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The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection

(oncogenesis/viral insertion/transcriptional activity/transient expression/chloramphenicol acetyltransferase)

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ABSTRACT **We characterized the transcriptional activity of the long terminal repeat (LTR) of Rous sarcoma virus by constructing a recombinant plasmid, pRSVcat, in which bacterial chloramphenicol acetyltransferase (CAT; acetyl-CoA:chloramphenicol 3-*O*-acetyltransferase, EC 2.3.1.28) coding sequences are placed under LTR control. We find that the LTR directs relatively high levels of CAT synthesis within 48 hr after calcium phosphate-mediated introduction of this plasmid into CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, or mouse NIH/3T3 cells. The level of CAT synthesis is 3-fold higher in CV-1 cells and up to 10-fold higher in HeLa and mouse NIH/3T3 cells than after transfection with a related vector, pSV2cat, carrying CAT sequences under control of the simian virus 40 early promoter. We have shown, by primer extension, that the amounts of CAT-specific mRNAs encoded by pRSVcat and pSV2cat correlate with the levels of CAT enzyme activity. By both S1 nuclease mapping and primer extension, we have demonstrated that the start site for RNA transcription within the LTR of pRSVcat corresponds to previous mapping data. We estimated transfection efficiencies by monitoring immunofluorescence induced by a rhodamine-labeled CAT antibody. Our results indicate that the Rous sarcoma virus LTR can direct synthesis of high levels of functional mRNA and has a wide expression range. The observed high transcriptional activity of the LTR is significant because it has been postulated that this LTR promotes activity of adjacent cellular oncogenes.**

There has been much interest in the class of tumor viruses known as retroviruses. Of particular interest are the long terminal repeats (LTRs) that flank either end of the integrated DNA copy of the viral genome. The structure of these LTRs has been reviewed by Temin (1) based on sequence data from several retroviruses. Though there are several subclasses of RNA tumor viruses, striking sequence similarities are found within these common flanking regions. In particular, the LTRs contain highly conserved sequences thought to be essential for the synthesis of viral DNA (2) and the integration of the viral genome into the host chromosome (3). LTRs also contain sequences involved in the regulation of transcription of the integrated viral genome. There are sequences important to the initiation of transcription ("C-C-A-A-T" and "T-A-T-A-A" boxes) and sequences related to polyadenylylation. By using *in vitro* transcription assays, the sites at which viral RNA synthesis initiates have been precisely mapped to regions within these terminal repeats (4–6).

Recently, it has been postulated that the LTRs of some avian viruses can serve directly as promoters to activate transcription of downstream cellular genes (7–9). In a few instances, however, the activation effect can result from integration of the LTR 3′ to the target gene (10). Repeat sequences that function similarly to the enhancer sequences found in simian virus 40 (SV40) (11–13) have been identified in the spleen necrosis virus and the murine leukemic and sarcoma viruses (14, 15). Thus, it has become important to distinguish between direct promoter activity and an enhancement effect seen *in vivo* with some retrovirus sequences (15).

To further understand the transcriptional activity of these long terminal repeats, we have chosen the LTR from the Rous sarcoma virus (RSV; Schmidt–Ruppin D strain), shown to be active transcriptionally *in vitro* (4). We subcloned this LTR from the plasmid pSR1 (16) into a derivative of pSV2cat, a recombinant vector that expresses chloramphenicol acetyltransferase (CAT; acetyl-CoA:chloramphenicol 3-*O*-acetyltransferase, EC 2.3.1.28) in eukaryotic cells (17). In the resulting plasmid, pRSVcat, a CAT encoding region is placed under transcriptional control of the RSV LTR. CAT assays in eukaryotic cells are specific and highly reproducible; thus, this system provides an excellent means of quantitating the expression of LTR activity. We found that pRSVcat is efficiently expressed after calcium phosphate-mediated transfections of five cell types: chicken embryo fibroblasts (CEF), CV-1 monkey kidney cells, Chinese hamster ovary (CHO) cells, HeLa cells, and mouse NIH/3T3 cells. The levels of CAT produced under RSV LTR control are higher than those produced by any other eukaryotic promoters that were tested, including the SV40 early region, Herpes simplex virus thymidine kinase (unpublished data), human β-globin (unpublished data), and CEF α2 (I) collagen (unpublished data) promoters. In addition, we measured plasmid specific mRNA levels and monitored transfection efficiencies by immunofluorescence with rhodamine-labeled antibody to CAT.

MATERIALS AND METHODS

Preparation of plasmid DNAs, DNA fragments, and the bacterial transformation protocol have been described in detail (17).

Eukaryotic Cell Transfection. Transfections were carried out by using calcium phosphate (18) followed by a glycerol shock (19). Precautions taken in preparing the precipitates have been described in detail (17). Cell lines were exposed to glycerol shock for 2–2.5 min at 37°C; however, the primary CEFs were found to be overly sensitive to this procedure. For CEFs, the glycerol treatment was at room temperature for 30 sec. Cells were transfected with either the plasmid pRSVcat (in which SV40 early promoter controls CAT expression) or a control plasmid pSVOcat, which contains no eukaryotic promoter (17).

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Abbreviations: LTR, long terminal repeat; RSV, Rous sarcoma virus; CAT, chloramphenicol acetyltransferase; CEF, chicken embryo fibroblast; CHO, Chinese hamster ovary; P_i/NaCl, phosphate-buffered saline; SV40, simian virus 40; bp, base pair(s).

Comparisons between the parental pSV2cat, pSVOcat, and pRSVcat vectors were made with 10 μg of DNA per plate (100 mm). Forty-eight hours after transfection, cells were harvested for assay of CAT activity (17) and for RNA isolation or were fixed for immunofluorescent staining.

Immunofluorescence Labeling. Goat anti-CAT antiserum was kindly provided to us by W. Shaw (Leicester, England) and was labeled directly with rhodamine using tetramethyl rhodamine isothiocyanate (Research Organics, Cleveland, OH). Screening cells for CAT activity with this fluorescent dye–antibody complex was performed as follows. Cells (10^5 in 35-mm dishes) transfected 48 hr earlier with various CAT plasmids were washed with phosphate-buffered saline (P_i/NaCl) and fixed in 3.7% formaldehyde in P_i/NaCl for 10 min. After a wash in P_i/NaCl, the cells were treated with 0.1% Triton X-100 in P_i/NaCl for 5 min. Cells were again washed in P_i/NaCl, and each plate was incubated in 1 ml of normal goat globulin containing 0.1% Triton X-100 in P_i/NaCl and 5 μl of rhodamine–goat anti-CAT antiserum. Plates were rocked gently for 30 min, washed with P_i/NaCl three times for 5 min (each wash) and mounted in glycerol under a 25-mm diameter no. 1 circular coverslip for fluorescence microscopy. Photographs were made with Kodak Tri-X film developed in Diafine and a Zeiss RA fluorescence microscope.

Preparation of RNA *in Vivo* and *in Vitro*. Cytoplasmic RNA was isolated from CV-1 monkey kidney cells. Three plates (100 mm) were used for each plasmid tested (pSV2cat, pRSVcat, and pSVOcat). Cells were washed with cold P_i/NaCl and lysed in ≈6 volumes of 10 mM Tris·HCl, pH 7.4/10 mM NaCl/3 mM MgCl/0.5% Nonidet P-40 for 5–10 min at 0°C (20). Nuclei were removed by centrifugation at 600 × *g* for 5 min (4°C), and RNA was isolated from the resulting supernatant by a modification of the procedure of Manley *et al.* (21). An equal volume of 7 M urea/0.35 M NaCl/10 mM Tris·HCl, pH 8.0/10 mM EDTA/1% sodium dodecyl sulfate and about 40 μg of tRNA per ml were added. The mixture was extracted twice with phenol/chloroform/isoamyl alcohol, 20:20:1 (vol/vol), and twice with chloroform/isoamyl alcohol. After precipitation with ethanol the RNA was collected by centrifugation and resuspended in water.

RNA was synthesized as described previously (4, 21, 22) with HeLa whole cell extracts. Reactions were terminated and RNA was prepared for primer extension and S1 nuclease mapping under conditions described by Weil *et al.* (23) and Merlino *et al.* (24).

Preparation of DNA Probes. For S1 nuclease mapping, a probe of the pRSVcat was prepared by digestion with *Pvu* II, which cleaves in the center of the CAT gene, 188 base pairs (bp) downstream of the start site of transcription in the RSV LTR (4). The DNA was treated with alkaline phosphatases, labeled by using polynucleotide kinase (25), and then digested with *Sph* I. This resulted in a probe of 324 bp, 5′ end-labeled at the *Pvu* II site. A 4% polyacrylamide/7 M urea gel was used to isolate this fragment. The probe DNA was eluted from the gel by the method of Maxam and Gilbert (26) and twice precipitated in ethanol and redissolved in water.

For primer extension, a 336-bp *Eco*RI fragment from pRSVcat was isolated and 5′ end-labeled as described above. After the end-labeling, the fragment was cut with *Pvu* II, and the resulting 102-bp probe was isolated with a 6.5% polyacrylamide/7 M urea gel.

Primer Extension. After transfection with the plasmids, cytoplasmic RNA was isolated and used for primer extension as described elsewhere (24, 25). One-third of each cytoplasmic RNA preparation was used for each primer extension experiment.

S1 Nuclease Mapping. S1 nuclease mapping was performed according to Berk and Sharp (27), including the modification of Weaver and Weissman (28). Details are given by Merlino *et al.* (24).

RESULTS

Construction of pRSVcat. The steps involved in subcloning RSV 3′ LTR sequences from the plasmid pSR1 are summarized in Fig. 1. These steps yielded a 524-bp fragment with one blunt *Pvu* II end and a cohesive *Hin*dIII end downstream to the RSV transcription start site. In addition to the LTR, this fragment has been shown to contain some carboxyl-terminal *src* sequences (16). The modified LTR fragment was joined to the plasmid pSV2cat (17) after removal of the pSV2cat SV40 early region promoter. In the resulting pRSVcat plasmid, the 3′ RSV LTR is juxtaposed as a promoter upstream from CAT encoding sequences (Fig. 1). The distance between the LTR transcriptional start site and the CAT initiation codon (the first AUG downstream from the start site) is about 70 bp.

Eukaryotic Cell Transfections and CAT Activity. To determine the *in vivo* expression levels of the RSV LTR, we assayed CAT activity in extracts from cells transfected with pRSVcat. Fig. 2 shows measurement of CAT activity in CEF extracts made 48 hr after introduction of pRSVcat DNA. To provide a comparison, this figure also shows the CAT produced after introduction of pSV2cat DNA, in which CAT expression is under

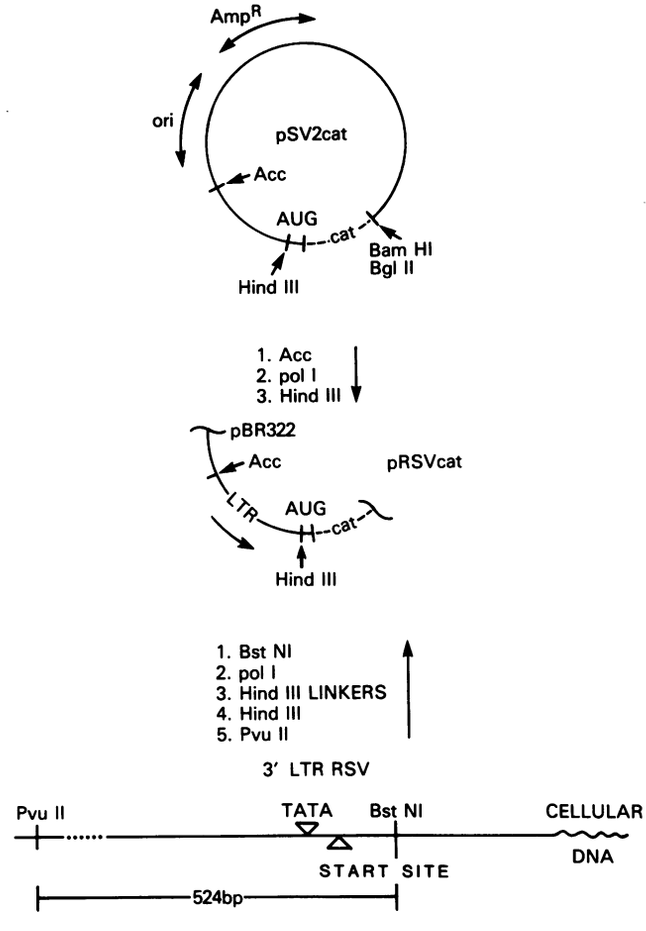

FIG. 1. Construction of pRSVcat plasmid, showing the steps involved in subcloning the RSV LTR from pSR1. The *Pvu* II–*Bst*NI fragment (524 bp) of the 3′ LTR was isolated by agarose gel electrophoresis and was inserted into pSV2cat after the removal of the SV40 early promoter region flanked by the *Acc* I (Acc) and *Hin*dIII sites. The position of the LTR relative to the CAT-encoding region is shown. Acc indicates the position of joining; the site was destroyed during construction.

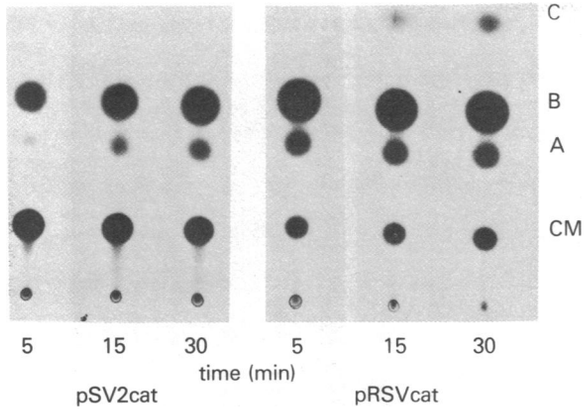

FIG. 2. CAT activity in CEF transfected with either pSV2cat or pRSVcat plasmids. The conversion of [^{14}C]chloramphenicol (row CM) to the monoacetate forms (rows A and B) or the diacetate form (row C) is shown with increasing reaction times. The above reactions used 10 μl of the standard 100-μl extract from 2×10^6 cells. CM and its acetylated forms are separated by ascending thin-layer chromatography.

the control of the SV40 early promoter. The thin-layer chromatogram demonstrates that there was considerably more CAT enzymatic activity in extracts of CEF transfected with pRSVcat than in CEF transfected with pSV2cat. To better quantitate the levels of enzyme activity, this experiment was repeated with cellular extract from 2×10^4 cells (1 μl of 100-μl extract prepared from 2×10^6 cells), so that the amount of monoacetate chloramphenicol formed was proportional to the amount of CAT present. The levels of monoacetylated [^{14}C]chloramphenicol produced were determined by scintillation counting (Fig. 3). Comparison of the linear parts of the curves in Fig. 3 shows that the pRSVcat plasmid yielded 5 times the level of CAT activity that was induced by pSV2cat DNA in CEF cells. The level of CAT in cells transfected with pSVOcat, a plasmid in which the SV40 promoter region is deleted, was less than 0.01% of the activity from pRSVcat. Fig. 3 further shows that the RSV LTR directed the accumulation of high levels of CAT activity not only in CEF (a host cell for RSV) but also in CV-1 monkey kidney cells (a host cell for SV40). Because the RSV LTR also induced more CAT synthesis than the SV40 early promoter in NIH/3T3 and HeLa cells (Table 1), this LTR seems to be exceptional in its capacity to function as a promoter in a particularly wide range of cell types.

Immunofluorescent Screening for CAT Activity. To determine the percentage of cells expressing these plasmids, transformation efficiency was monitored by immunofluorescence labeling with rhodamine-labeled antibody to CAT (Fig. 4). Up to 10% of both CV-1 cells and primary CEF showed a reaction with the CAT antibody when transfected with either pRSVcat or pSV2cat. There was considerable variability in the intensity of labeling from cell to cell. For this reason, it was not easy to compare absolute amounts of label in each cell. It was found that the seeding density had a large effect on the efficiency of transfection.

Analysis of RNA by Primer Extension. To determine the *in vivo* start site of RNA synthesis from the plasmid pRSVcat, we used plasmid-specific primer extension (24). When cytoplasmic RNA from pRSVcat-transfected cells was used as a template, the 102-bp 5′ end-labeled DNA primer was extended to 282 bp (Fig. 5, lane b). In Fig. 5, lane c, is the *in vivo* start site of RNA synthesis from the plasmid pSV2cat; here the same DNA primer was extended to 307 bp. This method allowed us to compare the relative amounts of RNA produced in CV-1 cells from the three plasmids used (pRSVcat, pSV2cat, and pSVOcat). Clearly

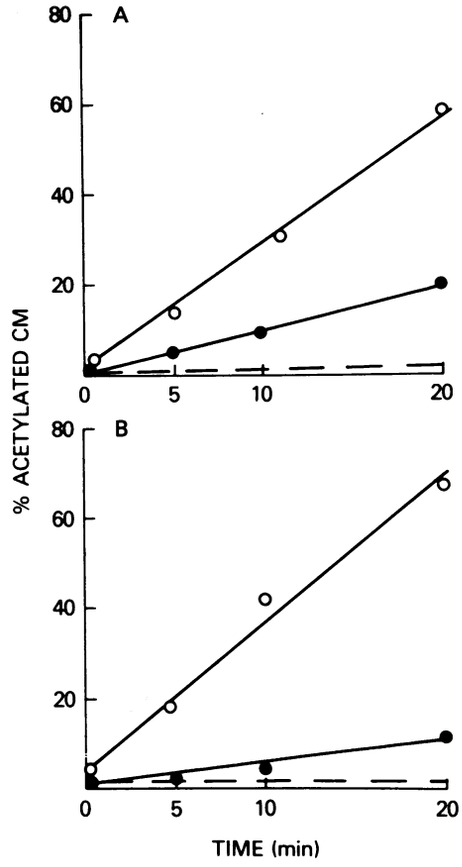

FIG. 3. Levels of CAT activity in CV-1 (*A*) and CEF (*B*) cells. The graphs show the percentage of chloramphenicol (CM) modified to the monoacetate forms. These linear reactions used 1 μl of the standard 100-μl cellular extract from 2×10^6 cells. ○, CAT activity in cells transfected with pRSVcat; ●, CAT activity in cells transfected with pSV2cat; ——, CAT activity in cells transfected with pSVOcat.

the amount of cytoplasmic RNA generated from the pRSVcat plasmid was greater than that from pSV2cat. A densitometer reading of the autoradiogram indicated that this difference was ≈10-fold. There was no detectable synthesis of RNA from the plasmid pSVOcat (Fig. 5, lane d).

S1 Nuclease Analysis of RNA. To compare the *in vivo* transcriptional start site, determined above, with the previously determined *in vitro* site of transcription (4), we used S1 nuclease mapping. Transcription of the cytoplasmic RNA from the pRSVcat plasmid initiated at the same point as was found *in vitro* (Fig. 6). The *in vitro* transcription of this plasmid was presumably carried out by RNA polymerase II because transcription was arrested in the presence of α-amanitin (Fig. 6, lane 3).

Table 1. Comparison of levels of chloramphenicol acetylated by cellular extracts

Cell type	pRSVcat	pSV2cat
CEF	100	19
CV-I	86	38
CHO	4.7	10.5
HeLa	2.5	0.3
NIH/3T3	1.5	0.15

Data have been normalized to the highest enzyme activity measured from 1 μl of a 100-μl extract after an incubation of 30 min; 68% of the chloramphenicol was acetylated by 1 μl of extract from pRSVcat-transfected CEF. It cannot be assumed that all of the above cell types have the same transfection efficiencies.

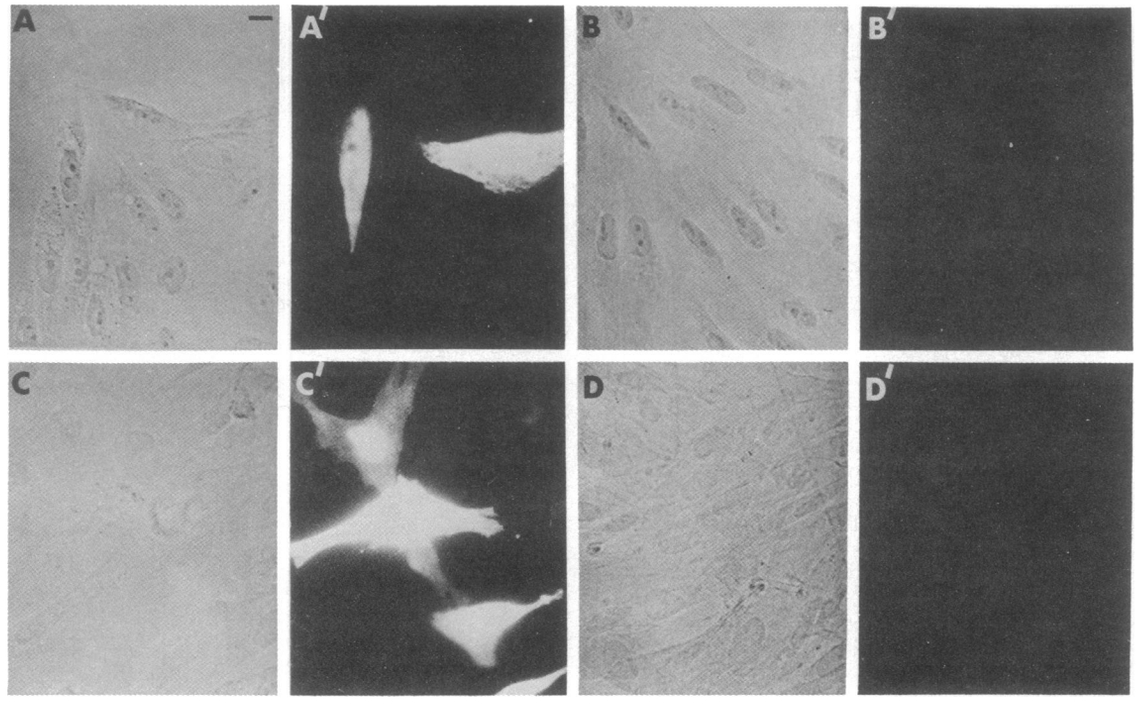

FIG. 4. Immunofluorescence localization of CAT in cells transfected with DNA. CV-1 cells (*A* and *B*) and CEF (*C* and *D*) were transfected as described with pRSVcat DNA (*A* and *C*) or calf thymus DNA (*B* and *D*). Forty-eight hours later, the cells were fixed, permeabilized, and incubated with rhodamine-labeled goat anti-CAT antibody. Paired phase (*A*, *B*, *C*, and *D*) and rhodamine fluorescence (*A'*, *B'*, *C'*, and *D'*) photographs were made using Tri-X film. The majority of positive cells occurred at the edge of the CV-1 islands as shown (*A* and *A'*). No labeled cells were seen in the calf thymus DNA controls (*B* and *B'*; *D* and *D'*). (×280; Bar = 10 μm.)

DISCUSSION

This study used a recombinant genome expressing chloramphenicol acetyltransferase to investigate the *in vivo* promoter strength of the distal 3' LTR of RSV. We found that the RSV LTR induces the accumulation of high levels of functional CAT mRNA in a variety of cell types. The estimate of apparent RSV LTR promoter strength was obtained by assay of CAT activity and measurement of steady-state CAT mRNA levels in CV-1

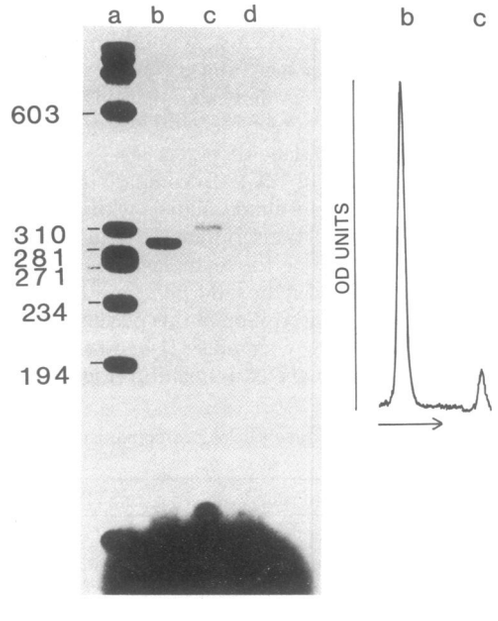

FIG. 5. *In vitro* primer extension of 102-bp 5' end-labeled fragment using cytoplasmic RNA from CV-1 cells as a template. (*Left*) Lanes: a, ΦX174 marker DNA; b, RNA from cells transfected with pRSVcat DNA; c, RNA from cells transfected with pSV2cat DNA; d, RNA from cells transfected with pSVOcat DNA. Sizes are shown in base pairs. (*Right*) Densitometer tracing of the autoradiogram shows the relative amounts of cDNA in lanes b and c.

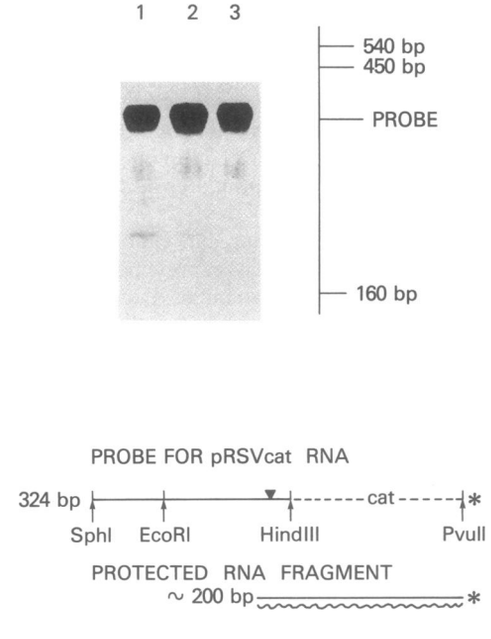

FIG. 6. S1 nuclease mapping of cytoplasmic RNA and *in vitro* synthesized RNA. (*Upper*) Lanes: 1, cytoplasmic RNA from pRSVcat-transfected CV-1 cells hybridized to pRSVcat-specific probe; 2, RNA synthesized *in vitro* with HeLa whole cell extracts and pRSVcat DNA, hybridized to pRSVcat specific probe; 3, same as lane 2 but in the presence of α-amanitin. (*Lower*) Diagram of probe used in mapping pRSVcat RNA.

cells and by CAT assay in CEF, CHO cells, HeLa cells, and mouse NIH/3T3 cells. We found that higher levels of CAT are produced from the pRSVcat plasmid than from the pSV2cat plasmid in all cell types tested except for CHO cells.

S1 nuclease mapping and primer extension were used to show that *in vivo* transcription of pRSVcat plasmid DNA in monkey kidney CV-1 cells initiates at the same point as determined in HeLa cell extracts *in vitro*. The primer extension experiments in addition confirmed that the relative amounts of CAT-specific RNA correlate with the expression of CAT protein, though the translational efficiency of mRNA transcribed from pSV2cat may be somewhat better than mRNA transcribed from pRSVcat.

Based on these results, we conclude that the RSV LTR is an efficient promoter in a variety of eukaryotic cells. This is consistent with results of others showing that RSV can transform both avian and mammalian cells (29–31). We consider less likely the following two alternative explanations for the high levels of CAT mRNA after pRSVcat transfection: the possibility that pRSVcat is expressed in an unusually large percentage of cells and the possibility that pRSVcat exists at an increased copy number in transfected cells. First, the percentage of cells expressing pRSVcat does not appear to be unusually high; as observed by immunofluorescence, there was no obvious difference between the number of cells expressing pRSVcat and pSV2cat plasmids. Second, we have no evidence suggesting that pRSVcat replicates or persists at a higher copy number than do other plasmids: Southern analysis of pRSVcat, pSV2cat, and pSVOcat DNAs 48 hr after transfection failed to reveal substantial differences (data not shown). It is also possible that the hybrid LTR–CAT RNA transcribed from pRSVcat could be exceptionally stable. Nevertheless, the finding that the RSV 3′ LTR induces high levels of functional mRNA, containing heterologous downstream coding sequences, is significant whether the mechanism is efficient transcription or mRNA stabilization.

It has been proposed that an integrated copy of the retroviral genome near a host oncogene acts to increase transcription of that oncogene (7, 9, 32). In support of this, it has been demonstrated that LTRs can induce expression of potential host transforming sequences in *in vitro* constructs (33, 34). Results obtained by Joyner *et al.* (35) in mouse cells are consistent with the function of the Friend spleen focus-forming virus LTR as a promoter for the heterologous Herpes simplex virus thymidine kinase gene. Similarly, Lee *et al.* (36) have shown that the mouse mammary tumor virus LTR can direct expression of a dihydrofolate reductase cDNA sequence. It is important to emphasize that our analysis focuses on the RSV LTR as a strong promoter in a wide variety of cells but does not address its potential function as a transcriptional enhancer. Because the majority of LTR insertions in avian lymphomas have been found to be 5′ to the host oncogene (7), it is quite possible that the LTR most frequently functions directly as a promoter in activating oncogene expression. However, the few instances in which the LTR has been shown to be downstream (7, 10) indicate that the avian LTR may be able to increase RNA synthesis by a secondary means as well.

Note Added in Proof. Levels of CAT activity in cells transfected with pRSVcat have been monitored also by a rapid spectrophotometric assay (unpublished data).

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