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Murine A type retroviruses promote high levels of gene expression in embryonal carcinoma cells

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Summary

The expression of Intracisternal A Particle (IAP) genes in the mouse embryonal carcinoma cell line PCC3 was investigated by cDNA cloning and transient gene expression assays. A group of 26 IAP cDNA clones, products of transcriptionally active IAP proviruses, were selected from a cDNA library made from undifferentiated PCC3 cell RNA. Several of these clones were characterized by restriction enzyme mapping and DNA sequence analysis. The DNA sequence in both the promoter and structural regions of two cDNAs closely resembles those of IAP genomic clones. Three new sequence elements were identified within the U3 region, an Sp1 transcription-factor-binding site, an adenovirus E1a enhancer sequence and a region of homology to a promoter element of adenovirus E4 gene. Hybrid constructs were made that place the U3/R region of the IAP cDNAs immediately 5′ to the chloramphenicol acetyl transferase (CAT) gene. IAP–CAT constructs were transfected into PCC3 cells, and cell extracts prepared and analysed for CAT enzyme activity and CAT RNA levels. IAP–CAT transfected cells were shown to contain substantial levels of CAT enzyme activity and to accumulate much greater levels of CAT RNA than two standard promoters, pRSVcat and pSV2cat. The ability of these A type retroviral promoters to function in PCC3 cells is in direct contrast to the near total restriction of normal C type retroviral expression in EC cells.

Key words: retrovirus, embryonal carcinoma cell, cDNA cloning, gene expression, intracisternal A particle.

Introduction

As an example of a gene family abundantly expressed during mouse embryogenesis, we have chosen to study the endogenous A type retroviruses known as Intracisternal A Particle (IAP) genes (Kuff *et al.* 1981; Lueders & Kuff, 1977; Ono *et al.* 1980). The structural organization of IAP genes is similar to that of retroviral proviruses, with long terminal repeats (LTR) of 300–500 bp flanking protein-coding regions (Christy *et al.* 1985). Individual IAP genes are members of a dispersed multigene family with approximately 1000 copies per haploid genome in *Mus musculus* (Lueders & Kuff, 1977; Ono *et al.* 1980; Shen-Ong & Cole, 1982). The expression of IAP genes has been extensively studied in mouse cell lines and normal mouse tissues, including mouse embryos (Kuff & Fewell, 1985; Morgan & Huang, 1984; Moshier *et al.* 1985; Piko *et al.* 1984; Wujcik *et al.* 1984; Yotsuyangi & Szollosi, 1981). In the case of mouse embryos, IAP antigens, IAP particles and IAP transcription have been observed in the preimplantation stage of mouse development, but not at later stages of development (Huang & Calarco, 1981*a*,*b*; Moshier *et al.* 1985; Piko *et al.* 1984; Yotsuyangi & Szollosi, 1981). Using murine embryonal carcinoma (EC) cell lines as a model system for the early mouse embryo, we and others have observed IAP transcripts in both pluripotent and nullipotent EC cell lines (Hojman-Montes de Oca *et al.* 1983; Morgan & Huang, 1987; Moshier *et al.* 1985).

The activity of IAP genes in EC cells and mouse embryos is directly opposite to the behaviour of murine C type retroviruses (e.g. Mo-MuLV). Few C

type retroviruses have been observed in preimplantation mouse embryos and infection with exogenous viruses leads to an initial integration event, but no viral gene expression occurs (Jaenisch *et al.* 1975; Kelly & Codamine, 1982). Analysis of the mechanisms controlling C type retroviral expression in EC cells has revealed that viral gene expression is blocked at the level of transcription (Gautsch & Wilson, 1983). When the promoter activity of the Mo-MuLV LTR was tested in the EC cell line F9, it was found to be totally inactive (Gorman *et al.* 1985; Linney *et al.* 1984). This block to expression can be overcome by the use of naturally derived host range mutants or by the selection for rare drug-resistant phenotypes in cell culture systems (Barklis *et al.* 1986; Seliger *et al.* 1986).

As a first step in an investigation of the mechanisms that control IAP gene expression in embryonic cells, we have isolated actively transcribed IAP gene from the pluripotent EC cell line, PCC3. These genes were characterized by restriction enzyme mapping and DNA sequence analysis and were shown to be similar to randomly isolated IAP genomic clones containing all the regulatory sequences essential for transcriptional control. By the use of transient gene expression assays, we report herein that IAP genes actively promote the expression of linked gene in undifferentiated EC cells.

Materials and methods

Cell culture

PCC3-A/1 cells (kindly provided by A. Rizzino) were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (50 i.u. ml^{-1}) and streptomycin (50 mg ml^{-1}), with the addition of glucose (4 g l^{-1}) and L-glutamine (4 mM). Care was taken to plate PCC3 cells as single cells and not to let the monolayer grow to more than 50% confluence.

cDNA library construction

The general procedures used in cDNA synthesis have been previously described (Watson & Jackson, 1985) and only specific modifications are presented. An IAP-specific oligonucleotide primer (5'TTTATTGCTTACATCTTCAGG-3') complementary to the extreme 3' end of the IAP RNA was annealed (at 10 μg ml^{-1}) to 10 μg of PCC3 poly(A)-containing RNA and used to initiate first strand synthesis. Double-stranded cDNA was dG tailed with terminal transferase and an oligo dC-*Eco*RI adaptor molecule (5'-AATTC$_{30}$-3') annealed. The cDNA was size fractionated and ligated to *Eco*RI-cut λgt10. The location of the primer used in cDNA synthesis and of the probe used to screen the resultant library are shown in Fig. 1.

CAT expression vectors

The vector used for CAT expression (pCAP) was constructed from commercially available plasmids (pcDV-1, M13 mp19 and pCM4, Pharmacia; R.J.C. & R.C.C.H., unpublished data. IAP cDNA clones containing promoter (U3/R regions) were isolated from the λgt10 vector by *Eco*RI digestion and preparative gel electrophoresis. These IAP fragments were then made blunt end with Klenow fragment and ligated to *Hin*dIII-cut blunt end pCAP. Clones in the proper orientation were isolated by restriction-enzyme mapping.

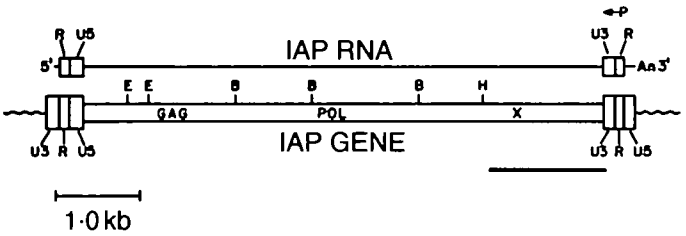

Fig. 1. IAP provirus showing cDNA primer and hybridization probe locations. A representative IAP provirus is shown in relation to its corresponding mRNA transcript. GAG and POL refer to regions of the provirus that code for the major IAP virion protein and reverse transcriptase, respectively, the 3' end of the IAP provirus has not been definitely assigned a gene product(s) and is referred to as X. The locations of three restriction enzyme sites (B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII) found in the majority of IAP genes are as indicated. The location of the oligonucleotide primer (←P) used to initiate first strand cDNA synthesis is shown above the IAP RNA. The region of the IAP provirus used to generate a hybridization probe for screening the cDNA library is indicated by a dark line and cellular flanking sequences indicated by a wavy line.

DNA-mediated gene transfer and CAT enzyme assays

Varying amounts of CsCl-banded CAT constructs were introduced into freshly plated cell monolayers (5×10^5 cells per 60 mm dish) by the calcium phosphate coprecipitation technique (Hutter *et al.* 1981). After exposure for 12–16 h, the culture medium was removed, the monolayer washed and fresh media added. Cell were harvested approx. 48 h after plating and cell extracts assayed for CAT enzyme activity as described by Gorman *et al.* (1982*b*). In each experiment, assays were performed on cell extracts from equal numbers of cells using the amount of extract titrated to fall within the linear range of CAT activity. Quantification of the conversion of [^{14}C]chloramphenicol to acetyl-[^{14}C]chloramphenicol was accomplished by excising the appropriate area of the TLC plate and counting in a scintillation counter. Data were normalized as described in the text.

CAT Northern blot analysis

RNA extractions were performed using the guanidinium isothiocyanate/CsCl procedure (Chirgwin *et al.* 1979). Formaldehyde gel electrophoresis and Northern blot transfer were as described previously (Wujcik *et al.* 1984). CAT transcripts were detected using a high specific activity ($>10^9$ cts min^{-1} μg^{-1}) single-strand RNA probe generated

from a CAT-pSP64 vector (a gift of J. Alwine). Hybridization and riboprobe preparation were as described by Melton *et al.* (1984).

Nucleotide sequence analysis

IAP cDNA clones EC26 and EC32 were subcloned into M13 vectors mp18 and mp19 and sequenced using the dideoxynucleotide chain-termination method (Sanger *et al.* 1977). Both strands of each clone were sequenced. DNA sequence comparisons were performed using the NUCALN and Los Alamos SEQH programs as previously described (Christy *et al.* 1985).

Results

cDNA cloning of IAP genes from the PCC3 EC cell line

As an initial step in the study of the developmental regulation of IAP gene expression, we have chosen to study IAP expression in murine embryonal carcinoma (EC) cell lines. Because IAP genes are members of a large multigene family, we decided to isolate IAP cDNA clones in order to limit our analysis to *bona fide* transcriptionally active genes. The nature of the retroviral transcription unit is such that the main determinant of the LTR promoter region (the U3 region) is copied at the 3′ end of each mRNA (Fig. 1). This fact permitted us not only to analyse the primary structure of the IAP cDNA clones but also to perform promoter function assays.

The pluripotent EC cell line PCC3 was chosen as a source of RNA to use in constructing a cDNA library (this cell line had previously been shown to express IAP genes as two moderately abundant RNAs of 5·3 and 7·2 kb (Hojman-Montes de Oca *et al.* 1981; Morgan & Huang, 1987). 26 IAP cDNA clones were obtained from this library (Fig. 1 shows the location of the first strand cDNA primer used to construct the library, see Methods for details). Fig. 2A shows the restriction enzyme maps of five of these clones. These cDNA clones have many of the conserved restriction enzyme sites found in randomly isolated IAP genomic clones (Christy *et al.* 1985). These include, an invariant *Pst*I recognition sequence found adjacent to the CAAT box plus *Hha*I and *Hae*III sites usually present within the U3 region. A more detailed restriction enzyme analysis (using the previously mentioned enzymes plus *Rsa*I and *Bst*NI) of a total of 17 clones revealed the majority of the IAP cDNA clones to be unique clones (data not shown). This suggests that IAP transcripts in the PCC3 cell line originate from multiple proviral loci.

To analyse further the similarity of the IAP cDNA clones to IAP genomic clones, we determined the nucleotide sequence of clones EC26 and EC32. Fig. 3

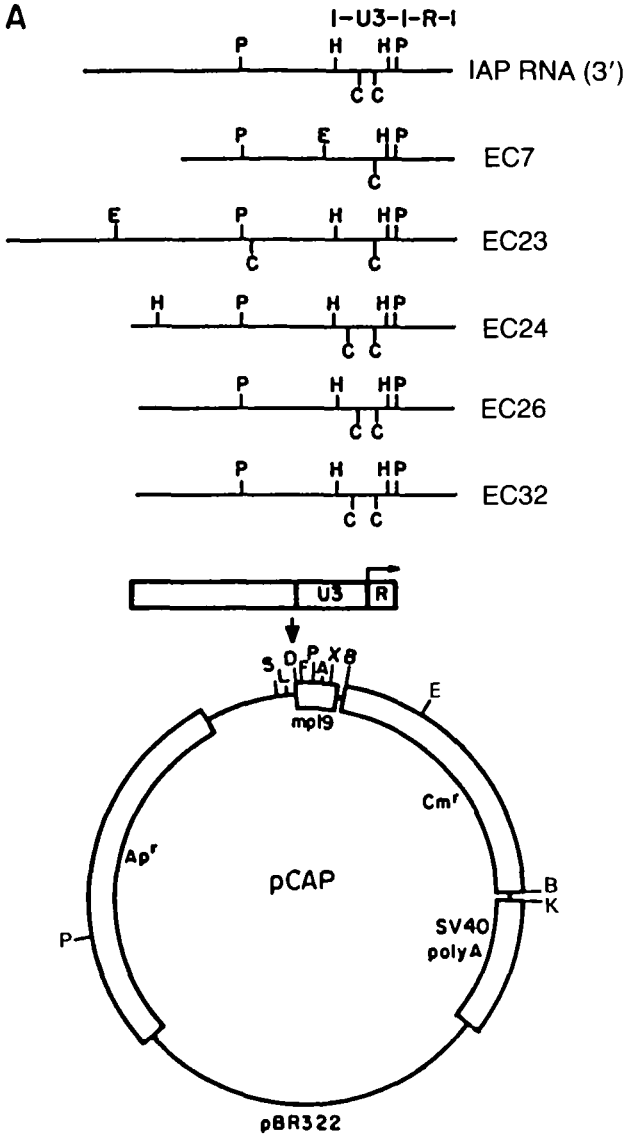

Fig. 2. Structure of IAP cDNA clones and CAT expression vector. Panel A shows the restriction enzyme maps of five IAP cDNA clones in relation to a consensus map of the 3′ end of IAP RNA generated from analysis of genomic clones. Only the first 1·2 kb of the clone EC23 is shown, its full length is 2·5 kb. Panel B is the restriction enzyme map of the pCAP CAT expression vector depicting the insertion of an IAP cDNA clone into the *Hin*dIII site. Clones pEC7cat, pEC23cat and pEC26cat were produced in the orientation shown, such that transcription (indicated by the arrow) is initiated at the U3/R boundary and reads through the CAT gene. Restriction enzyme sites are indicated as follows: C, *Hha*I; E, *Eco*RI; H, *Hae*III; P, *Pst*I; L, *Cla*I; X, *Xba*I; D, *Hin*dIII; F, *Sph*I; A, *Acc*I; *S*, *Sal*I, B, *Bam*HI; K, *Kpn*I.

shows the resultant sequence of these clones in relation to that from a randomly isolated IAP genomic clone, IAP 62. The cDNA clones are extremely well conserved both to each other (98 %) and to the IAP genomic clone (92 %) with no major insertions

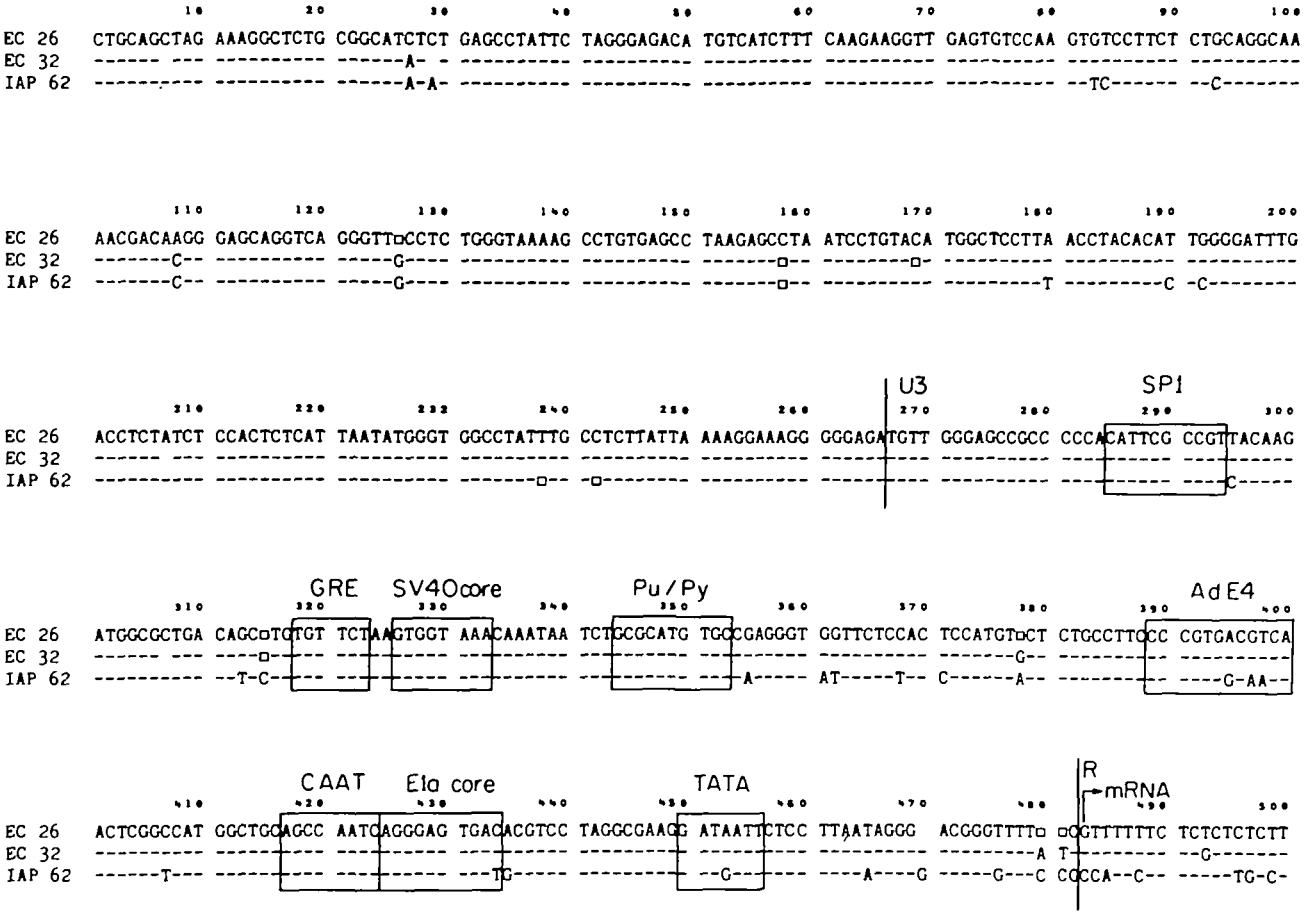

Fig. 3. DNA sequences of IAP cDNA clones EC26 and EC32. The DNA sequence is shown of the 3′ 500 bp of IAP cDNA clone EC26 (top line) in relation to IAP cDNA clone EC32 (middle line) and IAP genomic clone IAP 62 (bottom line). Nucleotide substitutions are as indicated (N), homologies are shown by dashed lines (– – –), gaps have been introduced where necessary to maintain homology. Sequences within the U3 region that have homology to proposed eukaryotic promoter elements are as indicated (boxed nucleotides, see text).

or deletions. Since our major interest in isolating these cDNA clones was to study IAP gene expression, we searched for possible consensus sequence elements which are thought to be important in the regulation of eukaryotic genes. Several of these elements have previously been identified in the IAP U3 region (Lueders *et al.* 1984; Ymer *et al.* 1986), these include a potential glucocorticoid-responsive element (GRE, Scheidereit & Beato, 1984), an SV40 core enhancer sequence (SV40 core, Weiher *et al.* 1983), an alternating purine/pyrimidine stretch (Pu/Py, Nordheim & Rich, 1983), a CAAT box (CAAT, Efstratiadis *et al.* 1980) and a TATA box homology (TATA, Benoist *et al.* 1980). In addition to these sequences, we have found a potential Sp1-binding site (Sp1, Briggs *et al.* 1986), a region of homology to one of the promoter elements of the adenovirus type 5 E4 promoter (Ad E4, Gilardi & Perricaudet, 1985), and an adenovirus E1a core enhancer sequence (E1a core, Hen *et al.* 1983).

Analysis of IAP promoter activity

To test the relative promoter activity of the IAP-containing cDNA clones, inserts containing IAP promoter regions (U3R) were cloned into the pCAP vector which permits the expression of the bacterial CAT gene (diagrammed in Fig. 2B). Three IAP cDNA–CAT constructs were assembled using clones EC7, EC23 and EC26. As an initial titration of the amount of DNA that could be introduced into PCC3 cells, we transfected 1, 3, 10, 15 and 20 μg of the EC7–CAT construct (pEC7cat) into PCC3 cells and, 60 h post-transfection, equal amounts of cell extracts were prepared and assayed for CAT enzyme activity. The results of this experiment (Fig. 4) show that IAP genes could promote CAT gene expression in PCC3 cells and that the linear range of DNA uptake/CAT expression under these conditions was between 3 and 15 μg of input DNA.

To analyse more thoroughly the ability of the IAP cDNA clones to promote expression in PCC3 cells, we carried out multiple independent transfections

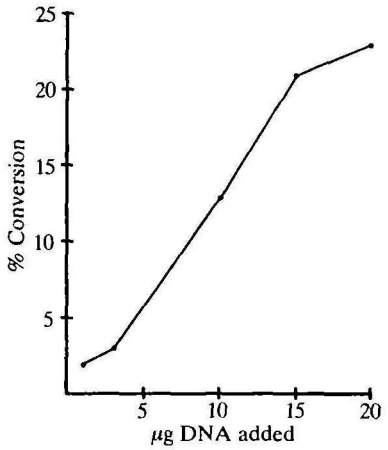

Fig. 4. IAP–CAT constructs promote CAT gene expression in PCC3 cells. 1, 3, 10, 15 and 20 μg of the pEC7cat vector were transfected into 5×10^5 PCC3 cells and 2 days later cells were harvested and assayed (30 min incubations) for CAT enzyme activity. Data on the percentage conversion of chloramphenicol to acetyl chloramphenicol was calculated and plotted *versus* amount of transfected DNA.

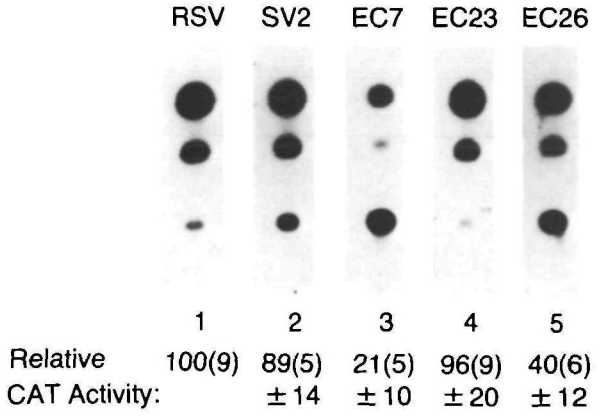

Fig. 5. Expression of IAP–CAT plasmids in PCC3 cells in relation to standard promoters. A typical autoradiogram is shown of the resultant TLC separation of acetylated (top two spots) from nonacetylated chloramphenicol (bottom spot) in a CAT enzyme assay (60 min incubations) of PCC3 cells (5×10^5) transfected with 5 μg of the indicated plasmids (1, pRSVcat; 2, pSV2cat; 3, pEC7cat; 4, pEC23cat; 5, pEC26cat). Following autoradiography, areas corresponding to chloramphenicol (Cm) and acetyl chloramphenicol (AcCm) were excised and quantified by scintillation counting. To obtain relative CAT activity, data were normalized by setting the percentage conversion of Cm to AcCm produced by pRSVcat equal to 100; number of repetitions (n) and standard deviations (+/− n) were as shown.

(using 5 μg DNA) of each IAP cDNA–CAT construct into PCC3 cells and compared their CAT gene expression to that promoted by two standard promoters, pRSVcat and pSV2cat (both of these two standard promoters had previously been shown to be active in EC cells, Gorman *et al.* 1986). After each transfection had been repeated a minimum of five times, the data were normalized by setting the % conversion of chloramphenicol to acetylated chloramphenicol produced by the pRSVcat-transfected cell extract equal to 100 (Fig. 5). The order of relative promoter strength of these genes in PCC3 cells (as measured by CAT enzyme activity) was found to be pRSVcat (100) > pEC23cat (96) > pSV2cat (89) > pEC26cat (40) > pEC7cat (21).

To assay directly the level of IAP-promoted CAT-gene expression, we next looked at transiently produced CAT RNA levels by Northern blot analysis (Fig. 6). Although CAT mRNA has been generally found to be difficult to detect by Northern blot analysis, we have found that the use of high specific activity ($>10^9$ cts min^{-1} μg^{-1}) CAT riboprobe can reproducibly detect very low levels of CAT RNA. The CAT riboprobe also has the property that it cross-hybridizes with mouse 18S ribosomal RNA, which in our analysis serves as an internal control for the amount of RNA loaded per lane (this is seen as a dark band in all lanes of Fig. 6). In this experiment, we transfected 10 μg of each of the IAP cDNA–CAT constructs, the two standard promoters and two control plasmids into PCC3 cells and, 48 h later, total RNA was isolated and subjected to Northern blot analysis (the two control plasmids, EC24 and EC32, were constructs in which IAP-coding regions, not U3, were placed adjacent to the CAT gene). Data in Fig. 6 clearly demonstrate that while the three IAP–CAT mRNAs (corresponding to clones EC7, EC23 and EC26) were readily detected in this blot, we were unable to detect CAT RNA in cells transfected with either pRSVcat or pSV2cat. CAT RNAs produced by pRSVcat and pSV2cat can be visualized if more DNA (20 μg) is used to transfect PCC3 cells (data not shown). Although no CAT RNA was observed in either of the two control lanes, a very small amount of CAT enzyme activity (>1 % conversion) was observed in the pEC32cat lane (Fig. 6, lane 7, top), perhaps due to a small amount of transcription induced by cryptic promoter elements.

Discussion

Activation of zygotic gene expression in the early mouse embryo occurs at the 2-cell stage and steadily increases during development (Piko & Clegg, 1982). Little is known about which genes are activated at this stage of development and nothing is known about the mechanisms that promote their induction. One of the few genes whose expression has been studied in detail in the early embryo is the IAP gene family (Huang &

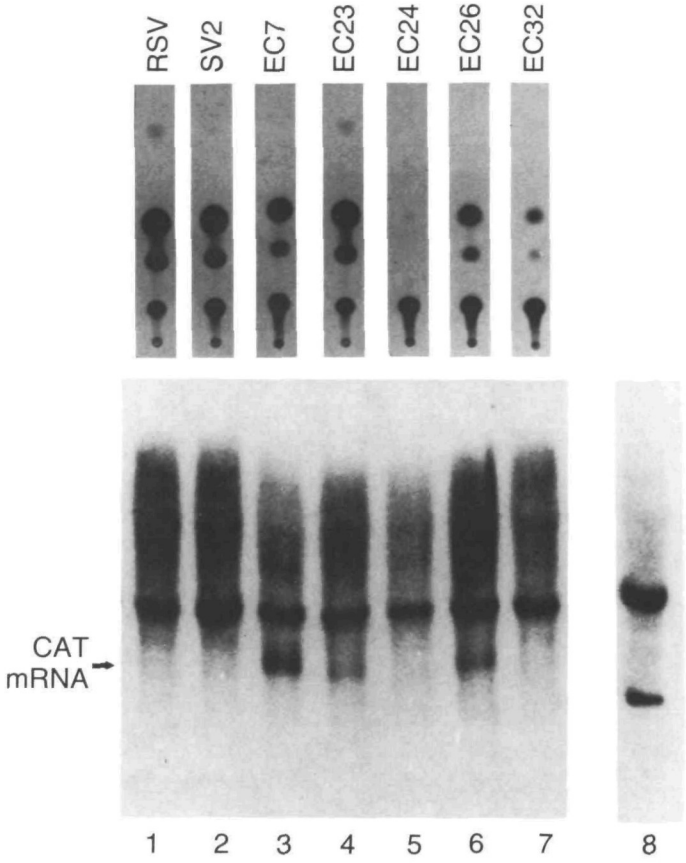

Fig. 6. CAT Northern blot analysis. 10 μg of each of the indicated CAT vectors (1, pRSVcat; 2, pSV2cat; 3, pEC7cat; 4, pEC23cat; 6, pEC26cat) were transfected into PCC3 cells (4×10^6 cells) along with two control genes (5, pEC24cat; 7, pEC32cat) and 60 h later cells were harvested and the bulk of the cell pellet used to isolate RNA. The isolated RNA (approx. 40 μg) was then subject to electrophoresis on a formaldehyde agarose gel followed by transfer to a nitrocellulose membrane and hybridized with a CAT riboprobe as described in methods. A small amount of each initial cell pellet (approx. 5 %) was used for the CAT enzyme analysis shown on top of the figure. As a positive control for the Northern blot (lane 8), 100 pg of a 780 bp CAT DNA fragment was added to RNA from mock-transfected cells. The arrow points to the location of the CAT mRNA. The band above the CAT mRNA in each lane is due to cross-reactivity of the CAT riboprobe with mouse 18S rRNA which serves as a internal standard for the amount of RNA loaded per lane.

Calarco, 1981*a*,*b*; Moshier *et al.* 1985; Piko *et al.* 1984; Yotsuyanagi & Szollosi, 1981). Using the pluripotent EC cell line PCC3 as a model for the early embryo, it has previously been demonstrated that endogenous IAP genes are actively transcribed in undifferentiated EC cells and that differentiation leads to a decrease in IAP transcription (Hojman-Montes de Oca *et al.* 1983; Morgan & Huang, 1987; Moshier *et al.* 1985).

We describe here the first report of the analysis of IAP promoter function using IAP cDNA clones which, by definition, originate from actively transcribed IAP proviruses. Previous reports of IAP promoter function used promoter elements from random IAP genomic clones (not all IAP genomic clones contain active LTRs, R.J.C. & R.C.C.H., unpublished data), or from IAP genes whose activity was implied from a transposition event (Horowitz *et al.* 1984; Lueders *et al.* 1984). IAP genes were cloned into a vector such that their U3 promoter regions could promote the synthesis of a linked CAT gene. All the IAP promoters tested were able to produce CAT enzyme activity and to accumulate substantial amounts of CAT RNA in the EC cell line PCC3 (Figs 5, 6). This result is in direct contrast to the near total lack of promoter function of murine C type retroviruses (Mo-MuLV) in EC cells (Gorman *et al.* 1985; Linney *et al.* 1984). These data also imply that the individual IAP proviruses, from which these clones derived, contained active promoter elements and argues against the possibility that transcription of IAP genes in this cell line resulted from promotion by upstream cellular sequences (e.g. Barklis *et al.* 1986).

There have been two reports demonstrating that the restriction of C type retroviral gene expression in EC cells can be overcome (Barklis *et al.* 1986; Seliger *et al.* 1986). Although these host range mutants are clearly functional in EC cells, they are still two to three orders of magnitude less active in EC cells than they are in differentiated cells such as 3T3 cells (Weiher *et al.* 1987). Our data indicate that IAP promoters mediate high levels of gene expression (relative to the two strong promoter elements of RSV and SV40) in EC cells and it has been shown previously that endogenous IAP gene are active in a wide variety of differentiated cell types including fibroblast and plasmacytoma cell lines and such adult mouse tissues as the thymus and pancreas (Kuff & Fewell, 1985; Leiter & Kuff, 1986; Wujcik *et al.* 1984).

DNA sequence analysis of the IAP U3 region revealed it to be composed of multiple sequence motifs which are similar to known promoter/enhancer elements (Fig. 3). The three new elements reported here are a potential binding site for the Sp1 transcription factor, an adenovirus E1a core enhancer sequence and a region of homology with one of the adenovirus E4 promoter regions. It is unlikely that all eight of these U3 sequence elements are simultaneously binding transcription factors (this would be particularly difficult for the CAAT box and E1a enhancer sequences, which directly abut each other). It is a possibility that the complexity of the IAP promoter permits it to function in a diverse set of tissues and at varying developmental stages. Preliminary data from deletion mutagenesis experiments indicate that the first 150 bp of the U3 region are essential for maintaining a high level of promoter

function in EC cells (R.J.C. & R.C.C.H., unpublished data).

In EC cells, the high levels of IAP-promoted gene expression may not only be a result of a high level of transcriptional activity, but may involve an enhancement of RNA stability. Fig. 6 demonstrates that substantial amounts of IAP–CAT RNA accumulate in transfected PCC3 cells, while little RSV- or SV40-promoted CAT RNA is detectable. It is possible that the presence of the IAP R region, as a leader to the CAT RNA, may increase CAT RNA stability in PCC3 cells. Similar mechanisms involving an increase in gene expression by the enhanced stability of retroviral transcripts has been suggested for the B2 host range mutant of Mo-MuLV and for the human immunodeficiency virus (Cullen, 1986; Rosen *et al.* 1986; Weiher *et al.* 1987). The ability of IAP promoters to express efficiently genes in undifferentiated EC cells and their natural occurrence in mouse embryos suggest the possibility that all or part of these genes may be adapted to generate vectors which, unlike the current Mo-MuLV vectors, could transcribe novel genes in the preimplantation mouse embryo.

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