

Functional Analysis of the Long Terminal Repeats of Intracisternal A-Particle Genes: Sequences within the U3 Region Determine Both the Efficiency and Direction of Promoter Activity

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The transcriptional activity of five intracisternal A-particle (IAP) long terminal repeats (LTRs) in mouse embryonal carcinoma PCC3-A/1 cells and in Ltk⁻ cells was determined. We tested the promoter activity of the LTRs by coupling them to the reporter gene chloramphenicol acetyltransferase (CAT) or guanosine-xanthine phosphoribosyltransferase (*gpt*). Each LTR was tested for promoter function in both the sense (5' to 3') and antisense (3' to 5') orientation preceding the reporter gene. The transcriptional activity of individual IAP gene LTRs varied considerably, and the LTR from IAP81 possessed promoter activity in both directions. The bidirectional activity of the IAP81 LTR was confirmed by monitoring *Ecogpt* expression in stably transfected Ltk⁻ cells, with the initiation sites for sense and antisense transcription being localized to within the IAP81 LTR by S1 nuclease mapping. Deletions of LTR81 show that for normal 5'-to-3' gene transcription (sense direction), the 3'U3/R region determines the basal level of transcription, whereas sequences within the 5'U3 region enhance transcription four- to fivefold. Deletion mapping for antisense transcription indicates that a 64-base-pair region (nucleotides 47 to 110) within the U3 region is essential for activity. These data indicate that the U3 region contains all the regulatory elements for bidirectional transcription in IAP LTRs.

Intracisternal A particles (IAPs) are endogenous retroviruslike structures containing a high-molecular-weight RNA (41, 48), a core protein (p73), and reverse transcriptase-like activity (50). IAP genes are members of a dispersed multi-gene family found in approximately 1,000 copies per haploid genome in *Mus musculus* (40). The structural organization of IAP genes is similar to that of retroviral proviruses, with 5' and 3' long terminal repeats (LTRs) (12, 30) flanking protein-coding sequences (9, 41). Studies with cloned IAP genes have identified two classes, type I and type II, differentiated by size and sequences at the 5' end of the gene (32, 40, 45).

IAPs are expressed during development in normal preimplantation embryos (6, 8, 27), but are also found in many transformed cell lines including embryonal carcinomas (22; R. M. Morgan and R. C. C. Huang, *Dev. Genet.*, in press), hybridomas (11, 21), and plasmacytomas (35, 49). The expression of IAPs during the preimplantation stages of early embryogenesis, but not during later stages of development, is in contrast to the expression of other viruses including murine leukemia virus and murine C-type virus, in which the earliest viral expression occurs postimplantation (25, 27). Embryonal carcinoma (EC) cells are similar to mouse preimplantation embryos in that IAP transcripts have been observed in both pluripotent (PCC3) and nullipotent (F9) EC cell lines (22, 36), with the amount of IAP-specific RNA and IAP-specific promoter activity declining after *in vitro* differentiation (22; Morgan and Huang, in press). In cell lines that actively express IAP genes, IAP elements have been shown to be integrated at new locations in the mouse genome and therefore can act as insertional mutagens. In the hybridoma *igk-1*, an IAP gene inactivated the kappa-light chain gene by inserting into an intron of the gene (20). An

IAP gene activated the *c-mos* gene in plasmacytoma XRPC20 by insertion into the 5' end of the *c-mos* gene coding region (7, 28). Activation of the *c-mos* gene by IAP proviral integration is unusual in that the IAP gene is inserted in a 3'-to-5' (head-to-head) orientation relative to the *c-mos* gene, indicating that the IAP LTR can promote and initiate transcription in two directions (23).

Like other retroviruses (e.g., murine leukemia virus), IAP LTRs contain the presumptive regulatory signals for promotion, initiation, and polyadenylation of transcription (10, 29). Sequence comparison of seven IAP gene LTRs showed a large variation in both size and sequence, with the variability in size being due to nucleotide insertion or deletion within the R regions of the LTRs (10). The LTRs of integrated exogenous and endogenous avian retroviruses have also been found to differ in size (26). In contrast to IAP genes, the size variation in avian retrovirus LTRs was found to be in the U3 region, with the endogenous viruses having their promoter and leader sequences deleted, making them transcriptionally inactive (13, 15). To determine the extent to which this size and sequence variation between randomly isolated IAP LTRs affects transcription and to determine whether all IAP genes were transcriptionally active, we tested five LTRs for promoter activity by coupling them to the bacterial chloramphenicol acetyltransferase (CAT) gene or the guanosine-xanthine phosphoribosyltransferase (*gpt*) gene. The LTRs were cloned in both the sense (5' to 3', U3-R-U5) and antisense (3' to 5', U5-R-U3) orientations preceding the reporter gene to determine whether they function as active IAP gene promoters and/or promote transcription to cellular flanking regions. The promoter activity of the IAP LTRs varied considerably, and several LTRs did promote transcription in the antisense direction. The ability of an IAP gene LTR to transcribe cellular flanking regions is similar to the previously reported activity by the LTRs of an IAP gene inserted adjacent to the *c-mos*

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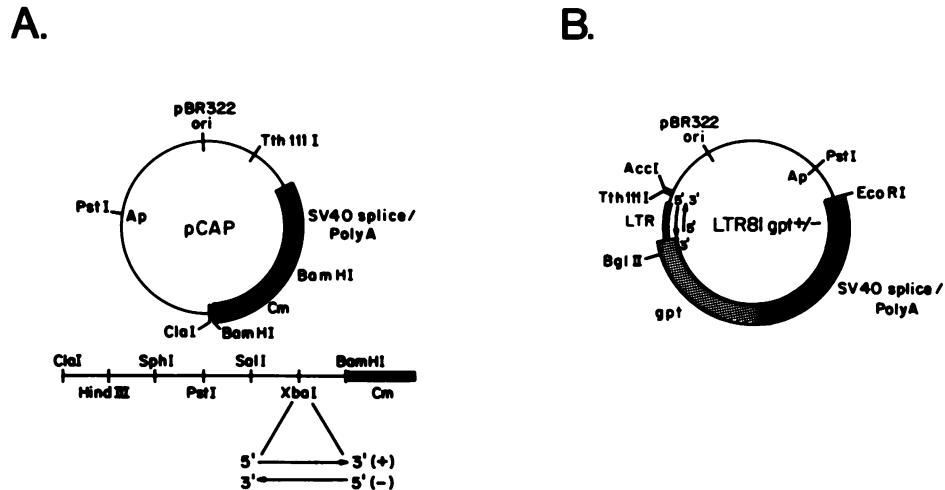






FIG. 1. Diagrammatic representation of the plasmids used in this study. (A) The pCAP vector used in cloning IAP LTRs to monitor their promoter activity by expression of CAT. The sequences of the chloramphenicol gene (Cm, ) , SV40 poly(A) signal () , pBR322 sequences (—), and relevant restriction endonuclease sites are indicated. The IAP gene LTR fragments were inserted, in both the sense (5' to 3') and antisense (3' to 5') orientations, into the *Xba*I site of the pCAP polylinker preceding the CAT gene. (B) The LTR81gpt vector was constructed by using SV2gpt (37) and the IAP81 LTR fragment shown in Fig. 2A. The SV40 early-region promoter and origin of replication were removed and replaced with the IAP81 LTR (—) to generate recombinant clones with the IAP81 LTR in both the sense (LTR81gpt⁺, 5' to 3') and antisense (LTR81gpt⁻, 3' to 5') orientations preceding the *gpt* gene () . The sequences of the SV40 poly(A) signal () , pBR322 (—), and restriction endonuclease sites used in the S1 mapping are indicated.

gene (7, 23, 28) and of IAP81 (K. M. Wujcik, Ph.D. thesis, The Johns Hopkins University, Baltimore, Md., 1982). Using S1 mapping of the LTR from IAP81, a bidirectional promoter, we localized the transcription initiation sites for both sense and antisense promoters within the LTR. The transcriptional activity of 5' and 3' deletion mutants of the IAP81 LTR was assayed, and regions important for bidirectional promoter activity were identified within the U3 region.

MATERIALS AND METHODS

General procedures. Restriction endonucleases and DNA modification enzymes were obtained from New England BioLabs, Inc., unless otherwise noted. Endonuclease digestions and DNA modifications were carried out as specified by the manufacturer. RNA was extracted by the guanidinium isothiocyanate-CsCl procedure (46), resolved on 1.5% agarose-formaldehyde gels (2.2 M formaldehyde, 0.02 M sodium phosphate [pH 6.8]), and transferred directly to nitrocellulose. RNA hybridizations were performed by the procedure of Melton et al. (33). DNA restriction fragments were radiolabeled by the procedure of Fienberg and Vogelstein (16).

Cell culture and DNA transfection. Mouse Ltk⁻ cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 50 U of penicillin per ml, and 50 mg of streptomycin per ml. PCC3-A/1 cells (kindly provided by A. Rizzino) were grown in the same medium supplemented with 0.4% glucose and 4 mM L-glutamine.

Transfections were performed by the calcium phosphate coprecipitation technique (19) as modified by Hutter et al. (24). For transient CAT assays, 10 to 20 μ g of CsCl-purified DNA (17) of CAT plasmid DNA was applied to 5×10^5 to 7.5×10^5 PCC3-A/1 cells per 60-mm dish. To obtain cell lines that contain stably integrated IAP LTRgpt constructs, 5 μ g of plasmid DNA was applied to 10^6 Ltk⁻ cells per 100-mm

dish. Transformants containing *Ecogpt* were selected (37), and individual clonal populations and a pooled sample were isolated.

CAT enzyme assays. Cell extracts were isolated 48 to 60 h posttransfection, and assays for CAT enzyme activity were performed as described by Gorman et al. (18), with 0.25 μ Ci of [¹⁴C]chloramphenicol (New England Nuclear Corp.) as substrate. Assays were performed on cell extracts from equal numbers of cells titrated to fall within the linear range of conversion of substrate to its acetylated forms. The percentage of conversion was determined by cutting out the appropriate region from the thin-layer chromatography plate and counting in a toluene-based scintillation fluid. The control plasmid, pSV2CAT, was obtained from the American Type Culture Collection.

S1 nuclease mapping of LTR81gpt transcripts. The S1 probes were made by digesting the appropriate LTR81gpt vector (Fig. 1) with *Acc*I and *Bgl*II. The fragments (20 pmol of ends) were 5' end labeled with T4 polynucleotide kinase (Promega Biotec) and 100 μ Ci of [γ -³²P]ATP (>3,000 Ci/mmol; Amersham Corp.). To obtain a single end label at the *Bgl*II site, fragments were further digested with *Tth*111I and the mixture was used for S1 mapping.

The S1 nuclease mapping was performed by the method of Berk and Sharp (3), with 20 μ g of total RNA and 0.1 μ g of end-labeled LTR81gpt DNA. The hybridizations were carried out at 52°C for 8 to 12 h, and S1 nuclease reactions (500 U of S1 nuclease per ml; Pharmacia, Inc.) were performed for 30 min at 37°C. The protected DNA fragments were analyzed on 6% acrylamide-8 M urea gels.

Construction of CAT and LTR81gpt expression vectors. The vector used for CAT expression (pCAP; Fig. 1A) was constructed from commercially available plasmids, pcDV1, M13mp19, and pCM-4 (Pharmacia). The pCAP vector was generated by ligating the *Hind*III-to-*Kpn*I vector fragment of pcDV1 (39) to the *Hind*III-to-*Kpn*I M13mp19 polylinker DNA. The bacterial CAT gene sequence (Tn9) isolated from

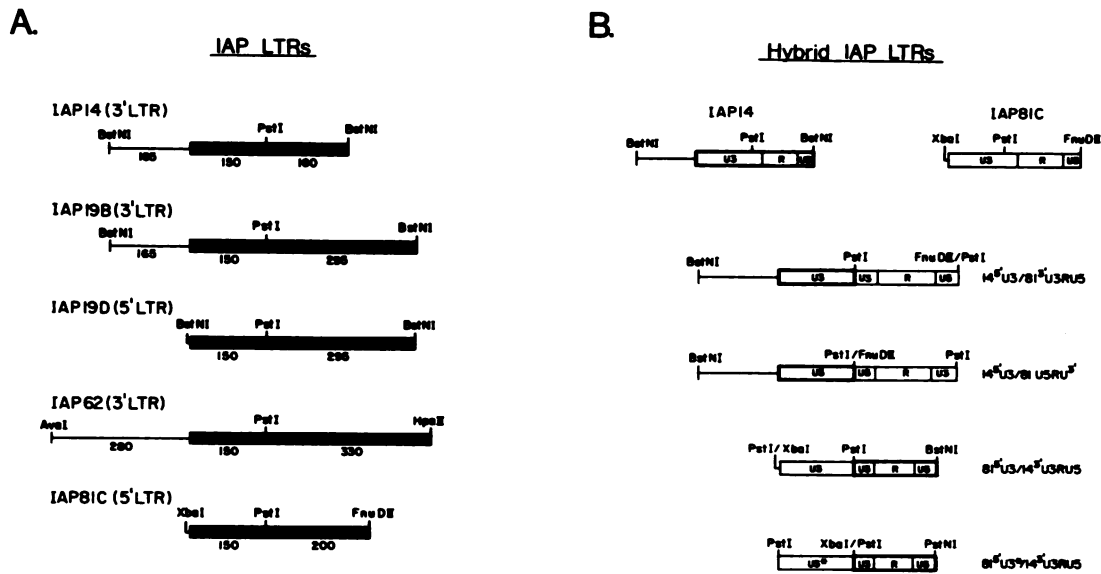


FIG. 2. (A) IAP gene LTR fragments that were inserted into the *Xba*I site of the pCAP polylinker to monitor CAT activity. The detailed physical maps and sequences of the LTRs have been described previously (10, 40). The IAP LTR sequences (■) and IAP gene sequences (—) are shown. The 3' LTRs (IAP14, IAP19B, and IAP62) each contain 165 to 280 bp of IAP gene sequence, and the 5' LTRs (IAP19D and IAP81) contain 7 to 12 bp of cellular flanking DNA. All of the LTRs have 12 to 20 nucleotides from the 3' end of the U5 region deleted. (B) Structures of the hybrid LTRs between IAP14 and IAP81 LTRs. Using the invariant *Pst*I site found in all the LTRs (10) (Fig. 2A), we switched the 5' and 3' ends of the IAP14 (■) and IAP81 (□) LTRs to generate four recombinant clones. $14^5U3/81^3U3RU5$ contains the first 150 nucleotides of the U3 region of LTR14 (5^5U3) and the 3' end of IAP81 LTR (3^3U3RU5). $14^5U3/81^5U3RU5$ contains the same 150 bp of the IAP14 LTR, but the IAP81 3^3U3RU5 fragment has been inverted. $81^5U3/14^3U3RU5$ contains the first 150 nucleotides of the IAP81 LTR U3 region (5^5U3) and the 3^3U3RU5 region from IAP14 LTR. $81^5U3/14^5U3RU5$ has the IAP81 U3 region inverted ($U3^*$) relative to the IAP14 LTR sequences. These hybrid LTRs were cloned into the *Xba*I site of the pCAP vector to give the corresponding hybrid LTRs in both the sense (e.g., $14^5U3/81^3U3RU5$ -CAT) and antisense (e.g., CAT- $14^5U3/81^3U3RU5$) orientation preceding the CAT gene.

pCM-4 (Pharmacia) was cloned into the *Bam*HI site of the polylinker in pcDV1-mp19, resulting in pCAP. The pCAP vector permits convenient insertion of promoter sequences into six restriction sites (*Cla*I, *Hind*III, *Sph*I, *Acc*I, *Sal*I, and *Xba*I) 5' to the CAT gene coding regions (Fig. 1A).

Isolation and subcloning of IAP genes have been described previously (40). The LTRs from four different IAP genes (IAP14, IAP19, IAP62, and IAP81) were isolated and cloned into the pCAP vector, and recombinant plasmids containing each LTR in the sense (LTRCAT⁺ [5' to 3', U3-R-U5]), and antisense (LTRCAT⁻ [3' to 5', U5-R-U3]) orientation preceding the CAT gene isolated (Fig. 1A).

The LTR81gpt vector was constructed by using the IAP81 LTR fragment shown in Fig. 2A and SV2gpt (kindly provided by C. W. Wu). The SV2gpt vector was digested with *Hind*III and *Pvu*II, and the simian virus 40 (SV40) early-region promoter was replaced with the IAP81 LTR in either the sense (LTR81gpt⁺, U3-R-U5gpt) or antisense (LTR81gpt⁻, U5-R-U3gpt) orientation preceding the *gpt* gene (Fig. 1B).

Construction of hybrid LTR-CAT clones. The hybrid LTRs of IAP81 and IAP14 were constructed by digesting M13mp18 subclones of IAP81 LTR or IAP14 LTR (+ and - orientations) with *Pst*I, a conserved site in all of the LTRs. This allowed the isolation of IAP81 LTR *Pst*I fragments containing either the 150-base-pair (bp) 5^5U3 or the 201-bp 3^3U3RU5 and IAP14 LTR *Pst*I fragments containing the vector plus either the 5^5U3 or the 3^3U3RU5 region. The IAP14 LTR-vector fragments were ligated with the corresponding IAP81 LTR *Pst*I fragments, generating IAP14/81 hybrid LTR clones: $81^5U3/14^3U3RU5$, $81^5U3/14^5U3RU5$, $14^5U3/81^3U3RU5$, and $14^5U3/81^5U3RU5$ (the inverted IAP81 5^5U3 fragment is denoted $U3^*$) (Fig. 2B). The hybrid LTR

mutants were subcloned in both the sense and antisense orientations into the pCAP vector (Fig. 1A) to monitor promoter activity.

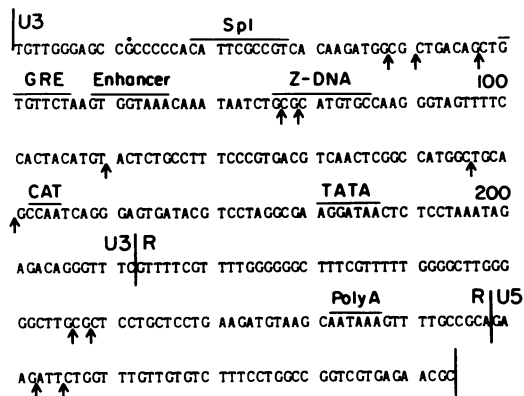
Construction of IAP81 LTR deletion mutants. Site-directed deletions of IAP81 LTR via novel restriction enzyme sites were used to generate 5'-to-3' and 3'-to-5' mutants. IAP81 DNA was digested with restriction endonucleases, and the fragments were isolated and cloned into the pCAP vector. The nucleotide sequence of the IAP81 LTR is shown in Fig. 3A, and the deletion points after ligation are marked by vertical arrows. Recombinant clones for all fragments were obtained in both orientations preceding the CAT gene and are shown schematically in Fig. 3B.

Nucleotide sequencing. The nucleotide sequence of recombinant pCAP and LTR81gpt constructs was determined by using plasmid DNA (51) and specific synthetic oligomers to the CAT and *Ecogpt* genes. The CAT oligonucleotide is complementary to nucleotides 15 to 34 of the coding sequence of the CAT gene ($5'$ -CAACGGTGGTATATC CAGGTG- $3'$) (1). The *Ecogpt* oligonucleotide is complementary to nucleotides -186 to -157 in the 5' noncoding region of the *Ecogpt* gene ($5'$ -CAAACATATCACGCAAGCCTG TCTTGTGTCC- $3'$), immediately 3' to the *Hind*III site in SV2gpt (42). Sequencing was done by the dideoxynucleotide chain termination method of Sanger et al. (43).

RESULTS

Comparative analysis of IAP LTR expression. In transient-expression assays of transfected plasmid DNA, the conversion of chloramphenicol to acetylchloramphenicol can be measured and is an indirect reflection of the promoter-driven transcriptional activity (18). To quantitate the promoter

A.



B.

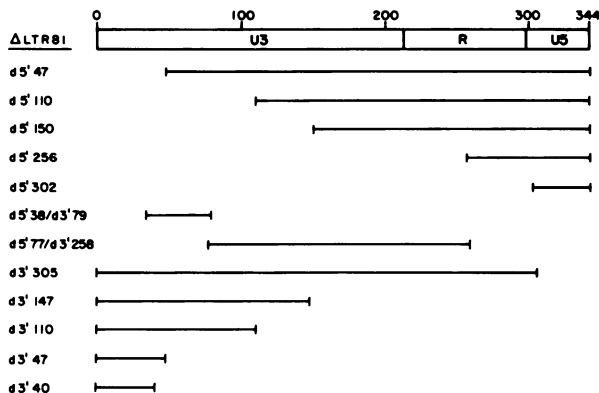


FIG. 3. IAP81 LTR sequence and schematic representation of the deletion mutants (A). Nucleotide sequence of LTR81 in a 5'-to-3' orientation. The putative signals for transcription initiation are shown (CAT, TATA, and PolyA) (10, 31). The invariant *Pst*I site found in all the LTRs is located at position 150. Other sequences found in the LTR of IAP81 that show homology to sequences that affect genes in other systems are shown. These include an inverted *Sp*I binding site (*Sp*I) (4; Morgan and Huang, in press), a glucocorticoid-responsive element (GRE) (5, 44), SV40 core enhancer element (Enhancer) (31, 47), and potential Z-DNA-forming sequences (Z-DNA) (31, 38). At nucleotide 12 (indicated by *), the sequence has a G residue that was originally reported as a C (10). The nucleotide location of the IAP81 LTR deletions relative to the IAP81 LTR sequence are indicated by the arrows. (B) Deletions mutants of IAP81 LTR are shown relative to their position in the U3-R-U5 regions of IAP81 LTR. The deletions are designated by where the deletion starts relative to the LTR81 sequence. Deletions from the 5' end of the LTR are denoted by d5', and deletions from the 3' end are denoted by d3'. Internal fragments are described by using the 5' and 3' deletion points for that fragment, d5'/d3'.

efficiency of IAP LTRs, five LTRs from four different IAP proviral genes (IAP14 [3' LTR], IAP19 [5' and 3' LTR], IAP62 [3' LTR], and IAP81 [5' LTR]) (Fig. 2A) were used in transient CAT assays. Each IAP LTR was cloned in both the sense (5' to 3' LTRCAT⁺) and antisense (3' to 5', LTRCAT⁻) orientations relative to the CAT gene and transfected into PCC3-A/1 cells.

The CAT activity of the extracts shows that IAP LTRs

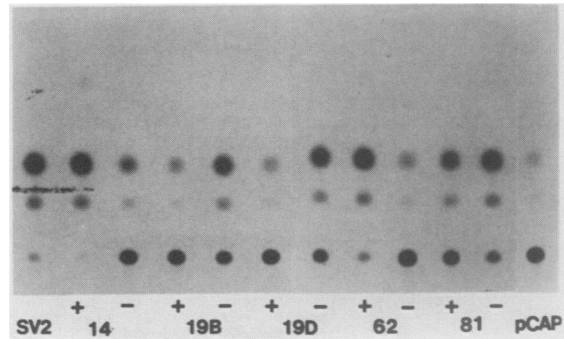


FIG. 4. Assay of CAT activity in PCC3-A/1 cell extracts. The LTRCAT clones were analyzed for CAT activity in both the sense (LTRCAT⁺) and antisense (LTRCAT⁻) orientations. PCC3-A/1 cells (7.5×10^5) were transfected with 20 μ g of the indicated LTR CAT DNA. Extracts (100 μ l) were prepared 48 h posttransfection, and 20 μ l of each extract was incubated for 30 min at 37°C. Results of multiple experiments assayed in the linear range are summarized in Table 1.

have a wide range of promoter activity in both the sense and antisense transcriptional orientations. The results of one experiment are shown in Fig. 4. The LTRs of IAP14 and IAP62 are efficient promoters in the normal direction of IAP gene transcription (LTR14CAT⁺ and LTR62CAT⁺), but have little or no activity in the opposite direction (LTR14CAT⁻ and LTR62CAT⁻) in PCC3-A/1 cells. With the 5' (LTR19D) and 3' (LTR19B) LTRs of IAP19 the converse is true, indicating that the IAP19 LTRs do not promote transcription efficiently in the 5'-to-3' direction (LTR19B/DCAT⁺) but that they actively transcribe in the 3'-to-5' direction (LTR19B/DCAT⁻) (Fig. 4). In PCC3-A/1 cells, the expression of LTR81 is unique in that it shows significant levels of CAT activity in both transcriptional orientations (LTR81CAT^{+/+}), indicating that it is a bidirectional promoter and may transcribe both the IAP81 gene sequences and the cellular flanking sequences where it is integrated.

The results of several experiments are summarized in Table 1 and were standardized by using the SV40 promoter in SV2CAT. For transcription in the 5'-to-3' (sense) direction, the LTRs can be ranked IAP14 > IAP62 > IAP81 > IAP19. The LTR of IAP14 is the strongest promoter (LTR14CAT⁺), being 1.8-fold more active than the SV40 promoter (SV2CAT), whereas the IAP62 and the IAP81 LTRs are about 30% and 20%, respectively, as active as the SV2CAT promoter. For transcription in the 3'-to-5' (anti-

TABLE 1. Comparison of IAP LTR CAT activity in PCC3-A/1 cells

Promoter	Relative activity ^a (no. of expt.) in:	
	Sense orientation	Antisense orientation
SV2CAT	100	
LTR14CAT	186.4 ± 26.2 (7)	4.6 ± 1.9 (8)
LTR19BCAT	6.4 ± 1.4 (6)	24.4 ± 5.1 (6)
LTR19DCAT	3.2 ± 1.7 (7)	29.1 ± 3.3 (6)
LTR62CAT	35.8 ± 19.0 (6)	4.8 ± 1.4 (5)
LTR81CAT	17.2 ± 5.2 (9)	34.0 ± 3.2 (6)
pCAP	3.5 ± 1.3 (5)	

^a Relative CAT activity was calculated by normalizing the percent conversion of chloramphenicol to acetylchloramphenicol for SV2CAT equal to 100.

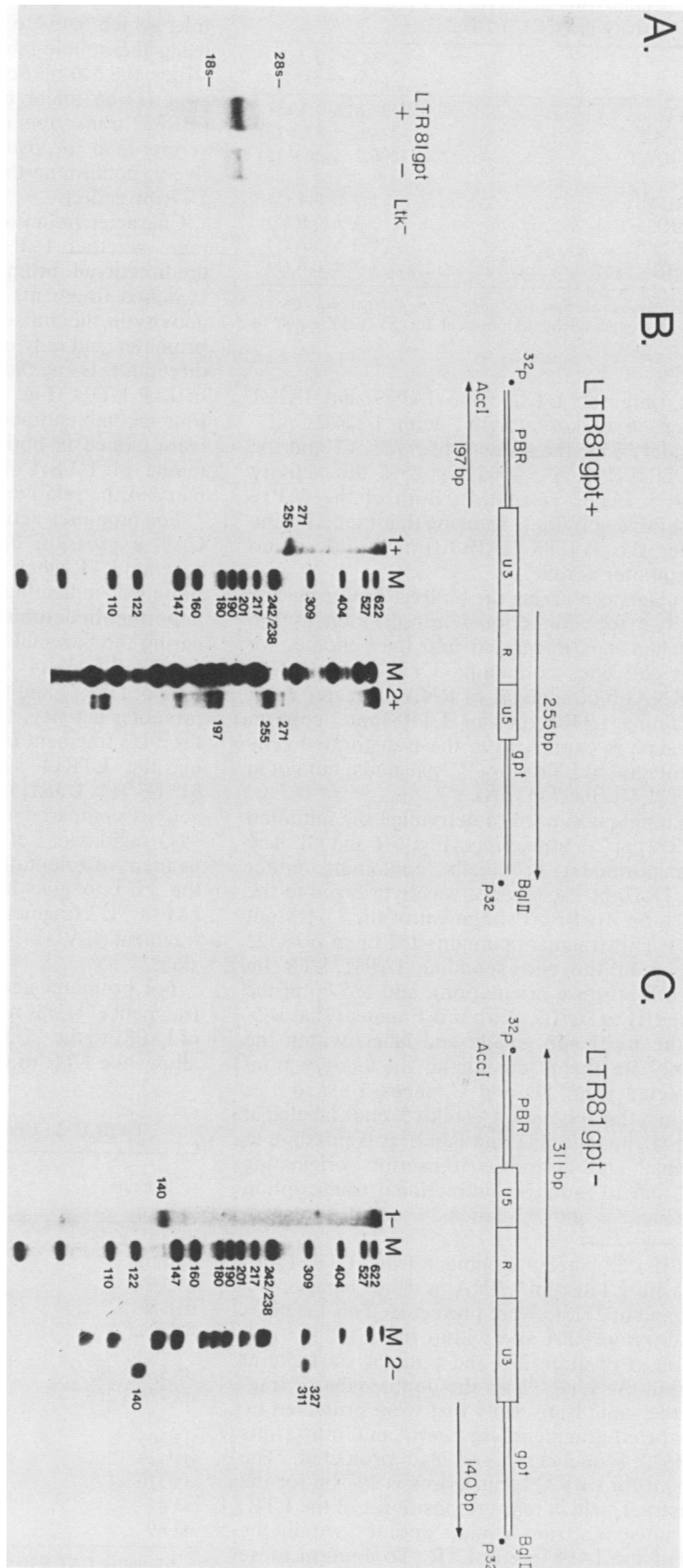


FIG. 5. (A) Northern blot analysis of LTR81gpt⁺/⁻ total RNA from LTR81gpt⁺/⁻ transformed cells. Total RNA (20 µg) was analyzed on a 1.5% agarose-formaldehyde gel and hybridized with the *Bgl*II-to-*Bam*HI fragment of the *Eco*gpt gene (Fig. 1B). LTR81gpt⁺ RNA from cells transformed with LTR81gpt⁺ (lane +), LTR81gpt⁻ RNA from cells transformed with LTR81gpt⁻ (lane -), and RNA from untransformed LTR81gpt⁻ cells (lane LTR81gpt⁻) are shown. The size of the *Eco*gpt transcript is approximately 2.3 kilobases. The schematic figures of LTR81gpt⁺/⁻ show the initiation sites for the major protected transcripts for each direction of transcription obtained from the S1 nuclease mapping studies. (B) S1 mapping of the transcription initiation sites of LTR81gpt⁺ (LTR81 in the sense orientation preceding *Eco*gpt [Fig. 1B]). RNA isolated from LTR81gpt⁺ cells that had been transformed with LTR81gpt⁺ was hybridized to either the single-end-labeled *gpt* probe (*Bgl*II site, lane 1+) or the double-end-labeled *gpt*/*pBR322* probe (*Bgl*II-*Acc*I site, lane 2+) as described in the text. (C) S1 nuclease mapping of the transcription initiation sites of LTR81gpt⁻ (LTR81 in the antisense orientation preceding *Eco*gpt). RNA from LTR81gpt⁻ cells transformed with LTR81gpt⁻ was hybridized to the corresponding single-end-labeled (*Bgl*II site, lane 1-) and double-end-labeled (*Bgl*II-*Acc*I site, lane 2-) probes. In panels B and C, M is the *pBR322* *Hpa*II marker.

TABLE 2. Comparison of hybrid LTR promoter activity in PCC3-A/1 cells

Hybrid promoter	% Conversion ^a (no. of expt)
14 ⁵ /81 ³ U3RU5-CAT	38.8 ± 4.1 (5)
81 ⁵ U3/14 ³ U3RU5-CAT	228.6 ± 81.4 (3)
81 ⁵ U3*/14 ³ U3RU5-CAT	206.2 ± 56.4 (3)
CAT-14 ⁵ U3/81U5RU3 ^{3'}	1.9 ± 0.4 (5)
CAT-14 ⁵ U3/81 ³ U3RU5	3.4 ± 0.9 (5)
CAT-81 ⁵ U3/14 ³ U3RU5	28.9 ± 5.6 (5)
CAT-81 ⁵ U3*/14 ³ U3RU5	1.7 ± 0.8 (5)

^a Relative CAT activity was calculated by normalizing the percent conversion of chloramphenicol to acetylchloramphenicol for SV2CAT equal to 100%.

sense) direction, only the LTRs from IAP19 and IAP81 showed significant levels of activity, with LTR81CAT⁻ having approximately 35% the activity of SV2CAT and the IAP19 LTRs (LTR19B/DCAT⁻) having 25% the activity (IAP81 > IAP19 > IAP62 = IAP14). Both of the IAP19 LTRs gave comparable activities, showing that the IAP gene sequences flanking the 3' LTR (IAP19B) have little or no effect on LTR promoter activity.

Stable transformants containing the bidirectional promoter IAP81 LTR. To test for bidirectional transcription by the LTR of IAP81 when it is integrated into the genome, we made permanent cell lines containing LTR81gpt^{+/-} (Fig. 1B). Northern (RNA) blot analysis of RNA extracted from Ltk⁻ cells containing LTR81gpt⁺ or LTR81gpt⁻ confirm that the *Ecogpt* gene is expressed in the transformed cells that contain the integrated LTR81gpt^{+/-} plasmids, but not in nontransformed Ltk⁻ cells (Fig. 5A).

S1 nuclease mapping was used to determine the initiation sites for the LTR81gpt^{+/-} transcripts (Fig. 5B and C). The RNA from transformed Ltk⁻ cells containing either LTR81gpt⁺ or LTR81gpt⁻ sequences was hybridized to the corresponding 676-bp *AccI*-*Bg*II fragment of the LTR81gpt plasmid (Fig. 1B). This fragment contains 181 bp of pBR322 sequences, 370 bp of the corresponding IAP81 LTR (in either the sense or antisense orientation), and 125 bp of the *Ecogpt* gene (*Hind*III to *Bg*II). With this fragment that is 5' end labeled at the *Bg*II site (single end label) within the *Ecogpt* gene, only fragments containing the *Ecogpt* transcripts are protected (Fig. 5B and C, lanes 1+ and 1-, respectively). When this fragment has both 5' ends labeled at the *AccI* and *Bg*II sites (double end label), it is possible to protect both sense and antisense transcripts originating within the LTR and to monitor bidirectional transcription simultaneously (Fig. 5B and 5C, lanes 2+ and 2-, respectively).

When the *Bg*II end-labeled fragment from LTR81gpt⁺ was hybridized with LTR81gpt⁺ RNA, a major band of 255 bp and a minor band of 271 bp were protected. This localizes the major transcription start site within the LTR for IAP gene transcription at position 228 and a minor start site at position 212 within the LTR. With the double-labeled fragment (lane 2+) the same transcripts that were protected by the *Bg*II end-labeled fragment are seen, and transcripts containing pBR322 sequences were also protected. The protected fragment for pBR322 transcripts is 197 bp for the LTR81gpt⁺ construct, which maps to position 1 of the LTR, indicating that antisense transcription initiates within the inverted repeat of the IAP81 gene LTR. To confirm these results, we used the *Bg*II end-labeled fragment (single end label) for LTR81gpt⁻ to hybridize to LTR81gpt⁻ RNA. This

Ecogpt transcript probe protects a fragment of 140 nucleotides which maps to position 1 at the 5' end of the LTR. By using the double-labeled fragment for the LTR81gpt⁺ construct, the 140-bp *Ecogpt* protected fragment is seen, as well as a 311-bp major band and a 327-bp minor band for the pBR322 transcripts of LTR81gpt⁻ RNA. These larger transcripts map to positions 228 and 212 of the LTR, respectively, confirming the sites of initiation for the normal IAP LTR promoter.

Characterization of IAP14/IAP81 hybrid LTRs. To determine whether IAP gene LTRs could be altered from a unidirectional promoter to a bidirectional promoter, we switched fragments from the LTRs of IAP14 (little or no activity in the antisense direction) and IAP81 (bidirectional promoter, but only one-sixth as active as IAP14 in the sense direction). Using the invariant *Pst*I site within the U3 region of IAP LTRs (Fig. 2A), we shuffled fragments, generating four recombinational clones (Fig. 2B). These hybrid LTRs were cloned in both orientations preceding the CAT gene (clone 14⁵U3/81U5RU3^{3'}-CAT was not isolated). A summary of the relative CAT activities is shown in Table 2.

For promoter activity in the sense direction, the levels of CAT expression do not change from the levels of the parental LTR when the 5'U3 regions of IAP14 and IAP81 are switched, indicating that the 3'U3RU5 of the LTR is most important in determining basal levels of transcription. Comparing the parental LTR14CAT⁺ (Table 1) with the hybrid mutant 81⁵U3/14³U3RU5-CAT (Table 2) shows that the 5'U3 of LTR81 and the 5'U3 of LTR14 give the same level of promoter activity. In fact, hybrids constructed by inserting the 5'U3 fragment from LTR81 in either orientation preceding the LTR14 3'U3RU5-CAT (81⁵U3/14³U3RU5-CAT, 81⁵U3*/14³U3RU5-CAT) gives equivalent or higher CAT activity compared with the LTR14CAT⁺ parental clone. The 5'U3 regions of both LTR14 and LTR81 can function independent of orientation, but they must be placed in front of the 3'U3 to give high promoter activity, since when the IAP14 5'U3 fragment is at the 5' end of the IAP81 U5RU3^{3'} fragment (CAT-14⁵U3/81U5RU3^{3'}), there is no CAT expression.

For promoter activity in the antisense direction the 5'U3 fragment (5'U3) is most important. Switching the 5'U3 region of IAP81 to the 3'U3RU5 of IAP14 (CAT-81⁵U3/14³U3RU5) allows the LTR to function as a bidirectional promoter, like

TABLE 3. Comparison of LTR81CAT deletion clones

Deletion	CAT activity ^a (no. of expt) for:	
	LTR81CAT ⁺	LTRCAT81 ⁻
d5'47	19.5 ± 5.0 (4)	89.3 ± 7.9 (3)
d5'110	10.3 ± 0.3 (2)	0.78 ± 0.2 (5)
d5'150	13.1 ± 4.9 (4)	30.0 ± 5.4 (4)
d5'256	2.27 ± 0.6 (3)	4.15 ± 1.5 (5)
d5'302	3.77 ± 0.7 (3)	3.81 ± 0.4 (5)
d5'38/d3'79	1.52 ± 0.2 (3)	0.86 ± 0.1 (5)
d5'77/d3'258	12.9 ± 2.2 (3)	47.3 ± 11.2 (3)
d3'305	234.0 ± 9.6 (3)	106.9 ± 9.8 (2)
d3'147	54.7 ± 21.2 (3)	113.0 ± 31.5 (4)
d3'110	9.0 ± 2.6 (4)	110.0 ± 11.3 (4)
d3'47	1.48 ± 0.5 (4)	6.95 ± 3.4 (3)
d3'40	0.97 ± 0.4 (4)	0.72 ± 0.2 (5)

^a Relative CAT activity was calculated by normalizing the percent conversion of chloramphenicol to acetylchloramphenicol for the parental LTRs (81CAT^{+/-}) equal to 100.

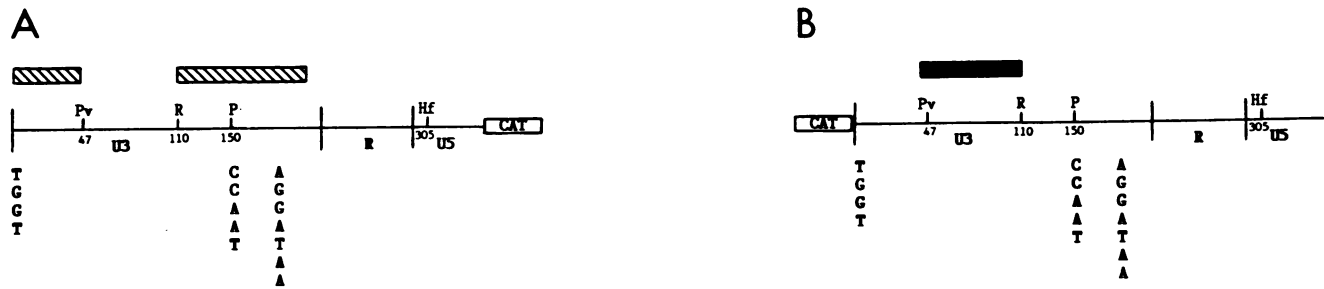


FIG. 6. Approximate locations of the IAP81 promoter elements for (A) sense (LTR81CAT⁺) and (B) antisense (LTR81CAT⁻) transcription. Regions important for sense transcription by the IAP81 are indicated (▨). Region 110 to 256 is important in promoter activity and contains the CAT and TATA sequences, with region 1 to 47 containing the sequences important for increased levels of transcription and an Sp1 binding site (4; Morgan and Huang, in press) and a core enhancer element (31, 47). The region important for antisense transcription (■) is from position 47 to 110. This region contains the core enhancer element and a Z-DNA-forming sequence (31, 38), but no consensus polymerase II promoter sequences were found.

LTR81CAT⁻. The hybrid mutant CAT-14^{5'}U3/81^{3'}U3RU5 (which does not contain the ^{5'}U3 of IAP81) and the parental LTR14CAT⁻ do not have antisense activity, indicating that the IAP81 ^{5'}U3 contains an antisense promoter. This expression is dependent on the orientation of the ^{5'}U3 fragment, since when the ^{5'}U3 fragment of IAP81 is inverted, the LTR no longer supports antisense transcription (CAT-81^{5'}U3^{7'}/14^{3'}U3RU5).

IAP81 LTR deletion analysis. IAP LTRs contain sequences that are essential for transcriptional regulation, which are shown with the sequence of IAP81 LTR in Fig. 3A. These putative regulatory sequences can be used to subdivide the LTR into three functional domains, U3-R-U5 (10, 32) and have been implicated in control or modulation of promoter activity in other systems.

To determine the functional importance of the different regions of the IAP81 LTR, we constructed deletion mutants. Mutations within the LTR consisted of deletions made from both 5' and 3' ends of the LTR (denoted d5' and d3', respectively) and internal fragments (d5'/d3'). The deletion points within the LTR are marked in Fig. 3A (with vertical arrows) and used to name the clone (e.g., d5'47, deletion from the 5' end to nucleotide 47). The deletion fragments are shown schematically in Fig. 3B. To determine the sequences important for bidirectional transcription, we placed each remaining fragment of LTR81 (LTR81) in front of the CAT gene in both the sense (CAT⁺) (e.g., d5'47/CAT⁺) and antisense (CAT⁻) (e.g., d5'47/CAT⁻) orientations.

Previous results indicated that sequences 3' to the *Pst*I site (^{3'}U3RU5) are necessary for a basal level of IAP LTR transcription in the sense orientation and that transcription is initiated in this region. To define which regions of the LTR enhance the normal transcription of IAP genes, we used deletions cloned in the sense orientation with respect to the CAT gene (CAT⁺). Deletion of the first 47 bp (d5'47) from the 5' end resulted in an 80% loss of transcriptional activity. Larger deletions from the 5' end (d5'110 and d5'150) and the internal fragment clone d5'77/d3'258 resulted in similar residual activity as that of d5'47. Only when all of the putative promoter elements were deleted (d5'256 or d5'302) was expression completely abolished. Deletions from the 3' end of the LTR81 showed that the terminal U5 region was not necessary (d3'302) for normal LTR activity and that deletion of these sequences slightly enhanced CAT expression. With these deletion data, the control region for a basal level of CAT⁺ transcription can be mapped between nucleotides 150 and 256 of the U3-R region. However, full activity was not

observed when the first 47 bp of the U3 region were missing, indicating that the first 47 bp may function to increase or control the level of promoter activity, although this region of the U3 has no promoter activity by itself (Table 3, d3'47 and d3'40). These two regions which are responsible for full transcriptional activity in the sense orientation are diagrammed in Fig. 6A.

To determine the regions of the LTR important for transcription by the antisense promoter, we analyzed CAT⁻ deletion mutants. Previous results from hybrid LTRs showed that the promoter activity was in the first 150 bp of the IAP81 LTR (^{5'}U3), and S1 nuclease studies showed that initiation was at position 1 of the LTR. Analysis of the 5' deletion mutants indicated that the U3 regions were necessary for activity, since clones d5'302 and d5'256, which contain R and U5 regions, showed no activity. When the terminal 47 nucleotides of the U3 region (d5'47) were deleted, the activity was not significantly decreased. If the first 77 bp (d5'77/d3'256) are deleted, the level of CAT activity decreases to 50%, and deletion of the first 110 nucleotides (d5'110) completely abolished CAT⁻ activity. By using the 3' deletion mutants, similar levels of CAT activity to those in the intact LTR were found in clones d3'147, d3'110, and d3'305, but no activity was found in mutants containing smaller fragments of the ^{5'}U3 region (d3'47, d3'40, and the internal fragment d5'38/d3'79). This indicates that the inverted repeat region is not necessary for initiation or promotion, that the region between nucleotides 47 and 77 is important in monitoring the level of promoter activity, and that nucleotides 77 to 110 are essential for promotion in the antisense direction. With these deletion data, the sequences essential for normal levels of CAT⁻ activity can be mapped between nucleotides 47 and 110 in the U3 region (Fig. 6B).

DISCUSSION

Using two independent assays, we showed that the LTRs of randomly isolated endogenous IAP genes are transcriptionally active in embryonal PCC3-A/1 and Ltk⁻ cells. This correlates with previous reports showing that two other IAP gene LTRs could function as promoter elements in mouse and heterologous cell types (23, 31).

Comparison of the LTR promoter activity was measured by using transient CAT assays with the CAT gene under the control of a specific IAP LTR. The LTRs from the different proviral IAP genes showed a wide variation of promoter

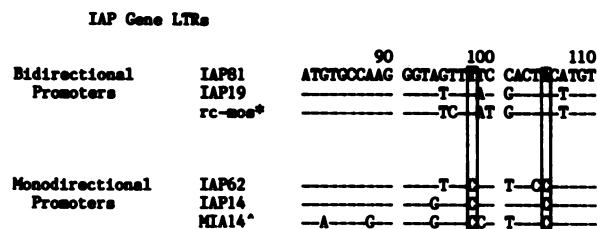


FIG. 7. Sequence of the region that is possibly responsible for antisense promoter activity. The nucleotide numbering corresponds to the sequence of IAP81 in Fig. 3A. All sequences are compared to the bidirectional promoter of IAP81. The LTRs that have been shown to promote CAT activity in the antisense direction are shown (IAP19B/D and *rc-mos** [20]), and sequences of the LTRs that do not have any antisense promoter activity (IAP14, IAP62, and MIA14* [29]) are shown. Two conserved nucleotide changes between antisense promoters and LTR promoters that function only in the correct orientation can be found and are boxed.

activity in both the sense and antisense directions. Unique among the IAP gene LTRs tested in this and previous studies (23, 31) was the LTR of IAP81, which functioned as a bidirectional promoter in the CAT assays and had higher activity in the antisense direction than in the sense direction. These data also indicate that the variation in the R region size and sequence has very little effect on the direction or levels of promoter function, since LTRs with both long (LTR62 and LTR19) and short (LTR14 and LTR81) R regions (10) actively promote CAT^{+/-} expression.

The permanently transformed mouse Ltk⁻ cell lines containing LTR81gpt indicate that the IAP81 LTR also transcribes bidirectionally when integrated. Both transcripts initiated within the LTR and transcribed *gpt* and pBR322 sequences simultaneously (Fig. 5B and C, lanes 2+ and 2-, respectively). It is possible that transcripts originate from different integrated plasmid copies and are not transcribed from the same LTR, but this is unlikely, since bidirectional activity of LTR81 is seen in transient CAT assays and previous studies with *in vivo*- and *in vitro*-labeled RNA and IAP81 (Wujcik, Ph.D. thesis).

The initiation site(s) for sense and antisense transcripts was determined by S1 nuclease mapping of the LTR from IAP81. For initiation in the 5'-to-3' direction, the major protected fragment is at position 228 of the LTR, with a minor band at position 212. This is similar to the result found in the S1 nuclease mapping of transcripts from MIA14 by Lueders et al. (31), who also found two protected fragments. S1 mapping of the antisense transcripts shows initiation at position 1, within the inverted repeat of the intact LTR: the same initiation site of the *c-mos* transcript of *rc-mos* (23). This initiation site is not essential for antisense transcription or initiation, since when the first 47 nucleotides of the U3 region are deleted (Table 3, d5'47), antisense transcription by the LTR still occurs. The ability of the antisense promoter to use other initiation sites is also evident in the double-label experiment, in which several bands below the 197-bp fragment (Fig. 5B) and 140-bp fragment (Fig. 5C) are seen, indicating initiation within the flanking pBR322 sequences and *gpt* gene.

To determine the importance of the LTR U3-R-U5 regions in bidirectional promoter activity of IAP LTRs, we switched fragments from the LTRs of IAP14, the strongest promoter in the sense direction, and IAP81, the bidirectional promoter (Fig. 2B). Experiments show that it is possible to make the

IAP14 LTR a bidirectional promoter by switching the 5'U3 regions, but this region has little effect in determining promoter activity in the 5'-to-3' direction. For promoter activity in the sense orientation, the 3'U3RU5 region determines the maximal level of activity and the 5'U3 region is necessary only to obtain this maximal activity. The most surprising result is that when the LTR81 5'U3 fragment is cloned in the opposite orientation (5'U3*) with respect to the LTR14 3'U3RU5 fragment (81'5'U3*/14'3'U3RU5-CAT), the level of CAT activity is comparable to that of the normal IAP14CAT⁺ promoter. It is possible that there is enough promoter sequence in the 3'U3RU5 (in front of the CAT box) to allow normal transcription by IAP14. This is unlikely, since deletion analysis indicated that the regions immediately upstream are important for activity (Table 3, d5'150) and analysis of the activity of the LTR14 3'U3RU5 fragment indicates that the level of residual transcription is approximately 13% of that of the intact IAP14 LTR (data not shown). This 150-bp 5'U3 may act as an enhancer for promoting transcription, making the orientation of this 5'U3 insert unimportant for activity, although its position 5' to the 3'U3RU5 fragment is critical (Table 2). The antisense promoter is located completely within the first 150 bp of the U3 region (5'U3). Switching the 5'U3 region of IAP81 to the 3'U3RU5 of IAP14 enables the IAP14 LTR to become a bidirectional promoter, and switching the 5'U3 region of IAP14 to the 3'U3RU5 of IAP81 inactivates antisense transcription by the LTR of IAP81. This correlates with the results showing that the region from 47 to 110 bp in the 5'U3 contains the antisense promoter (see deletion data).

The functional boundaries of the sense promoter elements (Fig. 6) were determined by using deletion mutants which scanned the LTR of IAP81 (Fig. 3B). Deletions from the 5' end (d5'47) decreased expression to approximately 20% of that of the intact LTR, and expression remained at this level until deletions extended past the putative CAT and TATA boxes (d5'256), indicating that sequences within the first 47 nucleotides are important for full promoter activity. This mutation is also near the putative enhancer core sequence found in IAP LTRs (31), and deletion of this region may disrupt an IAP enhancer element. The 3' deletion mutants also showed that the first 147 bp (d3'147) of the Δ 81LTR gives significant levels of CAT gene expression, even though it contains no putative control sequences. Activity of a similar fragment in the LTR of avian retrovirus was demonstrated (14), although it was not as high as that seen with d3'147 of the IAP81 LTR.

Sequence comparison between the LTRs to determine nucleotide changes responsible for promoter activity revealed no consistent pattern, except that the putative TATA boxes are variable in sequence in all the IAP LTRs studied (10, 29, 35). The TATA box is normally important only in determining the precise transcription initiation site, but mutations in this region have been shown to decrease the level of transcription 2- to 10-fold (34). The most active IAP LTR, LTR14CAT⁺, has the sequence AGGATAA as its putative TATA box, whereas the LTRs of IAP19 have TATA boxes similar to the consensus sequence AATATAA (2), but have little CAT⁺ activity (Fig. 4; Table 1).

Analysis of the antisense promoter gives a defined region that is important for promoter function which is bounded by nucleotides 47 and 110 (Fig. 6). The level of activity of the Δ 81LTR deletion clone d5'77/d3'256 is lower than that of the other deletions that contain the region from nucleotides 47 to 77 (d3'110, d3'147, and d5'40), indicating that this region may be important in determining the level of antisense

promoter activity. Comparison of the sequence in this region indicates that the 5' end of this region is well conserved in all LTRs (10), whereas consistent nucleotide changes which might be important in antisense activity are found between nucleotides 90 and 110 (Fig. 3A) and are compared in Fig. 7. It is not known which of these nucleotide changes are important in controlling antisense promoter activity. In vitro studies showed that the transcription of the cellular flanking regions by LTR81 were α -amanitin sensitive, indicating that antisense transcription was by RNA polymerase II (Wujcik, Ph.D. thesis). Analysis of the U3 region important to antisense transcription (nucleotides 47 to 110) has uncovered no sequences resembling a CAT or TATA box control sequence.

These results show that many endogenous IAP genes do have the potential to be active and may transcribe genes in both the sense and antisense directions. All of the IAP gene LTRs we (this study) and others (23, 31) have tested are transcriptionally active in at least one direction (sense or antisense) and have the potential to transcribe bidirectionally (IAP81). This fact indicates that many IAP genes could potentially cause aberrant expression of flanking cellular genes, independent of the orientation of the IAP gene, similar to the IAP gene integrated into the *c-mos* gene in the myeloma XRPC20 (*rc-mos*) (11, 23, 28). The major functional domains for transcriptional activity have been defined, and studies to analyze the specific regions and putative regulatory factors important in IAP regulation are currently in progress.

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LITERATURE CITED

- Alton, N. K., and D. Vapnek. 1979. Nucleotide sequence analysis of the chloramphenicol resistance transposon Tn9. *Nature (London)* **282**:864-869.
- Benoist, C., and P. Chambon. 1981. *In vivo* sequence requirements of the SV40 early promoter region. *Nature (London)* **290**:304-315.
- Berk, A. J., and P. A. Sharp. 1977. Sizing and mapping early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. *Cell* **12**:721-732.
- Briggs, M., J. T. Kadonaga, S. P. Bell, and R. Tjian. 1986. Purification and biochemical characterization of the promoter-specific transcription factor Sp1. *Science* **234**:47-52.
- Burt, D. W., A. D. Reith, and W. J. Brammer. 1984. A retroviral provirus closely associated with the Ren-2 gene of DBA/2 mice. *Nucleic Acids Res.* **12**:8579-8593.
- Calarco, P. G., and D. Szollosi. 1973. Intracisternal A-particles in ova and preimplantation stages of the mouse. *Nature (London) New Biol.* **243**:91-93.
- Canaani, E., O. Dreazen, A. Klar, G. Rechavi, D. Ram, S. B. Cohen, and D. Givol. 1983. Activation of the *c-mos* oncogene in a mouse plasmacytoma by insertion of an endogenous intracisternal A-particle genome. *Proc. Natl. Acad. Sci. USA* **80**:7118-7122.
- Chase, D. G., and L. Piko. 1973. Expression of A- and C-type particles in early mouse embryos. *J. Natl. Cancer Inst.* **51**:1971-1973.
- Chiu, I. M., R. C. C. Huang, and S. A. Aaronson. 1985. Genetic relatedness between intracisternal A-particles and other major oncovirus genera. *Virus Res.* **3**:1-11.
- Christy, R. J., A. R. Brown, B. B. Gourlie, and R. C. C. Huang. 1985. Nucleotide sequence of murine intracisternal A-particle gene LTRs have extensive variability within the R region. *Nucleic Acids Res.* **13**:289-302.
- Cohen, J. B., T. Unger, G. Rechavi, E. Canaani, and D. Givol. 1983. Rearrangement of the oncogene *c-mos* in mouse myeloma NSI and hybridomas. *Nature (London)* **306**:797-799.
- Cole, M. D., M. Ono, and R. C. C. Huang. 1981. Terminally redundant sequences in cellular intracisternal A-particle genes. *J. Virol.* **38**:680-687.
- Cullen, B. R., K. Raymond, and G. Ju. 1985. Transcriptional activity of avian retroviral long terminal repeats directly correlates with enhancer activity. *J. Virol.* **53**:515-521.
- Cullen, B. R., K. Raymond, and G. Ju. 1985. Functional analysis of the transcriptional control region located within the avian retroviral long terminal repeat. *Mol. Cell. Biol.* **5**:438-447.
- Cullen, B. R., A. M. Skalka, and G. Ju. 1983. Endogenous avian retroviruses contain deficient promoter and leader sequences. *Proc. Natl. Acad. Sci. USA* **80**:2946-2950.
- Fienberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**:6-13.
- Garger, S. J., O. M. Griffith, and L. K. Grill. 1983. Rapid purification of plasmid DNA by a single centrifugation in a two-step cesium chloride-ethidium bromide gradient. *Biochem. Biophys. Res. Commun.* **117**:835-842.
- Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* **2**:1044-1051.
- Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**:456-467.
- Hawley, R. G., M. S. Shulman, and N. Hozumi. 1984. Transposition of two different intracisternal A-particle elements into an immunoglobulin kappa-chain gene. *Mol. Cell. Biol.* **4**:2565-2572.
- Hawley, R. G., M. S. Shulman, H. Murialdo, D. M. Gibson, and N. Hozumi. 1982. Mutant immunoglobulin genes have repetitive DNA elements inserted into their intervening sequences. *Proc. Natl. Acad. Sci. USA* **79**:7425-7429.
- Hojman-Montes de Oca, F., L. Dianoux, J. Pewries, and R. Emanoil-Rovicovitch. 1983. Intracisternal A-particles: RNA expression and DNA methylation in murine teratocarcinoma cells. *J. Virol.* **46**:307-310.
- Horowitz, M., S. Luria, G. Rechavi, and D. Givol. 1984. Mechanism of activation of the mouse *c-mos* oncogene by the LTR of an intracisternal A-particle gene. *EMBO J.* **3**:2937-2941.
- Hutter, K. M., J. A. Barbosa, G. A. Scangos, D. D. Pratcheva, and F. H. Ruddle. 1981. DNA mediated gene transfer without carrier DNA. *J. Cell. Biol.* **91**:153-156.
- Jaenisch, R., H. Fan, and B. Croker. 1975. Infection of preimplantation mouse embryos and of newborn mice with leukemia virus: tissue distribution of viral DNA and RNA and leukemogenesis in the adult animal. *Proc. Natl. Acad. Sci. USA* **72**:4008-4012.
- Ju, G., L. Boone, and A. M. Skalka. 1980. Isolation and characterization of recombinant DNA clones of avian retroviruses: size heterogeneity and instability of the direct repeat. *J. Virol.* **33**:1026-1033.
- Kelly, F., and H. Condamine. 1982. Tumor viruses and early mouse embryos. *Biochim. Biophys. Acta* **651**:105-141.
- Kuff, E. L., A. Feenstra, K. Lueders, G. Rechavi, D. Givol, and E. Canaani. 1983. Homology between an endogenous viral LTR and sequences inserted in an activated cellular oncogene. *Nature (London)* **302**:547-548.
- Kuff, E. L., A. Feenstra, K. Lueders, L. Smith, R. Hawley, N. Hozumi, and M. Shulman. 1983. Intracisternal A-particle genes as movable elements in the mouse genome. *Proc. Natl. Acad. Sci. USA* **80**:1992-1996.
- Kuff, E. L., L. A. Smith, and K. K. Lueders. 1981. Intracisternal A-particle genes in *Mus musculus*: a conserved family of retrovirus-like elements. *Mol. Cell. Biol.* **1**:216-227.
- Lueders, K. K., J. W. Fewell, E. L. Kuff, and T. Koch. 1984. The long terminal repeat of an endogenous intracisternal A-particle

- gene functions as a promoter when introduced into eucaryotic cells by transfection. *Mol. Cell. Biol.* **4**:2128–2135.
32. Lueders, K. K., and E. L. Kuff. 1980. Intracisternal A-particle genes: identification in the genome of *Mus musculus* and comparison of multiple isolates from a mouse gene library. *Proc. Natl. Acad. Sci. USA* **77**:3571–3575.
 33. Melton, D. A., P. A. Krieg, M. R. Rebaglianti, T. Maniatis, K. Zenn, and M. D. Green. 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* **12**:7035–7056.
 34. McKnight, S. L., and R. Kingsbury. 1982. Transcriptional control signals of a eukaryotic protein-coding gene. *Science* **217**:316–325.
 35. Morgan, R. A., and R. C. C. Huang. 1984. Correlation of undermethylation of intracisternal A-particle genes with expression in murine plasmacytomas but not in NIH/3T3 embryo fibroblasts. *Cancer Res.* **44**:5234–5241.
 36. Moshier, J. A., R. A. Morgan, and R. C. C. Huang. 1985. Expression of two murine gene families in transformed cells and embryogenesis, p. 101–116. *In* B. Pullman, P. O. P. Ts'o, and E. L. Schneider (ed.), *Interrelationship among aging, cancer and differentiation*. D. Reidel Publishing Co., Dordrecht, The Netherlands.
 37. Mulligan, R. C., and P. Berg. 1981. Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. *Proc. Natl. Acad. Sci. USA* **78**:2072–2076.
 38. Nordheim, A., and A. Rich. 1983. Negatively supercoiled simian virus 40 DNA contains Z-DNA segments within transcriptional enhancer sequences. *Nature (London)* **303**:674–678.
 39. Okayama, H., and P. Berg. 1983. A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. *Mol. Cell. Biol.* **3**:280–287.
 40. Ono, M., M. D. Cole, A. T. White, and R. C. C. Huang. 1980. Sequence organization of cloned intracisternal A-particle genes. *Cell* **21**:465–473.
 41. Paterson, B. M., S. Segal, K. K. Lueders, and E. L. Kuff. 1978. RNA associated with the murine intracisternal type A particles codes for the main particle protein. *J. Virol.* **27**:118–126.
 42. Richardson, K. K., J. Fostel, and T. R. Skopek. 1983. Nucleotide sequence of the xanthine guanine phosphoribosyl transferase gene of *E. coli*. *Nucleic Acids Res.* **11**:8809–8816.
 43. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 44. Scheiderei, C., and M. Beato. 1984. Contacts between hormone receptor and DNA double helix within the glucocorticoid regulatory element of mouse mammary tumor virus. *Proc. Natl. Acad. Sci. USA* **81**:3029–3033.
 45. Sheng-Ong, G. L., and M. D. Cole. 1982. Differing population of intracisternal A-particle genes in myeloma tumors and mouse subspecies. *J. Virol.* **42**:411–421.
 46. Ullrich, A. J., J. Shine, J. Chirgwin, R. Pictet, E. Tischer, W. J. Rutter, and H. M. Goodman. 1977. Rat insulin genes: construction of plasmids containing the coding sequences. *Science* **196**:1313–1319.
 47. Weiher, H., M. Konig, and P. Gruss. 1983. Multiple point mutations affecting the simian virus 40 enhancer. *Science* **219**:626–631.
 48. Wilson, S. H., E. W. Bohn, A. Matsukage, K. K. Lueders, and E. L. Kuff. 1974. Studies of the relationship between deoxyribonucleic acid polymerase activity and intracisternal A-particles in mouse myelomas. *Biochemistry* **13**:1078–1094.
 49. Wujcik, K. M., R. A. Morgan, and R. C. C. Huang. 1984. Transcription of intracisternal A-particle genes in mouse myeloma and Ltk⁻ cells. *J. Virol.* **52**:29–36.
 50. Yang, S. S., and N. A. Wivel. 1974. Characterization of an endogenous RNA-dependent DNA polymerase associated with murine intracisternal A-particles. *J. Virol.* **13**:712–720.
 51. Zagursky, R. J., K. Baumeister, N. Lomax, and M. L. Berman. 1985. Rapid and easy sequencing of large linear double-stranded DNA and supercoiled plasmid DNA. *Gene Anal. Tech.* **2**:89–94.