Activation of gene expression is adversely affected at high multiplicities of linked simian virus 40 enhancer

(linked chloramphenicol acetyltransferase gene/reduced enhancement of expression/reduced competition for *trans*-acting factors/transient and integrated expression)

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Communicated by Hewson Swift, January 3, 1986

ABSTRACT We have cloned multiple copies of 72-base-pair (bp) repeat transcriptional enhancer element from simian virus 40 in plasmid vectors upstream from the bacterial chloramphenicol acetyltransferase gene under the control of the simian virus 40 early promoter. Two copies of the 72-bp repeat provided efficient activation of gene expression. Increasing the number of linked 72-bp units to four substantially improved the activation of gene expression, but further addition of enhancers diminished the activation of gene expression proportionally to the number of enhancers added. Enhancer regions containing 10 or more copies of the 72-bp sequence were very inefficient in gene activation. Plasmids containing such expanded enhancer regions also competed less efficiently *in vivo* for *trans*-acting enhancer-binding factors. These effects are specific for the enhancer element and are not produced by reiterations of the 21-bp promoter element. Multiple enhancer units placed upstream do not interfere with the accuracy of mRNA initiation directed by the simian virus 40 early promoter in these plasmid constructs but do severely inhibit the initiation of replication at the neighboring simian virus 40 origin of replication that overlaps the early promoter region. These results are consistent with the hypothesis that the structural alterations induced in the DNA by a large number of copies of the enhancer are not favorable for the activation of a linked gene or for the binding of factors believed to mediate the enhancement effect.

Transcriptional enhancer elements are key functional components of the regulatory regions of a number of viruses and some cellular genes in eukaryotes (for review see ref. 1). All enhancers share some common properties—their capability to activate transcription when linked in *cis* to homologous or heterologous genes in a position- and orientation-independent fashion and their shared structural motifs, such as the enhancer core sequences, and in some cases other sequences with a potential to assume a Z DNA configuration. The repetition of oligonucleotide sequences, such as the core elements or those with alternating purines and pyrimidines (2–4), and/or the duplication of an entire block having enhancer activity, such as 72-base-pair (bp) repeats of simian virus 40 (SV40) (5–7), the 68-bp repeats of the human papovavirus BKV (8, 9), or the 72- and 73-bp repeats within the long terminal repeats of murine leukemia or sarcoma viruses (10), are characteristic features of many enhancers.

In SV40, a number of point or deletion mutations in the enhancer region decreased virus infectivity drastically or caused loss of viability (2, 11). Revertants of such mutants have been isolated that compensate, to some extent, for the original mutation by duplicating either the region of the enhancer containing the core sequence (12) or other se-

quences located within or adjacent to the enhancer in the viral genome (13, 14). None of the point or deletion mutants within the enhancer region or any of the revertants isolated so far have been more infectious than wild-type SV40, and the enhancer regions of the variants have only a fraction of the activity of the wild-type enhancer. Thus, as yet no “up” mutations have been found in the enhancer sequence. It was of interest, therefore, to examine whether increased enhancement could be obtained by increasing the number of copies of the enhancer. Though redundancy is a major attribute of enhancer elements, there was the question of whether there was a limit to enhancer reiteration in a genome since the maximum known so far was three copies occurring in the genome of BK virus. To examine these questions, we have linked multiple copies of the SV40 enhancer to the bacterial chloramphenicol acetyltransferase (CAT) gene (15) in plasmid vectors and show that the expression of the linked gene is affected adversely at high multiplicities of the enhancer.

MATERIALS AND METHODS

Two parent plasmid vectors termed pA₁₀cat₂ (16) and pSV2-cat (15) (obtained from L. Laimins and B. H. Howard of the National Institutes of Health, Bethesda, MD, respectively) containing the CAT gene linked to the SV40 early promoter were used as vectors for cloning multiple copies of the SV40 72-bp repeat enhancer as described in Fig. 1. Standard cloning techniques (17), in accordance with the National Institutes of Health guidelines for recombinant DNA research, were used. The constructions were verified by diagnostic restriction analyses and limited DNA sequencing (18). Three series of plasmids, termed pA, pB, and pC, were constructed with the SV40 enhancer cloned upstream from the SV40 early promoter. The pB and pC series contained multiple copies of the enhancer arranged as tandem reiterations having head-to-tail joints in all plasmids tested. Both the early (E) and late (L) transcriptional orientations of the enhancer were obtained in several instances. From 1 to 14 copies of the 72-bp repeat enhancer were cloned in this fashion.

Analyses of CAT gene expression under transient conditions were performed by transfection of monkey CV-1 cells or human HeLa cells with the CAT plasmids either alone or along with competitor non-CAT-expressing plasmids by the calcium phosphate-coprecipitation technique and quantitation of CAT activity in extracts of cells harvested 48 hr later (15, 19). The 5' ends of CAT mRNA were determined by primer extension (20) using total cellular RNA isolated by the guanidinium isothiocyanate procedure (21) from monkey COS-1 cells (22) transfected with the plasmids as a source of

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Abbreviations: SV40, simian virus 40; CAT, chloramphenicol acetyltransferase; E, early; L, late; bp, base pair(s).

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FIG. 1. Construction of CAT plasmids containing multiple copies of the 72-bp repeat enhancer from SV40. DNA fragments from the SV40 enhancer region, with their ends modified to *Bam*HI or *Bgl*II ends, were cloned at the unique *Bgl*II site of plasmid $pA_{10}cat_2$ (16) upstream from the SV40 early promoter and the minimal replication origin (Ori) shown. Fragment A is 92 bp long and contains one full copy of the 72-bp repeat (shown boxed) and a 20-bp adjacent sequence up to SV40 nucleotide 270. Fragment B is 173 bp long and contains 142 bp of the SV40 enhancer region corresponding to approximately two copies of the 72-bp repeat (shown boxed) located between nucleotides 270 and 128 linked via an *Sph*I site with a 31-bp plasmid sequence (shown hatched) located between nucleotides 565 and 596 of pBR322. The enhancer core elements (2) and the stretches of alternating purines and pyrimidines capable of forming Z DNA structure (4) are indicated by the letters C and Z, respectively. Fragment A was cloned as a single copy and fragment B was cloned in 1, 2, 3, 4, 5, or 7 copies joined in a head-to-tail fashion corresponding to 2, 4, 6, 8, 10, or 14 copies, respectively, of the 72-bp repeat. One permuted copy of the SV40 72-bp repeat (shown boxed as fragment C) located between the *Sph*I sites at 128 and 200 was cloned between the two *Sph*I sites of plasmid pSV2-cat (15), resulting in constructs containing three or nine copies of the 72-bp repeat joined head-to-tail in the early (E) orientation.

the mRNA template. To study the effect of enhancer multiplicity under integrated conditions, murine N1E neuroblastoma cells were cotransfected with the CAT gene plasmids and plasmid pSV2neo having the bacterial *neo* gene (23). Populations of stably transformed cell clones resistant to the neomycin derivative G418 were isolated and propagated, and CAT activities of cell extracts were determined as described (15, 19).

RESULTS

Effect of Enhancer Multiplicity on Gene Expression. The results of transient expression assays in CV-1 and HeLa cells are shown in Fig. 2. The results indicate that the enhancerless parent plasmid $pA_{10}cat_2$ expressed the CAT gene poorly as described (16). Derivatives of this plasmid, containing 1, 2, or 4 copies of the enhancer, produced proportionally higher levels of CAT gene expression. Increasing the enhancer multiplicity to greater than four caused a decline in gene expression relative to the maximum value obtained with a copy number of four, again proportional to the number of copies added. There was little enhancement of gene expression observed with 10 or more copies of the enhancer present in the constructs in the cell lines tested.

Using the primer extension technique, we mapped the 5' ends of the CAT mRNAs from COS-1 cells transfected with these plasmids. The analyses for constructs with 4 or 10 copies of the enhancer are presented in Fig. 3. A typical cluster of five mRNA 5' ends was observed. The major initiation site indicated by the topmost band E corresponds to the major SV40 early mRNA 5' end located between nucleotides 5237 and 5233 (20). Minor initiation sites within SV40 sequences downstream from the promoter region (bands D, C, and B) and in the bacterial sequence upstream from the CAT coding region (band A) were also observed. Thus, all the mRNA 5' ends observed were located downstream from the SV40 early promoter in these constructs, and the upstream enhancer units did not contribute any initiation sites.

The CAT activities expressed by the murine N1E cell clones having the CAT gene integrated into the cell DNA are shown in Fig. 4. The integrated CAT gene of plasmid pRSV-cat (24) under the control of the Rous sarcoma virus promoter-enhancer was expressed efficiently and was used as a positive control. The CAT gene linked with four copies of the SV40 enhancer (pB4L-cat) was expressed more efficiently than those of plasmids pB2L-cat and pSV2-cat containing two copies of the SV40 enhancer. The CAT genes linked with no enhancer ($pA_{10}cat_2$) or 10 copies of the SV40

FIG. 2. Effect of enhancer multiplicity on gene expression. CAT gene plasmid constructs containing multiple copies of the 72-bp repeat (Fig. 1) were analyzed for CAT gene expression in CV-1 or HeLa cells as described earlier (15, 19). (a) Autoradiograph of a thin layer chromatography plate showing CAT activities of extracts of CV-1 cells harvested 48 hr after transfection with the plasmids. The number and orientation (E or L) of the enhancer repeats in the constructs are shown at the top. The plasmids used are as follows: pA1E-cat (lane 1), pA1L-cat (lane 2), pB2E-cat (lane 3), pB2L-cat (lane 4), pB4E-cat (lane 5), pB4L-cat (lane 6), pB6E-cat (lane 7), pB8E-cat (lane 8), pB10L-cat (lane 9), pB14L-cat (lane 10), and the enhancerless parent vector pA₁₀cat₂ (lane 11). The percent conversion of the ¹⁴C-labeled substrate chloramphenicol (CAP) to its acetylated products (aCAP) catalyzed by extracts of transfected cells in 30 min at 37°C is indicated at the bottom of each lane. (b) Kinetics of CAT activities in extracts of HeLa cells harvested 48 hr after transfection with the plasmids. The percentage of acetylation of chloramphenicol is plotted as a function of incubation time. The number of copies and the orientation of the enhancer(s) are indicated.

FIG. 3. Mapping the CAT mRNA 5' ends by using the primer extension technique. A 21-nucleotide synthetic oligonucleotide with the sequence CAACGGTGGTATATCCAGTGA, complementary to the CAT coding sequence located between 34 and 14 nucleotides downstream from the AUG start codon, was used as the primer. The primer 5' end-labeled with ³²P was annealed with the CAT mRNA made from COS-1 cells transfected with constructs pB4E-cat and pB10L-cat and extended by reverse transcription. The products were resolved on an 8% urea/polyacrylamide denaturing gel along with size markers (shown in lane M) and autoradiographed. The letters A to E denote the mRNA 5' ends, and the numbers at the right denote the nucleotide lengths of the markers.

enhancer (pB10L-cat) was not expressed at detectable levels. Thus, the enhancer present in a highly multimeric form affected gene expression adversely both in an autonomous plasmid and when integrated into the nuclear DNA.

Plasmids Containing Multiple Copies of the Enhancer Compete Inefficiently for Trans-Acting Factors *in Vivo*. The capacity of cotransfected non-CAT-expressing plasmids containing multiple copies of the SV40 enhancer to compete *in vivo* with the two copies of the SV40 enhancers present in

FIG. 4. CAT gene expression from plasmids integrated into the cellular DNA. Murine N1E neuroblastoma cells were cotransfected with plasmid pSV2-neo and each of the following other plasmids: pRSVcat, pSV2cat, pB2Lcat, pB4Lcat, pB10Lcat, and pA₁₀cat₂. Populations of G418-resistant cell clones were pooled, maintained in medium containing G418 (400 µg/ml), and grown up to a density of 5×10^6 cells. Cell extracts were assayed for their CAT activity as described in Fig. 2. The arrow indicates the direction of ascending thin layer chromatography performed to separate the substrate chloramphenicol (C) from its acetylated products (AC) following the CAT reactions.

pSV2-cat for *trans*-acting, enhancer-binding factors was quantitated under transient expression conditions. It has been postulated that such *trans*-acting factors are of limited availability (25–27). Since the multiple enhancer plasmid constructs contain several factor-binding enhancer domains in a tandem array, it is conceivable that they could sequester this limited supply of *trans*-acting factors and make them unavailable for the enhancer units linked to the CAT test gene.

The results shown in Fig. 5 indicate that plasmid pSV2-neo with two copies of the enhancer competed effectively with pSV2-cat causing a decrease by a factor of four in CAT gene expression at a competitor:template molar ratio of 2:1 and confirmed reports (25–27) that the *trans*-acting factors were of limited availability. At the same molar ratio, competitor plasmids pB8E-cat and pB14L-cat having 8 or 14 copies of the enhancer linked to a defective CAT gene competed less efficiently and decreased CAT gene expression by a factor of two, although at the 2:1 plasmid molar ratio used they contained an 8- or 14-fold excess of enhancer copies compared to the test gene plasmid. These results indicate that though they contain a large number of potential factor binding sequences, the enhancer regions of plasmids with a high enhancer multiplicity are less efficient in the binding of *trans*-acting factors required for enhancement. Plasmid pA₁₀cat₂ did not compete at all, showing that an enhancerless plasmid having only a promoter sequence cannot compete in this assay.

DISCUSSION

Our present results indicate that the enhancer copy number is a critical factor for determining the strength of activation of expression of a linked gene. Under transient expression conditions, increasing the enhancer multiplicity caused either

an increase or a decrease in the enhancement obtained depending on the number of copies of the enhancer already present in the transcriptional unit (Fig. 2). A plot of the transcriptional activation effect as a function of the enhancer multiplicity gave a characteristic bell-shaped curve (Fig. 6). Maximum transcriptional activation was obtained with four copies of the enhancer in both HeLa and CV-1 cells. Further increments in enhancer multiplicity resulted in reduction in the activation effect. This downward shift was more precipitous in the case of HeLa cells than in CV-1 cells for reasons that are not clear.

Similar to the transient expression results, under conditions where the CAT gene and the linked enhancer units were integrated into the cellular DNA, four copies of the enhancer provided maximum enhancement, while 8 or 14 copies provided practically no enhancement (Fig. 4). We also found that as in the case of transcription initiated from a proximal promoter, autonomous replication initiated at the neighboring SV40 origin of DNA replication was inhibited severely by high multiplicities of the SV40 72-bp enhancer. Even at low to moderate multiplicities of the enhancer, replication was inhibited to some extent in contrast with transcription that was activated substantially (R.K. and K.N.S., unpublished results). We also found that reiterations of the SV40 21-bp repeat promoter element of up to 15 copies did not exert a positive or negative influence on transcription or replication under transient expression conditions (data not shown). Thus, the inhibitory effects at high copy numbers appear to be specific for a transcriptional enhancer element.

One of the reasons for the inhibition of gene expression by multiple copies of an enhancer could be the generation of far upstream mRNA initiation sites within the enhancer units relegating the AUG start codon for the CAT protein to more internal positions in the CAT mRNAs produced. The results in Fig. 4 indicate that the accuracy of mRNA initiation

FIG. 5. Competition for *trans*-acting, enhancer-binding factors *in vivo* in CV-1 cells. (A) A dose-response curve for CAT expression from the test gene plasmid pSV2-cat. The CAT activity expressed (B_0) is plotted as a function of μg quantities of transfected pSV2-cat. (B) Subconfluent CV-1 cells were cotransfected with 5 μg of the test gene plasmid and various amounts of competitor plasmids pA₁₀cat₂ (1), pSV2-neo (2), pB8E- Δ cat (3), or pB14L- Δ cat (4). The latter two plasmids were constructed from plasmids pB8E-cat and pB14L-cat, respectively, by the deletion of a 984-bp sequence located between the *Bal* I and *Hpa* I sites including 174 bp of the carboxyl-terminal CAT coding sequence. Plasmid pBR322 was used as carrier to yield a total of 30 μg of DNA per plate. The CAT activities expressed were determined 48 hr after transfection (15, 19). CAT activity expressed by the test gene in the absence of the competitor is denoted as B_0 and that with the competitor as B . The ratio B/B_0 is plotted as a function of competitor:template molar ratio.

FIG. 6. A plot of the activation of CAT gene expression as a function of enhancer copy number. HeLa (dotted line) and CV-1 (solid line) cells were transfected with the plasmid constructs shown in Fig. 1. Cells were harvested 48 hr later, and CAT activities of cell extracts were assayed by measuring the percent conversion of chloramphenicol to its acetylated products (aCAP). The CAT activity expressed is plotted as a function of enhancer copy number.

directed by the SV40 early promoter was not affected by the linked enhancer units and that they themselves did not produce new initiation sites. Thus, the inhibition of gene expression by multiple copies of the enhancer is not due to incorrect initiation of transcription.

In wild-type SV40, the enhancer region is 164 bp long. The length of the enhancer region is expanded substantially in the plasmid constructs made in this study, spanning a distance of 1.2 kilobases in the construct containing 14 copies of the enhancer. Such an expanded enhancer region was not only deficient in activation of gene expression but was a poor competitor for enhancer-binding, *trans*-acting factors, in spite of having a repeated array of potential binding sites (Fig. 5). These results, coupled with the transcription initiation results and the inhibitory effect on replication, suggest that these adverse effects are perhaps related to structural features of the multiple copy enhancer region. The presence of the enhancer sequence is known to influence the chromatin structure assumed by the template *in vivo* (28). Our results can be explained by the hypothesis that a highly multimeric enhancer region produces a structural alteration in the template chromatin that is less favorable for the binding of *trans*-acting factors and for the activation of expression of a linked gene.

The results of our present study could explain the low frequency of reiteration of the enhancer in all known animal viruses. Using the SV40 enhancer as the example, we have shown that at high enhancer multiplicities, transcription of a linked gene and replication of the template were both reduced tremendously. At intermediate frequencies of reiteration (say, four copies), the transcriptional activation was high but replication was significantly inhibited. In the absence of the enhancer, the replication efficiency was high, but transcription was greatly reduced. A low reiteration frequency of the enhancer (such as two copies in SV40) has probably been

naturally selected to maintain a balance between replication and transcription leading to the successful propagation of the virus in the host.

We thank Dr. S. Hartzell for discussions and generous help with the figures, Drs. R. Kucherlapati and H. Reiter for critical reading of this manuscript, and Ms. Lenor I. Piedra for expert preparation of the manuscript. This research was supported by Research Grant NP 248E and F from the American Cancer Society. C.T.S. thanks Dr. Reiter for support. K.N.S. is the recipient of Faculty Research Award FRA-202 from the American Cancer Society. T.A.F. is supported by Training Grant PHS CA 09318-04 from the National Institutes of Health.

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