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The 3′ Long Terminal Repeat of a Transcribed yet Defective Endogenous Retroviral Sequence Is a Competent Promoter of Transcription†

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Although actively transcribed and present as multiple genomic copies, a distinct class of endogenous murine leukemia virus-related sequence does not give rise to infectious virus. Since the long terminal repeat at the 3′ terminus provides the transcriptional start site after reintegration, we determined the structure and potential promoter activity of that sequence obtained from cDNA of endogenous retroviral transcripts. These studies demonstrate that the distinctive 3′ long terminal repeat sequence of these transcripts could serve as an effective promoter of transcription and, therefore, may not be the primary defect in the infectious cycle of retroviral replication but may result in the propagation of these endogenous retroviral sequences in the genome as retrotransposons.

In addition to the more limited set of strain-specific, infectious ecotropic and xenotropic retroviral loci (8, 35), the genomes of all strains of mice contain multiple copies of endogenous retroviral sequence that do not give rise to detectable infectious virus, although the structure and sequence of these genomic elements are highly similar to those of retroviral RNA (9, 18, 22, 24) and may contribute to the de novo generation of recombinant leukemogenic viruses (24, 28). To examine the defect preventing infectivity, as well as the maintenance and evolution of these virus-related sequences, we have described the regulated expression and structure of apparent full-length and spliced transcripts of these loci in strain 129 mice (27, 28). The transcripts potentially encode proteins homologous to typical retroviral products, including both a gp70-p15E polyprotein whose amino-terminal half is virtually identical to that found in infectious leukemogenic mink cell focus-forming (MCF) viruses and a region at the 3′ portion of *pol* identical to the carboxy terminus of the Pr180 *gag-pol* polypeptide of AKR MCF 247 (28). Several features distinguish these defective retroviral sequences from infectious murine leukemia virus (MuLV). These include a glutamine rather than a proline tRNA-binding site (33, 36) and a *gag* sequence that eliminates a minor glycosylated gene product gPr80 *gag* (12). However, substitution of endogenous *gag* and a glutamine tRNA-binding site (12) or portions of *env* sequence during the generation of leukemogenic MCF virus (6, 19, 22, 25, 32) and spleen focus-forming virus (1, 2, 4, 11, 44) for sequence of ecotropic virus does not abolish the productive infectivity of such recombinant viruses.

The 5′ and 3′ termini of the majority, if not all, of these defective, endogenous proviral elements consist of long terminal repeat (LTR) sequences highly related to those of infectious virus, yet the termini differ by the insertion of a middle repetitive DNA element (23, 36). Since transcripts of these loci contain this LTR sequence at their 3′ termini (27) and since the 3′ LTR of viral RNA contributes the functional promoter of the integrated provirus in a typical infectious cycle (40) and therefore could aid in the diversification of these sequences by retrotransposon-mediated transposition, we wished to determine whether the 3′ LTR sequences of these transcripts were lacking in promoter function that would result in an inability to propagate as infectious virus.

Examination of independent cDNA clones of RNA transcripts expressed in epididymis, spleen, and liver of 129 G_{IX}^{+} mice revealed two 3′ LTR structures that differ in length by approximately 50 nucleotides as defined by a combined digestion with *Pst*I and *Kpn*I. Comparison of the nucleotide sequences of cDNA clones E2 and L62, each representing one of the endogenous LTR classes, with sequences of infectious ecotropic AKV (17), dualtropic, recombinant virus AKR MCF 247 (21), and NZB xenotropic virus (34) illustrates that, as several genomic copies of endogenous retroviral sequence that are not known to be transcriptionally active (23, 36), the 3′ termini of these transcripts contain several features typical for viral U3 sequence. By analogy with infectious virus (10), the endogenous sequences presented in Fig. 1 begin 1 nucleotide 3′ to the ochre codon ending the p15E open reading frame. Positions 1 to 32 in the endogenous sequence are homologous to the origin of plus-strand DNA synthesis (13, 39), with a polypurine stretch at positions 20 to 34. U3 begins at position 35 with an 11-nucleotide segment identical to the inverted repeat of Moloney MuLV (MoMuLV), AKV, MCF 247, or NZB xenotropic virus and extends to position 649. Positions 650 to 729 contain the viral RNA terminal redundancy, R. The CAT box equivalent sequence CAAC is located at positions 564 to 567 in the endogenous sequence, the TATA promoter element is 55 nucleotides further 3′, and the AATAAA polyadenylation signal is at positions 709 to 714. Despite these overall similarities, the two cDNA sequences differ substantially from the corresponding regions of infectious viruses, although they most closely resemble the sequence of xenotropic NZB LTR (34). Comparison of E2 with AKV shows 93 nucleotide substitutions, single-base deletions, or

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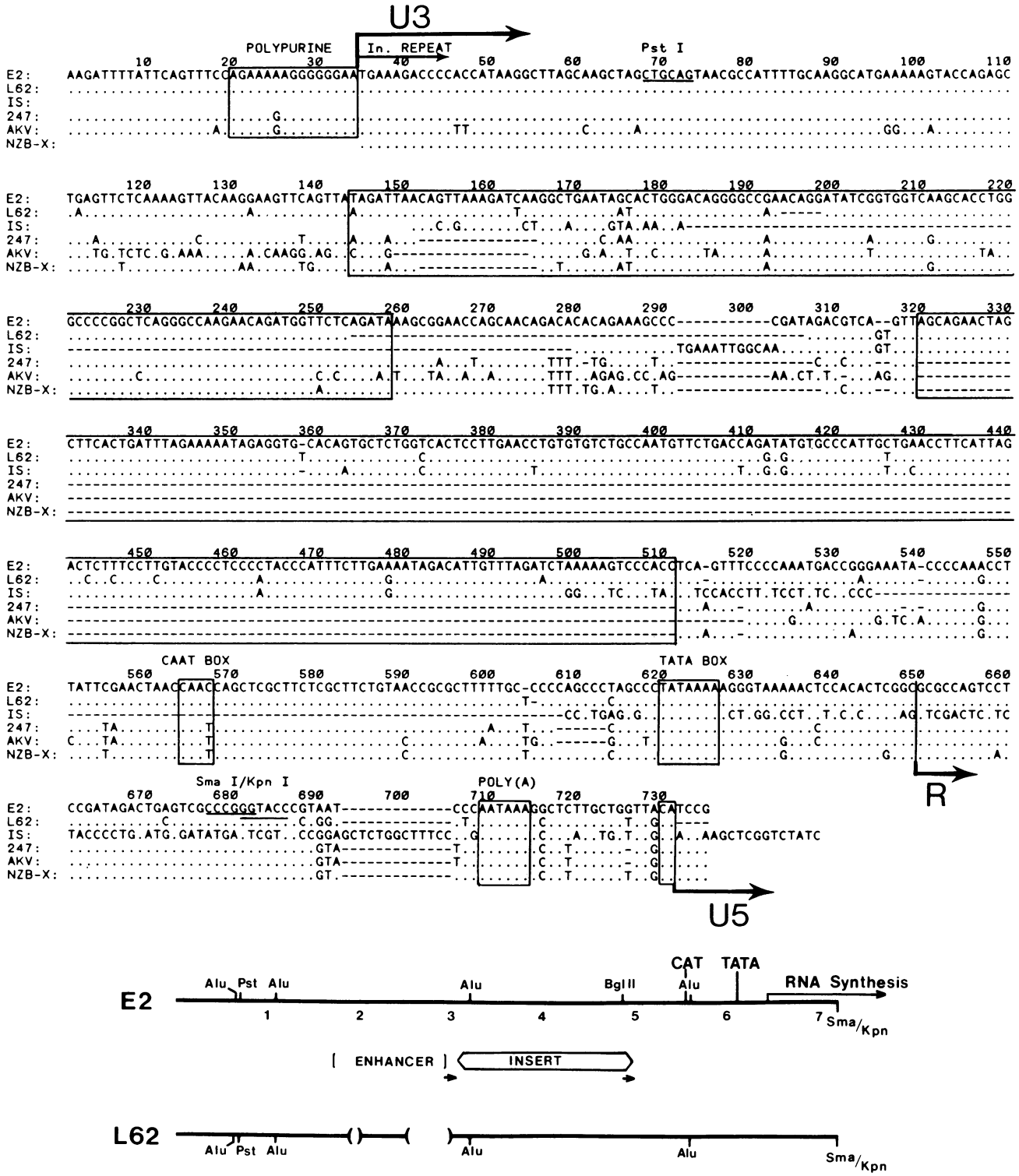

FIG. 1. Comparison of LTR sequences demonstrates the presence of the 192-bp middle repetitive element in the 3′ U3 sequence of the endogenous retroviral transcripts. The sequence of the endogenous 3′ LTR of cDNA clones E2 and L62 was determined by the dideoxy chain termination method (37) and compared with the 5′ LTR sequences of infectious MCF 247 (21), ecotropic AKV (17), and xenotropic NZB (34) viruses and the solitary LTR element B12 isolated from strain 129 genomic DNA (42), indicated as IS. E2 is a cDNA clone derived from an epididymal transcript deleted of the 5′ portion of p15E and the entire *env*-coding sequence (28); L62 is a cDNA sequence of the 3′ end of an apparently complete, undeleted liver transcript. Maximal alignment of the IS element required matching the first 32 nucleotides with the endogenous sequence and indicates a deletion in the IS sequence encompassing nucleotides at positions 183 to 279 and an insertion of 11 nucleotides at 692 to 704. Matched nucleotides are indicated by dots, and the spaces introduced for alignment are represented as dashes. The major conserved LTR sequences are boxed or indicated by arrows. The homologous regions containing potential enhancer activity are indicated as a boxed region, position 142 to 258. The 192-bp middle repetitive DNA insert within the expressed endogenous and IS LTR sequence at positions 320 to 511, flanked by the 6-bp repeat TCAGTT, is boxed.

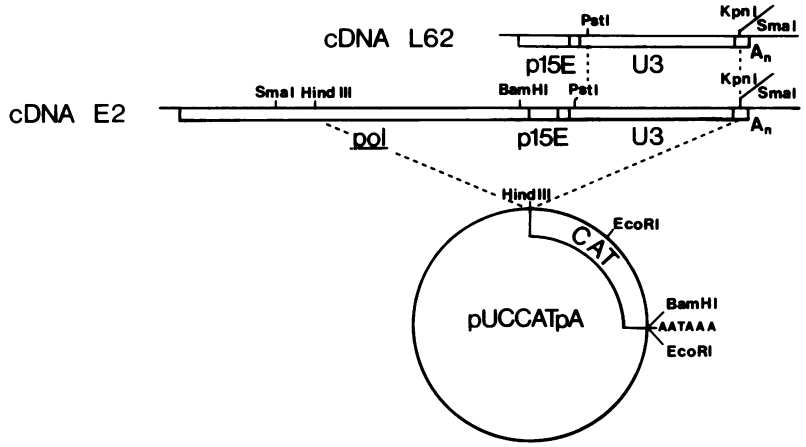

FIG. 2. Construction of 3′ LTR CAT expression plasmids. Two cDNA clones, E2 and L62, containing representative 600- and 550-bp *Pst*I-*Sma*I fragments of U3 sequence, respectively, are diagrammed. To generate the E2 CAT construct, *Hin*dIII linkers were ligated to the *Sma*I fragment of pE2. The U3-containing fragment, bounded at the 5′ *Hin*dIII site in the *pol* homologous region and at 3′ end with the *Hin*dIII linker at the *Sma*I site, which is conserved within the R region at the 3′ end of the LTR of both these endogenous and infectious proviral sequences as an overlapping *Sma*I-*Kpn*I decanucleotide, was then ligated into the *Hin*dIII site of the expression vector pUCCAT (30). The L62 CAT construct was made by ligating the *Sma*I-*Pst*I E2 *pol*-p15E fragment to the *Pst*I-*Sma*I U3 fragment of L62, followed by the addition of *Hin*dIII linkers and ligation into pUCCAT as indicated for pE2CAT.

insertions over the approximately 485 aligned nucleotides; comparison of E2 sequence with MCF 247 and NZB xenotropic sequences shows 44 and 40 nucleotide differences, respectively. In addition, both the E2 and L62 LTR sequences contain the tandem direct repeat AGCCCT found upstream from the TATA box in xenotropic but not ecotropic or MCF viral LTR sequence (34). However, although the cDNA sequences contain homologies to enhancer regions of infectious virus, the sequence TGGAAAGTCCC associated with viral enhancers and considered a core sequence in the simian virus 40 enhancer (41), present in both AKV (169 to 179) and MCF 247 (282 to 291), appears significantly diverged.

The two endogenous U3 regions, E2 and L62, differ by (i) 29 single nucleotide changes of 658 corresponding positions (4.4% divergence), (ii) two deletions of 5 and 42 nucleotides in clone L62 relative to E2, and (iii) two deletions of 2 and 1 nucleotides in E2 relative to L62. The 5-nucleotide deletion in L62 relative to E2 (194 to 198) is within endogenous sequence aligned by homology to enhancer sequences of MCF 247 and AKV. The larger deletion (254 to 306) removes further potential enhancer sequence. Also, both E2 and L62 3′ LTR regions include a 192-nucleotide insert (positions 320 to 511) of middle repetitive DNA not present in infectious virus. This insert, also found typical for LTR regions of endogenous BALB/c provirus (23, 36), is bounded by 6-nucleotide direct repeats (interrupted in L62 by a 2-nucleotide insertion) which are present in MCF 247 as tandem repeats but are present only as a single copy in AKV. This pattern of direct repeats is reminiscent of the action of transposable genetic elements which may be involved in the evolution of retroviral LTRs (23, 36). The 192-nucleotide insertion bears close similarity to a portion of a middle repetitive DNA element (IS in Fig. 1) which exists in the 129 mouse genome independent from retrovirus-coding regions and shows evidence of movement (42, 43). For the approximately 210 nucleotides (positions 304 to 513) that can be aligned well with E2 and L62 (Fig. 1), this repetitive element shares 91% sequence similarity with the endogenous transcripts.

To determine whether the endogenous LTR sequences can serve as efficient transcriptional promoters, the LTRs were joined at the conserved *Kpn*I-*Sma*I overlapping cleavage sites in the R region to the bacterial chloramphenicol acetyltransferase (CAT)-coding region of the expression vector pUCCATpA (30) and transient levels of mRNA expression were assayed by enzymatic CAT activity in cell extracts after $CaPO_4$-mediated DNA transfection (14, 15). To generate pE2U3CAT, the 1,450-base-pair (bp) *Hin*dIII-*Sma*I fragment of cDNA plasmid clone pE2 (28) was ligated after the addition of *Hin*dIII linkers at the *Sma*I site (Fig. 2). To insert the alternative LTR structure containing the 5- and 42-bp deletions, *Hin*dIII linkers were added at the *Sma*I site of cDNA clone L62 and the 550-bp fragment, resulting from cleavage at the *Pst*I site at the 5′ end of U3, was used to replace the 600-bp U3 region of the pE2U3CAT construct. Positive and negative orientation of the LTR with respect to CAT-coding sequence was determined by mapping *Bam*HI, *Pst*I, and *Hin*dIII cleavage sites. M1CAT, a construct containing the robust LTR promoter of MoMuLV (29), was used as a normalizing control. CAT activity was measured after transfection of mouse L cells and a cell line of strain 129 origin (SSLC) derived from 129 PCC4aza1 embryonal carcinoma cells (38).

The endogenous LTRs could provide substantial promoter activity (Table 1). The E2 LTR resulted in approximately 20% of the level of CAT obtained with M1CAT in L cells and 15 to 50% of M1CAT activity in SSLC cells. Substitution of the complete E2 U3 fragment with that of L62, which differs from E2 by deletions of 5 nucleotides at the 5′ and 42 nucleotides at the 3′ sides of the putative enhancer element, resulted in an activity approximately 40% of that found with E2 U3. Similar results were obtained with an additional independent clone of the L62 CAT construct (data not shown). As expected, the reverse orientation of either the E2 or L62 LTR resulted in no CAT expression, consistent with the CAT measurement reflecting relative promoter strength. The results show that the cDNA derived from endogenous proviral LTR transcripts could provide a functional promoter at least in a transient state as unintegrated, episomal DNA. In preliminary experiments, similar constructs driving the *neo*^{r} gene of Tn*5* in L cells showed promoter activity

TABLE 1. Promoter activity of endogenous LTRs assayed by transient expression of CAT activity

LTR	CAT activity in[a]:		
	L cells	SSLC cells	
		Expt 1	Expt 2[b]
M1CAT	1.00 (7.8%)	1.00 (10.5%)	1.00 (34.8%)
SV2CAT	0.35	1.17	ND
OCAT	0.07	0.02	ND
E2$\overrightarrow{U3}$ CAT	0.21	0.15	0.51
E2$\overleftarrow{U3}$CAT	0.02	ND	0.002
L62$\overrightarrow{U3}$ CAT	ND	ND	0.22
L62$\overleftarrow{U3}$CAT	ND	ND	0.002

[a] Mouse L cells or SSLC cells were plated at 5×10^6 cells per 100-mm dish and exposed to 20 μg (L cells and SSLC cells in experiment 2) or 10 μg (SSLC cells in experiment 1) of calcium-phosphate-coprecipitated plasmid DNA. Extracts were prepared at 40 h posttransfection, and CAT activity was assayed (14) by using 30% of the cell extract per point. CAT activity is expressed as the level of conversion to acetylated forms of chloramphenicol after 15 min (SSLC cells in experiment 2) or 30 min (L cells and SSLC cells in experiment 1) of incubation at 37°C relative to the expression of M1CAT. A time course indicated a linear rate of CAT enzymatic activity for each cell extract at these times. The actual percent conversion for extracts of M1CAT-transfected cells is shown in parentheses. M1CAT and SV2CAT contain the 3′ U3 promoter of the MoMuLV (29) or the simian virus 40 early promoter with enhancer (14), respectively. OCAT is the M1CAT deleted of MoMuLV sequence, and it corresponds to pUCCAT (30). The E2 CAT and L62 CAT constructs contain the 3′ LTR sequences of endogenous retroviral transcripts as diagrammed in Fig. 2. The orientation of the endogenous LTR promoter with respect to CAT-coding sequences is indicated by an arrow over U3.

[b] In this experiment, SSLC cells were exposed to 6 μg of M1CAT. As CAT expression is a linear response to the amount of input plasmid DNA, the activity of plasmids containing endogenous LTRs in SSLC cells was normalized relative to the amount (6 μg) of M1CAT DNA input.

from E2 LTR, as detected by S1 nuclease protection of transcripts in G418 resistant, stably transfected cell lines (data not shown).

We considered that the 192-nucleotide insertion element in 3′ LTR sequences might provide the block in infectious virus formation by affecting promoter activity at the 5′ end to limit the number of transcribed genes and at the 3′ end of transcripts to block transcription after regeneration of the 5′ LTR promoter through retroviral replication. Several observations now make this unlikely. First, we showed that the 3′ LTR bearing this 192-nucleotide sequence was a promoter as measured in transient expression assays. Second, since the vast majority of LTR sequences in the 129 strain genome include the 192-nucleotide middle repetitive DNA element (36; D.E.L., unpublished observations), it is likely that the transcription of abundant, polyadenylated endogenous retroviral RNA is initiated at LTR sequences bearing this 192-nucleotide element. In a finding consistent with the possibility that such LTRs serve as promoters, we recently isolated recombinant clones of transcriptionally active endogenous retroviral genes containing 5′ and 3′ LTR structures that include this repetitive DNA insertion element (P. Policastro, M. Fredholm, and M. C. Wilson, manuscript in preparation). Kohler et al., moreover, have reported (26) that solitary LTR IS elements similar to that shown in Fig. 1, which include a TATA box homolog downstream from the middle repetitive DNA element, can function as promoters when joined to a retroviral enhancer sequence. Therefore, it is probable that the primary defect in producing infectious virus in 129 mice is within the protein-coding regions upstream of the 3′ LTR (28), possibly in the gene for reverse transcriptase (20).

The demonstration of promoter activity at the 3′ LTR of endogenous retroviral transcripts suggests that reverse-transcriptase-mediated duplicative transposition could result in a new, active transcription unit and could therefore lead to a perpetuating process to generate the multiple copies of endogenous retroviral sequence present in the murine genome. For example, we have identified several copies of endogenous retroviral sequence, bearing identical deletions of *env* sequence, that appear to have transposed during the derivation of different strains of mice (Policastro et al., in preparation). Similarly, intracisternal A-type particles produce no infectious particles (31) but appear to transpose (16) as do rodent VL30 elements (7) and yeast Ty elements (5). Thus, the presence of a functional promoter element within an RNA transcript may be the minimal requirement for a retrotransposon (3) and may be sufficient for maintaining a large, dispersed population of sequences. The 192-nucleotide repetitive element within the endogenous LTR also shows evidence of transposon movement (23). The striking sequence conservation of this element both within intact proviral structures and in isolated, solitary LTR sequences (42) indicates an additional mechanism, such as gene conversion, in maintaining this homogeneity while allowing divergence in flanking enhancer and promoter regions. The possibility that this conserved sequence, flanked by 6-bp repeats, may function as a regulatory cassette influencing tissue-specific and strain-specific regulation of retroviral transcription (27) remains the subject for further study.

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