MOLECULAR AND CELLULAR BIOLOGY, July 1986, p. 2593–2601
0021-9193/86/072593-09$02.00/0

Vol. 6, No. 7

# Effects of the Position of the Simian Virus 40 Enhancer on Expression of Multiple Transcription Units in a Single Plasmid

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Received 27 January 1986/Accepted 7 April 1986

**We have examined the ability of the simian virus 40 72-base pair enhancer segment to simultaneously activate multiple transcription units with plasmids that contain one, two, or three simian virus 40-based transcription units in various arrangements. After transfection into CV1 cells, the expression of a marker gene, Ecogpt, was determined as a function of the position of that marker gene relative to the other transcription units and the position of the marker gene relative to enhancer elements on the plasmids. Two types of position effects were revealed by that analysis. The first, promoter occlusion, causes reduced transcription at a downstream promoter if transcription is initiated at a nearby upstream promoter. This effect does not involve enhancer elements directly, even though the effect is most pronounced when the downstream promoter lacks an enhancer element. The second effect stems from the ability of promoter sequences to reduce the effect of a single enhancer element on other promoters in the same plasmid. This latter effect is mediated by either promoters adjacent to the enhancer element or promoters interposed between the enhancer element and the other promoters on the plasmid.**

The DNA sequences required for eucaryotic class II promoter function are located about 20 to 110 base pairs (bp) upstream of the site of transcription initiation; in some cases this region can be subdivided into distinct groups of essential nucleotides (15, 31). Transcription from certain promoters can also be influenced by additional DNA sequences called enhancers. These elements act in *cis* to increase the rate of transcription from a variety of promoters (for review, see reference 26). The unique features of enhancers are that they often function in an orientation-independent manner and can act over distances of several kilobases (3, 17, 34). They are frequently associated with viral genomes such as simian virus 40 (SV40) (3, 15, 34), polyomavirus (12), BK virus (40), adenovirus (22), bovine papillomavirus (29), and retroviral long terminal repeats (5, 27), but have also been found in cellular DNA (3, 8, 18, 32). Some enhancers, particularly the SV40 enhancer, are able to activate the promoters of a variety of genes: herpes simplex virus thymidine kinase (28), beta-globin (3, 4, 24, 46), conalbumin (34), and the adenovirus major later region (34).

Very little is known about how enhancers function. Enhancers generally define sites of DNase hypersensitivity in chromatin (17, 25), and one has been associated with a region of "Z DNA" (38). Since enhancers exhibit various degrees of cell type specificity, it has been inferred that they interact with particular cellular components, perhaps proteins (3, 11, 18, 27). Comparisons of different enhancer elements have demonstrated only weak homologies at the level of DNA sequence (50).

Our present work focuses on how the SV40 early region promoter is influenced by the enhancer contained in the 72-bp repeat segment that is located 107 to 251 bp upstream from the start of transcription. In particular, we examined how the enhancer affects the activity of promoters associated with multiple transcription units on the same plasmid. Thus, plasmids with one, two, or three tandem transcription units associated with enhancer-containing or enhancer-deficient promoters were transfected into cells, and the expression of one of the transcription units was monitored as a function of its position relative to the other two. The same protocol allowed us to determine how the position of the enhancer, whether associated with the promoter or by itself, influenced the expression of the marker transcription unit. Our results indicate that there are indeed pronounced effects of enhancer position on the activation of these transcription units. The implications of these results are discussed in terms of possible mechanisms of enhancer function and some general features of eucaryotic transcription regulation.

## MATERIALS AND METHODS

**Plasmid DNAs.** All plasmids were constructed by using standard techniques (30).

Plasmid pSV2Agpt (see Fig. 1) was derived from pSV2gpt (35) as follows. The unique *Pvu*II restriction site of pSV2gpt was converted to a *Bam*HI restriction site by the introduction of a pBam$_{10}$ linker fragment (Collaborative Research). The 237-bp *Mbo*I G fragment of SV40 DNA which contains the early-region polyadenylation signal (6) was converted to head-to-tail concatemers by ligation of their *Bam*HI and *Bcl*I mutually cohesive termini. The dimer-length restriction fragments were purified by gel electrophoresis (47) and inserted into the synthetic *Bam*HI restriction site of the modified pSV2gpt. The head-to-tail dimer of the SV40 *Mbo*I G fragment in pSV2Agpt is oriented so that the direction of "early transcription" is counterclockwise toward the SV-gpt transcription unit. Note that a *Bam*HI restriction site is conserved at the junction of the dimer fragment and the SV40 promoter (see Fig. 1). Plasmid pSV232A is identical to pSV2Agpt except that the SV40 early promoter's enhancer is deleted; this promoter was previously described as the S-232 mutant by Fromm and Berg (15). The *Sal*I restriction site of S-232 was first converted to a *Bam*HI restriction site by linker insertion, and the resulting *Bam*HI-*Hin*dIII restric-

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tion fragment that carries the mutant promoter replaced the corresponding wild-type promoter fragment in pSV2Agpt (see Fig. 1).

The plasmids that contain the two transcription units gpt and neo (e.g., pSV2Aneo2Agpt; see Fig. 2) were made by inserting a neo transcription unit from either pSV2Aneo or pSV232Aneo (modified forms of pSV2neo [43]) into either of the two *Bam*HI restriction sites present in pSV2Agpt or pSV232Agpt.

Plasmids containing three transcription units neo, gpt, and dhfr (44) in tandem [pSV232A(neo/gpt/dhfr); see Fig. 5] were constructed by methods analogous to those described above. In this case plasmid pSV232Adhfr was first derived from pSV2dhfr (44). The *Bam*HI restriction fragment that contains the SV-dhfr transcription unit was then inserted into the most distal *Bam*HI restriction site of pSV232Aneo232Agpt.

The DNA fragment referred to as the SV40 "enhancer element" was derived from the X-100 deletion of Fromm and Berg (15). The 175-bp fragment obtained by digestion of X-100 DNA with *Xho*I and *Pvu*II restriction endonucleases, and containing the enhancer sequence, was incubated with *Escherichia coli* DNA polymerase I and the four deoxynucleoside triphosphates to destroy the *Xho*I cohesive end; $pBam_{10}$ linkers were ligated to each end of the DNA fragment, and then it was inserted into one of the *Bam*HI restriction sites in the aforementioned plasmids.

**Nomenclature of plasmids.** The plasmids are named according to the 5′ to 3′ transcriptional order of the genes contained on the plasmid. When the number 2 precedes the genes' names, it indicates that the promoter is the wild-type SV40 early promoter; the number 232 indicates a promoter lacking the enhancer; and a zero means that there is no promoter preceding the gene. "LR" indicates that the enhancer element is situated 3′ to the most "downstream" gene on the plasmid. For example, pSV2Aneo232Agpt-LR represents a plasmid whose neo transcription unit, containing a wild-type SV40 promoter, is upstream of the gpt transcription unit whose promoter lacks the enhancer but which contains the enhancer at its 3′ end (see Fig. 4D).

**Assays of transformation.** CV1 cells (33) were transfected with circular plasmid DNA (10 μg/10-cm dish) by the calcium phosphate coprecipitation technique of Graham and van der Eb (20) as modified by Parker and Stark (39). Cells were transferred 40 to 48 h before transfection at a density of approximately $10^6$ cells per 10-cm dish. Following transfection and 2 days of growth under nonselective conditions, the cells were split 1:10 and 1:20 into media selective for the $gpt^+$ phenotype (36). The cultures were fed every 4 to 5 days; after 2 to 3 weeks they were stained and the surviving colonies were counted. The transformation frequency for each plasmid is expressed relative to those obtained with pSV2Agpt in the same experiment (generally $1 \times 10^{-4}$ to $2 \times 10^{-4}$ per cell) and represents the number average obtained from at least four separate transfections.

**Assays of XGPRTase.** CV1 cells were transfected as described above, except that 5 μg of either pCH110 (for β-galactosidase [21]) or pSV2cat (for chloramphenicol acetyltransferase [19]) was added to each precipitation to normalize transfection efficiencies (see below). Two days after the transfections the cells were washed two times with ice-cold Tris-buffered saline and scraped into cold Tris-buffered saline containing 15% (vol/vol) glycerol and 10 mM dithiothreitol (0.4 ml/10-cm dish). After sonication 0.1 volume of 1% Nonidet P-40–0.5% deoxycholate was added, and following incubation for 30 min on ice, the cellular debris was removed by centrifugation. Protein extracts were stored at −70°C without noticeable loss of XGPRTase activity.

Each extract was assayed for β-galactosidase or chloramphenicol acetyltransferase activity as described in reference 21 or 19, respectively. The enzyme activities obtained from these assays were assumed to indicate relative transfection efficiencies and thus were used to determine the relative amounts of each extract to be loaded on the protein gels for determination of XGPRTase (generally 15 to 80 μl of extract was loaded per lane).

The cell extracts were electrophoresed on gels consisting of a 9-cm resolving gel (7.5% acrylamide, 0.2% bis, 70 mM Tris chloride, pH 7.5) and a 2-cm stacking gel (5% acrylamide, 0.14% bis, 50 mM Tris-$PO_4$, pH 5.5). Proteins in the extracts were loaded in 8% glycerol plus 0.005% bromophenol blue and stacked at 50 V and resolved at 250 V at 4°C in barbital buffer (5.52 g of diethylbarbituric acid, 1 g of Tris base, pH 7.0, per liter). At the completion of the electrophoresis the gel slabs were placed on the surface of a moistened sheet of polyethyleneimine-cellulose (Polygram Cel 300 PEI; Brinkmann Instruments, Inc.) and overlaid with a reaction cocktail cast in a slab of 0.5% agarose (1.5 mm thick). This reaction slab (33 mM Tris chloride, pH 8.7, 7 mM $MgCl_2$, 4 mM dithiothreitol, 1 mM PRPP, 0.2 mCi of [$^3$H]guanine) was cast between gel plates to mimic the shape of the acrylamide gel. The entire sandwich was incubated uncovered for 2 h at 37°C. The polyethyleneimine-cellulose sheet was removed from the sandwich, washed in water for 15 min, dried, dipped in 2-methylnaphthalene containing 0.4% PPO (2,5-diphenyloxazole), and autoradiographed at −70°C.

The XGPRTase activities, determined by densitometric tracing of the autoradiograms, were subject to only small variation (<20% from one experiment to another) and were linear with the amount of extract loaded on the gels. The XGPRTase activity with each of the experimental plasmids was compared with the activity obtained in a pSV2Agpt transfection carried out in parallel. Each value represents the number average obtained from at least four separate transfections.

**Analysis of transcribed RNAs.** Poly(A)-containing RNA was prepared from cells 2 days after transfections. Poly(A)-containing RNA corresponding to between 50 and 200 μg of total cellular RNA was denatured with 7% formaldehyde (65°C, 20 min.) and applied to nitrocellulose essentially as described by Thomas (45). DNA fragments corresponding to the coding sequences of the neo, gpt, and dhfr transcription units were labeled with [α-$^{32}$P]dCTP, using random hexanucleotide primers (13), and used as hybridization probes. RNA samples from each transfection were applied to the nitrocellulose in serial twofold dilutions to confirm the linearity of the autoradiographic signal.

## RESULTS

The essential design of the gpt transcription unit used in these studies is illustrated in Fig. 1. The transcription unit contains an SV40 early promoter, the Ecogpt gene (gpt), the intron corresponding to the SV40 small t-antigen mRNA, and the SV40 early-region polyadenylation signal. The plasmid that carries this and related transcription units, pSV2Agpt, differs from pSV2gpt (described in detail elsewhere [35]) in having two tandem DNA segments each containing the SV40 early polyadenylation signal immediately 5′ to the SV40 early promoter (see Materials and Methods). These fragments reduce the level of gpt expression in CV1 cells to essentially zero (as measured by

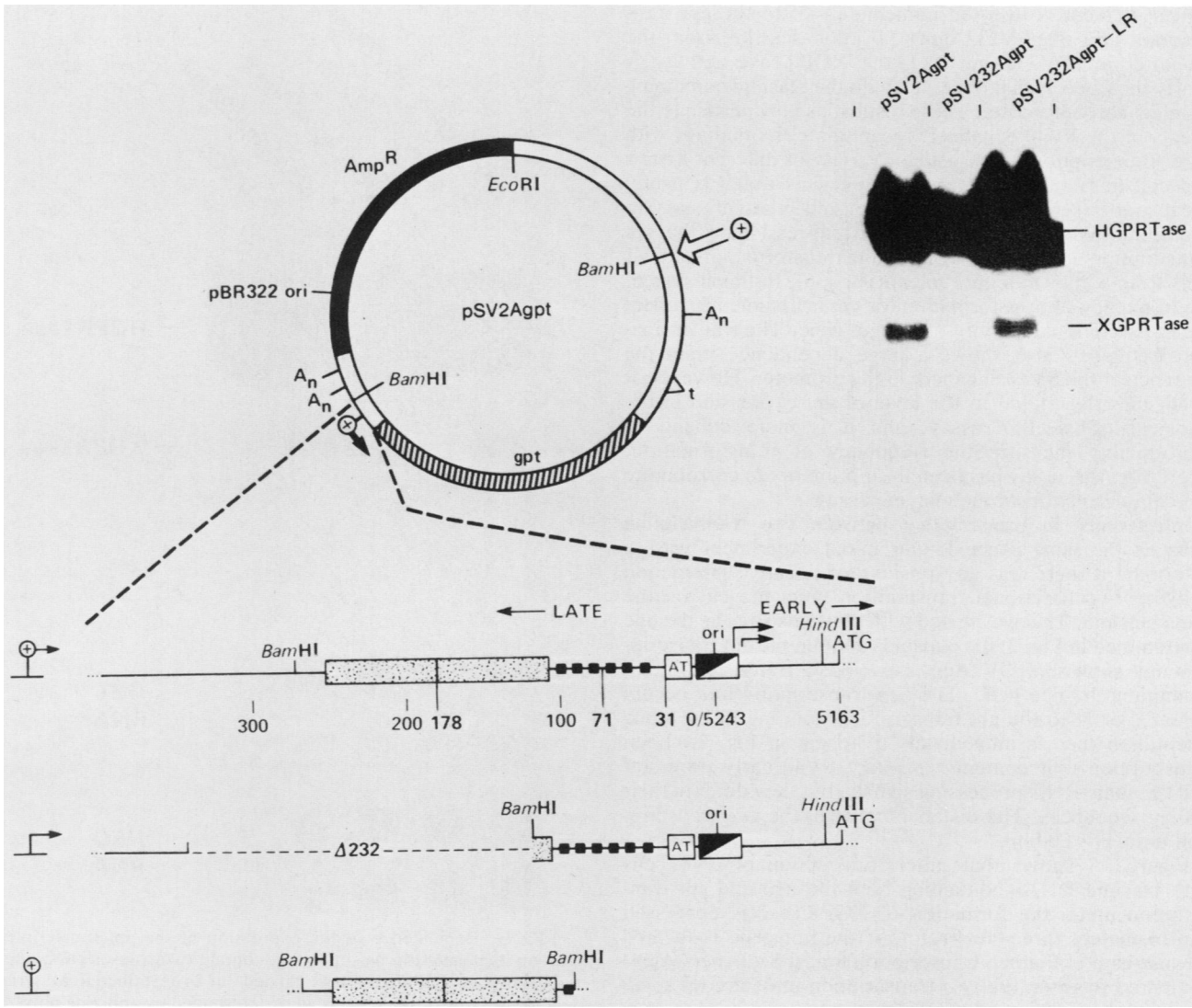


FIG. 1. Structure of a plasmid containing the gpt transcription units and expression of XGPRTase. The various segments of the plasmid are represented as follows: ⊙→, the wild-type SV40 early region promoter in pSV2Agpt; ⌐→, the enhancer-deleted SV40 promoter in pSV2323Agpt; and ⊕, the enhancer element inserted into the *Bam*HI site indicated on pSV232Agpt-LR. Autoradiogram at upper right indicates the relative XGPRTase activities in extracts 2 days after transfection of CV1 cells with the indicated plasmids (see Materials and Methods).

transformation frequency and XGPRTase levels) when there is either no SV40 promoter or a mutant SV40 promoter fused to the gpt gene (41; our unpublished observations). Low levels of gpt expression in the unmodified plasmids that carry mutant SV40 promoters probably results from the existence of weak transcription start sites in plasmid sequences or even in genomic DNA adjacent to the plasmids after integration. The polyadenylation signals presumably cause truncation of these transcripts and prevent incorporation of the gpt sequence into a stable mRNA. Hence, the transcripts containing expressible gpt genes are most likely to arise from transcription that initiates within the SV40 promoter region of pSV2Agpt and related plasmids (see below).

Three SV40 early region promoter elements will be discussed (Fig. 1). The pSV2A contains the intact or wild-type SV40 promoter which spans the SV40 DNA segment between nucleotides 5171 and 270; this region includes the major transcription start site, the origin of DNA replication, the adenine-plus-thymine-rich block, the quanine-plus-cytosine-rich repeats, and the enhancer element (the 72-bp repeats). pSV*232*A designates the plasmid whose promoter lacks the enhancer (i.e., the 72-bp repeat segment) and extends from nucleotides 5171 to 114. The third element, referred to as the enhancer or "72-bp repeat" element (-LR), consists of the segment of DNA between nucleotides 95 and 270 (with *Bam*HI linkers at each terminus).

Two assays have been used to assess the levels of gpt transcription in CV1 cells. The first measures XGPRTase production during transient transfections. These values are normalized to the activities of chloramphenicol acetyltransferase or β-galactosidase plasmids (see Materials and Methods). Figure 1 shows that this assay is sensitive and that there is a >50-fold dependence of XGPRTase activity on the presence of the SV40 enhancer in the promoter (cf. PSV2Agpt and pSV232Agpt). However, if the SV40 en-

hancer element is inserted immediately 3′ to the gpt transcription unit of pSV232Agpt (2.0 kilobases [kb] from the promoter in pSV232Agpt-LR), the XGPRTase activity is nearly the same as that observed with the plasmid containing an intact early promoter. These results, as they pertain to the effects of the SV40 enhancer, recapitulate the findings with other transcription units, using a variety of different assays and cell lines (3, 15–17, 28, 34, 46). This assay is useful because it is sensitive, reproducible, and relatively easy to quantitate. We have also measured expression of the gpt transcription unit by measuring stable transformation of CV1 cells from a $gpt^-$ to a $gpt^+$ phenotype (36). In this instance, the frequency of transformation for gpt is assumed to reflect the transcriptional activity of the gpt gene. The transformation frequency also shows a large dependence upon the presence of the SV40 enhancer in the promoter. However, it is not linearly related to the level of gpt expression in the transient transfection assay, and it is more difficult to reproducibly measure the frequency of transformation. Therefore, this assay has been used primarily to corroborate the data derived from the enzyme assays.

**Interference in transcription between two transcription units on the same plasmid.** Our initial experiment was to determine if there was any positive or negative interaction between two functional transcription units present on the same plasmid. This was tested with plasmids such as the one diagrammed in Fig. 2; the plasmids contain the gpt transcription unit shown in pSV2Agpt as well as a transcription unit containing the neo gene. The neo transcription unit occurs either 3′ or 5′ to the gpt transcription unit and in the same orientation (neo is immediately 5′ to gpt in Fig. 2). Each transcription unit contains an intact SV40 early promoter and the same RNA processing signals, but they differ in their coding sequences. The distance between the two transcription units is <200 bp.

Figure 3A shows that, after transfection of CV1 cells with plasmid DNAs containing both the neo and gpt transcription units, the formation of XGPRTase is depressed approximately three- to fivefold if the gpt gene is located downstream of the neo transcription unit (pSV2Aneo2Agpt) compared to when the two transcription units are on separate plasmids (pSV2Aneo + pSV2Agpt). This impairment of XGPRTase activity is not observed if the gpt transcription unit is upstream of the neo transcription unit (pSV2Agpt2Aneo) or when the upstream neo transcription unit lacks the SV40 early promoter (pSV0Aneo2Agpt). These results indicate that expression of the gpt transcription unit is inhibited because the upstream neo transcription unit is transcribed and not because the neo transcription unit contains an inhibitory sequence or because there is a second transcription unit on the same plasmid. An analysis of the RNA transcribed from the gpt and neo transcription units shows the same consistent trend: regardless of the particular coding sequence, the upstream transcription unit is expressed more efficiently than the downstream gene (Fig. 3B). A similar interference of transcription in tandemly arrayed transcription units has already been described in a bacterial

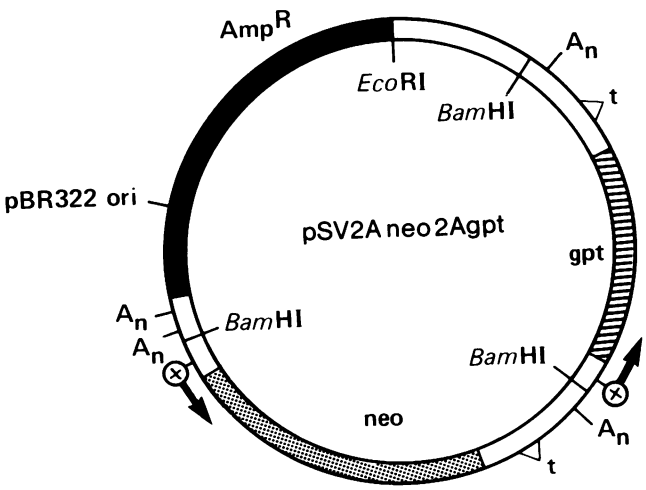


FIG. 2. Structure of pSV2Aneo2Agpt and related plasmids with two transcription units. Transcription units that contain the neo and gpt coding sequences are on *Bam*HI restriction fragments. The neo and gpt transcription units can occur in either order: neo upstream and gpt downstream or the reverse.

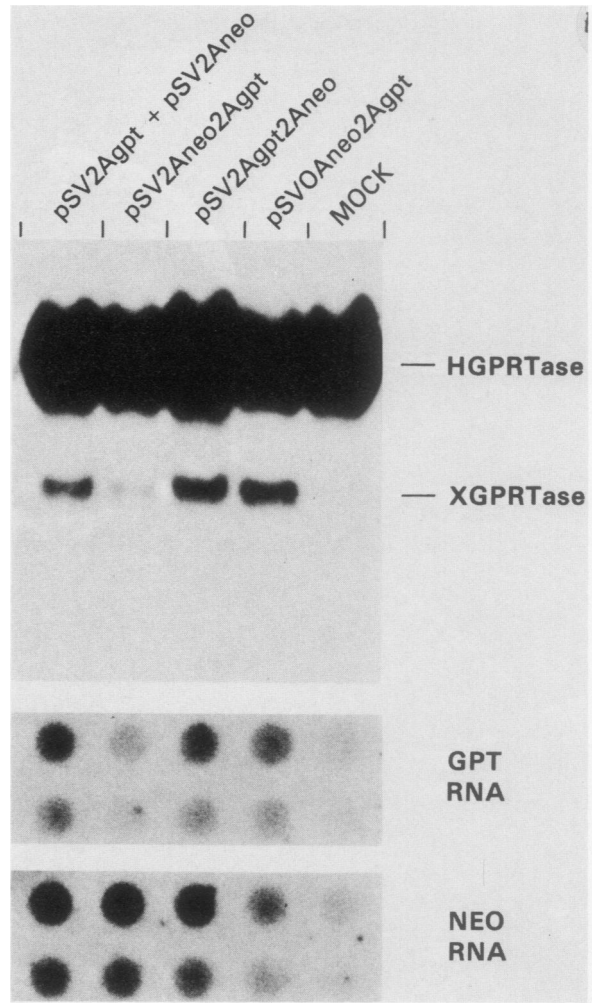


FIG. 3. Dependence of the expression of the gpt transcription unit on its position in plasmids that contain two transcription units. (A) Assays of XGPRTase were carried out as described in Materials and Methods. (B) RNA levels were determined by applying poly(A)-containing RNA corresponding to approximately 200 μg of total RNA (row 1) and serial twofold dilutions of that amount (row 2) to two separate nitrocellulose filters. RNA blots were then hybridized with DNA probes corresponding to gpt and neo coding sequences as indicated. "pSV2Agpt + pSV2Aneo" indicates transfections that were carried out with the neo and gpt transcription units on separate plasmids.

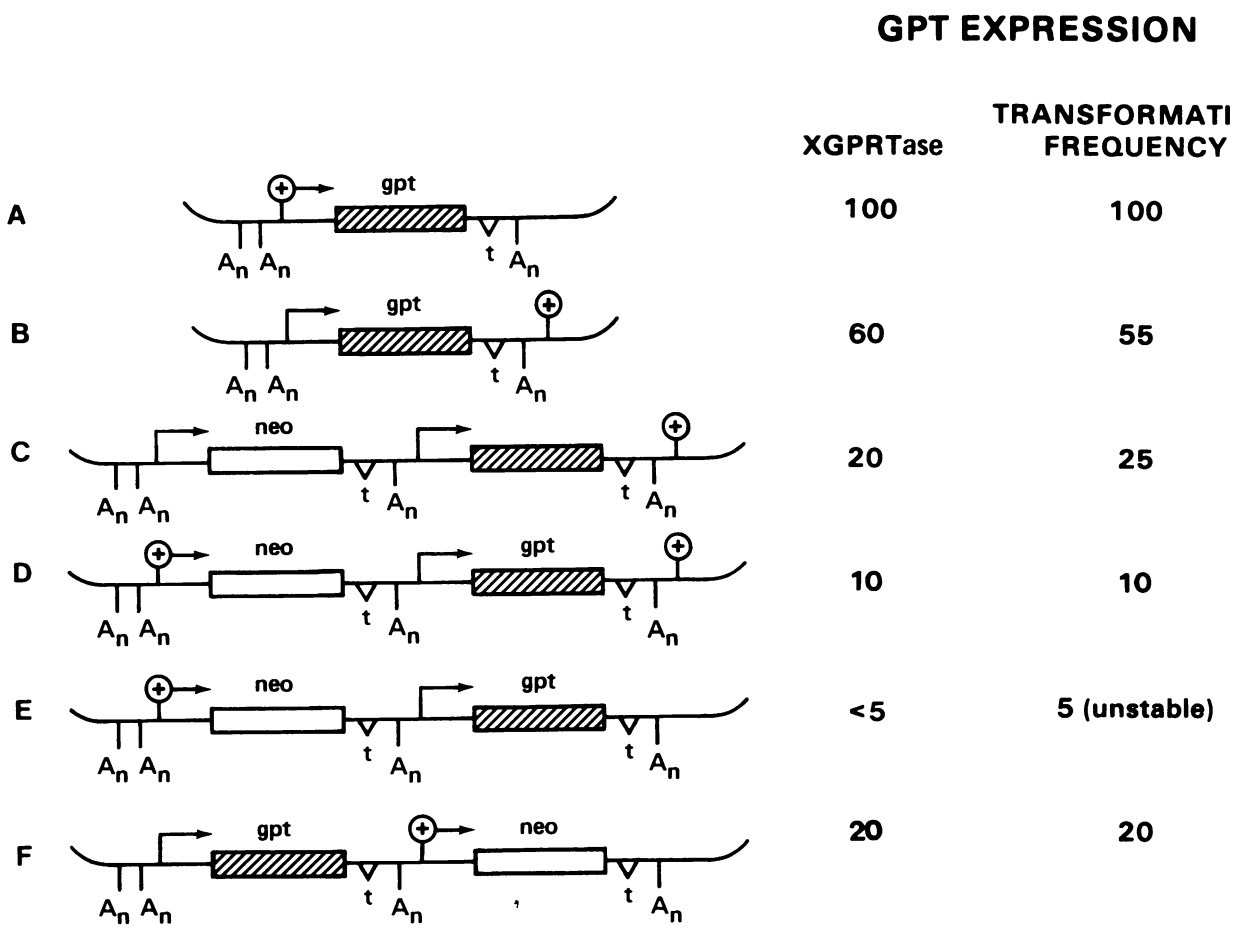


FIG. 4. Effects of the positions of the enhancer and transcription units on the expression of the gpt transcription unit in plasmids that contain two transcription units. The structure of each plasmid is represented schematically: ⊕→ indicates a wild-type SV40 early region promoter; ⌐→ indicates an enhancer-deleted SV40 promoter; and ⊕ indicates that SV40 enhancer segment alone (see Fig. 1). XGPRTase was determined in extracts obtained from cells transfected with the indicated plasmid and the autoradiograms were scanned by densitometry; the values shown are the number average from four separate experiments expressed to the nearest 5%. Transformation frequencies were determined as described in Materials and Methods and are also represented as number averages relative to the transformation frequencies obtained with pSV2Agpt. "Unstable" indicates that colonies stopped growing after reaching about 20 to 50 cells.

system (see Discussion) and is referred to as "promoter occlusion" (1).

The effect of tandem transcription units is quite pronounced if the promoter on the downstream gene lacks the 72-bp repeat segment. The level of expression from the downstream enhancerless SV40 promoter is 50- to 100-fold lower as measured by either XGPRTase formation during transient transfections or stable transformation (Fig. 4E). However, unlike the result obtained in Fig. 1 and 4B, where the insertion of the enhancer downstream of an enhancerless transcription unit stimulates expression to nearly the level of an intact promoter, there is only a marginal effect of a 3′ enhancer if the enhancerless transcription unit is preceded by an intact functional transcription unit. Note that expression of such a downstream gpt transcription unit is less impaired if the upstream transcription unit also lacks the associated enhancer sequence (Fig. 4B versus 4C).

The enhancement of the gpt transcription unit's expression by the 3′-enhancer segment is also diminished if that enhancer is part of a second functional promoter. Thus, when the enhancer-promoter combination occurs at the 3′ end of the enhancerless gpt transcription unit, the activation of gpt expression is not as great as with the enhancer alone (Fig. 4B versus 4F). These results indicate that enhancement of the SV40 early promoter is reduced when the enhancer sequence is associated with a second functional promoter and that the location of that promoter-enhancer unit relative to the position of the promoter is highly relevant.

**Effect of enhancer position on activation of three tandemly arranged transcription units.** To determine how an enhancer sequence affects multiple enhancer-dependent promoters on the same plasmid, we constructed a series of plasmids containing three tandem transcription units, each driven by an SV40 early region promoter lacking the enhancer. The structural features common to these plasmids [designated pSV232A(neo/gpt/dhfr)] are illustrated in Fig. 5. They resemble pSV2Aneo2Agpt except that, in addition to the gpt and neo transcription units, they contain a third transcription unit with the mouse dihydrofolate reductase cDNA (44) as the expressed function; the dhfr transcription unit is located 3′ to the gpt transcription unit. Furthermore, each of the promoters in the three transcription units lack the 72-bp enhancer element. Also, the 5′ noncoding sequences of the gpt segment (between *Hin*dIII and *Bgl*II) have been deleted to increase the translation efficiency of the RNA and increase the yield of XGPRTase (37). To test the effect of the

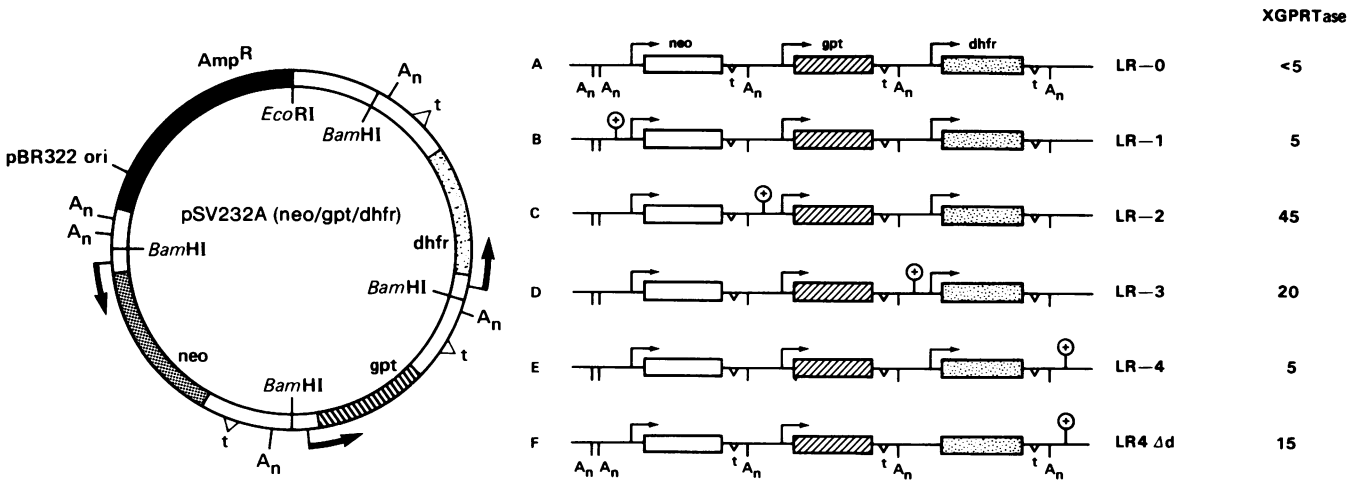


FIG. 5. Structure of pSV232A(neo/gpt/dhfr) plasmids and XGPRTase levels resulting from the insertion of the SV40 enhancer at various positions. Plasmid nomenclature is as described in the legend to Fig. 4 (see also Fig. 6). The XGPRTase activities are expressed relative to those obtained with pSV2Agpt (100%), and each is the number average of four separate experiments rounded to the nearest 5%.

enhancer position, the 72-bp repeat was inserted at the various *Bam*HI restriction sites flanking the three transcription units: upstream of the neo transcription unit, upstream of the gpt transcription unit, and upstream and downstream of the dhfr transcription unit.

Each of the plasmids was transfected onto CV1 cells, and the amount of XGPRTase activity relative to that obtained after transfection with pSV2Agpt was determined (Fig. 5 and 6). Lacking the 72-bp repeat segment, plasmid LR-0 [pSV232A(neo/gpt/dhfr)] fails to express gpt. When the enhancer segment is located upstream of the neo transcription unit (LR-1), the level of gpt expression is barely detectable, although the level of neo RNA is markedly increased (see below) (Fig. 7). When the enhancer segment occurs immediately upstream of the gpt transcription unit (LR-2) gpt is expressed quite efficiently. XGPRTase levels are significantly lowered, however, if the enhancer is located downstream of the gpt transcription unit (LR-3) and still lower if the enhancer is downstream of the dhfr transcription unit (LR-4). Measurements of the frequencies of transformation for the gpt marker with these same plasmids give the same qualitative results (data not shown).

Measurements of the amount of RNA transcribed from the three transcription units are shown in Fig. 7. Transfection of CV1 cells with plasmid LR-1 yields readily detectable amounts of neo RNA but only low levels of the gpt and dhfr RNAs. Transfection with plasmid LR-2 yields low levels of neo and dhfr RNAs, but detectable levels of gpt RNA. With plasmids LR-3 and LR-4 the levels of neo and gpt RNAs are very low, but there are detectable amounts of dhfr RNA. Generally, the results of the RNA analysis corroborate the measurements of XGPRTase formation. Thus, a gene is expressed most efficiently when the enhancer is situated either immediately upstream or immediately downstream of the transcription unit. The enhancer's action is considerably reduced if another transcription unit is interposed between the enhancer and the enhancer-dependent promoter.

In plasmids LR-1, LR-2, and LR-3 the enhancer element is organized with respect to the gpt transcription unit in much the same way that it is in the plasmids with two transcription units (see Fig. 4). Consequently, the results of XGPRTase expression from the plasmids with three transcription units must be interpreted with promoter occlusion in mind. Thus, enhancement of transcription of an upstream transcription unit inhibits transcription of the immediately downstream transcription unit, thereby obscuring any enhancement of the downstream promoter (see Discussion).

Plasmid LR-4, however, minimizes this complication in that increased transcription of the dhfr transcription unit should not inhibit gpt expression. Moreover, note that the expression of gpt by plasmid LR-4 is strikingly less than with plasmid LR-3. Conceivably the lower expression of gpt by plasmid LR-4 versus LR-3 is due to the greater distance between the enhancer and the gpt promoter in the two plasmids (1.9 kb in LR-3 versus 3.6 kb in LR-4), or the difference might be attributable to particular DNA sequences that intervene in plasmid LR-4. Plasmid LR4Δd was used to address this question; LR4Δd differs from LR-4 in that the enhancerless promoter associated with the dhfr transcription unit is absent (Fig. 5). Since the deletion involves only 194 bp, the distance from the enhancer in LR4Δd to the promoter of the gpt transcription unit is only marginally reduced (3.4 versus 3.6 kb). However, gpt expression in plasmid LR4Δd is increased significantly over LR-4; in fact, expression of XGPRTase after transfection with LR4Δd is nearly that with plasmid LR-3. This suggests that the transmission of the enhancer's effect on a distal promoter can be reduced by interposing certain DNA sequences between the two regulatory sequences; in this case, an enhancer-dependent promoter produced the attenuation.

## DISCUSSION

Perhaps the most intriguing characteristic of enhancers is their ability to act at a distance. Some of the earliest studies indicated that the 72-bp repeat sequence associated with the SV40 early-region promoter could also function if it was separated from the promoter by as much as 2 to 4 kb. Nevertheless, for the enhancer to act, it and the promoter it activates must be on the same DNA molecule (3, 17, 34). The explanation for this ability remains obscure. In the present study we set out to determine if a single enhancer could activate the promoters of multiple discrete transcription units. Furthermore, our experiments explore if there is

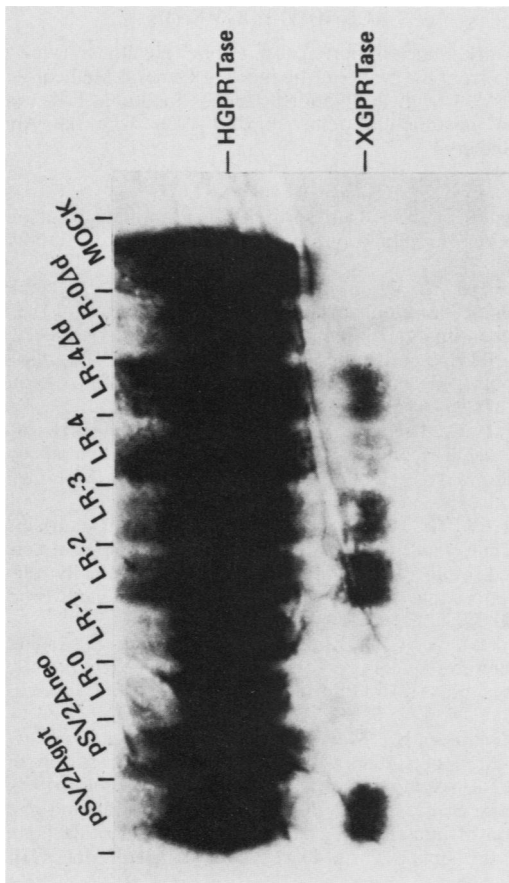


FIG. 6. Effect of position of the enhancer on the expression of the gpt transcription unit in pSV232A(neo/gpt/dhfr) plasmids. XGPRTase activities were determined as described in Materials and Methods. LR-0, No enhancer; LR-1, enhancer inserted at the *Bam*HI site immediately 5′ to the neo transcription unit; LR-2, enhancer inserted at the *Bam*HI site between the neo and gpt transcription units; LR-3, enhancer inserted at the *Bam*HI site between the gpt and dhfr transcription units; and LR-4, enhancer inserted at the *Bam*HI site immediately 3′ to the dhfr transcription unit. Δd indicates that the SV40 promoter between the *Bam*HI and *Hin*dIII sites of the dhfr transcription unit has been deleted. LR4Δd was derived from LR-4 and LR0Δd was derived from LR-0.

differential activation of the different promoters depending upon where the enhancer is located with respect to each transcription unit.

Our assay of enhancer activity relied on the expression of a gpt transcription unit in association with various other transcription units on the same plasmid. Thus, the plasmids contained three tandem transcription units encoding neo, gpt, and dhfr, each with an SV40 early-region promoter from which the enhancer segment had been deleted. The plasmids were modified by inserting an enhancer, the 72-bp repeat from the SV40 early-region promoter, at various locations relative to the three transcription units. After transfection of the plasmid DNAs into CV1 cells, expression of gpt as XGPRTase was measured to assess the activity of the gpt transcription unit. These plasmids do not replicate in CV1 cells. Moreover, since the structure of the gpt transcription units is the same in the various plasmids, alterations in mRNA stability and translatability are probably not a factor. Thus, we assume that the levels of XGPRTase reflect the efficiency of the promoter associated with the gpt coding sequence. Because of the extremely low levels of the various RNAs produced in these transfections, we have obtained only semiquantitative estimates of RNAs transcribed from the three transcription units. Overall, however, the data regarding mRNA levels and the gpt transformation data corroborate the conclusions based on XGPRTase formation.

During our experiments we found that expression of the downstream transcription unit of tandemly arranged transcription units is reduced. This phenomenon is similar to the transcriptional interference previously described by Adhya and Gottesman (1). They termed this effect promoter occlusion and attributed the inhibition to transcriptional readthrough from the upstream promoter. It seems likely that the inhibition of gpt expression that we observe is due to transcription emanating from upstream and continuing through the SV40 early-region polyadenylation signal at the end of the upstream transcription unit (14, 23) into the promoter region of the downstream transcription unit. We have detected a three- to fivefold inhibition of the downstream promoter when the two transcription units contain the same wild-type SV40 early promoter (Fig. 3). The effect is even more dramatic when the downstream promoter lacks the enhancer sequence as the expression of the gpt transcription unit from the enhancer-deleted promoter is virtually undetectable when it occurs downstream of the neo transcription unit, containing an enhancer (Fig. 4E; Fig. 6 and 7, LR-1). The inhibition we detect is clearly not a consequence of having two promoters in the same plasmid since the expression of gpt is normal if the orientation of the two transcription units is reversed (Fig. 4F).

Promoter occlusion in mammalian cells has also been described in the context of retrovirus transcription (9). In that case it appears that transcription initiation at the promoter in the 5′ long terminal repeat of an avian leukosis virus

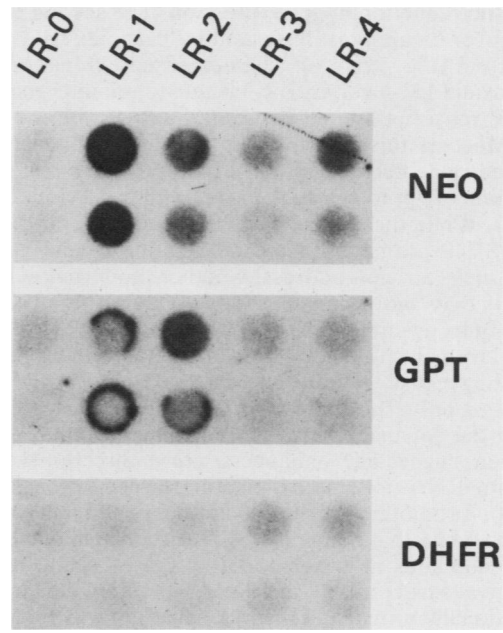


FIG. 7. Analysis of RNA levels produced after transfections with pSV232A(neo/gpt/dhfr) plasmids. The transfections of CV1 cells and analysis of RNA were carried out as described in Materials and Methods. Poly(A)-containing RNA corresponding to approximately 60 (row 1) or 30 (row 2) μg of total cellular RNA was applied to three separate nitrocellulose filters and hybridized with probes corresponding to neo, gpt, and dhfr coding sequences as indicated.

provirus directly inhibits transcription initiation within the 3′ long terminal repeat. It has been postulated that this interference is critical in the development of certain bursal lymphomas in chickens and must be eliminated before full activation of the c-*myc* oncogene can occur. Whether regulatory events in mammalian cells are influenced by promoter occlusion effects still remains to be seen.

While transcriptional interference between adjoining transcription units may have as yet unanticipated significance in gene regulation, it must also be considered in analyzing gene function out of their normal chromosomal context. For example, Wasylyk et al. (48) and de Villiers et al. (10) conclude that enhancer elements preferentially activate proximal promoters. These conclusions were drawn in both of their studies because enhancers preferentially stimulated promoters adjacent to them compared with those located further downstream. Our studies lead to the same inference (Fig. 4E and 7B), but we believe that promoter occlusion severely distorts the magnitude of that apparent preference.

Another position effect detected by our experiments is the inhibition of enhancer activity at a distance by particular DNA sequences. Two examples of "enhancer damping" were encountered. In one, the effect was mediated by an adjacent promoter sequence, and in the other an intervening promoter sequence was responsible. The first case is illustrated by the difference in gpt expression in the arrangement in plasmids pSV232Agpt-LR and pSV232Agpt2Aneo (Fig. 4B and F). Even though the location of the enhancer segment with respect to the promoter of the gpt transcription unit is the same in each plasmid, there is two- to threefold greater expression of gpt when the enhancer is not associated with a promoter (Fig. 4B) as when it is adjacent to an SV40 promoter (Fig. 4F). This is not due simply to the introduction of two promoters compared to a single promoter, since when each is introduced on separate plasmids there is no reduction of gpt expression. The second example of enhancer damping is illustrated by plasmids LR-3, LR-4, and LR4Δd (Fig. 5 and 6). The decreased expression of gpt with plasmid LR-4 versus LR-3 is due to the interposition of the dhfr transcription unit between the enhancer and the gpt gene. Most of this effect stems from the SV40 promoter sequence since deletion of the promoter region restores the expression of gpt to nearly the level with LR-3 (Fig. 5 and 6, LR4Δd). While these results focus specifically on the effect of the SV40 promoter in the attenuation of the enhancer effect, they do not address whether nonpromoter DNA elements may show similar effects (49). Furthermore, these results only describe this effect with the SV40 early promoter. It remains to be determined whether other promoters will also give rise to these phenomena.

We can only speculate as to why promoter sequences prevent the enhancer from affecting distal promoters. If, as has been suggested, enhancers are bidirectional "entry sites" for RNA polymerase or other transcriptional factors (34, 48), promoters might block the movement of these factors beyond that point. It is also conceivable that enhancers provide sites at which topoisomerases act to alter the topological structure of the DNA, thereby introducing a bidirectionally propagatable torsional strain which facilitates the binding of transcription proteins at or near the promoter. In that event, promoters could relieve the torsional strain or diminish the propagation of that strain by binding RNA polymerase or other transcription factors or both. Further studies are needed to explore these and other alternatives; in vitro transcription studies such as have recently been described (42) should prove useful in this regard.

## ACKNOWLEDGMENTS

This work was supported by Public Health Service grants GM-13235 from the National Institute of General Medical Sciences and CA 15513 from the National Cancer Institute. T.K. was supported by postdoctoral fellowship PF-1966 from the American Cancer Society.

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