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Functional RNA polymerase II promoters in solitary retroviral long terminal repeats (LTR-IS elements)

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ABSTRACT

LTR-IS elements are middle repetitive sequences in the mouse genome with structural features of solitary retroviral LTRs. In order to get some insight in the possible functional role of these sequences the promotor activity of two LTR-IS representatives differing by 105 bp in their U3 region was investigated. Gene fusions between LTR-IS sequences and the bacterial gene coding for chloramphenicol acetyl transferase (CAT) were transfected into mouse 3T6 cells and the expression of CAT was measured. It is shown that the LTR-IS sequences represent weak RNA polymerase II promoters which require enhancement by cis-or trans-activating factors.

INTRODUCTION

Retroviral proviruses exhibit striking similarities to transposable elements of prokaryotes, yeast and insects (for review see 1). This similarity encompasses structural as well as functional properties. Retroviral DNA contains two terminally repeated sequences (LTR) which resemble prokaryotic IS elements or delta sequences of the yeast transposon Ty-1. The structural similarities include a short (3 to 6 bp) direct repeat of host DNA at the junction between the provirus and host DNA, inverted repeats at both termini of each LTR and the presence of transcriptional control signals including regions implicated in the initiation and termination of transcription. Evidence is also accumulating for the existence of sequences with an enhancer-like function (2, 3) within the U3 region of the LTR known as cis-acting transcriptional activators (for review see 4).

We have previously described a new family of middle repetitive DNA elements in the mouse genome called LTR-IS (5). These sequences closely resemble insertion elements and have the

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structural features of solitary retroviral LTRs. They are 500 - 600 bp long, start and end with 11 bp inverted repeats and are flanked by a 4 bp duplicated sequence of host DNA at either end. The LTR-IS elements contain putative transcription regulatory signals, including a "TATA" box, and a polyadenylation signal. The functional significance of the LTR-IS elements remains still a target for speculations. Recent studies indicated that LTR-IS sequences have arisen in early mouse ancestors and were mobile at least at some point during their evolution (6). These findings suggest two alternative modes of action: The LTR-IS elements could act as mutagens by insertion into a gene, thus causing its inactivation, or they could function as mobile promoters by activating transcription of adjacent DNA regions. In order to prove this working hypothesis we investigated whether the LTR-IS sequences can function as promoters for RNA polymerase II *in vivo*. In this paper we show that gene fusions containing LTR-IS sequences in front of the bacterial gene coding for chloramphenicol acetyltransferase (CAT) are efficiently expressed in mouse cells provided that a cis-acting enhancer element is present in the recombinant plasmids.

MATERIALS AND METHODS

Recombinant plasmids

The control plasmids pSV2 CAT and pA10 CAT were described by Gorman et al. (7). As a recipient plasmid for insertion of LTR-IS sequences pCAT3M of Laimins et al. (8) was used. It lacks all transcriptional regulatory sequences but retains the AUG codon for initiation of CAT translation. LTR-IS fragment to be tested for promotor activity were inserted into the Bgl II site 37 bp in front of the CAT AUG codon by blunt-end ligation. The nucleotide sequences of the LTR-IS clones were published (5). The plasmids containing the collagen promoter without an enhancer (ColCAT) or with polyoma virus enhancer (pHB 20) were constructed and described by Herbomel and Yaniv (9).

Transfection procedure

Subconfluent cultures of cells were transfected with supercoiled plasmid DNA by a modification of the calcium phosphate coprecipitation technique (7, 10, 11). One day prior to trans-

fection the cells were plated at a density of 5 x 10^5 cells per 9 cm dishes in Dulbecco's MEM medium containing 10 % fetal calf serum. Cells were supplied with fresh medium 3 h before transfection. A transfection cocktail was prepared containing 50 mM HEPES, pH 7.1, 280 mM NaCl and 1.5 mM $NaHPO_4$. While vortexing 0.5 ml of this solution, 0.5 ml of the DNA-$CaCl_2$ mixture (20 μg DNA, 250 mM $CaCl_2$, 2 mM Tris-HCl, pH 7.6) was added dropwise. The DNA precipitates were allowed to stand for 30 min at room temperature and then applied to the cells. Transfected cells were incubated overnight at 37° C in the presence of 5 μg/ml amphotericin B (GIBCO), which has been shown to enhance transfection efficiency (12). 16 h after transfection the cells were refed with fresh medium and cultured for another day.

Assay for CAT activity

The cells were harvested 44 hrs after transfection and assayed for CAT activity by the method of Gorman et al. (7) with a few minor modifications. Cell extracts were prepared by sonicating the cells in 200 μl of 250 mM Tris-HCl (pH 7.8). The enzyme was assayed in a final volume of 180 μl of 250 mM Tris-HCl (pH 7.5) containing 30 μl of cell extract, 20 μl of 4 mM acetyl coenzyme A and 0.1 μCi of ^{14}C-chloramphenicol (50 μCi/mmole; New England Nuclear Corp.). The enzyme assay was carried out at 37° C for various times ranging from 5 to 90 min, and stopped by adding 0.5 ml cold ethyl acetate. The chloramphenicol was extracted from the reaction mixture by two more extractions with 0.2 ml ethyl acetate. The pooled solvent was evaporated, the chloramphenicol dissolved in 25 μl ethyl acetate and spotted onto silica thin-layer chromatography plates. The unreacted chloramphenicol was separated from the mono- and diacetylated forms by ascending chromatography in chloroform -methanol (95:5). After autoradiography, the radio-active spots were cut out, counted in a scintillation counter and the conversion of chloramphenicol into the acetylated forms was calculated.

ß-galactosidase assay

When CAT and ß-galactosidase activity were to be assayed simultaneously, 20 μg of CAT constructs were co-transfected with 2 μg of pCH110 (13), a plasmid where the SV40 promoter and en-

hancer sequences are fused to the lac Z gene of E. coli. ß-galactosidase was assayed in 60 µl of the cell extracts essentially as described by Miller (14).

RNA preparations

Cells from one to three plates of transfected cells were lysed in 2.5 ml 4 M guanidinium isothiocyanate, 10 mM EDTA, 50 mM Tris-HCl, pH 7.6, 2 % sarcosyl, 0.14 M ß-mercaptoethanol. 1 g CsCl was added and the solution layered over a 1.2 ml cushion of 5.7 M CsCl, 0.1 M EDTA. RNA was pelleted by centrifugation at 35,000 rpm in a Beckman rotor SW 55 for 16 hrs (15, 16). The RNA pellet was dissolved in 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1 % SDS and extracted with chloroform-butanol (4:1 v/v) before ethanol precipitation.

Primer extension analysis

To prepare the radioactive primer, pSV2 CAT was digested with Pvu II, which cleaves in the center of the CAT gene, 150 bp downstream of the 5' end of the CAT coding region. 1 µg of the digested DNA was preincubated for 10 min at 37° C with 2 units T4 DNA polymerase in 25 µl 33 mM Tris-acetate (pH 7.9), 66 mM Na-acetate, 10 mM Mg-acetate, 100 µg/ml bovine serum albumin and 0.5 mM DTT. This resulted in a partial digestion of the coding DNA strand by the 3'-5' exonuclease activity of the T4 DNA polymerase. The single-stranded regions were filled in by T4 DNA polymerase in the presence of 0.25 mM dGTP, dCTP, dTTP and 30 µCi of (^{32}P) dATP so that the labeled nucleotides spread over the length of the coding strand of the primer. After 10 min at 37° C the reaction was chased with 0.25 mM cold dATP for a further 20 min. After inactivation of the T4 DNA polymerase by heating for 5 min at 65° C, the DNA was cleaved with Eco RI. The 102 bp PvuII-EcoRI fragment was isolated after electrophoresis in a 8 % polyacrylamide gel. Approximately 100,000 cpm of the primer were mixed with 40 - 50 µg total cellular RNA from transfected cells, ethanol precipitated, and dissolved in 25 µl hybridization buffer (80 % formamide, 40 mM PIPES, pH 6.4; 0.4 M NaCl, 1 mM EDTA, 1 mM vanadyl ribonucleoside complex). After incubation for 5 min at 70° C the primer was annealed to the RNA by incubating for 3 hrs at 42° C. Hybrids recovered by ethanol precipitation were extended with reverse transcriptase as described by Hernandez and Keller (17).

RESULTS

Construction of the LTR-IS-CAT plasmids

Previously we have described a new family of middle repetitive sequences in the mouse genome which cross-hybridize with an endogenous retroviral LTR-probe (5). The nucleotide sequence of nine clones of these sequences, called LTR-IS elements, revealed that these 500 to 600 bp long elements are about 90 % homologous and exhibit structural features characteristic of solitary retroviral LTRs. There are two classes of LTR-IS elements, the long and the short one (18). The length differ ences are due to two deletions. One 12 bp deletion is located at position - 200 and another 93 bp deletion at position - 160 upstream from the putative cap site. Fig. 1 a shows schematically

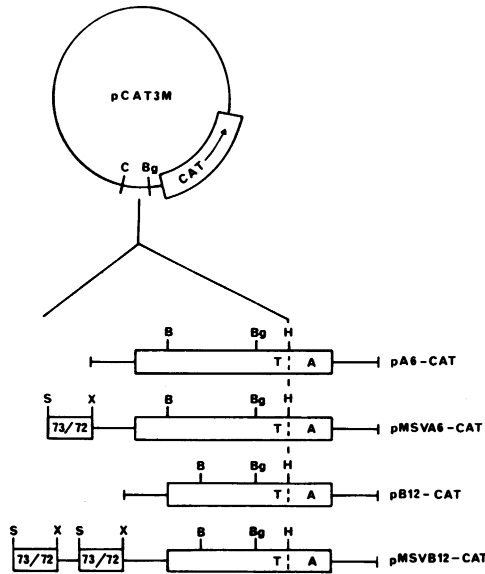

Fig. 1
The structures of plasmid constructs containing the LTR-IS elements
BglII/HindII fragment of the LTR-IS clones pA6 and pB12 was inserted into the BglII site of the recipient plasmid pCAT3M by blunt end ligation. pMSV A6 CAT and pMSV B12 CAT were constructed by inserting the subcloned 72/73 Moloney MSV enhancer into the pA6 CAT and pB12 CAT. B, BalI, Bg, BglII; C, ClaI; H, HindII; S, Sau 3A; X, XbaI, T indicates the position of the TATA box, A indicates the ATAA box.

the structure of the two classes of the LTR-IS elements with signal sequences implicated in the initiation of transcription by RNA polymerase II and RNA polyadenylation.

In order to investigate whether these solo LTR-IS elements could constitute functional promoters _in vivo_ we have inserted the LTR-IS sequences into a mammalian expression vector. The plasmids used in this study were constructed from pCAT 3 M (8), a plasmid which contains no eukaryotic promoter, the bacterial gene coding for chloramphenicol acetyltransferase (CAT) and the SV40 polyadenylation signal downstream of the CAT gene sequence. When a polymerase II promoters is placed in front of the CAT gene a properly processed, translatable mRNA is produced in eukaryotic cells. The amount of CAT enzyme activity directed by a given eukaryotic promoter has been shown to provide a quantitative measure of the transcriptional activity of this promoter. Therefore, if the two classes of LTR-IS elements differ in their promoter strength, they are expected to promote different CAT levels after introduction into animal cells. The clone pA6 was used as a representative of the long class of the LTR-IS elements, whereas the clone pB12 belongs to the shorter class of LTR-IS elements. In both cases the LTR-IS sequence was cut with Hind II, which cleaves 26 bp downstream of the TATA box and ligated to the blunt ended Bgl II site of the pCAT3M, 37 bp upstream of the CAT gene AUG codon. The hybrid genes were designated pA6 CAT and pB12 CAT, respectively (Fig. 1).

Expression of LTR-IS-directed CAT activity

The LTR-IS-CAT constructs were transfected into mouse 3T6 cells using the calcium phosphate procedure (7, 10, 11). After 40 hrs the CAT activity present in the cells was assayed. For quantitative comparison a number of CAT gene fusions was transfected in parallel with control plasmids which contained strong or weak RNA polymerase II promoters, respectively. As a control the plasmid pSV2 CAT was used which contains the SV40 early promoter including the 72 bp repeat enhancer sequences (7). The analogous plasmid without the SV40 enhancer is designated pA10 CAT (7). Furthermore we used two plasmids which contain a non-viral promoter in front of the CAT gene. In the plasmid pCol-CAT the promoter of the chicken collagen 2 type I gene was

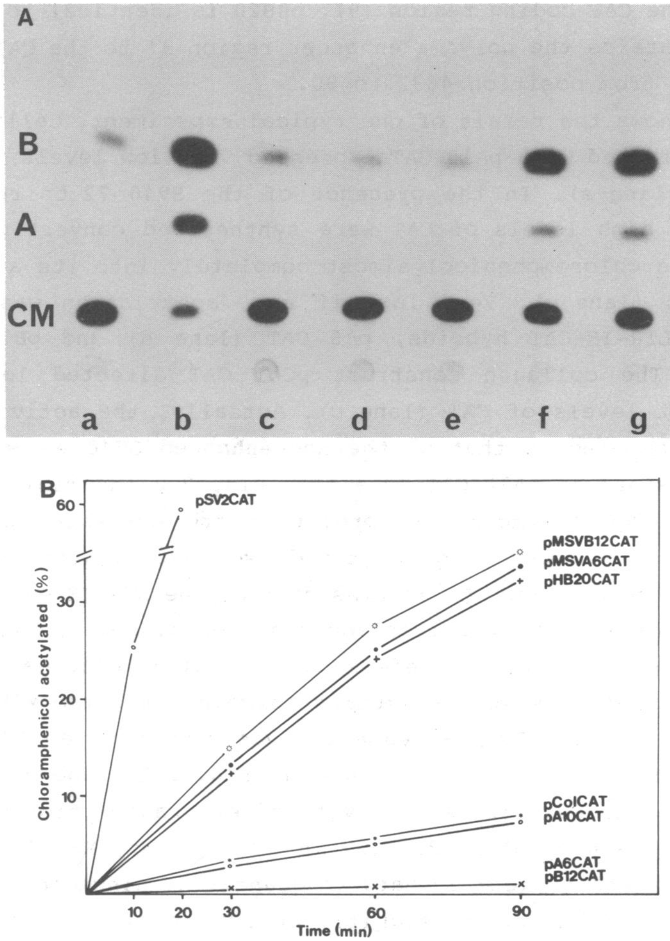

Fig. 2 A
CAT activity in 3T6 cells transfected with various CAT gene fusions
Transfections were done as described in Materials and Methods. The enzyme reaction was performed with 30 µl of extract at 37° C for 60 min. The ^{14}C-chloramphenicol (CM) was separated from the acetylated forms (A, B) by thin layer chromatography. a) pA10 CAT, b) pSV2 CAT, c) pCol CAT, d) pA6 CAT, e) pB12 CAT, f) pHB20 CAT, g) pMSV A6 CAT

Fig. 2 B
Time course of CAT enzyme activity in extracts from transfected cells
30 µl of extracts from cells transfected with different plasmids were incubated with ^{14}C-chloramphenicol and the CAT activity was determined in aliquots at different times.

fused to the CAT coding region (9), pHB20 is identical to pCol-CAT but contains the polyoma enhancer region 3' to the CAT-SV40 region (9) from position 4632 to 90.

Fig. 2 A shows the result of one typical experiment. Cells that were transfected with pA10 CAT expressed very low levels of CAT activity (lane a). In the presence of the SV40 72 bp repeats (pSV2 CAT) high levels of CAT were synthesized converting the radioactive chloramphenicol almost completely into its acetylated forms (lane b). Very low, if any, acetylation was seen with the LTR-IS-CAT hybrids, pA6 CAT (lane d) and pB12 CAT (lane e). The collagen construct pCol CAT directed low but significant levels of CAT (lane c). Actually, the activity of pCol CAT compared to that of the non-enhanced SV40 early promoter contained in pA10 CAT (see also Fig. 2 B and Table 1).

Since most RNA polymerase II promoters are activated by enhancers by 2 - 3 orders of magnitude we investigated whether transcription and thus CAT expression from the LTR-IS sequences could be augmented by viral enhancer elements, too. In view of the observed host-cell preference of viral enhancers the 72/73 bp repeat from Moloney murine sarcoma virus (Mo-MSV) was inserted 5' to the LTR-IS sequences yielding pMSV A6 CAT and pMSV B12 CAT, respectively. As shown in Fig. 2 A (lane g) there was a large increase in CAT activity after transfection of the enhancer-containing plasmids, MSV A6 CAT into and MSV B12 CAT. The level of enhancement is hard to quantitate since in the absence of the enhancer no significant levels of CAT activity were seen. The fact that the low transcriptional activity of the LTR-IS-CAT constructs could be augmented by the viral enhancer suggest that the LTR-IS elements may constitute weak RNA polymerase II promoters.

Quantitation of CAT activity

In order to compare the LTR-IS directed CAT expression quantitatively with the enhanced or non-enhanced SV40 and collagen promoter activity, kinetic experiments have been carried out. Fig. 2 B shows the time course of CAT activity in extracts from cells transfected with pSV2 CAT, pA10 CAT, pA6 CAT, MSV A6 CAT, MSV B12 CAT, pCol CAT and pHB20. The highest CAT levels were consistently found in cells transfected with pSV2 CAT. 50 % of

Table 1

CAT activity in extracts from 3T6 and 293 cells transfected with various CAT gene fusions

PLASMID	CAT ACTIVITY* (%) IN EXTRACTS FROM 3T6 CELLS	293 CELLS
pSV2 CAT	100.0	100.0
pA10 CAT	1.9	3.9
pCol CAT	2.4	19.8
pHB20	8.1	15.1
pA6 CAT	0.6	13.1
pMSV A6 CAT	8.2	13.9
pB12 CAT	0.3	7.5
pMSV B12 CAT	11.3	N.D.
pCAT 3M	0.6	6.4

*3T6 and 293 cells were cotransfected with CAT hybrid genes and plasmid pCH110. The CAT activities from different experiments were normalized to the ß-galactosidase activity, and compared to the CAT activity of pSV2-CAT transfected cells (= 100 %). The data shown are mean values of at least three independent experiments with different plasmid preparations.

the input chloramphenicol was acetylated within 15 min of incubation. CAT activity directed by pA10 CAT, and pCol CAT was low, yielding at most 7 % conversion of chloramphenicol into its acetylated forms after an incubation time of 90 min. CAT activity directed by LTR-IS CAT constructs was even an order of magnitude lower than pA10 CAT or pCol CAT.

A summary of the quantitative data derived from kinetic measurements of CAT activity in 3T6 cells is shown in Table 1. The conversion rates of chloramphenicol into its acetylated forms were calculated from mean values of different experiments performed with at least two different plasmid preparations. To achieve accurate comparison from one experiment to another and to eliminate variabilities in transfection efficiency from one plate to another we co-transfected the CAT constructs with an internal marker, the plasmid pCH110 (13). This plasmid expresses a ß-galactosidase fusion protein under the control of

the SV40 early promoter. The molar ratio of the CAT constructs to pCH110 was 10 : 1. The cell extracts were tested for both the CAT and ß-galactosidase activities. As a final set of data, the mean normalized conversion of chloramphenicol from the different plasmids was compared to pSV2 CAT.

Mapping of the CAT specific transcripts by primer extension

In order to investigate whether the LTR-IS promoted CAT transcripts originated from the presumptive initiation site 26 bp downstream of the TATA box, the 5' end of the hybrid transcripts were mapped. For this, a 102 bp Pvu II/Eco RI fragment derived from pSV2 CAT was labelled and hybridized to total cellular RNA. The primer was extended with reverse transcriptase and the cDNAs analyzed on sequencing gels. Fig. 3 A shows the primer extension analysis of RNA from cells transfected with pSV2 CAT and MSV A6 CAT, respectively. RNA from 3T6 cells transfected with pSV2 CAT yielded three bands characteristic for transcripts that were initiated at the SV40 early cap sites (lane 1). RNA from cells transfected with the MSV A6 CAT hybrid gene produced a reverse transcript approximately 260 nucleotides long (lane 2 and 3). This length corresponds to the distance from the presumptive cap site of the LTR-IS element to the 3' end of the primer, indicating the CAT-specific RNA had been initiated within the LTR-IS element downstream of the TATA box.

Transactivation of the LTR-IS promoter by the adenovirus E la gene product

Recently it has been shown that the Ela gene product of adenovirus can exert a positive regulatory effect on some cellular promoters when they are present in transfected DNA molecules (19, 20). In order to investigate whether this transactivating effect can also be observed on the LTR-IS directed CAT expression, we transfected pA6 CAT and pB12 CAT into human kidney 293 cells. These transformed cells express the adenovirus immediate early protein constitutively and have been shown to activate heterologous genes in an enhancer independent fashion (19, 20). Table 1 compares the CAT activity of the LTR-IS gene fusions after transfection into 3T6 or 293 cells, respectively. pA6 CAT and pB12 CAT which were quasi silent in 3T6 cells were acti-

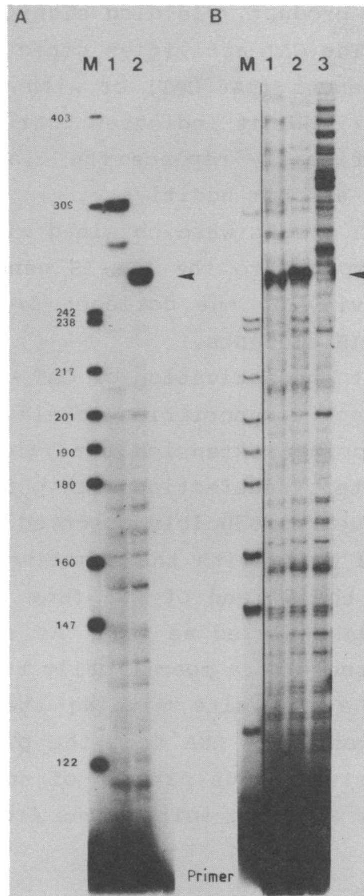

Fig. 3
Primer extension analysis of CAT-specific RNA synthesized in 3T6 or 293 cells
50 µg of total cellular RNA were hybridized to a labelled 102 bp PvuII/EcoRI fragment derived from the CAT gene and the hybrids were transcribed by reverse transcriptase. Following reverse transcription, RNA was degraded by RNAase treatment and the ^{32}P-cDNAs were electrophoresed on a 6 % denaturing polyacrylamide gel along with ^{32}P-DNA markers (M) from a HpaII digest of pBR322 DNA. A) cDNA transcripts of RNA from 3T6 cells transfected with pSV2 CAT (lane 1) and pMSVA6 CAT (lane 2) are shown. B) cDNAs of RNA from 293 cells transfected with pB12 CAT (lane 1), pA6 CAT (lane 2) and pCAT3M (lane 3). The arrow indicates the position of the reverse transcript of LTR-IS-CAT direct RNAs.

vated by the Ela gene product, yielding significant CAT enzyme levels in 293 cells. The CAT activities directed by the LTR-IS construct without enhancer (pA6 CAT) or with the MSV enhancer (pMSV A6 CAT) were cells. This indicates that the transactivation by Ela can functionally replace the cis-acting enhancer and that both effects are not additive.

Once again, higher CAT levels were obtained with the collagen-CAT constructs as compared to the LTR-IS gene fusions, indicating that the activity of the collagen promoter is higher than that of the LTR-IS elements.

In order to prove that the activation of CAT expression in 293 cells was due to correct transcription initiations at the presumptive cap site a primer extension analysis of RNA synthesized in 293 cells after transfection with pA6 CAT or pB12 CAT was performed. Since we reproducibly observed a stimulation of CAT expression in 293 cells with the negative control plasmid pCAT3M (see Table 1) the 5' end of RNA from 293 cells transfected with pCAT3M was analyzed as well. As shown in Fig. 3 B both LTR-IS-CAT constructs were specifically transcribed in 293 cells starting from a cap site withing the LTR-IS element (lanes 1 and 2). In contrast, RNA from the pCAT3M-transfected 293 cells showed a heterogenous pattern of non-specific bands which must result from abortive initiations from the vector DNA (lane 3).

DISCUSSION

Sequence analysis revealed the presence of transcription regulatory signals within the LTR-IS elements and raised the possibility that these repetitive sequences may function as "portable" promoters. In this study we have examined the transcriptional competence of two cloned LTR-IS elements. When inserted into the eukaryotic expression vector pCAT3M and transfected into NIH3T6 cells, no significant levels of CAT were produced. If, however, a viral enhancer was inserted into the LTR-IS-CAT constructs high amounts of CAT were synthesized. A similar stimulation of CAT expression was observed in 293 cells in the absence of a viral enhancer. Therefore, the LTR-IS elements could be classified as "weak" promoters which require either a

cis-linked enhancer sequence or a trans-activating factor(s) for transcriptional activity.

Previous similar investigations have identified a RNA polymerase II initiation site in the LTR of several retroviral proviruses (4). Regulatory signals could be mapped to at least three domains within the LTR. The first contains the CAAT box, the second contains the TATA box and downstream sequences including the RNA initiation site and the third - present in the U3 region of some retroviruses - contains enhancer elements. Most likely the presence of a strong enhancer determines both the level of viral transcription and the oncogenic potential of retroviruses.

A high copy number of the LTR-IS elements in the genome with functional promoter activity would require an additional regulatory mechanism in order to keep them under control. On the other hand, a weak promoter located on a movable element might be phylogenetically advantageous, for instance by creation of a novel transcriptional unit if inserted into a proper location. Recently Cullen et al. (21) have shown that the 3' LTR of a retrovirus is unable to act as an efficient promoter of transcription when a transcriptionally active 5' LTR is present. Such inhibition known as transcriptional overlap interference is relieved by the deletion of the 5' LTR or by an interruption of the 5' LTR-directed transcription. This observation could explain why only deleted proviruses have been found inserted adjacent to the c-myc in the ALV-induced lymphomas (22, 23). The above mentioned transcriptional interference (21) displayed by proviral structures with two LTRs underlines the potential of solo LTR-IS elements as possible activators of transcriptionally silent genes by promoter insertion. In addition to the positive effects of LTR-IS elements on transcription, they could also inactivate cellular genes simply by insertion into an essential gene region or by providing termination signals.

It is likely that not all LTR-IS elements are transcriptionally active. The transcriptional inactivity of some elements may be due to the 10 % sequence heterogeneity found between different elements (5) or may be controlled by external factors. The ex-

pression of the LTR-IS elements would be a consequence of their integration into transcribed or non-transcribed DNA regions. In order to distinguish between these possibilities it is necessary to isolate the LTR-IS elements that are active within the cell. These active elements could be structurally and functionally different from the majority of LTR-IS sequences. We are currently characterizing cellular transcripts and cDNA clones which hybridize to LTR-IS sequences to get more information about the functional significance of LTR-IS elements.

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