Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 6453–6457, November 1982
Biochemistry

Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus

(enhancers/gene expression/transcriptional regulation)

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Communicated by Wallace P. Rowe, July 28, 1982

ABSTRACT **The simian virus (SV40) 72-base pair (bp) tandem repeated sequences have recently been shown to function as activators or enhancers of early viral transcription. A recombinant viral genome was recently constructed by inserting 72-bp tandem repeats from the Moloney murine sarcoma virus (MSV) in place of the 72-bp repeats of SV40. Although this genome replicates in monkey kidney cells, its rate of large tumor antigen expression and replication is considerably slower than that of wild-type SV40. In mouse cells, however, equivalent levels of large tumor antigen appear to be expressed from both wild-type and recombinant genomes, suggesting a relationship between the level of enhancer activity and the host cell. To confirm this observation, we have applied a sensitive quantitative assay for gene expression based on the conversion of chloramphenicol to its acetylated forms. The gene encoding the enzymatic function chloramphenicol acetyltransferase was inserted into two vectors in which the enhancer sequences from SV40 or MSV were placed adjacent to the early SV40 promoter. The SV40 tandem repeats appear to activate gene expression to significantly higher levels in monkey kidney cells, but the MSV repeats are more active in two lines of mouse cells. These findings suggest that the tandem repeat elements may interact with host-specific molecules and, furthermore, may constitute one of the elements determining the host range of these eukaryotic viruses.**

Characterization of the nucleotide signals that constitute eukaryotic promoters is essential to an understanding of gene regulation. In addition to the Goldberg–Hogness sequence (or T-A-T-A box) which participates in the precise positioning of the 5′ ends of RNA molecules (1–3), upstream elements have been implicated in the activation or enhancement of transcription for certain viral or eukaryotic genes by a number of studies (4–12). It was initially demonstrated that the simian virus 40 (SV40) 72-base-pair (bp) repeats activate early viral gene expression (5, 6). Further studies indicated that these sequences could also function when they were removed from their original location and placed at positions distant from the other promoter elements (8, 9). The tandem repeats also retained activity when inserted in an inverse orientation (9, 13). Other studies have indicated that the 72-bp repeats of SV40 could increase the transformation efficiency of the herpes simplex virus thymidine kinase gene (10). An analogous fragment from the polyoma virus genome was shown to enhance the transcriptional activity of heterologous genes such as rabbit β-globin gene (11).

We have recently demonstrated that a retrovirus, the Moloney murine sarcoma virus (MSV), contains sequences in the long terminal repeats (LTRs) which can functionally replace the 72-bp repeats of SV40 (12). Preliminary data on large tumor antigen (T antigen) expression has suggested the possibility that the tandem repeats of SV40 and MSV activate gene expression in a host-specific manner.

In this study we used a sensitive and quantitative assay of gene activity to determine the effect of enhancers on the early SV40 promoter. The results suggest that host specificity is one property of certain activator elements.

MATERIALS AND METHODS

Virus Strains and Cell Lines. Small-plaque wild-type (WT) SV40 (strain 776) and the SV40 recombinant containing the MSV tandem repeats (SVr^{MSV}) (12) were used to assay T antigen production. The cell lines were CV-1 monkey kidney cells, secondary African green monkey kidney (AGMK) cells, $LMTK^-$ mouse cells, MEFC mouse cells (kindly provided by J. Tevethia), and NIH 3T3 mouse cells.

Viral Infection and T-Antigen Analysis. Equal amounts of WT SV40 (10 plaque forming units per cell) and SVr^{MSV} as determined by dot blot analysis were inoculated into cells grown to confluency in 6-cm dishes. At 24 hr after infection, the cultures were placed in methionine-free medium and labeled with [^{35}S]methionine at 100 μCi/ml (1 Ci = 3.7×10^{10} becquerels). Viral proteins were extracted and immunoprecipitated with hamster anti-SV40 T antigen antiserum provided by G. Jay or S. Tevethia. The immunoprecipitated proteins were analyzed by electrophoresis on 10% polyacrylamide gels (14).

Construction, Growth, and Purification of Plasmids. Construction of plasmids was performed as indicated in the text according to described methods (15). Plasmids were transfected onto cells 70% confluent by a modification of the Ca^{2+}-precipitation method (16, 17). The ratio of plasmid activity remained constant over the range 15–35 μg per dish, and 25 μg was chosen as the optimal concentration. After incubation at 37°C for 48 hr [the optimal time for incubation (unpublished results)], the cells were harvested and cell extracts were incubated with [^{14}C]chloramphenicol and 4 mM acetyl-CoA in 250 mM Tris (pH 7.8) (17). After analysis by ascending thin-layer chromatography, the rates of conversion were determined by scintillation counting.

RESULTS

Viral Expression. SVr^{MSV} is a recombinant virus constructed from WT SV40 by replacement of the 72-bp tandem repeats with those from Moloney murine sarcoma virus (Fig. 1). In a previous study (12) we observed that the recombinant virus grows more slowly than WT SV40, presumably due to the relative level of enhancement of T antigen by the SV40 (versus the MSV) activator sequences. To assess the relative production of T antigen in monkey and mouse cells, aliquots of both stock

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Abbreviations: SV40, simian virus 40; bp, base pair(s); MSV, Moloney sarcoma virus; LTR, long terminal repeats; T antigen, large tumor antigen; WT, wild type; CAT, chloramphenicol acetyltransferase.

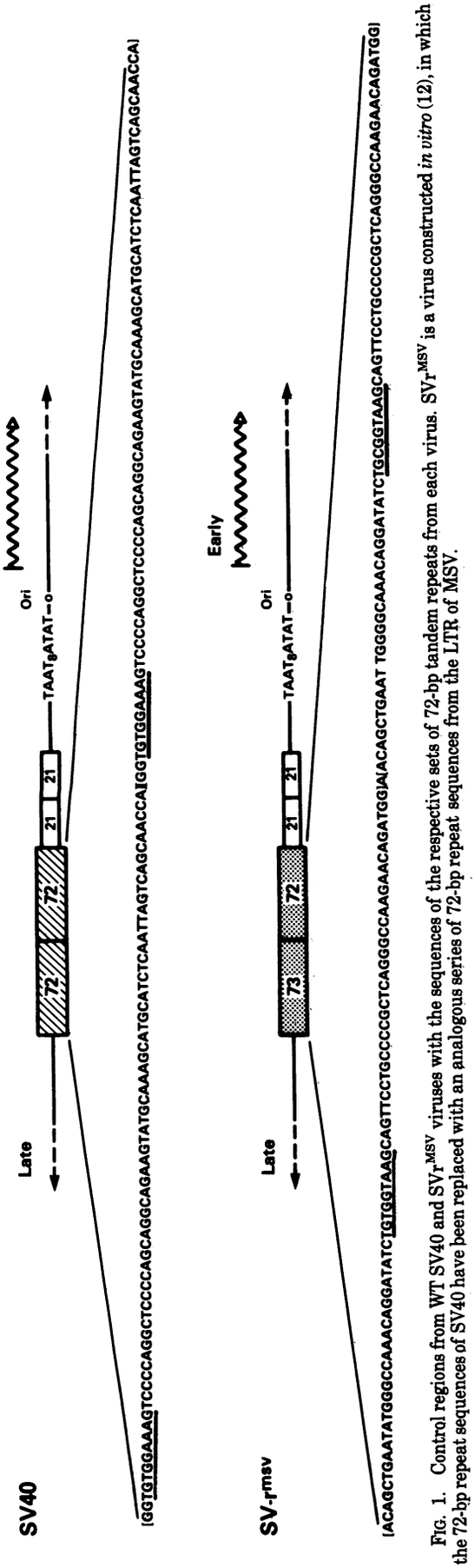

FIG. 1. Control regions from WT SV40 and SVr^{MSV} viruses with the sequences of the respective sets of 72-bp tandem repeats from each virus. SVr^{MSV} is a virus constructed *in vitro* (12), in which the 72-bp repeat sequences of SV40 have been replaced with an analogous series of 72-bp repeat sequences from the LTR of MSV.

viruses were inoculated in parallel into either CV-1 monkey kidney cells or MEFC mouse cells. To prevent a disproportionate increase in the template numbers as a result of replication, infections of permissive monkey kidney cells were performed in the presence of hydroxyurea (0.01 mg/ml), at levels known to inhibit viral DNA replication. No inhibitors were added to nonpermissive mouse cells. The relative amount of T antigen synthesized after infection of monkey kidney cells by WT SV40 relative to the recombinant virus was approximately 5:1 (Fig. 2) as determined by scanning the fluorograph of the polyacrylamide gel. In contrast, after infection of mouse cells, roughly equal amounts of T antigen were synthesized by these viruses. Because the only significant difference between these two viral genomes resides in the tandem repeats, the level of expression of T antigen might be dependent in part upon this activator element and its relationship to the species of infected cell.

Transient Plasmid Expression. The insensitivity in detection and quantitation of T antigen in the above study led us to adopt an alternative method of demonstrating the relative "activator strength." We (17) recently had constructed a plasmid, pSV2 cat (Fig. 3), containing the early SV40 transcriptional promoter adjacent to the coding sequences for a bacterial enzyme, chloramphenicol acetyltransferase (CAT; acetyl-CoA:chloramphenicol 3-*O*-acetyltransferase, EC 2.3.1.28) which provides a number of advantages in assaying gene activity. The production of CAT in eukaryotic cells (which is dependent upon the eukaryotic promoter and enhancer sequences preceding this gene) can be measured sensitively and accurately. Furthermore, the level of CAT activity in cell extracts has been found to correlate with gene activity at the level of RNA production (unpublished data). Because the enzyme CAT is apparently absent from eukaryotic cells, background levels of gene expression are effectively zero.

The plasmid pSV2 cat (Fig. 3) contains the SV40 repeats, the SV40 early promoter, the CAT-encoding region in place of the former T antigen sequences, and the SV40 poly(A) addition site. A corresponding plasmid carrying the MSV repeats (pSrM2 cat) and a control plasmid lacking either set of repeats but retaining other promoter elements ($pA_{10}cat_2$) were constructed as described in Fig. 3.

Equivalent amounts of the control plasmid ($pA_{10}cat_2$) or the CAT plasmids containing the SV40 (pSV2 cat) or the MSV (pSrM2 cat) tandem repeats were introduced into the CV-1 line of monkey kidney cells by the calcium precipitation method (16, 17). Forty-eight hours after transfection, equivalent amounts of cells were harvested and the CAT activity of the cell lysates was assayed by thin-layer chromatography. An example of this assay at 30 min is shown in Fig. 4. Similar experiments were performed by transfecting these three plasmid constructs into mouse L cells. The percentage of [^{14}C]chloramphenicol converted to an acetylated form was determined by scraping spots from the plates and measuring the radioactivity in a liquid scintillation counter. These results, expressed as percentage conversion during the time of a standard reaction, are shown in Fig. 5.

In monkey kidney cells and in mouse cells, the gene activity of plasmids with no activator elements ($pA_{10}cat_2$) was extremely low (<0.5%). This level of conversion, however, was higher than that seen with either a mock cell lysate or with a recombinant containing no promoter elements (pSVO cat, Fig. 3). It is not clear whether the low level of residual gene activity exhibited by the control plasmid $pA_{10}cat_2$ reflects the retention of 20 nucleotides from one SV40 repeat or is due to some inherent activity elsewhere in this control region (for example, the 21-bp repeats). Nevertheless, the constructs containing

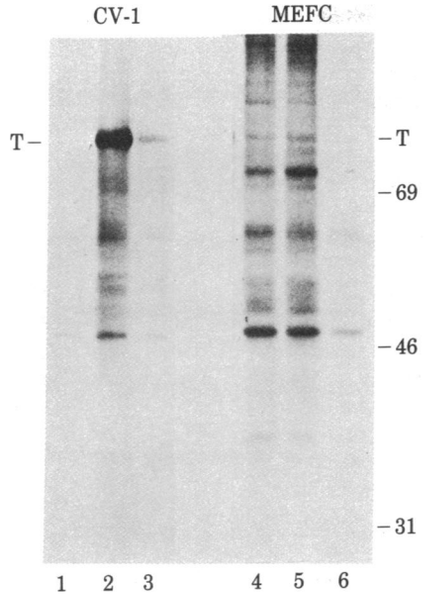

FIG. 2. Expression of SV40 T antigen after infection of CV-1 monkey kidney cells and MEFC mouse cells with WT SV40 and SVr^{MSV}. At 24 hr after infection, T antigen was labeled with [^{35}S]methionine, immunoprecipitated with anti-T antiserum, and analyzed by gel electrophoresis. A fluorograph of a typical gel is shown. Lanes: SV40 infection of CV-1 cells (lane 2) and MEFC cells (lane 5); SVr^{MSV} infection of CV-1 cells (lane 3) and MEFC cells (lane 4); immunoprecipitation of SV40-infected CV-1 cells (lane 1) and MEFC cells (lane 6) with normal hamster serum. The proteins observed in MEFC infections at molecular weight 73,000 and 45,000 were present in lane 6 (normal serum), although not clearly visible in this photograph. These bands are not related to T antigen. Numbers are molecular weight markers shown $\times 10^{-3}$.

complete enhancer elements showed significantly increased levels of *CAT* gene activity as assayed in extracts from either monkey (CV-1) or mouse (L) cells. In a standard 60-min reaction, the extracts from CV-1 cells transfected with the recombinant containing the SV40 repeats (pSV2 cat) converted approximately 11% of the chloramphenicol to an acetylated form while the plasmid containing the MSV repeats (pSrM2 cat) converted only 2% of the substrate (Fig. 5). Thus, the ratio of enhancement in CV-1 cells was approximately 5 times as great with the SV40 repeats as with the MSV repeats. Similar results were found in separate experiments using secondary African green monkey kidney cells (data not shown). Transfections of all three plasmids were also performed in human (HeLa) cells, and the ratio of SV40 expression to MSV expression was found to be approximately 5:1, similar to results observed in monkey cells.

The results obtained from transfection of mouse cells with the three plasmids are shown in Fig. 5. During the 60-min *in vitro* reaction, 6% of the substrate was converted by an extract of L cells transfected with pSV2 cat; the extract obtained after transfection of L cells with pSrM2 cat, which contains the MSV repeats, resulted in a 15% conversion; therefore, the ratio of activity for the SV40 to the MSV enhancers in mouse cells was approximately 2:5. Similar results were obtained when these plasmids were transfected into NIH 3T3, another line of mouse cells, (data not presented).

The percentage conversion of chloramphenicol with extracts obtained in four separate experiments was found to vary by 20%. Nevertheless, the ratios of activity for the MSV tandem repeats compared to the SV40 tandem repeats remained approximately the same. To show that similar amounts of DNA from each plasmid were transfected into tissue culture cells, we analyzed serial dilutions of cell extracts by hybridization with a nick-translated SV40 probe. These results demonstrated that there is no preferential uptake of one plasmid by a given cell line (data not shown). Thus, we conclude that, with a common SV40 promoter element, the *CAT* gene activity depends on the source of the activator sequences. The 72-bp tandem repeats from MSV stimulate gene activity to a higher level in two lines of mouse cells whereas the SV40 tandem repeats enhance gene activity to a greater extent in two lines of monkey kidney cells.

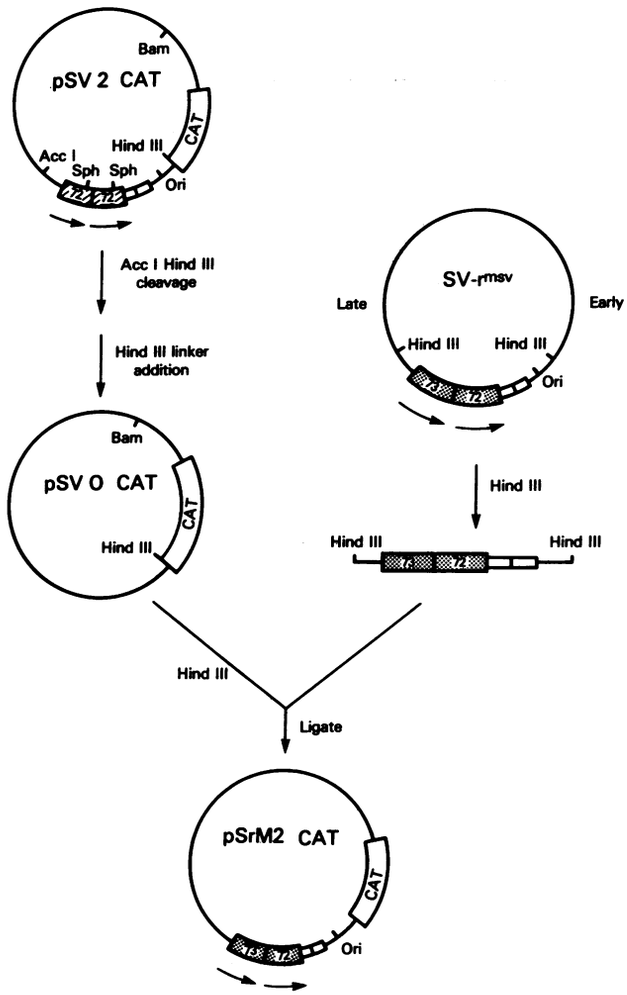

FIG. 3. Construction of plasmids carrying the SV40 and SVr^{MSV} transcription regulatory signals coupled to the SV40 chloramphenicol acetyltransferase gene (*CAT*). The plasmid pSV2 cat (17) contains the *Pvu* II/*Hin*dIII fragment of SV40 coupled to *CAT* in a plasmid carrying the *Pvu* II/*Eco*RI fragment from pBR322. The plasmid pSV2 cat was constructed by joining a fragment coding for chloramphenicol acetyltransferase (17) to the prokaryotic/eukaryotic vector pSV2 (18) which contains the pBR322 origin and ampicillin-resistance gene, a SV40 early transcription unit with promoter, T-antigen encoding sequences, and polyadenylylation site. The plasmid pSV0 cat was constructed by deleting the *Acc* I/*Hin*dIII fragment from pSV2 cat and religating the plasmid after the addition of *Hin*dIII linkers. The *Hin*dIII fragment from SVr^{MSV}, carrying the 72-bp tandem repeats from the LTR of MSV coupled to the 21-bp repeats and Goldberg–Hogness box of SV40, was ligated into the *Hin*dIII site of pSV0 cat. The resultant plasmid, pSrM2 cat, coupled the transcription regulatory sequences of SVr^{MSV} to *CAT* in a manner similar to that of pSV2 cat. A plasmid, $pA_{10}cat_2$, carrying the 21-bp repeated sequences and Goldberg–Hogness box coupled to *CAT* lacking tandem repeated sequences was generated by joining the *Sph* I/*Bam*HI fragment of pA_{10} (a pBR322 derivative plasmid) with the *Sph* I/*Bam*HI fragment of pSV2 cat.

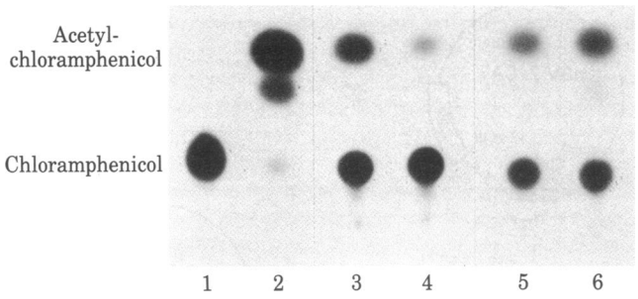

FIG. 4. Expression of CAT as determined by thin-layer chromatography. After incubation of [^{14}C]chloramphenicol and acetyl-CoA with cell extracts containing unknown quantities of CAT, the acetylated forms of chloramphenicol were separated from the nonacetylated form by thin-layer chromatography in chloroform/methanol, 95:5 (vol/vol). A typical autoradiograph of the developed thin-layer plate is presented in which the two monoacetylated forms of chloramphenicol migrate faster than the unmodified substrate. Samples represent chloramphenicol treated with: mock extract (lane 1), a commercially obtained CAT enzyme (lane 2), extracts from pSV2 cat-transfected CV-1 cells (lane 3) and L cells (lane 5); and extracts from pSrM2 cat-transfected CV-1 cells (lane 4) and L cells (lane 6).

DISCUSSION

The 72-bp tandem repeats of SV40 represent a eukaryotic regulatory element that is involved in the activation or enhancement of early viral transcription (5–9) and is capable of enhancing the transformation of LMTK$^-$ cells by the herpes simplex virus thymidine kinase gene (10). An unusual feature of the activator sequence as a control element is that it retains activity when inverted or when moved in either direction from its normal position (refs. 9, 11, 13, and 19; unpublished data). These features clearly differentiate the activator sequence from the classical elements of a promoter (e.g., the T-A-T-A box or binding site) which appear to bear a fixed relationship to the 5′ end of the transcription unit.

Although a number of viral genomes—such as SV40 (5, 6), polyoma virus (19, 20), BK virus (unpublished data), bovine papilloma virus (M. Lusky and M. Botchan, personal communication), and certain retroviruses (12, 21, 22)—contain activator elements, there is little sequence homology among these various elements. Nevertheless, we recently found that one set of core nucleotides frequently recurs. The sequence T-G-T-G-G-A-A-A-G in SV40 has a potential counterpart, T-G-T-G-G-T-A-A-G, in MSV (underlined in Fig. 1). Similar sets of nucleotides also can be found in the activator regions of BK virus, polyomavirus, and bovine papillomavirus. Although the role of such core sequences remains to be determined, preliminary data suggest that these nucleotides reside in a critical region of the activator element (unpublished data). We recently found that a recombinant viral genome in which the MSV 72-bp tandem repeats are substituted for the SV40 tandem repeats remains viable in monkey kidney cells (12). It was noted, however, that the recombinant virus grows more slowly in monkey kidney cells than does the WT SV40. Yet, in nonpermissive mouse cells, equivalent amounts of T antigen were synthesized. Because the essential difference between the two viruses resides in their activator sequences, we considered the possibility that cellular macromolecules might interact with these sequences in a host-specific fashion. In this study, the coding sequences for CAT were cloned into plasmids downstream from the SV40 promoter sequences and either the activator from SV40 or MSV. It was demonstrated that increased amounts of the CAT enzyme were synthesized in mouse cells transfected with the plasmid harboring the MSV activator and in monkey kidney cells transfected with a plasmid containing the SV40 activator. Because the amount of DNA uptake was similar for both plasmids, this result suggests that the repeats from the monkey virus (SV40) are more active in monkey cells whereas those from the mouse virus (MSV) are more active in mouse cells.

These experiments were designed to test the relative strength of two activator sequences as they affect the SV40 promoter. It is clear that the overall transcriptional strength of a viral activator–promoter complex in a specific cell will not necessarily reflect the host range of the virus. It has been found that a plasmid containing the entire Rous sarcoma virus LTR is more active in the CAT assay in monkey kidney cells than is pSV2 cat (unpublished data). Several studies have recently provided evidence that indicates that endogenous cellular genes can be activated by the insertion of promoter elements from retroviral LTRs (23–26). The demonstration that certain LTRs contain enhancer elements (ref. 12; M. Kriegler and M. Botchan, personal communication) suggests that activation of endogenous genes, which already have promoters, also might be regulated by the insertion or modification of enhancer sequences.

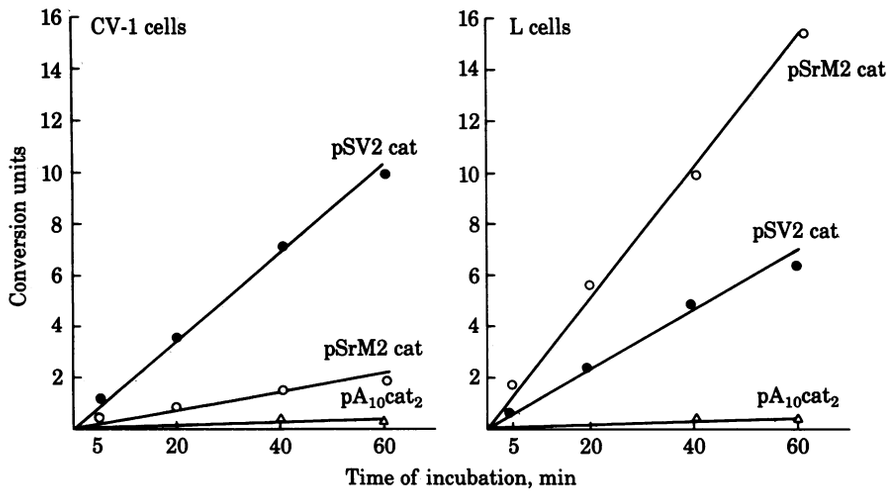

FIG. 5. Host-specific activation of the *CAT* gene by the 72-bp repeat sequences from MSV and from SV40. The amount of chloramphenicol acetylated by extracts from cells transfected with pSV2 cat (SV40 repeats), pSrM2 cat (MSV repeat), and $pA_{10}cat_2$ (no repeats) is shown as a function of time. One conversion unit represents acetylation of 1% of the substrate [^{14}C]chloramphenicol by one-fifth of an extract from a 10-cm dish of cells that had been transfected with 25 μg of plasmid DNA.

The finding that enhancer sequences act in a host-specific manner suggests that they preferentially recognize host-cell factors. Further insight into the potential host-specific nature of activator elements is provided by recent studies on the growth of polyomaviruses in embryonal carcinoma cells. Although WT polyomavirus is unable to grow in undifferentiated embryonal carcinoma cells, a number of viral mutants have been selected that can overcome what is presumably a block at the level of early viral gene expression (27–30). In all cases to date, the alterations in these mutants appear to reside in the region of the virus harboring elements analogous to the SV40 72-bp repeats. This would suggest that undifferentiated embryonal carcinoma cells contain host functions that cannot interact with the WT sequences but can recognize the mutant virus DNA.

Speculation on the nature of these host-cell factors is limited by our lack of understanding of the mechanism of activation. One model suggests that the activator sequence provides a DNA entry site for RNA polymerase II or a polymerase-associated molecule (e.g., a σ-like factor). As a second possibility, activators may provide binding sites for host-specific molecules that induce a change in the inherent conformation or chromatin structure of the template. The resulting structural alterations could render the DNA more active transcriptionally. Alternatively, the host-specific molecule reacting with enhancer sequences could simply sequester the transcriptional template in a cellular compartment that enhances its transcriptional activity.

The mechanism of action of enhancers remains unclear, yet the ability of the tandem repeats from both SV40 and MSV to function in monkey cells and mouse cells suggests that molecules capable of interacting with the various enhancer sequences may reside in all cells. The relative host specificity of this enhancement suggests, however, that a preferential interaction occurs between the host cell macromolecules and a specific activator element.

We thank M. Kessel, D. Cosman, N. Rosenthal, and R. Muschel for helpful discussions and M. Priest for the preparation of the manuscript. L.A.L. is a Damon Runyon–Walter Winchell Cancer Fund Fellow.

1. Benoist, C. & Chambon, P. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3865–3869.
2. Ghosh, P. K., Lebowitz, P., Frisque, R. J. & Gluzman, Y. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 100–104.
3. Gluzman, Y., Sambrook, J. & Frisque, R. J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3898–3902.
4. Grosschedl, R. & Birnstiel, M. L. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1432–1436.
5. Benoist, C. & Chambon, P. (1981) *Nature (London)* **290**, 304–310.
6. Gruss, P., Dhar, R. & Khoury, G. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 943–947.
7. Mellon, P., Parker, V., Gluzman, Y. & Maniatis, T. (1981) *Cell* **27**, 279–288.
8. Lee, F., Mulligan, R., Berg, P. & Ringold, G. (1981) *Nature (London)* **294**, 228–232.
9. Moreau, P., Hen, R., Everett, R., Gaub, M. P. & Chambon, P. (1981) *Nucleic Acids Res.* **9**, 6047–6068.
10. Capecchi, M. R. (1980) *Cell* **22**, 479–488.
11. Banerji, J., Rusconi, S. & Schaffner, W. (1981) *Cell* **27**, 299–308.
12. Levinson, B., Khoury, G., VandeWoude, G. & Gruss, P. (1982) *Nature (London)* **295**, 568–572.
13. Fromm, M. & Berg, P. (1982) *J. Mol. Appl. Genet.*, in press.
14. Laemmli, U. (1970) *Nature (London)* **227**, 680–685.
15. Goodman, H. & MacDonald, R. (1979) *Methods Enzymol.* **68**, 75–89.
16. Graham, F. & van der Eb, A. (1973) *Virology* **52**, 456–467.
17. Gorman, C., Moffat, L. & Howard, B. (1982) *Mol. Cell Biol.*, in press.
18. Howard, B., Southern, P., Mulligan, R. & Berg, P. (1982) *J. Mol. Appl. Genet.*, in press.
19. deVilliers, J. & Schaffner, W. (1981) *Nucleic Acids Res.* **9**, 6251–6254.
20. Tyndall, C., LaMantia, G., Tacker, C. M., Favaloro, J. & Kamen, R. (1981) *Nucleic Acids Res.* **9**, 6231–6250.
21. Huang, A. L., Ostrowski, M. C., Bernard, D. & Hager, G. L. (1981) *Cell* **27**, 245–255.
22. Chang, E. H., Ellis, R. W., Scolnick, E. M. & Lowy, D. R. (1980) *Science* **210**, 1249–1251.
23. Blair, D. G., Oskarsson, M., Wood, T. G., McClements, W. L., Fishinger, P. J. & VandeWoude, G. F. (1981) *Science* **212**, 941–943.
24. Hayward, W. S., Neel, B. G. & Astrin, S. M. (1981) *Nature (London)* **290**, 475–480.
25. Payne, G. S., Bishop, J. M. & Varmus, H. E. (1982) *Nature (London)* **295**, 209–214.
26. Neel, B. G., Hayward, W. S., Robinson, H. L., Fang, J. & Astrin, S. M. (1981) *Cell* **23**, 323–334.
27. Katinka, M., Yaniv, M., Vasseur, M. & Blangy, D. (1980) *Cell* **20**, 393–399.
28. Katinka, M., Vasseur, M., Montreau, N., Yaniv, M. & Blangy, D. (1981) *Nature (London)* **290**, 720–722.
29. Fujimura, F. K., Deininger, P. L., Friedmann, T. & Linney, E. (1981) *Cell* **23**, 809–814.
30. Sekikawa, K. & Levine, A. J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6556–6560.