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The Long Terminal Repeat of the Intracisternal A Particle as a Target for Transactivation by Oncogene Products

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It has been shown recently that the c-*mos* oncogene becomes activated in myeloma XRPC-24 via insertion of an intracisternal A particle (IAP) long terminal repeat (LTR). The inserted LTR serves as a promoter from which transcription of the 3′ rearranged c-*mos* initiates. The insertion is in a head-to-head orientation such that the transcriptional orientations of the IAP and the 3′ rearranged c-*mos* are opposite. It has already been shown that this IAP LTR has two promoters, one transcribing the IAP genome and the other transcribing the rearranged c-*mos*. Since the IAP genomes are actively transcribed in mouse myelomas but not in normal cells, it was interesting to test whether transcriptional activation of the IAP occurs in the presence of active oncogene products, especially nuclear ones. The 5′ LTR of the IAP inserted in myeloma XRPC-24 was chosen as a convenient model to test the effect of viral and cellular oncogene products. These included simian virus 40 (SV40) large-T antigen, the adenovirus early 1A (E1A) gene product, the *myc* gene product, and p53. The LTR was coupled to the bacterial gene coding for chloramphenicol acetyltransferase (CAT) in two orientations, and the levels of CAT directed by the LTR promoters were assayed in either the presence or the absence of the oncogene products. The levels of CAT directed by the 5′ LTR promoter transcribing the IAP were significantly elevated in the presence of SV40 large-T antigen, the adenovirus E1A and *myc* gene products, and p53. The promoter transcribing the rearranged c-*mos* was transactivated by SV40 large-T antigen and the adenovirus E1A gene product. The results indicate that oncogene products may have an important role in turning on promoters of other genes. The IAP LTR may serve as a useful model for studying the effect of various gene products on promoters which are known to be activated in the malignant state.

It has recently been shown that transformation of embryonic cells is achieved when they are cotransfected with two oncogenes or, alternatively, immortalized with carcinogens and then transfected with an oncogene that is capable of transforming NIH 3T3 cells (21, 25, 28). The oncogenes effective in this system could be divided into two groups, one consisting of oncogenes specifying nuclear proteins (polyomavirus and simian virus 40 [SV40] large-T antigens, the adenovirus early 1A [E1A] gene product, v-*myc*, and c-*myc*) and the other consisting of oncogenes specifying cytoplasmic proteins (polyomavirus middle-T antigen and mutated H-*ras*). It was recently shown that p53 can also complement activated H-*ras* in transforming rat embryo fibroblasts into tumorigenic cells (8, 26). However, Spandidos and Wilkie reported on the successful transformation of rat embryo fibroblasts with the mutated H-ras alone linked to transcriptional enhancers (30).

Different studies have shown that products of the so-called nuclear oncogenes enhance the transcription of other promoters. A protein encoded in the E1A region of adenovirus DNA (289 amino acids long) enhances the transcription of other adenovirus early genes (3, 18, 23, 32), the HeLa cell 70-kilodalton heat shock protein promoter (24), the SV40 early promoter (31), the β-globin promoter (15), and the preproinsulin promoter (10). *myc* enhances transcription of the adenovirus E2 promoter (19). Therefore, we decided to analyze the effects of the SV40 large-T antigen, the adenovirus E1A and *myc* gene products, and p53 on the promoter activity of a long terminal repeat (LTR) of an intracisternal A particle (IAP).

It has already been shown that c-*mos* became activated in myeloma XRPC-24 via insertion of an IAP genome within its coding sequences. The IAP split the gene into 5′ rearranged c-*mos* (rc-*mos*) and 3′ rc-*mos* (6, 20, 27). The insertion was in a head-to-head orientation, such that transcription of the IAP and the 3′ rc-*mos* is in opposite directions. The activation of the 3′ rc-*mos* is due to insertion of the 5′ LTR of the IAP in juxtaposition to the 3′ rc-*mos*, such that the transcription of the 3′ rc-*mos* initiates within the 5′ LTR and proceeds throughout the 3′ rc-*mos* (17). The 5′ LTR was shown to have promoter activities in both directions: the normal IAP promoter and a promoter from which transcription of the 3′ rc-*mos* was initiated. Since IAP genomes are actively transcribed in mouse myelomas but not in normal cells (22), it was interesting to test whether products of oncogenes that specify nuclear proteins would have any effect on the transcriptional activity of the IAP LTR. This LTR was coupled to the bacterial gene coding for chloramphenicol acetyltransferase (CAT) in two opposite orientations, and the levels of CAT directed by the promoters of the LTR were assayed in either the absence or the presence of oncogene products. We found that the products of the SV40 large-T antigen, the adenovirus E1A and *myc* gene products, and p53 enhance the activity of the 5′ LTR promoter transcribing the IAP. SV40 large-T antigen and the adenovirus E1A gene product enhance the activity of the promoter transcribing the 3′ rc-*mos*.

MATERIALS AND METHODS

Cells. CV-1 cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% calf serum (GIBCO Laboratories) at 37°C with 5% CO_2. HeLa, 293, COS-M6, Rat-1, and IIB4 cells were grown in DMEM supplemented with 10% fetal calf serum at 37°C with 5% CO_2.

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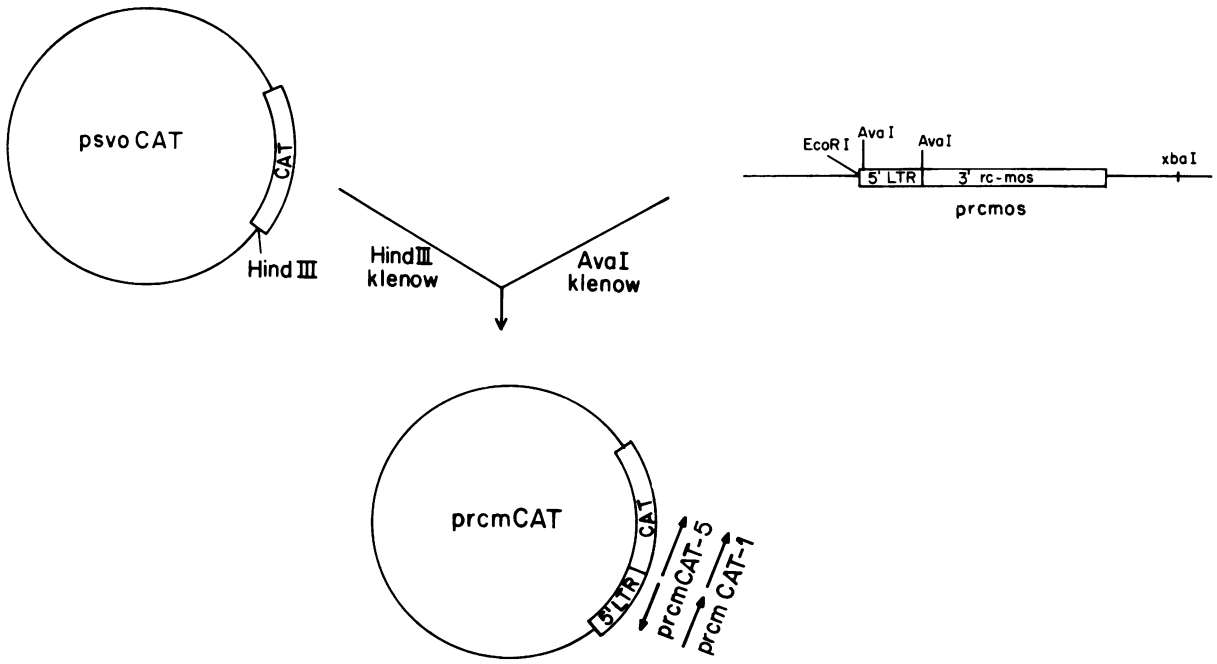

FIG. 1. Construction of plasmids containing the 5' LTR of an IAP coupled to the bacterial CAT gene. A 430-base-pair *Ava*I fragment was cleaved from a plasmid containing the active rc-*mos* (prcmos), treated with the Klenow fragment of *Escherichia coli* DNA polymerase I to flush the ends, and ligated to pSV0CAT previously linearized with *Hin*dIII and treated with the Klenow fragment of *E. coli* DNA polymerase I. For more details, see Horowitz et al. (17).

Virus. Strain 776 of SV40 was used for the infection experiments. Subconfluent CV-1 cells were infected with 50 to 100 PFU of the virus per cell in 5 ml of DMEM supplemented with 2% calf serum. After 2 h, the medium was removed, and 5 ml of DMEM supplemented with 10% calf serum was added.

Transfection. Transfections were carried out by a modification of the calcium phosphate coprecipitation technique (13). DNA (20 μg) was mixed with 0.5 ml of 2× HeBs, pH 7.05 (10 g of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], 16 g of NaCl, 0.74 g of KCl, 0.25 g of $Na_2HPO_4 \cdot 2H_2O$, and 2 g of dextrose per liter). 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 0.5×10^6 to 2×10^6 cells. After 4 h, the cells were subjected to a 2-min glycerol shock (10% glycerol in DMEM supplemented with 10% calf serum).

CAT assay. At 40 h after transfection, cultures were washed twice with phosphate-buffered saline. The cells were removed from the dish in 1 ml of 0.15 M NaCl–10 mM Tris (pH 7.5)–1.5 mM $MgCl_2$, precipitated in a microcentrifuge, and suspended in 100 μl of 0.25 M Tris (pH 7.8). Cell debris was removed by centrifugation in a microcentrifuge. The protein concentrations in the various cell extracts were determined by the method of Bradford (5). Amounts of cell extracts corresponding to 150 μg of protein were incubated with [^{14}C]chloramphenicol (New England Nuclear Corp.) in the presence of 4 mM acetyl coenzyme A at 37°C in a total volume of 140 μl for various times (20 to 60 min). The reaction was terminated by the addition of 1 ml of ethyl acetate. The organic phase was dried, and the dried material was resuspended in 30 μl of ethyl acetate, spotted onto a thin-layer chromatography plate (Merck & Co., Inc.), and chromatographed in chloroform-methanol (95:5).

The plate was dried and exposed to an Agfa (Curix) film. 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, Inc. Conditions were in accordance with the recommendations of the supplier.

RNA and DNA preparation and analysis. Poly(A)-containing RNA was prepared from cells at 48 h after transfection and analyzed by the nuclease S1 mapping technique as described elsewhere (17). For preparation of the probe, prcmCAT-1 was digested with *Eco*RI, labeled at its 5' end with T4 polynucleotide kinase, and digested with *Bgl*I. A 2.4-kilobase fragment was isolated from a 5% polyacrylamide gel and electroeluted.

RESULTS

Effect of T antigen on the levels of CAT directed by the 5' LTR promoters. The 5' LTR of the IAP inserted within the c-*mos* in myeloma XRPC-24 was coupled to the bacterial gene coding for CAT (Fig. 1). The 5' LTR of the inserted IAP is contained within an *Ava*I fragment of 430 base pairs. This fragment was isolated and ligated with the *Hin*dIII-cleaved plasmid pSV0CAT (12) in two orientations. In the plasmid designated prcmCAT-1, the LTR and the CAT gene are joined tail to head, as in the IAP, and have the same transcription orientation. In prcmCAT-5, the joining is head to head, as in the 3' rc-*mos*, and the transcriptional orientations of the LTR and the CAT gene are opposite. It has already been shown that both promoters direct the synthesis of CAT, although prcmCAT-1 was 20- to 30-fold more efficient than prcmCAT-5 (17).

These constructs were used to test whether the SV40 large-T antigen has an effect on the levels of CAT directed by the two LTR promoters. As the source of SV40 large-T

antigen, we used COS-M6 cells (11, 16) or, alternatively, CV-1 cells infected with SV40. CV-1 cells at 2 h after infection with SV40 or COS-M6 cells were transfected with prcmCAT-1, prcmCAT-5, and pSV0CAT. After 40 h, extracts were prepared, and CAT activity was assayed at different incubation times (Fig. 2 and 3). COS-M6 cells and CV-1 cells infected with SV40 have in common the expression of SV40 large-T antigen. Therefore, it is plausible that any enhanced activity was due to the presence of this protein. In CV-1 cells infected with SV40 or in COS-M6 cells, in which T antigen is expressed constitutively, CAT activity directed by the two LTR promoters was enhanced 10-fold. CAT activity directed by the promoter of plasmid pSV0CAT was only slightly higher in the presence of T antigen than in its absence. These results imply that the increase in CAT activity directed by the 5′ LTR promoters is due to the presence of T antigen.

Effect of the adenovirus E1A gene product on the levels of CAT directed by the 5′ LTR promoters. One of the adenovirus E1A gene products, a polypeptide of 289 amino acids, has been shown to enhance the activity of viral and nonviral promoters (32). Moreover, this gene product causes transformation of rat embryo fibroblasts in cotransfection experiments with either mutated H-*ras* or polyomavirus middle-T antigen (21, 28). Therefore, its effect on the promoters of the 5′ LTR of the inserted IAP was tested. HeLa and 293 cells were used for the transfection experiments. HeLa cells were derived from a human cervix carcinoma. 293 cells are human kidney embryonal cells that constitutively express the E1A proteins from an integrated copy of the left 14% of the adenovirus genome (1, 10, 14). 293 and HeLa cells were transfected with prcmCAT-1, prcmCAT-5, or pSV0CAT. After 40 h, extracts were prepared, and CAT activity was measured (Fig. 4 and 3). The activity of CAT induced by the 5′ LTR promoter in the tail-to-head orientation (prcmCAT-1) was at least 100-fold higher in 293 cells than in HeLa cells. Extracts from 293 cells containing 150 μg of protein converted almost 100% of the [^{14}C]chloramphenicol to the acetylated forms after 2 min of incubation at 37°C. This is a remarkable enhancement in activity. The CAT activity directed by the other 5′ LTR promoter (prcmCAT-5) was 20-fold higher in 293 cells than in HeLa cells. The effect of the adenovirus E1A gene product on the promoterless plasmid (pSV0CAT) was barely detectable. To prove that the adenovirus E1A gene product was responsible for the elevation in CAT activity in 293 cells, we used a plasmid (pLA8) containing the adenovirus E1A region. It consists of 9.1% of the left end of the adenovirus 2 genome, a transforming segment containing E1A, and most of E1B (28). HeLa cells were cotransfected with pLA8 in combination with prcmCAT-1, prcmCAT-5, or pSV0CAT (Fig. 5). Elevated levels of activity were observed when HeLa cells were cotransfected with the different plasmids together with pLA8 relative to the levels found in HeLa cells transfected with the

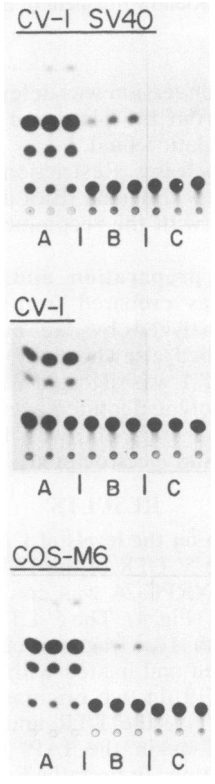

FIG. 2. Effect of T antigen on the levels of CAT directed by prcmCAT-1 (A), prcmCAT-5 (B), and pSV0CAT (C). CV-1 cells (10^6) were infected with 50 PFU of SV40 strain 776 per cell described in Materials and Methods. After 2 h, the medium was removed, and the cells were transfected with 20 μg of DNA. At the same time, 10^6 uninfected CV-1 cells and 10^6 COS-M6 cells were transfected with 20 μg of DNA. After 40 h, cell extracts were prepared in 100 μl of 0.25 M Tris (pH 7.8). Extracts containing 150 μg of protein were assayed for CAT activity at 20, 40, and 60 min of incubation.

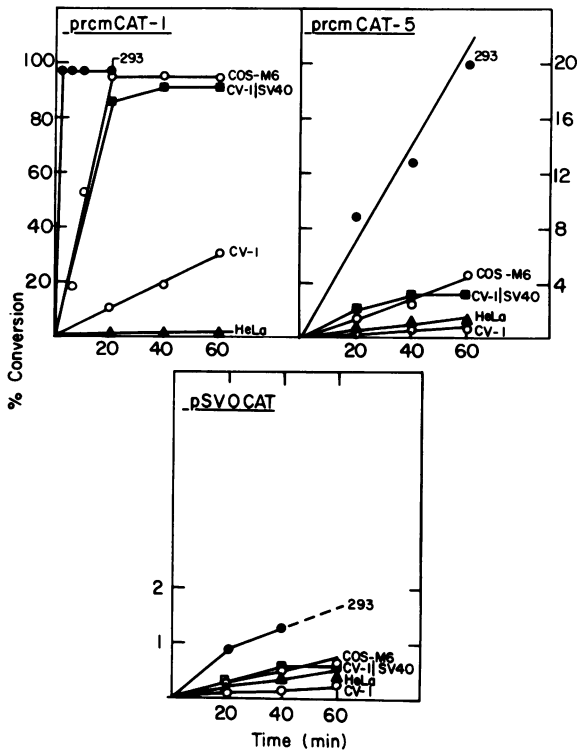

FIG. 3. Effect of SV40 large T antigen and adenovirus E1A gene product on the expression of CAT directed by prcmCAT-1, prcmCAT-5, and pSV0CAT. Details are as described in the legends to Fig. 2 and 4. The percentage of conversion of [^{14}C]chloramphenicol to the acetylated forms was determined by cutting spots from the plate and measuring the radioactivity in a liquid scintillation counter with toluene-based scintillation fluid.

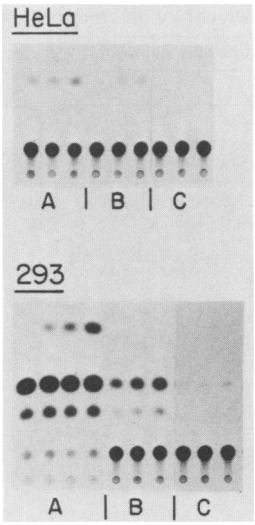

FIG. 4. Effect of the adenovirus E1A protein on the levels of CAT directed by prcmCAT-1 (A), prcmCAT-5 (B), and pSV0CAT (C). HeLa cells (10^6) or 293 cells (10^6) were transfected with 20 μg of DNA. After 40 h, cell extracts were prepared in 100 μl of 0.25 M Tris (pH 7.8). Extracts containing 150 μg of protein were assayed for CAT activity at 20, 40, and 60 min of incubation except that extracts of 293 cells transfected with prcmCAT-1 were assayed at 2, 5, 10, and 20 min of incubation.

same plasmids alone. In the presence of pLA8, there was a 10-fold increase in CAT activity directed by prcmCAT-1 and a 5-fold increase in CAT activity directed by prcmCAT-5. These results corroborate the results with 293 cells and suggest that an E1A product enhances the promoter activity of the modular plasmids.

Effect of p53 and the *myc* gene product on the CAT activity directed by the 5′ LTR promoter. p53 and *myc* are cellular oncogenes of the nuclear complementation group. They complement the mutated H-*ras* in transfection assays of rat embryo fibroblasts (8, 26). *myc* is rearranged in mouse plasmacytomas (29), in which IAP sequences are extensively transcribed (22). Therefore, we tested its ability to transactivate the IAP LTR promoter. The similarity between p53 and *myc* prompted us to test p53 as well. NIH 3T3 cells were transfected with prcmCAT-1 and pSV0CAT or cotransfected with those plasmids and an SV40 vector expressing either *myc* or p53 (Fig. 6 and Table 1). Cell lysates were prepared 48 h after transfection, and CAT activity was measured in samples containing 100 μg of proteins. p53 and *myc* enhanced the CAT activity directed by the 5′ LTR promoter (Fig. 6 and Table 1). *myc* increased the CAT activity promoted from the 5′ LTR 10-fold; p53 enhanced the CAT activity 5-fold. pSV0CAT had low activity in the absence or the presence of any of the tested oncogenes.

Analysis of CAT mRNA. The effect of the adenovirus E1A gene product and p53 on the steady-state levels of CAT mRNA was tested. 293 cells, IIB4 cells (RAT-1 cells constitutively expressing p53) (7), or RAT-1 cells were transfected with prcmCAT-1. After 48 h, poly(A)-containing RNA was extracted and analyzed by the nuclease S1 mapping technique with a 5′-end-labeled probe. The 2,400-base-pair hybridization probe used in this analysis generated a 450-nucleotide-long nuclease-resistant DNA fragment when hybridized to the CAT mRNA (Fig. 7).

Under the given experimental conditions, no CAT mRNA could be detected in RAT-1 cells transfected with prcmCAT-1 (Fig. 7a). However, in RAT-1 cells constitutively express-

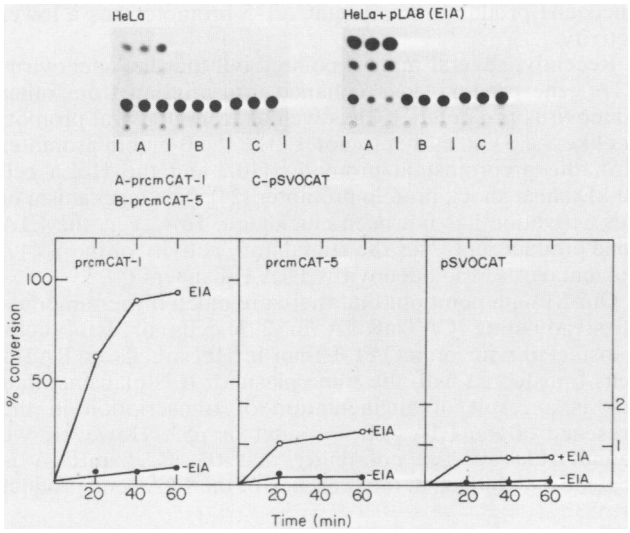

FIG. 5. Effect of the adenovirus E1A gene product on the levels of CAT directed by the IAP promoters. Transfection of HeLa cells was done as described in the legend to Fig. 4. Cotransfection was performed with 20 μg of DNA from the different plasmids and 20 μg of pLA8. After 40 h, the cells were processed, and CAT activity was assayed as described in the legend to Fig. 4. Radioactivity was measured as described in the legend to Fig. 3.

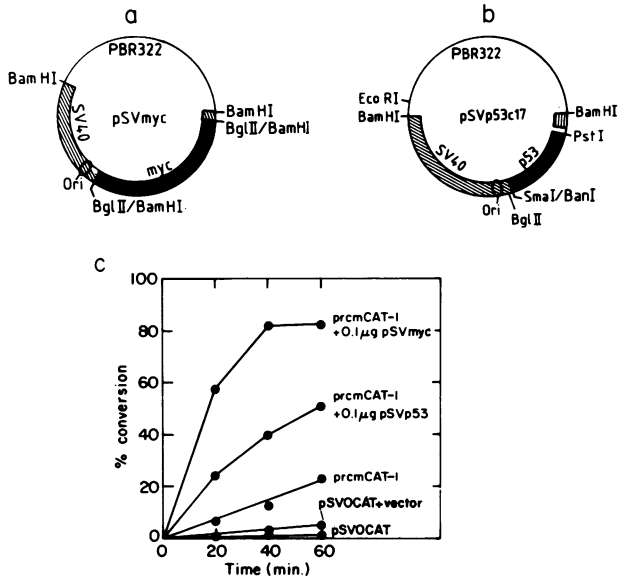

FIG. 6. Effect of the *myc* gene product and p53 on the expression of CAT directed by prcmCAT-1. (a) A *myc* expression vector (pSV*myc*) was constructed by coupling a 5.5-kilobase *Bam*HI fragment of c-*myc* containing the second and third exons to an SV40 early replacement vector as described by Eliyahu et al. (8). (b) The p53 expression vector pSVp53c17 was described elsewhere (8). (c) NIH 3T3 cells (10^6) were transfected with 0.1 μg of expression vector and 10 μg of either prcmCAT-1 or pSV0CAT. After 40 h, cell extracts were prepared, and samples containing 100 μg of protein were assayed as described in the legends to Fig. 2 and 3. Ori, Origin of replication.

TABLE 1. Conversion of [^{14}C]chloramphenicol to ^{14}C-acetylated chloramphenicol by different cell extracts

Plasmid	Conversion of [^{14}C]chloramphenicol (S) to ^{14}C-acetylated chloramphenicol (P) at[a]:											
	20 min				40 min				60 min			
	S	P	S + P	% Conversion [P/(S + P)]	S	P	S + P	% Conversion [P/(S + P)]	S	P	S + P	% Conversion [P/(S + P)]
prcmCAT-1	2.040	138	2,178	6.3	1,225	175	1,400	12.5	1,121	270	1,391	19.4
prcmCAT-1 + pSVp53	1,328	430	1,758	24.4	945	629	1,574	39.9	751	794	1,545	51.4
prcmCAT-1 + SVmyc	806	1,155	1,961	58.9	247	1,224	1,471	83.2	193	919	1,124	81.7
pSVOCAT	1,898	2.5	1,900	0.10	1,813	3	1,816	0.12	1,492	4	1,496	0.26
psVOCAT + pSVp53	1,083	1.2	1,084	0.1	1,255	2	1,257	0.13	1,328	4	1,332	0.3
pSVOCAT + pSVmyc	1,309	2	131	0.15	1,319	3	1,322	0.22	1,043	5	1,048	0.47

[a] Cell extracts were prepared as described in the legend to Fig. 6. Amounts of S, P, and S + P are reported in counts per minute × 10^2. The percentage of conversion of S to P was determined by cutting spots from the plate and measuring the radioactivity in a liquid scintillation counter with toluene-based scintillation fluid.

ing p53 (IIB4 cells), CAT mRNA levels increased concomitantly with the increase in CAT activity in the same cells (Fig. 7a). In 293 cells, CAT mRNA levels increased dramatically, as compared with the levels in HeLa or IIB4 cells (data for HeLa cell CAT mRNA are not shown).

DISCUSSION

The results presented in this paper show that SV40 large-T antigen, p53, and the adenovirus E1A and *myc* gene products markedly enhance the promoter activity of the LTR of IAP, as measured by CAT activity. SV40 large-T antigen increased the activity of prcmCAT-1 and prcmCAT-5 promoters by 20-fold and 5-fold, respectively. The adenovirus E1A gene product increased the level of CAT activity promoted from prcmCAT-1 and prcmCAT-5 100-fold and 10-fold, respectively, in 293 cells. p53 and *myc* increased the level of CAT activity directed by the promoter of prcmCAT-1 by 5-fold and 10-fold, respectively. It is plausible that the great increase in the level of CAT in the presence of T antigen or the E1A gene product in COS-M6 or 293 cells is due to their constitutive expression in these cell lines or stabilization of the transfected plasmids (2). Therefore, when a plasmid containing the E1A region (pLA8) was used to cotransfect HeLa cells instead of to transfect 293 cells, the level of CAT activity of the plasmids containing the two 5′ LTR promoters was elevated but did not reach the level obtained with 293 cells transfected with the same plasmids. In this case, the adenovirus E1A gene product increased the level of activity promoted from prcmCAT-1 and prcmCAT-5 by 10-fold and 3- to 5-fold, respectively. Since the activation of prcmCAT-1 was only 10-fold, it was expected that the activation of prcmCAT-5 would be low.

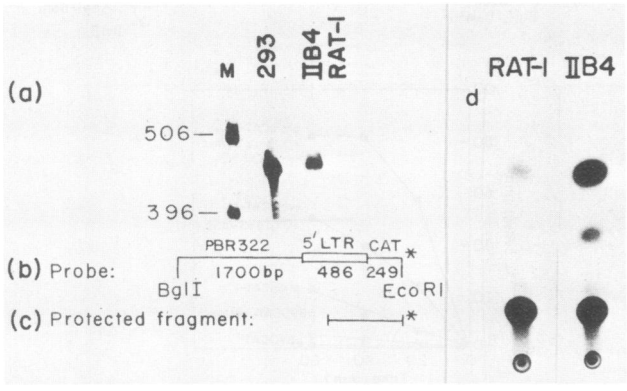

FIG. 7. Effect of p53 and the adenovirus E1A gene product on the transcription of prcmCAT-1. (a) 293, IIB4, and RAT-1 cells were transfected with 10 μg of prcmCAT-1. After 48 h, poly(A)-containing RNA was isolated and hybridized to a 5′-end-labeled probe for 18 h at 49°C. After nuclease S1 digestion, the protected fragments were resolved on a 7 M urea–5% polyacrylamide gel. The gel was exposed overnight to an Agfa film and developed. M, Markers. (b) Size of probe. bp, Base pairs. (c) Size of protected fragment. (d) Conversion of [^{14}C]chloranphenicol to the acetylated forms in the presence of 100 μg of lysates prepared from IIB4 or RAT-1 cells transfected with prcmCAT-1 as described in panel a.

The results presented above indicate that prcmCAT-1 is most suitable for the detection of transactivation by oncogene products. The prcmCAT-5 promoter has a lower activity.

Recently, several reports pointed out that the adenovirus E1A gene product does enhance transcription from other adenovirus promoters (32) as well as from nonviral promoters like the SV40 early promoter (31), the β-globin promoter (15), the preproinsulin promoter (10), and the HeLa cell 70-kDa heat shock protein promoter (24). The mechanism of this activation has not been elucidated. However, the E1A gene product represses the stimulatory activity of the SV40, polyomavirus, and adenovirus E1A enhancers (4).

Our S1 data point out that there are much higher amounts of steady-state CATmRNA in 293 cells or IIB4 cells transfected with prcmCAT-1 than in HeLa cells or RAT-1 cells transfected with the same plasmid. It is plausible that this is a result of augmentation of transcription in the presence of the E1A gene product or p53. However, we cannot rule out the possibility that the CAT mRNA is somehow stabilized in the presence of the E1A gene product or p53.

The induction of an IAP LTR by the *myc* gene product resembles the increased level of IAP transcripts in mouse tumors. It has already been shown that in mouse myelomas there is a 1,000-fold increase in the abundance of the IAP transcripts and that this is due mainly to enhanced transcription (22). It is worth mentioning that transcription of the VL30 sequences, members of another retroviruslike

multigene family, is rapidly and specifically induced by epidermal growth factor stimulation of quiescent cells (9). Since in the malignant state as well as in cotransfection experiments with oncogenes that specify nuclear proteins the IAP promoter is turned on, it seems that the gene products of these oncogenes enhance the transcription of such elements. During normal development of the mouse embryo, IAP has been found to be activated at the stage of four to eight cells (33). It may well be a result of activation of a proto-oncogene(s) that turns on, via its gene product, the expression of IAP by interacting with the LTR of these genes. Therefore, the activation of IAP may serve as an indicator of the activity of proto-oncogenes during embryonic development and as a convenient model system to test the activation of cellular promoters by gene products of nuclear oncogenes.

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