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The Long Terminal Repeat of an Endogenous Intracisternal A-Particle Gene Functions as a Promoter When Introduced into Eucaryotic Cells by Transfection

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We describe experiments designed to determine whether an endogenous intracisternal A-particle (IAP) gene randomly selected from a mouse embryo library has the potential to be transcriptionally active. Assays for IAP gene transcription were done with permanently transformed rat cells and transiently transfected monkey and mouse cells. The rat cells, which had integrated IAP gene copies, contained IAP RNA. A start site within the IAP 5′ long terminal repeat (LTR) was localized by S1 mapping. The promoter activity of the IAP LTR was also measured in cells 48 h after the introduction of recombinant plasmids in which bacterial chloramphenicol acetyl transferase (CAT) encoding sequences were under the control of the LTR. The IAP LTR promoted CAT activity in mouse and monkey cells. In mouse L-cells, the levels of CAT activity were 10 to 25% of those promoted by an analogous recombinant containing the Moloney murine sarcoma virus LTR as the promoter. In contrast to the Moloney murine sarcoma virus LTR, the IAP LTR was five- to eightfold more active in monkey cells than in mouse cells. The 5′ and 3′ LTRs were equally active, and promoter activity was dependent on having the orientation of the LTRs with respect to the CAT gene the same as their orientation with respect to the IAP gene. A 5′-flanking sequence containing a member of the highly repetitive R-sequence family increased CAT activity in COS cells 11-fold when present along with the LTR. Our results indicate that the LTR of an endogenous mouse IAP gene can function as an efficient promoter in heterologous as well as homologous cells.

Intracisternal A-particles (IAPs) are retrovirus-like entities abundantly expressed in many mouse tumor cells (25). Endogenous genetic elements homologous to the high-molecular-weight RNA of IAPs are reiterated at ca. 1,000 copies per haploid genome in *Mus musculus* (26, 31, 32, 41).

Until recently it could be questioned whether the IAP sequence elements had any potential functional significance since the IAPs themselves appeared to be so severely defective (see reference 24). However, IAP proviral elements have now been shown to appear in novel locations in mouse cellular DNA (5, 6, 12, 19, 23, 24, 47) and to affect the function of known genes at the target sites (19, 44). Insertion of IAP sequences into the introns of two κ light-chain genes resulted in defective gene function (19). In another case, insertion of IAP sequences (23) resulted in activation of the cellular oncogene c-*mos* (5, 44). These observations show that the large family of endogenous IAP elements can be a significant source of genetic variation in the mice.

A number of endogenous IAP provirus-like elements have been cloned and analyzed (26, 32, 41, 46, 47). Both long terminal repeats (LTRs) of a 7.2-kilobase (kb) IAP gene (MIA14) isolated from a mouse embryo genomic library have been sequenced (24) and have been shown to share many structural properties of proviral LTRs of known infectious retroviruses (48). Analysis of other retroviral LTRs has shown that they provide functions essential for expression of eucaryotic genes such as a promoter, initiation site, and signal for polyadenylation of the RNA. Some viral LTRs have also been shown to have the ability to enhance transcription (1, 11, 27–30), and sequences essential for this function have been defined (27, 50). Thus far, the LTRs shown to have these functions have all been derived from proviruses which were known to be active in the cells from which they were isolated. In this paper we describe experiments which investigate whether an endogenous IAP genetic element randomly selected from a mouse embryo library has the potential to be transcriptionally active. Evidence is presented that the IAP LTR is a strong promoter in mouse cells as well as in heterologous eucaryotic cells.

MATERIALS AND METHODS

Construction of plasmid vectors. All enzymes were from New England Biolabs, Beverly, Mass. DNA fragments were purified from agarose gels by electroelution, followed by extraction with phenol and chloroform-isoamyl alcohol. Conditions for ligation, transformation of bacterial cultures, and identification of colonies containing the appropriate inserts by colony hybridization have been described previously (32).

The derivation and structure of the plasmids used in the present study are shown in Fig. 1. We have previously described a phage clone λMIA14 (Fig. 1A, top) containing a 7.2-kb IAP gene which was isolated from a BALB/c mouse genomic library (26, 32). This recombinant contains a 0.45-kb element of the highly repetitive R-sequence family (13, 35) upstream of the 5′ IAP LTR (Fig. 1A, R). The 5′ *Hin*dIII-*Bam*HI and 3′ *Bam*HI-*Eco*RI fragments from this clone were sequentially inserted into a variant of pBR322 from which the *Bam*HI site had been removed. Ligation of the *Bam*HI ends yielded a construct containing a single *Bam*HI site inside the IAP sequence and lacking the usual 1.4-kb internal *Bam*HI fragment. The clone pMIAtk-1 shown on the second line was derived by inserting a 3.4-kb *Bam*HI fragment carrying the herpes thymidine kinase (*tk*) gene and associated control region (38) into this site. The orientation of

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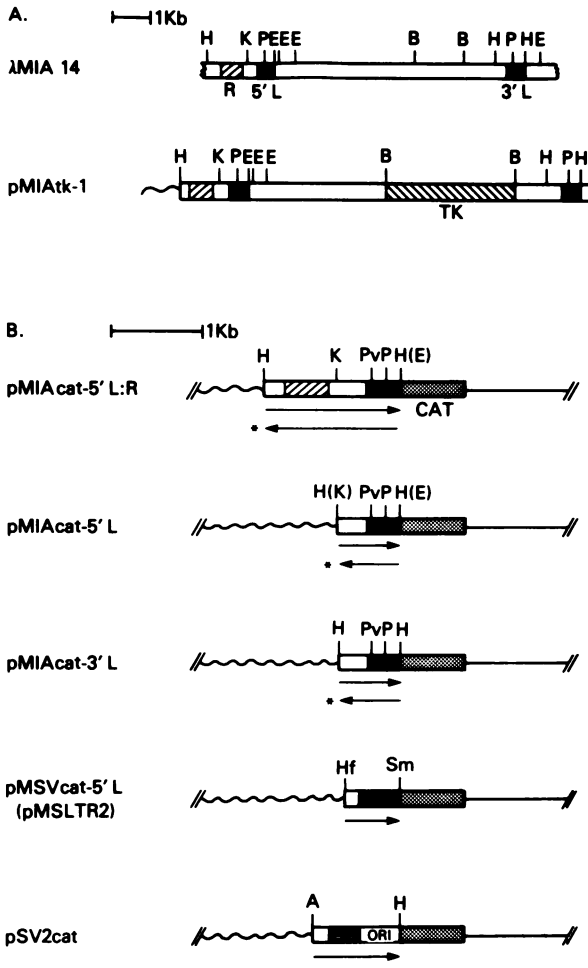

FIG. 1. Physical maps of cloned DNAs used for transfection experiments. (A) A partial restriction map of a phage clone λMIA14 containing a 7.2-kb IAP-specific region is shown at the top; the ends of the gene are delineated by LTRs (■). A highly repetitive R-sequence element (▭) upstream of the 5' IAP LTR is designated R. A map of pMIAtk-1, a construct in pBR322 containing the IAP and some flanking sequence from MIA14, is shown on the second line. The internal 1.4-kb *Bam*HI fragment of the IAP gene has been removed, and a fragment carrying the herpes *tk* gene (▨) inserted into this site (see text). (B) Maps of constructs made by cloning segments from the 5' and 3' ends of the MIA14 IAP gene into the plasmid pSV0cat containing the CAT gene (▩). Fragments derived in the following manner were isolated from agarose gels and cloned into the *Hin*dIII site of pSV0cat after attachment of *Hin*dIII linkers. The insert in pMIAcat-5'L:R is a 1.5-kb *Eco*RI-*Hin*dIII fragment containing the 5' LTR and flanking sequences (including R) cut from a previously characterized subclone of λMIA14, pMIA7 (26). The 0.7-kb fragment in pMIAcat-5'L contains the 5' LTR without flanking R-sequence and was isolated by cutting the insert in pMIAcat-5'L:R with *Kpn*I and *Eco*RI. The 0.7-kb fragment containing the 3' LTR in pMIAcat-3'L was isolated from plasmid pMIA6 (26) by cutting with *Hin*dIII. The orientation of all inserts is in the same direction as the CAT gene (→); constructs with inserts in the opposite direction (←) are designated with an asterisk after the name. pMSVcat-5'L contains an *Sma*I-*Hin*fI fragment of the MSV LTR (27). pSV2cat contains the early promoter region of SV40, including the 72-bp repeats (▩) (16). Restriction sites are as follows: H, *Hin*dIII; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; B, *Bam*HI; Pv, *Pvu*II; Hf, *Hin*fI; Sm, *Sma*I; A, *Acc*I. Sites in parentheses indicate sequences filled in during the attachment of linkers.

transcription of the IAP and *tk* sequences (5' → 3', left to right in Fig. 1A) is the same in this construct.

Figure 1B shows a series of constructs made by cloning segments from the 5' and 3' ends of the MIA14 gene into the plasmid pSV0cat (16) obtained from C. Gorman, National Institutes of Health (NIH). The derivation of the inserted fragments is given in the legend to Fig. 1. The plasmid pSV2cat (16) was provided by C. Gorman. DNA was isolated by a lysozyme-Triton X-100 procedure from bacterial cultures transfected with the constructs after growth for 48 h (without chloramphenicol amplification) and banded twice in CsCl as described by Gorman et a. (16). The pMSLTR2 construct (27) (here designated pMSVcat-5'L) carrying the Moloney murine sarcoma virus (MSV)-LTR was made by L. Laimins, NIH, who also provided the isolated DNA.

Eucaryotic cell transfection. A *tk*$^-$ line of Buffalo rat liver (BRLtk$^-$) cells (obtained from H. Coon, NIH) was used for experiments with pMIAtk-1. Cells were transfected by the calcium phosphate precipitation technique (17) with either supercoiled plasmid alone or plasmid that had been digested with *Bam*HI and mixed with calf thymus DNA. Clones which had stably incorporated the *tk* gene were selected by growth in HAT medium (51).

Monkey kidney cell culture lines CV1 and COS7 and mouse 3T3 and L-cell fibroblasts were used for transient transfections with the pMIAcat plasmids. The M6 subline of COS7 (14) cells developed by M. Horowtiz, Weizmann Institute, was obtained from C. Queen, NIH. NIH-3T3 cells (CCl 92) used were obtained from the American Type Culture Collection. L-cells were from L. Laimins, NIH. Cells were plated the day before transfection at 5.8×10^5 cells per 10-cm-diameter dish. Transfections were performed according to the calcium phosphate precipitation procedure of Graham and van der Eb (17) as modified by Parker and Stark (42), using 5 to 25 μg of supercoiled plasmid DNA.

S1 nuclease mapping. RNA was isolated from 10-cm-diameter plates of cells by the hot phenol-sodium acetate lysis procedure described by Queen and Baltimore (43). Polyadenylated RNA was prepared as previously described (36) with recycling over the oligodeoxythymidylate column. The probe was derived from plasmid pMIAcat-5'L:R* (Fig. 1B). The plasmid was cut with *Eco*RI to produce three fragments (2.2, 2.1, and 1.75 kb) which were end labeled with γ-^{32}P. The unfractionated mixture was used for S1 experiments since only the 1.75-kb fragment from the 5' end of the IAP gene (labeled at an *Eco*RI site 11 base pairs (bp) downstream from the 3' end of the LTR) could react with IAP transcripts. S1 mapping was performed by the method of Berk and Sharp (2) as modified by Weaver and Weissman (49). The hybridization was carried out at 50°C. S1 was from Miles Laboratories, Inc., Elkhart, Ind. The protected DNA was analyzed on polyacrylamide-urea gels (37). For sequencing reactions, the mixture of end-labeled fragments was further digested with *Pvu*II. This enzyme cut the IAP LTR at position 49 to produce a 306-bp *Pvu*II-*Eco*RI fragment which was isolated from a 5% polyacrylamide gel by electroelution. Sequencing was done by the method of Maxam and Gilbert (37).

Assay of chloramphenicol acetyl transferase activity in eucaryotic cells. At 48 h after transfection, cell extracts were prepared by sonication and assayed as described by Gorman et al. (16), using the conversion of [^{14}C]chloramphenicol (47 mCi/mmol; New England Nuclear Corp., Boston, Mass.) to the acetylated form. The samples were spotted on silica gel thin-layer plates and run in ascending chromatography. After autoradiography, the amount of acetylated chloram-

phenicol was quantitated by cutting out and counting the spots.

Miscellaneous procedures. Preparation of cellular DNA (31) and blot and dot hybridization with labeled probes (32, 33) have been described previously. Immunofluorescent staining of cells for T antigen was done with a hamster anti-simian virus 40 (SV40) antiserum from Huntingdon Research Center, Brooklandville, Md.

RESULTS

Generation of rat cell lines carrying cloned mouse IAP sequences. To test for transcription of cloned IAP sequences, we studied the IAP gene contained in λMIA14, a recombinant randomly selected from a mouse embryo genomic library (26, 32). This recombinant contains a full-length 7.2-kb IAP-specific region flanked by LTRs (26) which have been sequenced previously (24). A deleted form of this MIA14 gene was cloned into a modified pBR322 from which the *Bam*HI site had been removed (see above). This construct contained a single *Bam*HI site within the IAP sequence, into which we subsequently inserted a 3.4-kb *Bam*HI fragment containing the herpes *tk* gene (38). DNA of the resultant plasmid (pMIAtk-1, Fig. 1A), was used to transfect cells of a tk^- cultured rat liver line (BRLtk^-). Colonies were selected for ability to grow in HAT medium, and DNA from the transformed cells was analyzed by blot hybridization for the presence of integrated mouse IAP sequences. Although rat cells contain multiple copies of the sequences related to the mouse IAP genes (33, 34), the divergence between the two species makes it possible to specifically detect the mouse IAP sequences against the rat background. Restriction patterns and intensity of labeling of fragments relative to standards run in parallel were used to estimate copy numbers (data not shown). Two rat cell clones were selected for further analysis. One clone, BRL2, derived from cells transfected with supercoiled pMIAtk-1 DNA, contained a single copy of the IAP sequence. Another, BamCT6, derived from cells transfected with plasmid that had been cut with *Bam*HI and mixed with calf thymus DNA, contained three copies of the IAP sequence.

Transcription of mouse IAP genes in rat cells. Hybridization of labeled IAP DNA probes to dot blots of RNA from BRL2 and BamCT6 cells showed that IAP sequences were expressed as polyadenylated transcripts in both of the transformed clones. The plasmid DNA used to transfect the cells contained three potential promoter regions (pBR322, *tk*, and IAP LTR). We used S1 nuclease mapping to determine whether the IAP transcripts were initiating within the IAP LTR sequences. RNA from the rat cell clones was hybridized to the 1.5-kb fragment from the 5′ end on the IAP gene end labeled at the *Eco*RI site downstream from the 3′ end of the LTR (see above). RNA from both sources protected a major 130-bp fragment (Fig. 2A), placing the start of transcription at position 225 in the LTR sequence (Fig. 3). We did not assay for *tk* gene transcripts but assume that the gene was expressed with its own promoter.

The initiation position of the S1-protected fragment was also mapped relative to the Maxam and Gilbert sequence ladder of the 306-bp *Pvu*II-*Eco*RI fragment (see above). The major protected fragment comigrated in a position corresponding to the second C in a run of five pairs of CTs. This C lies at position 225 of the LTR sequence (Fig. 3).

RNAs from the two cell lines also protected a quantitatively minor band of 140 bp (Fig. 2A), corresponding to a G residue which is known to lie at position 218 of the LTR sequence (24). Five of the six nucleotides between these positions are T residues (Fig. 3). We believe that initiation may actually occur at position 218 but that the majority of the hybrids are shortened by S1 owing to the destabilizing effect of these T residues on the RNA-probe hybrid.

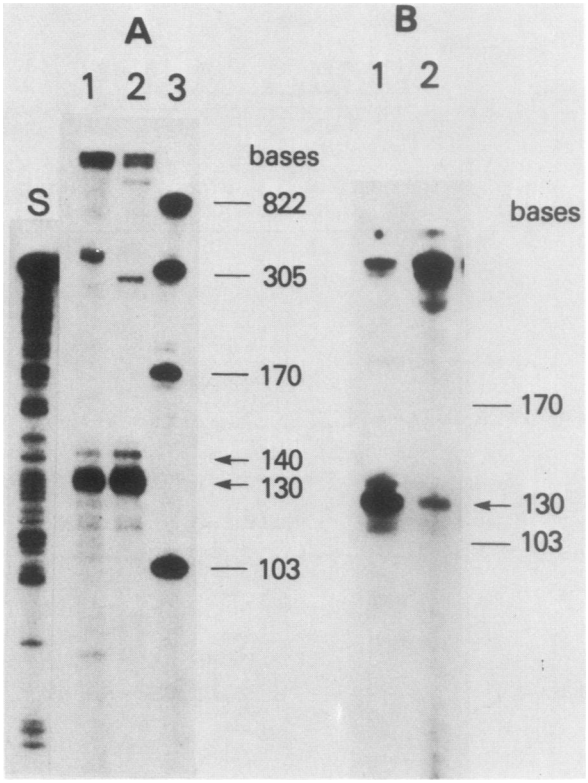

FIG. 2. S1 nuclease analysis of RNA from transfected cells. The probe was prepared from plasmid pMIAcat-5′L:R*, in which the insert is in an orientation opposite to that shown in Fig. 1B. Digestion of the plasmid with *Eco*RI resulted in three fragments which were end labeled with ^{32}P. Only a 1.75-kb fragment (including the IAP insert plus 0.25 kb of CAT gene sequences) could react with IAP transcripts. This fragment was labeled at the *Eco*RI site 11 bp downstream from the 3′ end of the LTR shown as H(E) on the map of pMIAcat-5′L:R in Fig. 1B (see also Fig. 3). The reaction products were analyzed on polyacrylamide-urea gels. (A) DNA protected from S1 digestion by 30-μg of total RNA from BRL2 cells (lane 1) and 1 μg of polyadenylated RNA from BamCT6 cells (lane 2) run in parallel with markers (lane 3). The end-labeled 306-bp *Pvu*II-*Eco*RI probe (see text) was sequenced by the method of Maxam and Gilbert (37) and analyzed by electrophoresis alongside the S1-protected DNA fragments on the same gel. The reaction specific for G residues is shown in the lane marked S. Arrows indicate sizes of the major (130-bp) and minor (140-bp) protected fragments. The 130-bp fragment comigrated with the second G in a set of five G residues in the sequence ladder; this corresponds to the C at position 225 in the sequence shown in Fig. 3. (B) DNA protected from S1 by 30 μg of total RNA from BamCT6 cells (lane 1) or from COS7 cells transfected with pMIAcat-5′L:R (lane 2).

Quantitative assay for gene expression. To examine the IAP LTR promoter sequences in more detail, we took advantage of a vector system which does not rely on selection of stably transformed cells and in which transient expression of plasmid DNAs can be conveniently measured (16). In the prototype plasmid, pSV2cat, the bacterial gene encoding chloramphenicol acetyl transferase (CAT) is cloned between the SV40 early promoter and an SV40 splice site and transcriptional termination signal. By modification of this

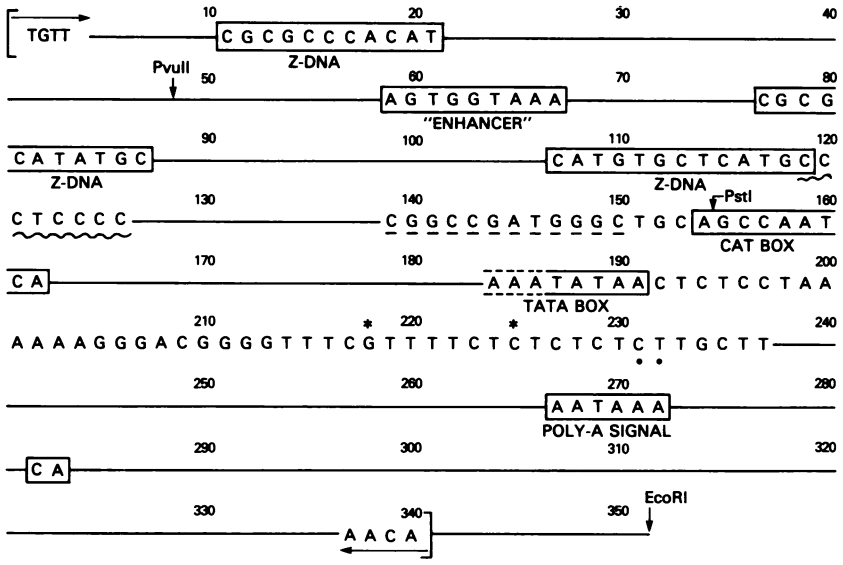

FIG. 3. Partial nucleotide sequence of the 5′ LTR of the IAP gene in MIA14. Positions are numbered from the 5′ end of the LTR sequence which has been previously published in its entirety (24). The inverted repeats at the end of the LTR are marked with arrows. Various signal sequences are boxed and labeled. A GC-rich region (positions 139–150) is underlined. The *Eco*RI site 11 bp downstream from the 3′ end of the LTR which was labeled in the probe used for S1 protection experiments is shown. The *Pvu*II site used to prepare the fragment for the sequence ladder in Fig. 2 is also shown. This ladder shows a CT pair (indicated by dots) at positions 231–232 which was not included in the previously published sequence (24). The five CT pairs between positions 223 and 232 correspond to the set of five Gs read on the negative strand on the sequencing ladder in Fig. 2. The apparent sites of initiation of RNA transcription are shown by asterisks at position 225 and 218 (see text).

plasmid, the CAT coding sequences can be placed under control of various other promoter sequences. Because eucaryotic cells do not contain this enzyme, the amount of enzyme, based on conversion of chlorampenicol to the acetylated form, reflects the strength of the promoter in the plasmid used to transfect the cells.

We used the recombinant pSV0cat in which the entire SV40 promoter region has been removed and a unique *Hin*dIII site is available for insertion of other promoter sequences with *Hin*dIII linkers (16). We made a construct (pMIAcat-5′L:R) which contained the IAP 5′ LTR as well as flanking sequences upstream of it. The 1.5-kb *Hin*dIII-*Eco*RI fragment was inserted as a promoter 5′ to the CAT gene (Fig. 1B). In this construct the orientation of the LTR with respect to the CAT gene is the same as it normally would be with respect to IAP gene transcription. Monkey CV1 cells were used as recipients, and the pSV2cat plasmid was used as a positive control for the assay. Extracts were prepared from cells 48 h after the introduction of the plasmid DNAs and tested for CAT activity. The acetylated and unacetylated forms were separated by thin-layer chromatography (Fig. 4). To quantitate the levels of CAT activity, the amount of acetylated substrate was determined by scintillation counting of the spots.

The CAT activity in extracts from cells transfected with pMIAcat-5′L:R was 2.6-fold higher than that in pSV2cat-transfected cells in the experiment shown in Fig. 4, lane 1 versus that in lane 2 (see also Table 1, experiment 1 with CV1 cells). However, this difference may not be significant since the activities of these constructs varied considerably when tested in two independently maintained CV1 cell lines (Table 1, experiment 2). The extent of variation in transfection experiments will be discussed below. These results indicated that the IAP LTR is capable of promoting transcription in monkey cells as well as rat cells, and that it is a strong promoter in heterologous cells.

The conditions generally used for transfection of cells by the calcium phosphate precipitation method result in uptake

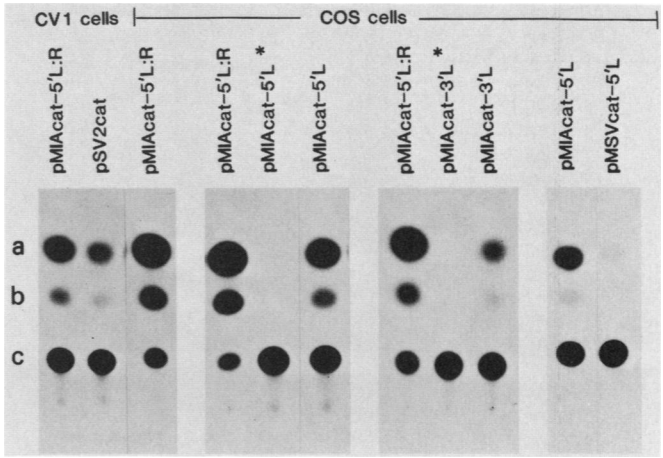

FIG. 4. Assay of CAT activity in extracts from transfected monkey kidney cells. Extracts (100 μl) were prepared from cells 48 h after transfection with the indicated plasmid DNA. CAT activity was measured by using conversion of [^{14}C]chloramphenicol (c) to its acetylated forms (a and b); these products were separated by thin-layer chromatography and detected by autoradiography as shown. Extracts prepared from transfected CV1 cells (lanes 1 and 2) or COS cells (lanes 3 to 11) are shown. The extracts, 20 μl (lanes 1 to 6), 10 μl (lanes 7 to 9), or 30 μl (lanes 10 and 11), were incubated for 30 min (lanes 1 to 3) or 60 min (lanes 4 to 11). Extracts were derived from cells transfected with the indicated DNAs.

of DNA by only ca. 10% of the cells (17, 42). We confirmed this for the CV1 cells we used by immunofluorescent assay of T antigen after transfection with SV40 DNA (data not shown). To improve the sensitivity of the assay, we tested a subline of COS7 monkey cells with a reportedly higher transfection efficiency (C. Queen, personal communication). After transfection of the COS7 cells with the pMIAcat-5'L:R plasmid, we found CAT activity fourfold higher than that in CV1 cells (Fig. 4, lane 1 versus lane 3). These results are summarized in Table 1, experiment 1. When the insert was inverted in orientation, as in pMIAcat-5'L:R* (Fig. 1B), CAT activity in CV1 or COS cells was extremely low.

Transcription initiated at identical sites in the LTR in two heterologous cell types. We used S1 nuclease mapping to locate the transcriptional start site in the pMIAcat-5'L:R plasmid in COS cells. The probe used was identical to that used to map the RNA in rat cells. RNA from COS cells protected a fragment the same size as that in the rat cells (Fig. 2B). Thus, in two different heterologous cell types the IAP LTR is transcriptionally active with the identical start site(s).

The probe in the S1 experiments was derived from a CAT construct and consisted of a mixture of fragments of which only one was specific for IAP transcripts (see above). RNA from COS cells transfected with the CAT plasmid protected two large non-IAP specific fragments which appear in Fig. 2B at the top of lane 2. The large material was present in S1 experiments with RNA from all cells which had been transfected with the CAT plasmids and did not correlate with CAT activity. It is likely to have resulted from reaction of the probe with RNA initiated at sites other than the LTR promoter. In the experiments with RNA from the transformed rat cells, there was little material at the top of the gel (Fig. 2B, lane 1). RNA from these cells cannot protect the additional probe fragments because the appropriate sequences were not included in the transforming DNA.

In comparing the amount of probe protected by the RNAs from the rat cells and COS cells in S1 mapping experiments (Fig. 2B, lanes 1 and 2), it was clear that the rat cells contained considerably higher levels of IAP RNA. An obvious reason for this is that in the transiently transfected COS cells only a fraction of the cells actually take up the DNA template, whereas in the permanently transformed rat cell clones, all cells contain IAP DNA.

Activation of IAP transcription by upstream sequences. The IAP LTR constructs thus far described contained mouse flanking sequences 5' to the LTR. Included in this region was a 450-bp repetitive element of the R-sequence family (13, 35). We made constructs which do not contain this region by cutting the *Hin*dIII-*Eco*RI fragment in pMIAcat-5'L:R with *Kpn*I (Fig. 1B). The 720-bp *Kpn*I-*Eco*RI fragment containing the LTR (but lacking R-sequence) was then inserted into pSV0cat in both orientations. In pMIAcat-5'L the orientation of the LTR was the same with respect to the CAT genes as that with respect to the IAP gene, whereas in pMIAcat-5'L* it was opposite. We compared these constructs with pMIAcat-5'L:R containing the R sequence as well as LTR in COS cells (Fig. 4, lanes 4 to 6, and Table 1, experiment 4) and in CV1 cells (Table 1, experiment 2). CAT activity in COS cells transfected with pMIAcat-5'L:R was considerably higher than that in cells transfected with pMIAcat-5'L. Cells transfected with pMIAcat-5'L* showed very low levels of activity. The activities promoted by pMIAcat-5'L in CV1 and COS cells were similar. The effect of R-sequence on LTR activity was considerably less in CV1 than in COS cells.

We also tested constructs containing the IAP 3' LTR sequence for promoter activity in COS cells and compared these with pMIAcat-5'L:R (Fig. 4, lanes 7 to 9). Cells transfected with pMIAcat-3'L, in which the 3' LTR is inserted in the same orientation as the 5' LTR in pMIAcat-5'L, showed CAT activity, and the activity relative to pMIAcat-5'L:R was the same for the 3' and 5' LTR constructs (Table 1, experiments 4 and 5). The 3' LTR inserted in the opposite orientation was inactive. Thus, both 5' and 3' LTRs appeared to be active as long as their orientation with respect to the CAT gene is the same as their orientation with respect to the IAP gene.

IAP LTR promoter activity in transfected mouse cells. We wanted to compare the promoter activity of the IAP LTR with that of a known mouse retrovirus LTR. We used the pMIAcat-5'L construct containing the IAP 5' LTR to compare with pMSVcat-5'L (pMSLTR2), which contains the MSV 5' LTR inserted in the analogous position in pSV0cat (see Fig. 1B). Initially we chose mouse L-cells as recipients. The CAT activity for the IAP 5' LTR construct was about 25% of the level given by pMSVcat-5'L (Table 2, experiment 1). The CAT activity promoted by the MSV LTR was in the range expected (L. Laimins, personal communication). In another transfection with low DNA levels in the absence of carrier, the IAP LTR construct had an activity 10% of the MSV LTR construct (Table 2, experiment 2). In this case, transfection of duplicate plates with each construct shows that the results are reproducible when the same cells are used on a given day. Comparison of the IAP construct with pSV2cat in L-cells indicated that the IAP LTR promoted

TABLE 1. CAT expression in transfected monkey cells[a]

Expt	Construct	Cells	Added DNA (μg/plate)	CAT activity (pmol/h per 20 μl)
1	pMIAcat-5'L:R	CV1	17	4,200
	pSV2cat	CV1	17	1,600
	pMIAcat-5'L:R	COS	17	17,500
2	pMIAcat-5'L:R	CV1	17	1,600
	pMIAcat-5'L:R*	CV1	17	10
	pSV2cat	CV1	17	3,960
	pMIAcat-5'L	CV1	17	900
	pMIAcat-5'L*	CV1	17	30
	pMSVcat-5'L	CV1	17	30
3	pMIAcat-5'L	CV1	17	580
	pSV2cat	CV1	17	918
4	pMIAcat-5'L:R	COS	5	4,400
	pMIAcat-5'L	COS	5	600
	pMIAcat-5'L*	COS	5	2
5	pMIAcat-5'L:R	COS	17	8,000
	pMIAcat-3'L	COS	17	1,000
	pMIAcat-3'L*	COS	17	None detected
6	pMIAcat-5'L	COS	10	500
	pMSVcat-5'L	COS	10	100
7	pMIAcat-5'L	COS	10	854
	pSV2cat	COS	10	2,300

[a] The amounts of chloramphenicol converted to the acetylated forms by 20 μl of a total 100 μl of extract from 2×10^6 cells are shown. Counts determined by scintillation counting of spots scraped from thin-layer chromatography plates such as shown in Fig. 4 were used to calculate picomoles, using the specific activity of the substrate. Only reactions in the linear range were used for the calculations.

TABLE 2. CAT expression in transfected mouse cells

Expt	Construct	Cells	Added DNA (μg/plate)	CAT activity (pmol/h per 20 μl)
1	pMIAcat-5'L	L-cells	17	247
	pMIAcat-3'L	L-cells	17	227
	pMSVcat-5'L	L-cells	17	950
2	pMIAcat-5'L	L-cells	5	52
	pMIAcat-5'L	L-cells	5	48
	pMSVcat-5'L	L-cells	5	590
	pMSVcat-5'L	L-cells	5	550
3	pMIAcat-5'L	L-cells	17	120
	pSV2cat	L-cells	17	131
4	pMIAcat-5'L	NIH-3T3	17	147
	pSV2cat	NIH-3T3	17	329
5	pMIAcat-5'L	NIH-3T3	10	92
	pSV2cat	NIH-3T3	10	175

CAT activity at a level similar to that promoted by the SV40 early promoter (Table 2, experiment 3).

A direct comparison of constructs containing the IAP 5' and 3' LTRs in L-cells (Table 2, experiment 1) confirmed our earlier conclusion from experiments with COS cells that the promoter activities of the two LTRs were comparable. Comparison of CAT activities promoted by pMIAcat-5'L in Table 2, experiments 1 to 3 shows that when transfections are carried out with the same constructs on different days, more variation can be expected.

We also tested the activity of the IAP LTR and pSV2 constructs in NIH-3T3 cells. The IAP LTR promoted activity at a level 50% of that promoted by the SV40 promoter in two transfections (Table 2, experiments 4 and 5).

High promoter activity of IAP LTRs in heterologous cells. The MSV CAT constructs were found to show higher activity in mouse cells than in monkey cells (28). We found a similar result for the MSV LTR in these experiments (Tables 1 and 2). In contrast, the IAP LTR promoted higher CAT activity in monkey cells than in mouse cells. We compared the relative effectiveness of the IAP LTR and MSV LTR directly in monkey COS cells (Fig. 4, lanes 10 and 11) and CV1 cells. In both types of monkey cells the IAP LTR was markedly more active in promoting CAT activity than was the MSV LTR (Table 1, experiments 2 and 6). When DNAs extracted from cells transfected with pMIAcat-5'L and pMSVcat-5'L were hybridized on filters with a labeled pBR322 probe, we found that the two preparations contained similar amounts of plasmid sequence. The sizes of the inserts in the constructs were also very similar. It is likely, therefore, that the relative CAT activities reflect different transcriptional activities promoted by the two types of LTR.

A comparison of pMIAcat-5'L with pSV2cat in CV1 and COS cells is shown in Table 1, experiments 2, 3, and 7. The IAP LTR-promoted CAT activities in these experiments were 23, 63, and 37% of the activities promoted by pSV2cat. pMSVcat-5'L promoted less than 1% of the activity of pSV2cat (Table 1, experiment 2).

Statistical analysis of CAT activities in transiently transfected cells. Given the variations in levels of CAT activity obtained in the transfections, how certain can we be that the observed differences between the various constructs are significant? Table 3 shows a summary of the activities measured for four of the constructs in different cells expressed as mean values and standard deviations per 10 μg of transfected DNA. Except in the cases in which the number of experiments was very small, the standard deviation ranged from 30 to 60%. pMIAcat-5'L was five- to eightfold more active in monkey cells than in mouse cells and had an activity 30 to 40% that of pSV2cat in monkey cells. The pMSV-cat-5'L was 10 to 20-fold less active in monkey cells than in mouse cells and also had considerably lower activity relative to pSV2cat in monkey cells. In mouse cells pMIAcat-5'L had levels of activity similar to pSV2cat, whereas pMSVcat-5'L was sixfold more active than pSV2cat. The differences in activity between the constructs in the various cell types are far larger than the variations seen between transfection experiments. We believe our data support the conclusion that the IAP LTR differs from another mouse viral LTR in functioning more efficiently as a promoter in heterologous cells than in homologous cells.

Table 3 also summarizes data showing that R-sequence increased the transcriptional activity of the IAP LTR significantly more in COS cells than in CV1 cells. The effect of R-sequence is the subject of current investigation.

DISCUSSION

We used two independent assays to show that an endogenous IAP gene is transcriptionally active in mouse cells as well as heterologous cells. We believe this is the first instance in which an endogenous provirus-like sequence has been shown to have activity when introduced into cells by transfection. An integrated murine leukemia virus copy (Mov-3) which proved to be transcriptionally active when cloned and transfected into BALB/3T3 cells (18) had been introduced into the germ line DNA by infection of embryos with exogenous murine leukemia virus (21). Two endogenous avian retroviruses were found to contain deficient promoter and leader sequences, resulting in RNA levels of only 10% of that transcribed from integrated exogenous provirus (8).

Since the gene in λMIA14 was randomly selected from a mouse embryo library and structurally resembles many other isolated IAP elements, one might suppose that a significant number of IAP elements could have a functional capacity under certain circumstances. One functionally significant modification of the IAP gene expected to result from

TABLE 3. Comparison of CAT activities in heterologous and homologous cell extracts

Construct	CAT activity (pmol/h per 20 μl per 10 μg of DNA) in:[a]				
	CV1 cells	COS cells	Mouse cells	CV1/mouse	COS/mouse
pMIAcat-5'L	440 ± 130 (2)	690 ± 350 (5)	90 ± 30 (8)	4.9	7.7
pMSVcat-5'L	20 (1)	100 (1)	950 ± 340 (3)	<0.1	0.1
pSV2cat	1,050 ± 640 (6)	2,300 (1)	150 ± 60 (3)	7.0	15.0
pMIAcat-5'L:R	1,710 ± 1,080 (2)	7,930 ± 2,890 (3)			

[a] CAT activity values are given as the mean ± the standard deviation. The numbers of independent transfections are given in parentheses.

cloning in bacterial vectors is a change in the state of methylation. Hojman-Montes de Oca et al. (20) have presented evidence linking IAP expression to the demethylation of endogenous IAP genes. IAP 5′ LTRs contain an *Msp*I site which is known to be methylated in most of the endogenous mouse IAP gene copies but demethylated in the case of the transcriptionally active MIA14 sequences integrated in the transformed rat cell lines BRL2 and BamCT6 (A. Feenstra, personal communication). MIA14 could be an example of an IAP gene element that is ordinarily methylated in the mouse genome but which can be transcriptionally activated by appropriate demethylation.

The IAP LTR appeared to be a relatively stronger promoter in monkey cells than in mouse cells. In contrast, the MSV LTR showed a strong specificity for mouse cells over monkey cells, an observation consistent with previous results showing that the MSV LTR enhancer can increase activity of the SV40 promoter sevenfold better in mouse cells than in monkey cells (28). A number of viral enhancers have strong species specificity (1, 4, 9, 11, 22, 45). On the other hand, the Rous sarcoma virus LTR, which has been shown to be a promoter in a variety of heterologous cells, was nearly as active in monkey cells as it was in chicken cells (15). The IAP LTR may prove to be another versatile promoter.

The permanently transformed rat BRL2 cells contained a single integrated copy of the pMIAtk-1 DNA, whereas the BamCT6 cells contained three copies in different locations. The pMIAcat-5′L:R DNA in COS cells was probably extrachromosomal. The start of transcription was mapped to the same position(s) in the MIA14 LTR in all three different situations. In an earlier mapping study with primer extension, the start site for IAP RNA in mouse myeloma cells was placed "near the *Pst*I site" in another cloned 5′ LTR (7). Since the fragment sizing was imprecise in these experiments, we believe the result is consistent with the present observations which place the start site at least 60 bp downstream from the *Pst*I site.

Figure 3 shows the relationship between the assigned start sites in the 5′ LTR of MIA14 and various sequence elements which have been thought to be important for transcription in other systems, even though their mode of action may be only speculative. Our results suggest that the most likely major start site in the LTR may be the G at position 218. This G is 31 bp downstream from the likely promoter sequence TATAA (187–191) and 64 bp downstream from a CAT box AGCCAATCA (154–162). Similar spatial relationships between these signals have been found in a variety of eucaryotic genes (3).

The MIA14 LTR contains a number of other sequences which have been implicated in control or modulation of promoter activity in cellular as well as viral genes. The sequence AGTGGTAA (59–77) is nearly identical to the enhancer sequences found in the SV40 72-bp repeat (50) and MSV LTR (28), and there are three regions capable of forming Z-DNA (11–21, 77–87, and 107–119) (40). The sequence CCCTCCCC (119–126) located 92 bp upstream from the initiation site resembles the sequence CCCCGCCC found 94 bp upstream from the *tk* gene and shown to be essential for expression (39). A GC-rich region between 139 and 150 has the same spatial relationship to the TATA box as similar regions which have been found upstream from a number of other genes.

Although the IAP and MSV LTRs both contain sequences homologous to the enhancer core (28) and regions of potential Z-DNA (40), the distribution of these sequences is quite different in the two LTRs. In the IAP LTR the three Z-DNA regions are all located upstream of the promoter, and the enhancer sequence is between two of the Z-DNA regions (Fig. 3). The MSV LTR contains two potential Z-DNA regions; these straddle the promoter and are far downstream from the enhancer sequence (10).

Since only a few IAP LTRs have been sequenced, we do not know how general these signal sequences are. The IAP LTR inserted 5′ to the c-*mos* gene in the rearranged rc-*mos* (44) gene had 88% homology with the LTR in the MIA14 IAP gene (23). The polyadenylation signal, enhancer sequence, and two of the three Z-DNA regions in the MIA14 LTR are located in corresponding positions in the rc-*mos* LTR, but no obvious TATA box is present (23). It should be pointed out, however, that since the LTR in rc-*mos* is in a head-to-head orientation with respect to the *mos* gene, the comparable signals may not have the same functional significance. A construct with the MIA14 5′ LTR in head-to-head orientation relative to the CAT gene was essentially inactive in COS cells but did show a low level of activity when tested in the transient expression assay in CV1 cells (Table 1, experiment 2). This was 30-fold lower than the activity seen when the LTR was in the same orientation as the CAT gene.

We have demonstrated by S1 protection experiments in COS cells that when the R-sequence is present upstream from the IAP 5′ LTR there is an increase in RNA transcripts originating in the LTR. R-sequence did not promote activity when present 5′ to the CAT gene without the IAP LTR. Preliminary experiments indicate that the R-sequence in the 5′-position also increases transcription from the SV40 early promoter in COS cells.

In conclusion, the present experiments show that the endogenous IAP gene LTR in MIA14 can function as an efficient promoter in heterologous as well as homologous cells. The results also show that initiation of transcription occurs at the same site in the IAP LTR regardless of the cell type or state of integration.

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