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Stable Growth of Simian Virus 40 Recombinants Containing Multimerized Enhancers

BRIAN ONDEK[1,2]† AND WINSHIP HERR[1]*

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724,[1] and Genetics Program, State University of New York at Stony Brook, Stony Brook, New York 11794[2]

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Multiple copies of each of three genetically defined simian virus 40 protoenhancers, A, B, and C, were able to substitute for the wild-type simian virus 40 enhancer. Although the recombinant viruses grew poorly, they could be propagated without the accumulation of enhancer rearrangements that might improve viability. Mutations that inactivate the multimerized B and C protoenhancers abolished virus growth, but, unexpectedly, a mutation that inactivates the octamer-enhanson within the B protoenhancer increased virus viability. This positive effect may reflect loss of repression of the B protoenhancer by the ubiquitous octamer-motif-binding protein Oct-1.

The simian virus 40 (SV40) regulatory region directs both early and late transcription from a bidirectional promoter. Early transcription results in the synthesis of T-antigen protein which, in turn, down-regulates early transcription and activates late transcription (for a review, see reference 20). Within the SV40 regulatory region lies the SV40 enhancer, the first of a class of transcriptional regulatory elements shown to activate transcription over large distances (1, 22). The SV40 enhancer region is required for virus viability because viruses lacking this region do not grow unless neighboring sequences are duplicated to restore enhancer function (26, 30).

The SV40 enhancer is a structurally complex regulatory element composed of multiple organizational levels. The two principal units of organization are enhansons and protoenhancers (8, 23). Enhansons are the fundamental units of enhancer structure and correlate with protein binding sites (6). Protoenhancers are functional units that have the ability to activate transcription over large distances when duplicated or further multimerized without strict spacing requirements. They are often composed of juxtaposed enhansons such as the SV40 sph, coreA/GTIIC, and GTI motifs shown in Fig. 1, but may also consist of a single enhanson, as is the case for the SV40 octamer and κB/TCII protoenhancers (8, 16, 23, 27).

Three SV40 protoenhancers, called A, B, and C (Fig. 1), were first identified by genetic selection. Phenotypic revertants of SV40 strains carrying deleterious point mutations within one or two of the A, B, and C protoenhancers arose after passage of mutant virus stocks in the African green monkey kidney cell line CV-1. These SV40 growth revertants always contained duplications of the remaining wild-type protoenhancer(s), which restored enhancer function (4, 12, 13). Multimerization of synthetic oligonucleotides representing each individual A, B, and C protoenhancer and assay for enhancer function by transient expression showed that multiple copies of each element are sufficient for enhancer function (24, 25). Thus, the SV40 enhancer is a composite of multiple elements that can functionally substitute for one another.

Because of the complex structure of the SV40 regulatory region, it is not known whether enhancer activity per se is the only essential function supplied by the SV40 enhancer region during virus growth. For example, sequences within the enhancer region, which in some cases have been shown to differ from the A, B, and C protoenhancers, are implicated in late promoter function (2, 3, 7, 9, 11, 14, 17, 19, 21). To address this issue, we replaced the wild-type SV40 enhancer with active and inactive multimerized enhancers and assayed virus growth and genome stability.

Figure 1 shows the sequence of the SV40 enhancer region and the boundaries of the A, B, and C protoenhancers as originally defined (12, 13). The labeled arrows indicate the sequences multimerized in each synthetic enhancer. Because of terminally redundant sequences (GTG in the A21 protoenhancer and an A in the B17 protoenhancer) the 21-bp A and 17-bp B oligonucleotides recreated 24 and 18 bp of the wild-type sequence, respectively. The broken arrows show the nucleotide changes in the matched mutant oligonucleotide constructions B17*dpm*2, B17*dpm*7, and C17*dpm*6. Except for B17*dpm*7, multimerized copies of these oligonucleotides have been described and assayed for enhancer function previously (24); the 6XB17*dpm*7 enhancer was constructed in the same way as the others. The B protoenhancer mutations *dpm*2 and *dpm*7 separate the activities of two different, but overlapping, protoenhancers (27). By inactivating the sphI enhanson, the *dpm*2 mutation inactivates the B protoenhancer, which is normally active in CV-1 cells. The *dpm*7 mutation, which has little effect on the activity of the sph enhansons and hence on the activity of the B protoenhancer in CV-1 cells, debilitates the octamer enhanson, which is a B cell-specific protoenhancer that is normally inactive in CV-1 cells (5, 8, 27).

To create the recombinant SV40 genomes containing multimerized protoenhancers, blunt-ended *Hin*dIII-*Pst*I fragments from the pβ series of plasmids described previously (24) were ligated into the SV40 enhancer replacement vector pSVER. Construction of this vector will be described in detail elsewhere. Briefly, it is a derivative of pAO (31) which contains a *Bam*HI site between the 72- and 21-bp repeats and a *Sac*I site immediately *ori* proximal to the

* Corresponding author.
† Present address: Department of Biology, B-022, University of California, San Diego, La Jolla, CA 92093.

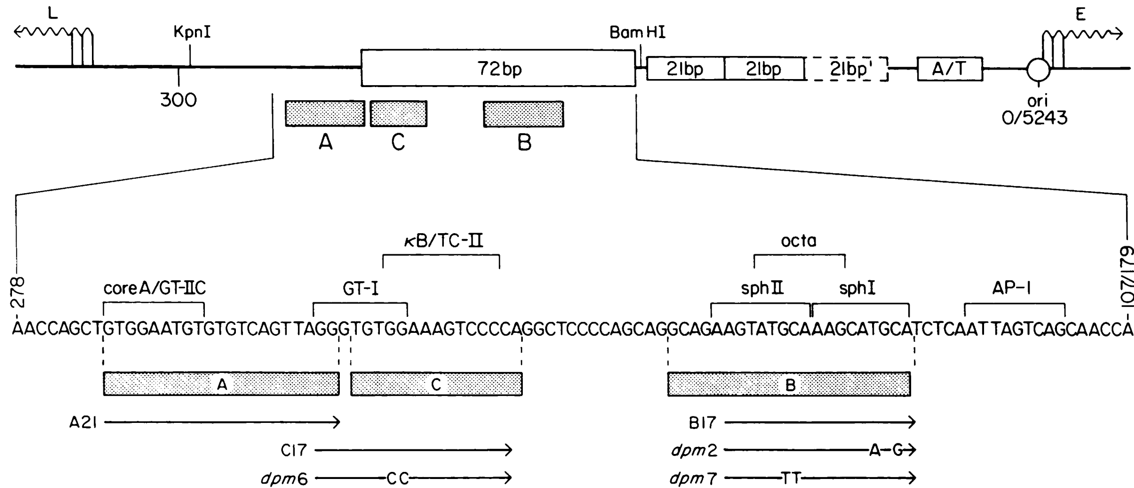

FIG. 1. SV40 enhancer region and the A, B, and C protoenhancer oligonucleotides. A diagram of the SV40 regulatory region at the top shows from right to left, the early transcriptional start sites (E), origin of replication (*ori*), AT-rich TATA-like element, one imperfect (dashed box) and two perfect 21-bp repeats, a single copy of the 72-bp element, and the major late start sites (L). The wild-type *Kpn*I restriction site and the engineered *Bam*HI restriction site in the pAO expression vector (31) are shown above, and the nucleotide sequence of the SV40 enhancer region with the position of the protoenhancers (stippled boxes) as described previously (12, 13), is shown below. The position of enhansons is shown above the sequence. The sequence of the six synthetic wild-type and mutant protoenhancer oligonucleotides used here is indicated by the arrows below the sequence of the enhancer; mutations are indicated by the base changes in the broken arrows. Note that multimerization of A21 extends the sequence identity to the SV40 enhancer by 3 bp (GTG) and multimerization of B17 extends the identity by 1 bp (A) to regenerate both sph enhansons. The orientation of the multimerized enhancers within the SV40 recombinants is indicated by + when oriented in the wild-type direction as shown in the figure and − for the opposite orientation.

unique SV40 *Kpn*I site (Fig. 1). The multimerized enhancers with six (6X) or in one case eight (8X) tandem protoenhancer copies were ligated to *Sac*I- and *Bam*HI-digested and end-repaired pSVER DNA. The recombinant SV40 regulatory regions between the unique SV40 *Kpn*I and *ori Bgl*I sites were then transferred to the plasmid pK1K1. pK1K1 contains 1.27 copies of the SV40 genome; a 0.27 terminal redundancy of the SV40 late region permits excision of unit-length SV40 genomes upon transfection into cells permissive for SV40 replication (10). To assay virus viability, pK1K1 plasmid DNAs were transfected into confluent CV-1 cells by treatment with DEAE dextran as described previously (12). The number and size of plaques were measured 14 and 18 days posttransfection. Results were similar at both time points.

Table 1 shows a comparison of the viability, as measured by plaque number, of SV40 containing one (1X72) or two (2X72) 72-bp elements and the recombinant viruses carrying multimerized enhancers. The SV40 enhancer mutant *dpm*126 is a nonviable 1X72 derivative with three sets of double point mutations that debilitate all three of the A, B, and C protoenhancers (12). Although the 6XA21, -B17, and -C17 recombinant viruses produced 100- to 10,000-fold fewer plaques than the wild-type SV40 viruses, each was more productive than the *dpm*126 mutant. Orientation (the + orientation indicates that the protoenhancers are oriented in the same direction as in the wild-type enhancer) does not affect the 6XC17 enhancer but unexpectedly has an effect on the 6XB17 enhancer, as the (−) orientation is 10-fold less active in this assay. Active protoenhancers were required for plaque formation because SV40 strains carrying the inactive B and C protoenhancer mutants *dpm*2 and *dpm*6, respectively, could not form plaques. The ability of three different short multimerized protoenhancers to support SV40 growth suggests that enhancer function is the only activity arising from the SV40 enhancer region that is essential for viral growth. Nevertheless, the relatively poor growth of these SV40 recombinants suggests that nonessential activities of the SV40 enhancer region may augment SV40 growth.

In Table 1, the previously described ability of the different multimerized enhancers to activate the human β-globin promoter in CV-1 cells (24) is compared with the results of

TABLE 1. Recombinant virus viability and enhancer activity of multimerized enhancers

Enhancer	Relative infectivity[a]	Relative enhancer activity[b]
2X72	1	1
1X72	0.4	0.4
*dpm*126	—	0.02
$6XA21^-$	7×10^{-4}	0.25
$6XB17^+$	8×10^{-4}	0.7
$6XB17^-$	7×10^{-5}	0.7
8XMB17/*dpm*2$^+$	—	0.02
6XMB17/*dpm*7$^+$	6×10^{-3}	0.6
$6XC17^+$	6×10^{-3}	0.2
$6XC17^-$	6×10^{-3}	0.2
6XMC17/*dpm*6$^+$	—	0.02

[a] Relative infectivity compared with wild-type SV40 strain 776 virus was determined by plaque assay as described previously (12). The results shown are from duplicate samples in one transfection series in which 10-fold dilutions from 400 ng to 40 pg of plasmid DNA were transfected. Plaques were counted on days 14 and 18. A second series of transfections gave similar results. The wild-type 2X72 pK1K1 construct produced 8×10^4 plaques per μg of plasmid DNA. —, No plaques observed.

[b] Except for the 6XB17*dpm*7 enhancer, which was assayed as described previously (23), the relative enhancer activities are from Ondek et al. (23). The enhancer activity represents the relative levels of β-globin reporter RNA after expression in CV-1 cells. The enhancerless plasmid pβe$^-$ produces 2% the level of β-globin transcripts produced by the 2X72 construct.

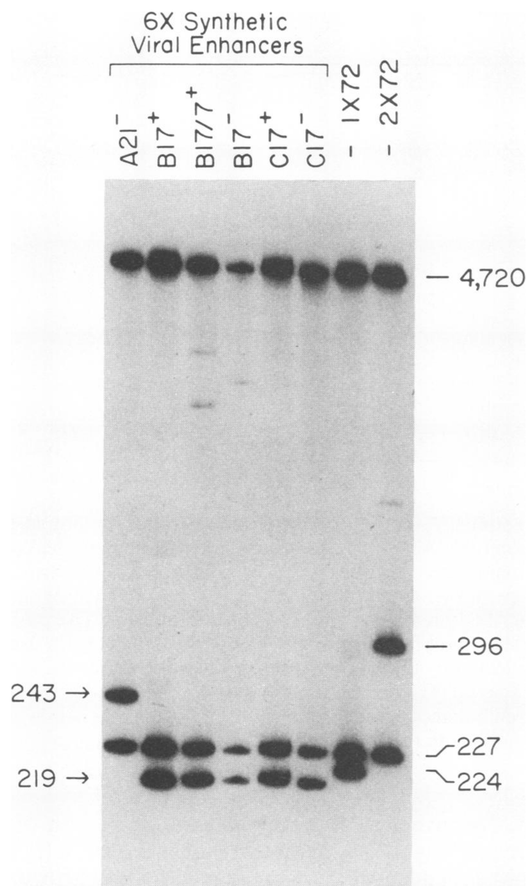

FIG. 2. Propagated recombinant SV40 with multimerized enhancers does not display enhancer rearrangements. Radiolabeled *Nco*I fragments from viral DNA isolated by Hirt extraction are shown after electrophoretic fractionation and autoradiography of a 6% native polyacrylamide gel. SV40 control DNA samples containing one or two copies of the 72-bp element, generating the 224- and 296-bp fragments, respectively, are shown at the right. The 227- and 4,720-bp fragments abut the enhancer containing *Nco*I fragment and are generally unaffected by rearrangements in the enhancer region. Six copies of the 17- and 21-bp oligonucleotides result in *Nco*I enhancer fragments of 219 and 243 bp, respectively, as indicated in the figure. The identities of the synthetic enhancers are indicated above each lane.

plaque formation by the recombinant viruses. This comparison shows that the relative potency of the active enhancers differs in the two different assays. Most significantly, the C protoenhancer can support better virus growth relative to its enhancer potential to the transient β-globin assay. The C protoenhancer probably represents the κB protoenhancer (16, 24a), a motif that is activated by virus infection (18, 29). Thus, this virus may stimulate its own enhancer upon infection.

A surprising result of the analysis of the 6XB17 series was that the 6XB17/*dpm*7$^+$ recombinant virus grew nearly 10-fold better than the wild-type parent 6XB17$^+$. This positive effect observed in plaque number was also reflected in an increased average size of the plaques (data not shown), further substantiating the positive effect of the *dpm*7 mutation. The *dpm*7 mutation had only a minimal effect, if any, on the ability of the wild-type 6XB17 enhancer to activate transcription by using a β-globin reporter gene in CV-1 cells (Table 1). But *dpm*7 is known to have a severe deleterious effect on the ability of the B protoenhancer octamer motif to activate both U2 small nuclear RNA transcription, an activity probably mediated by the ubiquitous octamer-motif-binding protein Oct-1, and β-globin transcription in B cells, an activity probably mediated by the lymphoid factor Oct-2 (27, 28, 28a). The ubiquitous Oct-1 protein is an unusual transcription activator because it does not normally activate mRNA promoters, and indeed overexpression of Oct-1 can repress the ability of the B protoenhancer to activate transcription; this repression is alleviated by the *dpm*7 mutation (28). Thus the improved viability of the 6XB17/*dpm*7$^+$ recombinant virus may reflect relief of repression by Oct-1. These results suggest that in wild-type SV40 the octamer motif could serve to regulate the activity of the B protoenhancer.

When 1X72 SV40 strains carrying debilitating point mutations within the A, B, or C protoenhancers are propagated, revertant viruses with tandem duplications arise readily (4, 12, 13). To test the genomic stability of the recombinant SV40 strains with multimerized enhancers, virus stocks were prepared by infection of CV-1 cells with virus isolated directly from the plaques arising from the transfections of pK1K1 DNA. Viral DNAs were subsequently isolated by Hirt extraction (15) 48 h postinfection of fresh CV-1 cells with the virus stocks. The isolated viral DNAs were digested with *Nco*I, and the resulting fragments were end-labeled and separated by polyacrylamide gel electrophoresis. The results of such an experiment are shown in Fig. 2. The relative amounts of DNA shown in Fig. 2 are not directly reflective of the amount of DNA recovered from infected cells; recovered DNAs from the different virus stocks varied by only about two- to fivefold (data not shown), suggesting the recombinant viruses are not very defective for early transcription or DNA replication.

In each sample shown in Fig. 2, the enhancer containing *Nco*I fragments (243 bp for 6XA21 and 219 bp for the other recombinants) are of the expected size and few, if any, rearrangements can be detected. (The faint, more slowly migrating fragments probably resulted from defective viral genomes, as these also appeared with wild-type SV40 virus; see the 2X72 sample.) This result is in sharp contrast to the ready appearance of rearrangements of the 1X72 point mutant derivatives (e.g., *dpm*12 and *dpm*6) even though the 1X72 mutants grow 10- to 100-fold better than the viruses carrying multimerized enhancers (12, 13). In the mutated 1X72 viruses, the rearrangements amplify the number of wild-type protoenhancers and thus create more potent enhancers. Perhaps, because the multimerized enhancers already contain a large number of protoenhancers, there is little advantage gained by rearrangement of such enhancers. The poor viability of the multimerized enhancer viruses would then probably reflect the absence of other critical functions such as efficient late promoter activity. Nevertheless, the ability to propagate SV40 with several different synthetic enhancers attests to the extremely plastic nature of this regulatory region.

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