do not stimulate the transforming activity of v-*mos*. These results show that the region of the LTR responsible for enhancement consists of sequences present in the U3 region, a sequence that contains the 73-bp tandem repeat.

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