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Mechanism of activation of the mouse c-*mos* oncogene by the LTR of an intracisternal A-particle gene

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In the mouse myeloma XRPC-24 the DNA of an intracisternal A-particle (IAP) is inserted within the coding region of c-*mos*. This insertion splits the c-*mos* into a 3′ rc-*mos* and a 5′ rc-*mos* separated by ~4.7 kb of IAP DNA. The insertion is in a head-to-head orientation and brings the 5′ LTR of the IAP in juxtaposition to the 3′ rc-*mos* such that the IAP and the 3′ rc-*mos* are transcribed in opposite directions. The intact c-*mos* gene is usually dormant, whereas the 3′ rc-*mos* is actively transcribed and is capable of transforming NIH3T3 cells. In an effort to understand the nature of this activation we mapped the 5′ ends of the 3′ rc-*mos* mRNA present in XPRC-24. We found two main mRNA start sites, one mapping to the junction of the 3′ rc-*mos* and the 5′ LTR, and the other located 10 nucleotides upstream to this junction, within the 5′ LTR. This result indicates that the 3′ rc-*mos* in XRPC-24 was activated by insertion of a promoter provided by the LTR of an IAP genome. Furthermore, the 5′ LTR appears to possess promoter activities in two directions. This conclusion was confirmed by the fact that this 5′ LTR, in both orientations, was able to activate the bacterial gene coding for chloramphenicol acetyltransferase (CAT) in the modular vector pSVOCAT.

Key words: c-*mos* oncogene/activation/long terminal repeat/intracisternal A-particle

Introduction

Accumulated data suggest that genetic changes in cellular oncogenes cause malignant transformation of cells. Various transforming oncogenes of the *ras* family differ by a single point mutation from their normal counterpart (Tabin *et al.*, 1982; Reddy *et al.*, 1982; Capon *et al.*, 1983). In Burkitt's lymphoma lines and mouse plasmacytomas c-*myc* has been translocated into one of the immunoglobulin loci (Shen-Ong *et al.*, 1982; Erikson *et al.*, 1983), resulting in the activation of the c-*myc* either *via* transcriptional activation or by a loss of attenuation at the translational level (Saito *et al.*, 1983; Perry, 1983). Another DNA rearrangement has been shown recently to involve the insertion of an endogenous retrovirus-like element, an intracisternal A-particle (IAP), within the c-*mos* oncogene in the myeloma XRPC-24 and NS-1 (Rechavi *et al.*, 1982; Kuff *et al.*, 1983; Cohen *et al.*, 1983; Gattoni-Celli *et al.*, 1983). In XRPC-24 the insertion was in a head-to-head orientation and split the c-*mos* into the 5′ rc-*mos* and 3′ rc-*mos* (Canaani *et al.*, 1984). The c-*mos* is usually dormant in all tested cells (Muller *et al.*, 1982), whereas the 3′ rc-*mos* is actively transcribed into 1.2-kb mRNA and is able to transform NIH3T3 cells (Rechavi *et al.*, 1982).

The activation of the c-*mos* could be due to the removal of inhibitory sequences upstream to the 5′ end of the gene or to the insertion of an element that either enhances transcription from a pre-existing promoter within the c-*mos* sequences or provides a new promoter. To resolve this question we mapped the ends of the 3′ rc-*mos* mRNA in the myeloma XRPC-24 by the S1 mapping technique (Berk and Sharp, 1978). We show that one major 5′ terminus maps at the junction of the 3′ rc-*mos* and the 5′ LTR and the other one within the 5′ LTR implying that the promoter resides within the 5′ LTR. Therefore the 5′ LTR of the inserted IAP contains promoters in two directions: the normal one, transcribing the IAP gene and a cryptic promoter from which transcription of the 3′ rc-*mos* initiates. To prove this notion unequivocally we coupled the 5′ LTR, in both orientations [head-to-head (as in the 3′ rc-*mos*) or tail-to-head (as in IAP)] to the bacterial gene coding for chloramphenicol acetyltransferase (CAT). We show that this element, in both orientations, promotes transcription of CAT mRNA, the tail-to-head orientation being more efficient. These results explain the mechanism whereby the mouse c-*mos* gene in XRPC-24 became activated by a head-to-head insertion of an IAP.

Results

Subcloning the 3′ rc-mos

The structure of the c-*mos* and the rc-*mos* genes is shown in Figure 1. The large *Eco*RI fragment (~14 kb) contains the activated 3′ rc-*mos* which is transcribed into a 1.2-kb mRNA and can transform NIH3T3 cells (Rechavi *et al.*, 1982). According to published results the 3′ end of the c-*mos* mRNA should map downstream to the *Hin*dIII site shown in Figure 1, at the 3′ end of the c-*mos* coding sequences (Van Beveren *et al.*, 1981). Therefore we decided to subclone the *Eco*RI-*Xba*I fragment harboring this *Hin*dIII site from the phage containing the 3′ rc-*mos*. We used a derivative of pBR322, designated pCh108b (Givol *et al.*, 1981) that contains a unique *Xba*I site. The *Eco*RI-*Xba*I fragment containing the 3′ rc-*mos* was cloned between the *Eco*RI and *Xba*I sites of pCh108b to yield the plasmid designated prcmos.

Characterization of the 3′ rc-mos mRNA

To determine the 5′ and 3′ ends of the 3′ rc-*mos* mRNA in the myeloma XRPC-24 we used the Weissman and Weaver modification of the Berk and Sharp S1 mapping technique (Berk and Sharp, 1978; Weaver and Weissman, 1979). The 5′ end-labeled probe was prepared by labeling prcmos with T4 polynucleotide kinase at the *Ban*I site [*Ban*I cleaves within the 3′ rc-*mos* sequences, at the *Kpn*I site (see Figure 1)], cleaving with *Eco*RI and isolating a 800-bp *Ban*I-*Eco*RI fragment. This end-labeled probe was hybridized to poly(A)-containing RNA extracted from XRPC-24 tumor and treated with S1 nuclease. The S1 nuclease protected fragments were resolved on a 6% acrylamide urea gel (Figure 2, panel B). Two major fragments, one 289–291 and the other 300–301 bp long,

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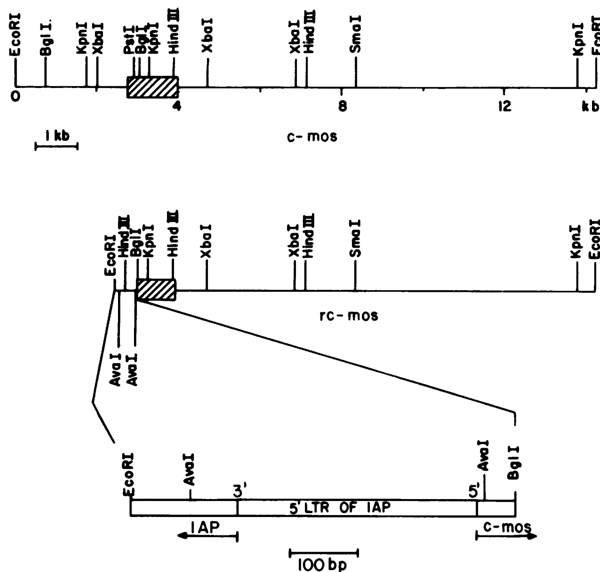

Fig. 1. Structure of the mouse c-*mos* and rc-*mos* genes isolated from the plasmacytoma XRPC-24. Isolation and characterization of the genes is described elsewhere (Rechavi *et al.*, 1982). The arrows indicate the direction of transcription.

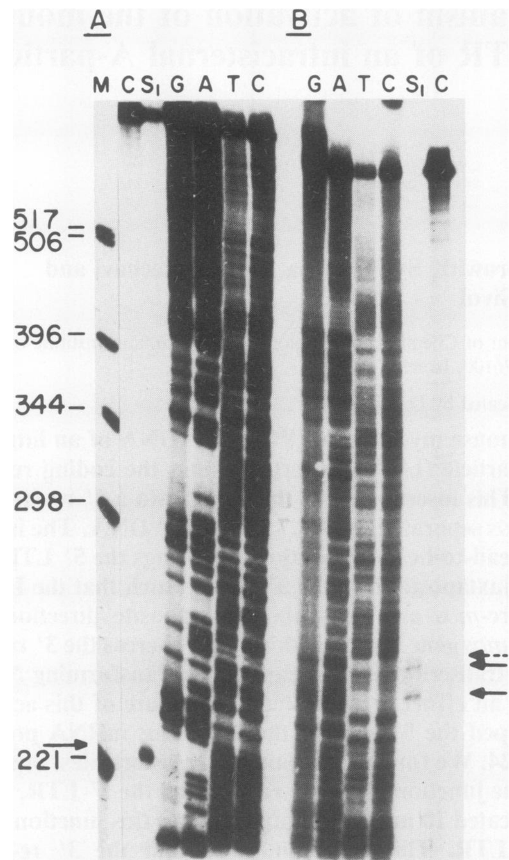

Fig. 2. S1 mapping of the 3′ rc-*mos* mRNA from the myeloma XRPC-24. Poly(A)-containing RNA was extracted from the tumor XRPC-24, as described in Materials and methods. 0.5 μg of 3′ or 5′ end-labeled probe was hybridized to 30 μg of poly(A)-containing RNA at 50°C for 16 h. Digestion with S1 was for 30 min at 45°C. The hybrids were extracted with phenol chloroform, precipitated with ethanol and resolved on a 6% acrylamide-8 M urea gel. Electrophoresis was for 8 h at 30 mA. The 3′ and 5′ end-labeled probes were sequenced according to Maxam and Gilbert (1980) and the reactions were run in parallel. **Panel A**: 3′ end-labeled probe. **Panel B**: 5′ end-labeled probe. M = markers. C = end-labeled probes. G,A,T,C = sequencing reactions of the corresponding probes.

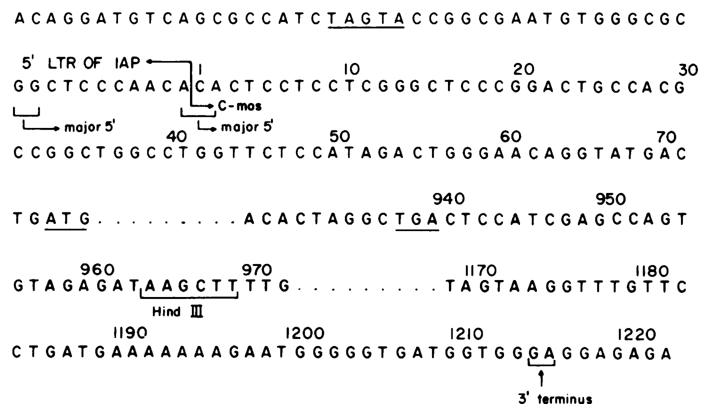

Fig. 3. Sequences around the 5′ and 3′ termini of the c-*mos* RNA in the myeloma XRPC-24. Sequences were obtained in the course of characterization of the 3′ rc-*mos* (G. Rechavi and D. Givol, unpublished results) and compared with the sequences obtained in the course of this work. The putative 'TATA' box, the putative initiation codon and the termination codon are underlined.

were protected from S1 nuclease. Hence, the two main start sites for the 3′ rc-*mos* mRNA map 289–291 and 300–301 nucleotides upstream from the *Ban*I site. The location of these start sites is at the junction of the 3′ rc-*mos* and the 5′ LTR and 10 nucleotides upstream to this junction, respectively (Figure 3). The 3′ end-labeled probe was prepared by labeling prcmos with avian myeloblastosis virus (AMV) reverse transcriptase at the *Hind*III site [*Hind*III cleaves within the 3′ rc-*mos* sequences and there is another *Hind*III site within the 5′ LTR sequences (see Figure 1)], cleaving with *Xba*I and isoalting the 900-bp *Hind*III-*Xba*I fragment. The results of hybridization of poly(A)-containing XRPC-24 RNA to the 3′ end-labeled probe are shown in Figure 2, panel A. The 3′ end-labeled probe protected a 247–248 bp fragment (Figure 2, panel A:S1). This result means that the 3′ end of the 3′ rc-*mos* mRNA in the myeloma XRPC-24 maps 247–248 nucleotides downstream to the end-labeled *Hind*III site. The location of the 3′ end on the sequence is shown in Figure 3. We could not find any consensus polyadenylation signal 19–30 nucleotides upstream to the 3′ end of the mRNA. There is an AAAAAAAGAA sequence 16 nucleotides upstream to the 3′ end.

The results presented here suggest that the 5′ LTR has promoter activities in two directions: one transcribing the IAP sequences and the other, transcribing the 3′ rc-*mos* sequences in XRPC-24. Both promoters are probably potentiated by the same enhancer element (see Discussion).

Activation of the CAT gene by the promoters of the 5′ LTR

To ascertain that the 5′ LTR of the inserted IAP has promoter activity on both strands we coupled the 5′ LTR, in both orientations, to the bacterial gene coding for CAT. This gene catalyses the conversion of chloramphenicol to acetyl forms in the presence of S-acetyl CoA (Cohen *et al.*, 1980).

The 5′ LTR of the inserted IAP is contained within a single *Ava*I fragment of 430 bp (Figure 1). This fragment has been isolated and ligated with the *Hind*III-cleaved plasmid pSVOCAT (Gorman *et al.*, 1982b), in two orientations, as shown in Figure 4. The orientation of the *Ava*I fragment with respect to the CAT gene was determined by cleavage of the plasmids with *Hind*III and *Nco*I and sequencing the appropriate fragments (results not shown). In the plasmid designated prcmCAT-1 the 5′ LTR and the CAT gene are

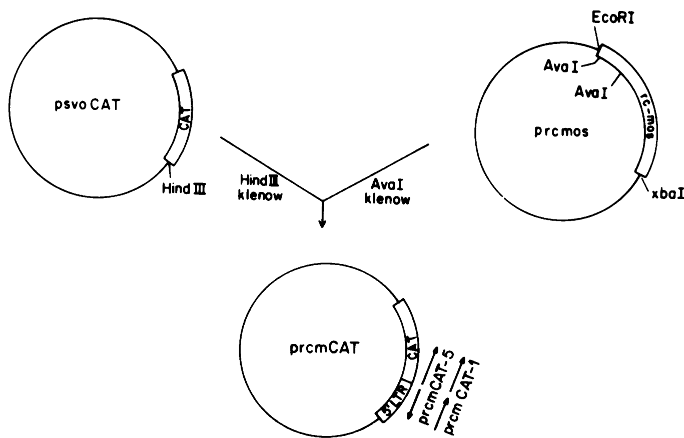

Fig. 4. Construction of plasmids containing the 5′ LTR of an IAP coupled to the bacterial CAT gene. DNA of plasmid prcmos that contained the *Eco*RI-*Xba*I fragment of 3′ rc-*mos* (including the 5′ LTR of the inserted IAP) was cleaved with *Ava*I and treated with the Klenow fragment of *Escherichia coli* DNA polymerase I to flush the ends. The 430-bp *Ava*I fragment was recovered from an agarose gel, using glass beads (Vogelstein and Gillespie, 1979). This fragment was ligated to pSVOCAT that had been linearized with *Hind*III and treated with the Klenow fragment of *E. coli* polymerase I to flush the ends. The ligation was at 16°C, overnight, in a total volume of 15 μl, in the presence of 10 mM $MgCl_2$, 10 mM Tris pH 7.8, 10 mM ATP and 100 units of T4 DNA ligase (Bio Labs). Bacteria transformed with the ligated DNA were screened with the nick-translated 430-bp *Ava*I fragment as a probe. To determine the orientation of the inserts, DNA from positive colonies was treated with *Hind*III and *Nco*I, that cleave in the 5′ LTR and the CAT gene sequences, respectively. The orientation was confirmed by sequencing.

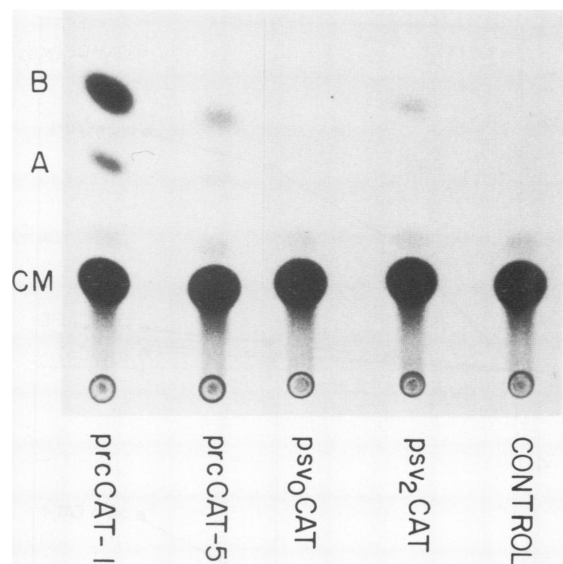

Fig. 5. Expression of CAT in NIH3T3 cells assayed by t.l.c. 10 cm dishes containing 10^6 cells/dish were transfected with 20 μg DNA as explained in Materials and methods. The cell extracts were prepared in 100 μl of 0.25 M Tris pH 7.8. 30 μl of extracts were used for CAT assay, in the presence of 1.5 μl of [^{14}C]chloramphenicol (Amersham) and 4 mM acetyl CoA in 0.25 Tris pH 7.8. The acetylated forms were separated from the non-acetylated form by t.l.c. in chloroform/methanol (95:5 v/v). **CM:** cloramphenicol, **A:** 1-acetylchloramphenicol, **B:** 3-acetyl chloramphenicol. Control: mock transfected NIH3T3 cells.

joined tail-to-head (as in IAP) and have the same transcriptional orientation while in prcmCAT-5 the joining is head-to-head (as in rc-*mos*) and the 5′ LTR and the CAT gene have opposite transcriptional orientations.

To test the ability of the 5′ LTR to activate the modular gene we assayed for CAT activity. NIH3T3 cells were transfected with prcmCAT-1, prcmCAT-5, pSV2CAT (Gorman *et al.*, 1982b) and pSVOCAT by a modification of the calcium-phosphate precipitation technique. Forty hours later cells were harvested and the CAT activity in lysates obtained from equal numbers of cells was assayed as explained in Materials and methods. In pSV2CAT the SV40 promoter element is coupled to the CAT gene while pSVOCAT does not contain any promoter. As shown in Figure 5, the level of conversion of [^{14}C]chloramphenicol (CM) to the acetylated forms (A,B) was much higher with prcmCAT-1 than that seen with either prcmCAT-5 or pSV2CAT. The activity of the 5′ LTR as a promoter in the head-to-head orientation (prcmCAT-5), though low, is significant in comparison with the negative control (pSVOCAT). The observation that the 5′ LTR in a head-to-head orientation could activate a modular gene can explain why insertion of an IAP, a possible endogenous movable element in the mouse genome, could activate the c-*mos* gene that is otherwise dormant.

Are the 5′ LTR promoters host specific?

Many genes contain, upstream to the mRNA start site, enhancer sequences (Khoury and Gruss, 1983), that augment transcription of RNA from the promoter. Laimins *et al.* (1982) showed that the MSV and SV40 enhancers are host specific. The transcriptional activity induced by the MSV enhancer from the MSV promoter is higher in mouse L cells in comparison with monkey cells, while the SV40 enhancer augments transcription from the SV40 promoter much better in monkey cells than in mouse cells.

To test for host specificity of the promoters of the 5′ LTR we transfected monkey cells (CV-1) and mouse cells (NIH3T3) with prcmCAT-1, prcmCAT-5, pSV2CAT and pSVOCAT. The results of such an experiment are shown in Figure 6 and Table I. The 5′ LTR promoter, in the tail-to-head orientation directed the synthesis of comparable amounts of CAT in either NIH3T3 or CV-1 cells. In NIH3T3 cells transfected with prcmCAT-5, the CAT activity was slightly higher (x 1.8 − 3.4) compared with that found in extracts of transfected CV-1 cells. The SV40 early promoter-enhancer element worked in CV-1 cells better than in NIH3T3 cells though the difference is not remarkable (x 5, for comparison see Gorman *et al.*, 1982a). We therefore conclude that there is no differential host-specific expression of prcmCAT-1 and prcmCAT-5 in the two lines that were tested.

Discussion

The c-*mos* gene is an intronless gene whose transcripts have not been detected in any mouse tissue tested in either adult animal or embryo (Muller *et al.*, 1982). Nevertheless, the c-*mos* sequence is well conserved between human and mouse (Watson *et al.*, 1982) suggesting that it has some yet unknown role. In the myeloma XRPC-24 the gene became activated *via* insertion of the genome of an IAP within the coding region of c-*mos*. This activation could be due to removal of inhibitory sequences or insertion of an element from which RNA transcription could initiate. We mapped the 5′ ends of the mRNA in XRPC-24 and found two main start sites: one at the junction of the 5′ LTR and the 3′ rc-*mos* sequences and the other 10 nucleotides upstream to the junction. The results imply that promotion takes place from a sequence residing

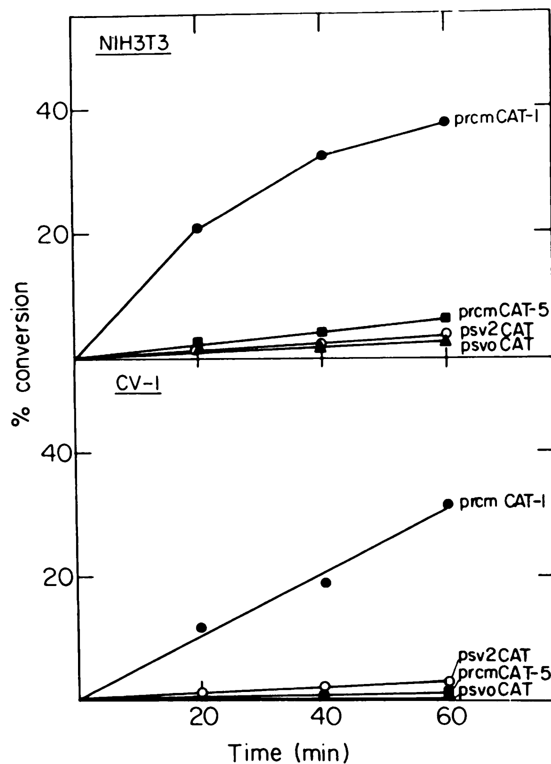

Fig. 6. Expression of CAT in NIH3T3 and CV-1 cells assayed by t.l.c. 10^6 CV-1 or NIH3T3 cells were transfected with 20 μg of the appropriate DNA. 40 h later extracts were prepared in 100 μl of 0.25 M Tris pH 7.8. Extracts containing either 100 μg protein (NIH3T3) or 150 μg protein (CV-1) were assayed for CAT activity at 20, 40 and 60 min of incubation. The percentage of [^{14}C]chloramphenicol converted to acetylated forms was determined by cutting spots from the plates and measuring the radioactivity in a liquid scintillation counter using toluene based scintillation fluid. The percentage of conversion to acetylated forms was normalized to 150 μg protein. **(A)** NIH3T3 cells, **(B)** CV-1 cells. pSVOCAT in this experiment is the plasmid designated pA10CAT2 by Laimins *et al.* (1984).

within the 5′ LTR. The sequence upstream to the mRNA start site does not contain a consensus 'TATA' but rather a TAGTA sequence (Figure 3). The 3′ terminus of the c-*mos* mRNA in XRPC-24 maps ~280 nucleotides downstream to the termination codon of the c-*mos* (Van Beveren *et al.*, 1981). There is no obvious consensus polyadenylation signal which is another puzzling characteristic of this gene. According to the above results the 5′ LTR of the inserted IAP is unique in the sense that it has a promoter activity in two directions. The normal activity is the one directing transcription of the IAP message while the other promotor resides on the other strand and directs transcription of the 3′ rc-*mos* in the myeloma XRPC-24. To obtain further evidence for this notion we coupled the 5′ LTR, in both orientations, to the modular vector pSVOCAT containing the gene for the bacterial enzyme CAT. The enzymatic assay is very sensitive, allowing detection of weak promoters as there is no background activity of CAT in the transfected cells. The strength of any promoter coupled to the CAT gene can be quantitatively assayed by the levels of [^{14}C]chloramphenicol converted to the acetylated forms in extracts of transfected cells. The results showed that the 5′ LTR in both orientations directed synthesis of active CAT. The promoter in the tail-to-head orientation (the one active in IAP) was 15- to 30-fold stronger than the one responsible for activation of the 3′ rc-*mos* in XRPC-24 (Table I). This 5′ LTR thus contains at least three elements: an enhancer like those found in LTRs of retroviruses and other retrovirus-like elements (Groner and Hynes, 1982), and two promoters for RNA polymerase II, in two directions. The 5′ LTR also contains a Z-DNA-like structure that has been correlated with enhancer regions (Nordheim and Rich, 1983). Though low, the activity of the 5′ LTR in the head-to-head orientation is significant. Since no more than 10 molecules of mos-specific mRNA are present in MSV-transformed cells (G. Vande Woude, personal communication), such a promoter activity could be sufficient to activate the c-*mos in vivo*, in the myeloma XRPC-24. It has already been shown (Hayward *et al.*, 1981; Payne *et al.*,

Table I. Levels of CAT directed by different plasmids

Plasmid \ cell	Time (min)	% conversion of [^{14}C]chloramphenicol to acetylated forms				
		NIH3T3	CV-1	NIH3T3 (−pSVOCAT)	CV-1 (pSVOCAT)	NIH3T3 / CV-1
prcm CAT-1	20	20.7	11.7	18.7	11.55	1.6
	40	32.9	18.9	30.8	18.73	1.6
	60	37.8	31.7	34.7	31.42	1.1
prcm CAT-5	20	0.9	0.5	0.9	0.35	1.8
	40	3.9	0.7	1.8	0.53	2.6
	60	6.2	0.9	3.1	0.69	3.4
pSV2CAT	20	2.4	1.3	0.4	1.15	0.3
	40	2.7	2.7	0.6	2.53	0.2
	60	3.7	2.9	0.6	2.62	0.2
pSVOCAT	20	2.0	0.15	0	0	–
	40	2.1	0.17	0	0	–
	60	3.1	0.28	0	0	–

Experimental details are described in the legend to Figure 6. The values obtained for pSVOCAT in each cell line were subtracted and the ratio of activity in NIH3T3 cells *versus* CV-1 cells was calculated for each time point.

1982) that in some of the bursal lymphomas that contain proviral avian leukosis virus (ALV) upstream to c-*myc*, transcription of c-*myc* initiates within the proviral LTR positioned upstream from the c-*myc* in the same transcriptional orientation.

In short, the results presented here show that activation of the 3′ rc-*mos* results from insertion of an IAP such that its 5′ LTR serves as an enhancer-promoter, from which transcription initiates and proceeds along the 3′ rc-*mos* sequences.

Materials and methods

Cells

CV-1 and NIH3T3 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% calf serum (Gibco). The cells were grown at 37°C with 5% CO_2.

RNA extraction

10–20 g of the tumor were homogenized in a Waring blender, in a cold urea-LiCl solution (3 M LiCl, 7 M urea). After being kept at 4°C overnight, the homogenate was precipitated in a Sorval centrifuge. The supernatant underwent phenol-chloroform extractions and ethanol precipitation. Selection of poly(A)-containing RNA was performed according to the method of Aviv and Leder (1972). Pellets were resuspended in 0.5 M NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 0.1% SDS and loaded onto an oligo(dT)-cellulose column. After several washes, the poly(A)-containing RNA was eluted with 10 mM Tris pH 7.5, 1 mM EDTA, 0.1% SDS and precipitated in ethanol. The pellets were washed twice with 0.2 M NaCl, 80% ethanol and once with 80% ethanol.

DNA probes

3′ end-labeled probe. 5–10 μg of *Hind*III-cut prcmos DNA was incubated with reverse transcriptase (Beard, Life Sciences) in the presence of 50 mM NaCl, 10 mM Tris pH 7.5, 5 mM $MgCl_2$, 5 mM dithiothreitol and 100 μCi of [^{32}P]dATP (Amersham, 3000 Ci/mmol) for 1 h at 37°C. Following heat inactivation of the enzyme the plasmid was cleaved with *Xba*I and the 900-bp *Hind*III-*Xba*I fragment was isolated from a 5% polyacrylamide gel by electroelution and ethanol precipitated.

5′ end-labeled probe. 5–10 μg of *Ban*I-cut prcmos DNA was treated with calf intestine alkaline phosphatase (Boeringer-Mannheim) in the presence of 10 mM Tris pH 8.0, 10 mM $MgCl_2$ for 90 min at 37°C. After phenol-chloroform extraction the DNA was ethanol precipitated. The resuspended DNA was labeled at its 5′ ends with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (Amersham, >3000 Ci/mmol) for 1 h, at 37°C. Following cleavage with *Eco*RI, the 800-bp *Eco*RI-*Ban*I fragment was isolated from a 5% polyacrylamide gel by electroelution.

S1 analysis

30 μg poly(A)-selected RNA was hybridized with 0.5 μg of DNA at the appropriate temperature in 20 μl of 80% formamide, 0.4 M NaCl, 0.04 M Pipes pH 6.5, and 1 mM EDTA. At the end of the incubation, 180 μl of S1 buffer was added (0.03 M sodium acetate, pH 4.5, 0.25 M NaCl, 1 mM $ZnSO_4$) with 200 units of S1 endonuclease (Miles). After incubation at 45°C for 30 min, 20 μg tRNA was added followed by phenol-chloroform extractions and the hybrids were precipitated with ethanol and washed with 80% ethanol.

DNA sequencing

The DNA probes were sequenced according to Maxam and Gilbert (1980) and electrophoresed through a 6% acrylamide-8 M urea at 30 mA.

Transfection

Transfection was performed by a modification of the Graham and Eb (1973) calcium-phosphate precipitation technique. 20 μg of DNA were mixed with 0.5 ml of 2 x HeBs (10 g Hepes, 16 g NaCl, 0.74 g KCl, 0.25 g Na_2HPO_4 $2H_2O$, 2 g dextrose/l). An equal volume of 0.4 M $CaCl_2$ in 10 mM Tris pH 7.6, 1 mM EDTA was added. The mixture was applied to $0.5-2 \times 10^6$ cells. After 4 h they were subjected to a 2 min glycerol shock (10% glycerol in DMEM supplemented with 10% calf serum).

CAT assay

40 h after transfection cultures were washed twice with phosphate-buffered saline (PBS). The cells were removed from the dish in 1 ml of 0.15 M NaCl, 10 mM Tris pH 7.5, 1.5 mM $MgCl_2$, resuspended in 100 μl of 0.25 M Tris pH 7.8 and sonicated. Cell debris was removed by centrifugation in a microfuge. The protein concentration in the various cell extracts was determined according to Bradford (1976). Amounts of cell extracts corresponding to 100–150 μg of protein were incubated with [^{14}C]chloramphenicol (New England Nuclear) in the presence of 4 mM acetyl CoA at 37°C in a total volume of 140 μl for various periods of time (20–60 min). The reaction was stopped by addition of ethyl acetate to extract the chloramphenicol and its acetylated derivatives. The ethyl acetate was dried and the residue was resuspended in 30 μl ethyl acetate, spotted onto a t.l.c. plate (Merck) and chromatographed in chloroform-methanol (95:5). The plate was dried and exposed to an Agfa film (curix RP2). The percentage of conversion was determined by cutting the appropriate spots from the plate and counting them in a toluene based scintillation fluid.

Restriction endonucleases

Restriction endonucleases were purchased from New England Biolabs unless otherwise indicated. Conditions were according to the supplier's recommendations.

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