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Murine Leukemia Virus Long Terminal Repeat Sequences Can Enhance Gene Activity in a Cell-Type-Specific Manner

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We tested the ability of sequences in the long terminal repeat (LTR) of a mink cell focus-forming (MCF) murine leukemia virus to function as an enhancer in a cell-type-specific manner. In a stable transformation assay, the MCF or Akv LTR and the simian virus 40 enhancer had similar activities in murine fibroblasts. In contrast, the MCF LTR had a significantly greater activity in murine T lymphoid cells than did either the simian virus 40 enhancer or the Akv LTR.

One of the regulatory signals for transcription of eucaryotic genes is *cis*-acting enhancer elements that can augment the transcriptional activity of different genes (for review, see references 14 and 18). Enhancer elements were first identified in the simian virus 40 (SV40) genome but have subsequently been found in other viruses, including retroviruses (17, 23, 24), and have recently been identified for cellular genes (1, 13, 29). Unique characteristics of these enhancer elements include their ability to function in an orientation-independent manner and over relatively long distances (2, 12, 25). Enhancer activity can also occur in a tissue- or species-specific manner (10, 21). Enhancers have been identified in the U3 region of the long terminal repeats (LTRs) of retroviruses (17, 19, 20). More recently, the LTRs of murine leukemia viruses (MLVs) have been implicated in the specific type of neoplasia caused by these viruses (5, 9, 22, 28), and it is possible that the sequences resembling enhancers are responsible for this specificity (4).

We have examined the LTR of a mink cell focus-forming (MCF) MLV (MCF-13 [35]) to study the role that these terminal repeated sequences play in the specificity of tumor formation by MCF viruses. Lymphomagenic MCF MLVs have been implicated in the development of thymic lymphomas in strain AKR mice because of their ability to accelerate the onset of tumorigenesis (6, 27). In contrast, this pathogenic characteristic is not possessed by a parental virus (Akv MLV [hereinafter referred to as Akv]) of the MCFs. The MCF MLVs have been shown to be recombinant viruses (11, 30).

We have determined the nucleotide sequence of the LTR of an infectious clone (unpublished data) of MCF-13 (Fig. 1) by the dideoxy method of Sanger et al. (32). Besides containing consensus sequences which are important in the regulation of transcription [CAT and TATA boxes and the poly(A) addition signal], this LTR included a 69-base-pair (bp) repeat (Fig. 1, in brackets) in the U3 region. From a comparison of the nucleotide sequence of the LTRs of MCF-13 and Akv (Fig. 1), it seems clear that the greatest differences are present in the U3 region. This is a region in the LTR where duplicated sequences resembling enhancer elements have been identified for other retroviruses, and these sequences also appear to be present in the case of MCF-13 and Akv (Fig. 1). We wished to determine whether the repeated sequences for MCF-13 and Akv could also function as enhancer elements and whether this activity could occur in a cell-type-specific manner. Tissue-specific differences in the activities of these enhancerlike elements could help to account for the different pathogenic properties of these two viruses.

To test this idea, we compared the LTRs of MCF-13 and Akv, along with the prototypical SV40 enhancer, in plasmid constructs containing each enhancer and the selectable gene conferring neomycin resistance (Neo^r) in murine cells in culture. A clone generated by Southern and Berg (33) which contains the SV40 origin of replication was used to test the SV40 enhancer (Fig. 2). The SV40 sequences that are present in this clone (pSV2-neo) are the 72-bp repeat that functions as an enhancer, the 21-bp repeat, and the promoter (TATA) for transcription of early viral genes. A similar clone missing all but 22 bp of one of the SV40 72-bp repeats was called pSV1-neo (15). This deletion has been shown to eliminate the enhancer activity of these repeated sequences (3, 16).

We inserted a permuted form of the LTR of MCF-13 into the single *Nde*I site of pSV1-neo (Fig. 2B). This LTR fragment was generated by an *Sma*I restriction enzyme digestion of an infectious clone containing two tandemly repeated LTRs of MCF-13 (35). Hence, this fragment was composed of the U5 and part of the R regions of one LTR and the U3 and part of the R regions of the second LTR. We constructed a similar fragment containing the Akv LTR (34). Since there was no available clone of the Akv MLV possessing tandemly repeated LTRs, we took 155 bp, consisting of the U3 and part of the R regions from the *Sma*I to the *Pst*I site, from the tandem LTRs of MCF-13 for the 5′ end of this fragment. We believed that it was important to maintain equal amounts of LTR sequences for both viral fragments because of reports that LTR sequences outside of the direct repeats can influence enhancer activity (19, 20). However, any differences between the two viral LTRs that may be detected will have to be attributed to sequences not included in the 155 bp shared by these two constructs. The LTR fragments were inserted in both orientations. Clones pMCF1 and pAkv1-neo have the direction of transcription from the promoter of the LTR in the same orientation as transcription of the *neo* gene. The LTRs of pMCF2 and pAkv2 are oriented in the opposite direction.

To test enhancer activities, we transfected the molecular clones described above into mouse cells by a modified protoplast fusion technique originally described by Sandri-

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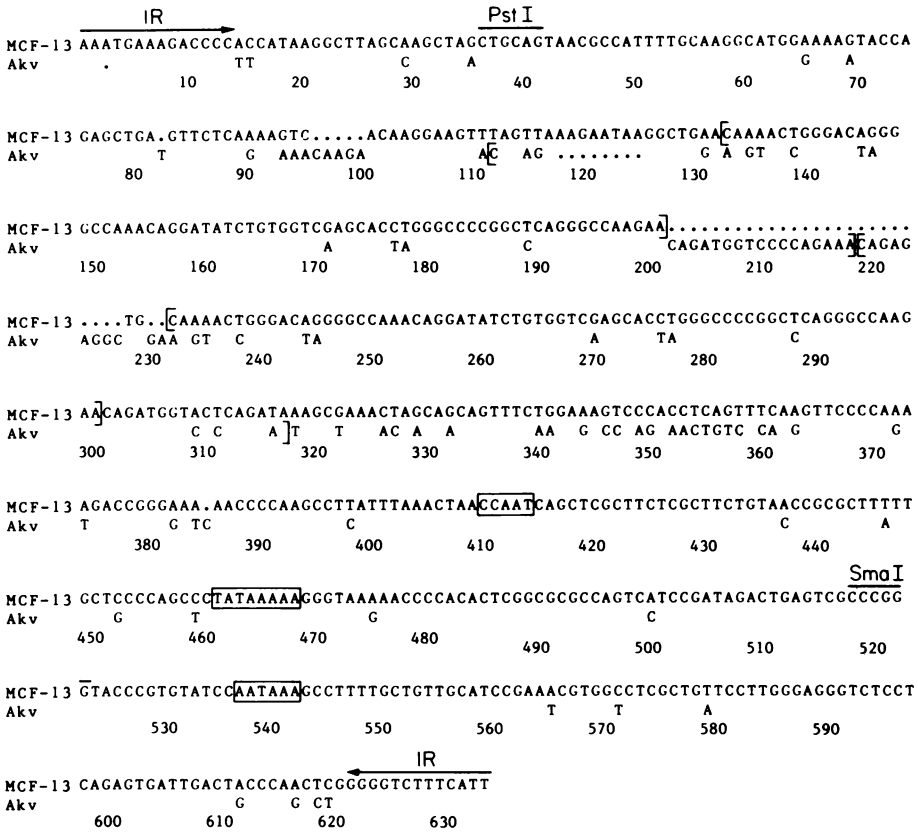

FIG. 1. Comparison of the nucleotide sequence of the LTRs of MCF-13 and Akv. Nucleotide differences are shown for the Akv LTR, and blank spaces correspond to identical bases. Deletions are indicated by dots. Arrows delineate the sequences composing the inverted repeats in the LTR. Duplicated sequences are enclosed in brackets. Boxed sequences correspond to the consensus CAT, TATA, and poly(A) addition sequences. The first digit of a number is placed below the base to which it corresponds. The LTR U3 region extends between nucleotides 1 and 491, R extends between nucleotides 492 and 559, and U5 extends between nucleotides 560 and 634. the *Pst*I and *Sma*I restriction endonuclease cleavage sites are shown because these enzymes were used in the construction of the LTR-containing clones.

Goldin et al. (31). The activities of the three clones containing enhancer sequences were compared with the enhancer minus clone pSV1-neo. To determine whether these viral enhancers had variable activities in different mouse cell types, we used a fibroblast (NIH/3T3) and a T lymphoid (SL-3) cell line for the transfections. The T lymphoid line that we used was originally established from a spontaneous thymoma in a strain AKR mouse (26).

Equal numbers of protoplasts (0.5×10^9 to 1×10^9) were applied to a total of 5×10^6 cells. In each experiment 10 tissue cluture plates (diameter, 10 cm) of NIH/3T3 cells grown in minimal essential medium and 10% heat-inactivated fetal calf serum were exposed to 400 μg of G418 per ml (GIBCO Laboratories or Sigma Chemical Co.) 48 h after the fusion. Immediately after fusing 5×10^6 SL-3 cells, 5×10^4 of these cells were plated into each well of a 24-well cloning plate. These cells were grown in RPMI and 10% heat-inactivated fetal calf serum and were transferred, at 48 h postfusion, to this medium containing 250 μg of G418 per ml.

Results of the transfection experiments are presented in Table 1. Transformation frequencies were calculated by counting the number of stable colonies of each cell type that were able to grow in selective media. Transformation frequencies for the SL-3 cells were calculated by a formula for the statistical testing of the Poisson distribution at 5% confidence level. Four different clones were compared in each experiment. They were MCF-13 and Akv clones with LTRs in the same orientation, the clone with the SV40 enhancer, and the enhancer-minus clone. The absolute transformation efficiencies for clone pSV1-neo differed from one experiment to the next (Table 1). However, the relative difference between the efficiency of a clone containing an enhancer sequence and that of the clone without an enhancer remained similar for different experiments. These relative values are shown in Table 1 for the clones containing viral enhancers.

We observed that clone pSV2-neo and those clones with the MCF-13 or Akv LTR in either orientation increased the efficiency of transformation from three- to ninefold compared with the enhancer-minus clone in NIH/3T3 fibroblasts. A similar comparison of the clones in the T cell line revealed increases comparable to those observed in fibroblasts, for the clones with either the SV40 enhancer or the Akv LTR. In contrast, the increase in transformation frequency that we observed for the clones containing the MCF-13 LTR in both orientations was significantly greater in T lymphoid cells (30- to 90-fold).

These results demonstrated that MCF-13 LTR sequences had a greater activity than either the Akv LTR or the SV40 enhancer in establishing stable transformants of murine T lymphoid cells. The activities of the two viral LTRs and the SV40 enhancer were similar in murine fibroblasts. Although we tested the entire LTR instead of the enhancerlike se-

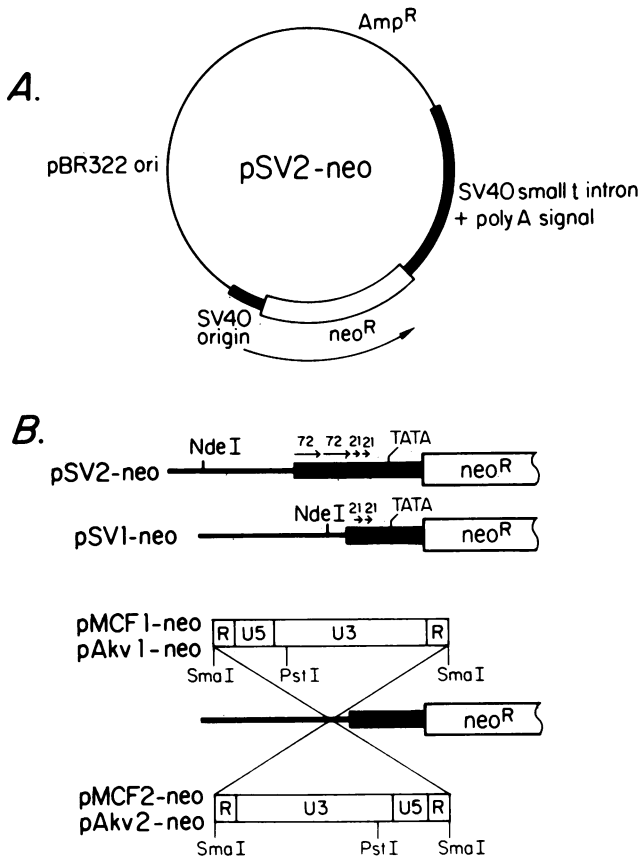

FIG. 2. Plasmid constructs used for transfection experiments. (A) pSV2-neo (33) is a pBR322 plasmid that contains the SV40 origin of replication, which includes the repeated 72-bp enhancer sequence, the 21-bp repeat, and the promoter (TATA) for early viral genes, upstream of the Tn5 phosphotransferase gene (neo^R) encoding neomycin resistance (also shown in Fig. 2B). The SV40 small-t intron and the poly(A) signal have been placed 3′ to this gene. This plasmid also contains the bacterial origin of replication and a gene encoding ampicillin resistance (Amp^r). (B) pSV1-neo (15) is a construct, derived from pSV2-neo, which is missing all but 22 bp of the 72-bp repeated sequences. pMCF1 and pAkv1-neo have the LTR fragment from the MCF-13 and Akv, respectively, inserted into the *NdeI* site in pSV1-neo in the same orientation as transcription of the *neo* gene. Constructs with the same LTR fragments inserted in the opposite orientation are pMCF2 and pAkv2-neo. Restriction endonuclease sites (*PstI* and *SmaI*) which were used in the generation of the LTR fragments are shown.

quences alone, it appears that these sequences were responsible for these results mainly because the large increase in transformation frequency was obtained regardless of the orientation of the LTR. This result would argue for an enhancer effect since one of the hallmarks of these elements is their ability to act in an orientation-independent manner. From our analysis of the cloned DNAs in stably transformed cell lines (F. Yoshimura and L. Ng, manuscript in preparation), it does not appear that the mechanism by which the MCF-13 LTR was able to increase transformation frequencies involved an increase in the copy number of transfected DNA. This observation is supported by similar results from other investigators who have examined the effect of other retroviral LTRs on the amount of transfected DNA stably integrated in a cell (17).

Although we have not directly demonstrated an effect of the MCF-13 LTR on transcriptional activation of the *neo* gene, we speculate that the enhancerlike activity that we have observed could augment transcription of viral genes in thymocytes. An increase in viral gene transcription in this cell type would result in higher titers of MCF-13, thereby accounting for the thymotropism of this virus (27). By a similar mechanism, MCF-13 proviral integration could increase the transcription of adjacent cellular genes in thymocytes. The increased transcription of specific chromosomal loci implicated in the development of AKR and related thymomas (7, 8) could be effected by adjacent proviruses possessing enhancers with similar activities to that of MCF-13. In conclusion, the cell-specific activity that we have observed for the enhancerlike element of the MCF-13 LTR could account for its oncogenic potential compared with that of the Akv MLV.

TABLE 1. Effect of viral enhancers on transformation frequencies[a]

Cell line (type) and experiment no.	Transformation frequency of pSV1 (10^{-6})	Transformation frequency relative to pSV1 of plasmid:				
		pSV2	pMCF1	pAkv1	pMCF2	pAkv2
NIH/3T3 (fibroblast)						
1.	8.4	3.4	3.7	8.7		
2.	3.2	2.6	3.3	3.3		
3.	0.81	6.0			6.6	5.6
4.	0.96	5.9			7.6	3.2
SL-3 (lymphoid)						
1.	1.24	3.6	28.0	1.9		
2.	0.14	5.7	90.0	6.7		
3.	0.29	5.2			45.9	6.9
4.	0.29	7.6			70.7	1.3

[a] NIH/3T3 or SL-3 cells were transformed by protoplast fusions with the different plasmid constructs. After 48 h of transformation, cells were grown in the presence of the antibiotic G418. Stable colonies were counted, and the transformation frequency was calculated by dividing this number by the total number of initially transfected cells. Actual transformation frequencies are presented only for clone pSV1-neo. Transformation frequencies relative to those obtained for pSV1-neo are shown for the plasmids containing either the SV40 enhancer (pSV2) or viral LTRs (pMCF and pAkv).

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