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Activation of Mutated Simian Virus 40 Enhancers by Amplification of Wild-Type Enhancer Elements

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We show that duplication of any one of three separate simian virus 40 enhancer elements, A, B, or C, can compensate for loss of function in the remaining two. Simian virus 40 revertants containing point mutations within the A and C (*dpm*16) or B and C (*dpm*26) enhancer elements contain tandem duplications that include the remaining wild-type element. These simple tandem duplications can create enhancers 25-fold more active than that of the parental mutant. These revertants can arise by illegitimate recombination between heterologous viral genomes. This was demonstrated by the recombinants resulting from a mixed infection with the viruses *dpm*16 and *dpm*2, which contain mutations in the A and C elements and the B element, respectively.

Dissection of eucaryotic promoters has shown that they consist of different classes of promoter elements which can be distinguished by their characteristic activities. For example, upstream promoter elements and enhancer elements each confer promoter specificity, but only enhancer elements are capable of controlling transcription from a position distal to the transcriptional initiation site (for reviews, see references 6 and 13). The positional flexibility of enhancer elements was first described for the simian virus 40 (SV40) early promoter, in which, in the prototypic SV40 strain 776, the enhancer region is characterized by a 72-base-pair (bp) tandem repeat (2, 14). Although the 72-bp duplication increases the activity of the SV40 enhancer, this duplication is not required for virus viability (21).

Mutational studies of a nonduplicated SV40 enhancer have shown that this enhancer contains multiple elements (8, 24). Genetic experiments in which growth revertants were isolated from SV40 carrying point mutations within the enhancer region have identified three separate enhancer elements, A, B, and C (Fig. 1), which range in length from 15 to 22 bp (8, 9). In the first such experiment 18 independent revertants of the SV40 enhancer mutant *dpm*12, which contains two sets of double point mutations (*dpm*1 and *dpm*2; Fig. 1), were characterized; each revertant carried a tandem duplication 45 to 135 bp long (9). The nucleotide sequence at the junction of each duplication did not recreate the sequences mutated by the *dpm*1 or *dpm*2 mutations nor did the junctions share any obvious homology. Furthermore, the mutated sequences themselves were not always duplicated, indicating that the revertant phenotype was not due to amplification of mutated elements. Instead, the most striking result of the duplication patterns was that a 15-bp sequence spanning the SV40 core element (11, 22) was common to all of the duplications (9).

The significance of the common 15-bp core region was tested by designing a set of double point mutations (*dpm*6) within the core element that debilitated SV40 growth and enhancer function (8; Fig. 1). Thirteen revertants of this virus were isolated, and they contained tandem duplications that consistently duplicated either one or both of the regions that were mutated in the *dpm*12 mutant. Together these experiments identified the A, B, and C elements, which span the wild-type sequences that are altered by the *dpm*1, *dpm*2, and *dpm*6 mutations, respectively. Of the *dpm*6 revertants, six of the duplications spanned both the A and B elements, six duplications contained the B element, and a single revertant, with a 21-bp duplication, duplicated the A element only. This last *dpm*6 revertant duplication defined the 21-bp A element. To test the significance of this single A-element duplication and to determine whether duplication of the A or B element alone would restore enhancer function when the other two elements are mutated, we isolated revertants of the *dpm*16 and *dpm*26 mutants in which either the A and C or B and C elements, respectively, are mutated. We found that duplication of any single element can compensate for loss of function in the other two elements by restoring both virus viability and enhancer function.

(A discussion of some of these results was included in M. Botchan, T. Grodzicker, and P. Sharp (ed.), *Cancer Cells 4/DNA Tumor Viruses*, p. 95–101, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1986.)

MATERIALS AND METHODS

Enhancer mutant constructions and isolation of revertants. The construction of the *dpm*1, *dpm*2, and *dpm*6 SV40 enhancer mutants by site-directed oligonucleotide mutagenesis has been described previously (8, 9). To construct *dpm*16 and *dpm*26, single-stranded M13 bacteriophage DNA containing a single 72-bp repeat and either the *dpm*1 or *dpm*2 mutations was used as the template for mutagenesis by the *dpm*6 oligonucleotide. The *dpm*16 and *dpm*26 enhancers were cloned into the SV40-containing plasmid pK1K1 (5, 8). This plasmid contains a terminal repetition of nucleotides 346 to 1782 of the SV40 genome, which allows for excision of the SV40 genome upon transfection of cells permissive for SV40 replication. The presence of mutations *dpm*1 and *dpm*2 were monitored by restriction enzyme site polymorphism: *dpm*1 creates a *Hin*fI site, and *dpm*2 destroys a *Sph*I site.

Revertants were isolated after DEAE-dextran-mediated transfection of CV-1 cells (60-mm Falcon plates) with 0.4 μg (for *dpm*16) or 1 μg (for *dpm*26) of mutant pK1K1 plasmid DNA. Higher levels of pK1K1 *dpm*26 DNA were used because *dpm*26 grows more poorly than *dpm*16 and it is more difficult to obtain revertant viruses. Resulting lysates were passaged once, and revertant isolates were purified until homogeneous by one to four rounds of plaque isolation. DNAs were purified by the Hirt procedure (10) and analyzed by restriction enzyme digestion and nucleotide sequence

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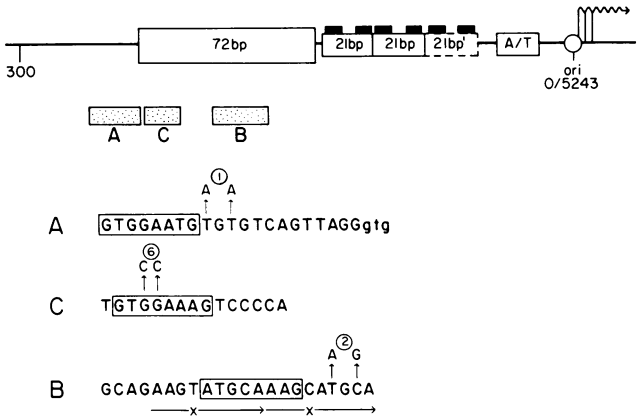

FIG. 1. Locations and nucleotide sequences of the A, B, and C enhancer elements within the SV40 early promoter. The diagram of the SV40 early promoter shows, from right to left, the transcriptional initiation sites, origin of replication, A+T-rich TATA-box-like region, six potential Sp1 binding sites (filled boxes) within two perfect and one imperfect 21-bp repeat, and a single 72-bp sequence that is tandemly duplicated in the wild-type strain 776 of SV40. The boxes labeled A, B, and C show the location of the three SV40 enhancer elements, as described previously (8). The nucleotide sequence of each element, along with the base changes caused by the *dpm*1, *dpm*2, and *dpm*6 double point mutations, is shown. The terminal sequence of the A element is ambiguous because of a 3-bp terminal redundancy, shown by the lowercase gtg. The boxed sequences identify the 8-bp sequences homologous to the core consensus sequence in the A and C elements and to the octamer sequence in the B element. The broken arrows identify two nearly perfect 9-bp repeats within the B element (see the text).

analysis as described previously (8). Except for three *dpm*26 revertants, each *dpm*16 and *dpm*26 revertant was independently isolated from separate transfections of the initial pK1K1 plasmid DNA. The *dpm*26 revertants *rd*62a, *rd*62b, and *rd*301 were isolated from plaques formed directly after DEAE-dextran-mediated transfection.

During the isolation of *dpm*16 revertants it became apparent that some of the viruses were derived from the *dpm*2 mutant (most probably because of a small amount of pK1K1 *dpm*2 DNA in the pK1K1 *dpm*16 plasmid preparation). From these isolations arose 10 *dpm*16 revertants, 11 *dpm*2-derived viruses, and 3 *dpm*2/*dpm*16 recombinants (*dpm*2/6 *rd*64, 1X72 *rd*4, and a 1X72 isolate). The nucleotide boundaries of each *dpm*2 duplication are as follows, with the size of the position ambiguity caused by homology at the endpoints indicated in parentheses (note that nucleotides 179 to 250 are missing from the *dpm* series of enhancer mutants because there is only one copy of the 72-bp repeat): *rd*131, 88 to 290 (1); *rd*117, 105 to 293 (0); *rd*91, 106 to 268 (1); *rd*90, 109 to 270 (0); *rd*87, 114 to 272 (0); *rd*73, 136 to 276 (1) (this revertant has an insertion of CCGC at the duplication junction); *rd*54, 161 to 284 (4); *rd*18, 155 to 272 (3); *rd*11, 161 to 171 (1); *rd*9, 112 to 120 (0). One *dpm*2-derived virus, *dl*18, contains an 18-bp deletion of nucleotides 279 to 296. The nomenclature system for revertants is as follows: the name of the original mutant is followed by *rd* (revertant duplication) and the total number of additional nucleotides created by the duplication(s); when different revertants of the same mutant are of the same size they are differentiated by the suffixes a, b, etc.; and when two independently derived revertants are identical in structure they are given the designations 1 and 2.

Assay of enhancer function and virus viability. Enhancer function was assayed by the ability of each enhancer to activate transcription of the human β-globin gene during transient expression in CV-1 cells. Mutant and revertant enhancers were cloned into plasmid πSVHSβΔ128, generating the πSVHHβΔ128 series (9). These plasmids contain the SV40 early promoter, from the *ori*-proximal *Hin*dIII site to the unique SV40 *Hpa*II site, positioned about 1.1 kilobases upstream of the β-globin transcriptional initiation site. Transfections of the experimental β-globin plasmid along with an internal control α-globin gene-containing plasmid (πSVHPα2) were performed by calcium phosphate coprecipitation as described previously (15, 20); cytoplasmic RNAs were isolated and probed by hybridization and nuclease protection of single-stranded, internally labeled RNA probes generated by SP6 polymerase as described previously (9). Relative levels of β-globin RNA expression were measured by scintillation counting and normalization to the α-globin internal control as described previously (15).

To assay for virus viability, mutant and revertant enhancers were cloned into the SV40 genome-containing plasmid pK1K1 (see above) as described previously (9). Plaque assays were performed using 60-mm plates of CV-1 cells after DEAE-dextran-mediated transfection of 400, 40, 4, or 0.4 ng of pK1K1 plasmid DNA. Duplicate plates were used for each dilution.

RESULTS

The location of the SV40 enhancer elements A, B, and C within an SV40 early promoter containing a single 72-bp element (called 1X72) and the nucleotide sequence of each element, as described previously (8), are shown in Fig. 1. The A and C elements each contain a sequence (boxed in Fig. 1) homologous to the core consensus sequence GTGG$^{A}/_{T}$ $^{A}/_{T}$ $^{A}/_{T}$G (22) which is found in a number of different viral and cellular enhancers. The B element shares homology with the octamer consensus sequence found in a number of cell-specific (e.g., immunoglobulin) and universally transcribed (e.g., histone H2B) gene promoters (see discussion in reference 4). The B element contains two nearly perfect 9-bp repeats which have been referred to as Sph motifs (24) (Fig. 1). The three sets of double point mutations (*dpm*) that we used to debilitate SV40 viability and enhancer function (*dpm*1, *dpm*2, and *dpm*6) are shown above the sequence of each element. We describe the structures of SV40 revertants of the double mutants *dpm*16 and *dpm*26 and recombinants between *dpm*2 and *dpm*16 viruses below. We then describe the results of plaque and enhancer assays of the various mutants and revertants.

***dpm*16 and *dpm*26 revertants.** We have previously shown that the *dpm*6 mutations in the C element and the combination of A-element *dpm*1 and B-element *dpm*2 mutations in *dpm*12 debilitate SV40 growth (8, 9). As expected, the combinations of *dpm*1 or *dpm*2 with *dpm*6 in *dpm*16 and *dpm*26 also had a severe effect on virus growth. Revertants of the *dpm*16 and *dpm*26 SV40 mutants were isolated by transfection of large amounts of cloned mutant SV40 DNA into the African green monkey kidney cell line CV-1. Mutant viruses were passaged to amplify revertant virus stocks. Revertant viruses were then plaque purified, and their structures were analyzed by both restriction enzyme digestion of viral DNA and nucleotide sequence analysis of the revertant enhancer regions (see Materials and Methods).

The structures of 10 *dpm*16 revertants and 12 *dpm*26 revertants are shown in Fig. 2. As with *dpm*6 and *dpm*12 revertants (8, 9), each of these new revertants contains a tandem duplication, here ranging in size from 31 bp in *dpm*16

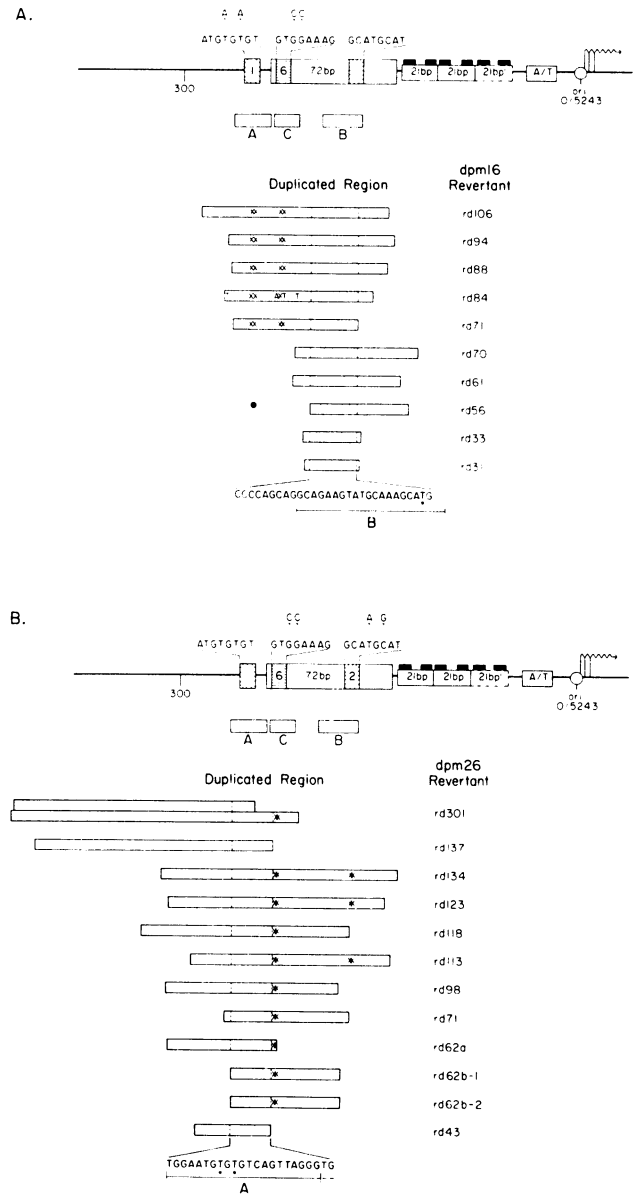

FIG. 2. Tandemly duplicated sequences in revertants of the *dpm*16 and *dpm*26 SV40 enhancer mutants. The duplicated regions of 10 *dpm*16 revertants (A) and 12 *dpm*26 revertants (B) are shown as rectangular boxes under the diagrams of the SV40 early promoter. The early promoter diagram is as described in the legend to Fig. 1. The sets of *dpm*16 and *dpm*26 point mutations are shown at the top of each panel above the wild-type SV40 sequences in these regions. The rectangular boxes aligned with the early promoter diagram identify the sequences that are tandemly duplicated in the revertants. XX identifies the locations of the *dpm*6 and either the *dpm*1 (panel A) or *dpm*2 (panel B) point mutations when contained in the duplicated sequences. The 29-bp sequence (nucleotides 129 to 157) common to all of the *dpm*16 duplications and the 23-bp (nucleotides 175/247 to 269) commonly duplicated sequence of the *dpm*26 revertants are shown at the bottom of panels A and B, respectively. The dots below these sequences show the positions that are mutated in the *dpm*2 (panel A) and *dpm*1 (panel B) mutants. The bars identify the A- and B-element sequences within the common regions. The nucleotide boundaries of each *dpm*16 revertant are as follows, with the size of the ambiguity in position caused by homology at the endpoints indicated in parentheses (note that nucleotides 179 to 250 are missing from the *dpm* series of enhancer mutants): *rd*106, 112 to 289 (0); *rd*94, 109 to 274 (1); *rd*88, 112 to 271 (4); *rd*84, 121 to 276 (2); *rd*71, 129 to 271 (0); *rd*70, 95 to 164 (2); *rd*61, 105 to 165 (0); *rd*56, 100 to 155 (2); *rd*33, 127 to 159 (0); *rd*31, 128 to 158 (2). The *dpm*16 *rd*84 duplication contains four base transitions: C → T at positions 163, 172, and 275 and G → A at position 175. The nucleotide boundaries of the *dpm*26 revertant duplications are as follows: *rd*301, 257 to 393 (0) and 160 to 395 (0) (the order of these two duplications has not been determined); *rd*134, 104 to 309 (1); *rd*123, 111 to 305 (0); *rd*118, 131 to 320 (0); *rd*113, 108 to 292 (0); *rd*98, 137 to 306 (1); *rd*71, 131 to 273 (2); *rd*62a, 172 to 305 (0); *rd*62b-1 and *rd*62b-2, 136 to 269 (0); *rd*43, 175 to 289 (0). The *rd*62b-1 and *rd*62b-2 revertants are identical in structure but were isolated independently.

*rd*31 to over 130 bp. In an unusual case, the largest revertant, *dpm*26 *rd*301, contains two separate duplications of 137 and 164 bp, the order of which is not known. Except for one revertant, *dpm*16 *rd*84, the revertant duplications do not contain any point mutations. The exception contains three C → T transitions and one G → A transition within the 84-bp duplicated sequence (Fig. 2). Because the four point mutations are duplicated, they probably arose during or soon after transfection of the viral DNA. Although this revertant exhibited restored enhancer function (see below), we do not know the relative contribution of the point mutations and the tandem duplication.

The different sequences duplicated in the *dpm*16 revertants all share a 29-bp region (Fig. 2), which overlaps all but the 3′-terminal CA dinucleotide of the 22-bp B element defined previously with the *dpm*6 revertants (8; compare the sequences in Fig. 1 with those in Fig. 2). These results suggest that the B element can be refined further and that the C-to-G transversion in the *dpm*2 mutation (Fig. 1) is not responsible for the debilitating effects of *dpm*2. These interpretations must be viewed with caution, however, because these revertant analyses cannot rigorously define the exact boundaries of enhancer elements. This is because we do not know the exact contribution of flanking sequences brought in by the junction created by the tandem duplication. These flanking sequences may restore a functional element but not the exact sequences of the original element.

The structures of the *dpm*26 revertants are of particular interest because the B element was preferentially duplicated in revertants of the *dpm*6 mutant and hitherto the A element had been defined by a single 21-bp duplication in the relatively weak *dpm*6 revertant *rd*21 (8). The structures of the 12 *dpm*26 revertants show that when the B element is mutated in combination with the C element, duplication of the A element is best able to compensate for loss of virus viability. These *dpm*26 revertants define a 23-bp common region that spans the entire 21-bp A element. The exact structure of the A element as defined by *dpm*6 *rd*21 is ambiguous because of a 3-bp terminal redundancy (Fig. 1). The 23-bp sequence common to the *dpm*26 revertants does not contain the 5′-terminal G residue, suggesting that this nucleotide, which lies within the aforementioned core homology (Fig. 1), is not a part of the A element.

In combination with the structures of the *dpm*12 revertants described previously (9), the structures of the *dpm*16 and *dpm*26 revertants show that the A, B, and C elements are each capable of compensating for loss of function in the other two. This contrasts with the structures of revertants of the triple ABC mutant *dpm*126, most of which contained complex rearrangements and in which no other elements could be identified by simple duplication patterns (8).

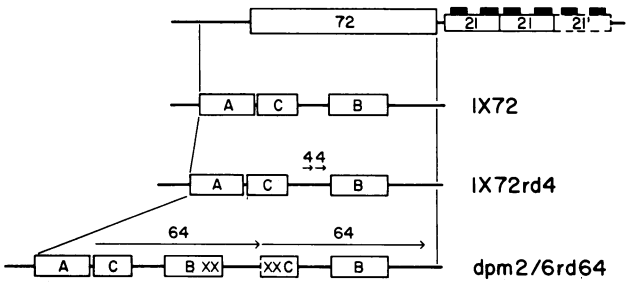

FIG. 3. Structures of recombinants between SV40 enhancer mutants *dpm*2 (B-element mutant) and *dpm*16 (A- and C-element mutant). The enhancer regions from the three recombinants, a 1X72 isolate, 1X72 *rd*4, and *dpm*2/6 *rd*64, are shown below a diagram of the SV40 early promoter region showing the 21-bp repeats and a single 72-bp element. The positions of the A, B, and C elements in each recombinant are shown. In *dpm*2/6 *rd*64 the central B and C elements contain the *dpm*2 and *dpm*6 mutations (XX), respectively. The recombinants are aligned on the B element. The locations and sizes of the duplications in the 1X72 *rd*4 and *dpm*2/6 *rd*64 recombinants are indicated by arrows and numbers (base pairs). The duplicated sequences are 145 to 148 with a 4-bp ambiguity due to terminal redundancy in 1X72 *rd*4 and 112 to 175 in *dpm*2/6 *rd*64.

During the process of isolating revertants a set of matched 71-bp revertant duplications has arisen: *dpm*12 *rd*71 (9), *dpm*16 *rd*71 and *dpm*26 *rd*71 (this report), and *dpm*126 *rd*71 (8). Except for *dpm*16 *rd*71, the same sequences were duplicated in each instance. This 71-bp duplication spans the A and C elements and nearly all of the B element. In *dpm*16 *rd*71 the duplication is shifted two nucleotides downstream to encompass all but two nucleotides of the previously defined B element, presumably because the B element is the only functional wild-type element in the *dpm*16 mutant. These matched revertants may have arisen because they are effective enhancers (see below) or because this structure is favored by the recombination process.

Generation of revertant enhancers by recombination between heterologous SV40 genomes. During the isolation of the *dpm*16 revertants it became apparent that our virus stocks also contained *dpm*2-derived viruses. Thus, 11 rearranged *dpm*2 viruses were isolated along with the *dpm*16 revertants (see Materials and Methods for the structures of these *dpm*2 derivatives). Except for *dpm*2 *rd*87 (see below), these *dpm*2-derived viruses were not examined in detail. Some of these *dpm*2-related viruses contain small duplications (9 to 18 nucleotides) or, in one instance, an 18-bp deletion, but because the parental *dpm*2 enhancer is reasonably strong, it is difficult to establish the extent to which these small rearrangements improve growth potential and enhancer function. The copropagation of the *dpm*2 and *dpm*16 viruses resulted serendipitously, however, in the isolation of three probable *dpm*2/*dpm*16 recombinant viruses.

The structures of these putative *dpm*2/*dpm*16 recombinant viruses, 1X72, 1X72 *rd*4, and *dpm*2/6 *rd*64, are shown in Fig. 3. The 1X72 virus probably arose by homologous recombination within the 41-bp region that separates the *dpm*6 and *dpm*2 mutations, whereas the 1X72 *rd*4 and *dpm*2/6 *rd*64 isolates probably resulted from nonhomologous recombination between the *dpm*16 and *dpm*2 viral genomes. Only for *dpm*2/6 *rd*64, in which the *dpm*2 and *dpm*6 mutations are linked, can we be absolutely confident that the recombinant arose from the *dpm*16 and *dpm*2 viruses. Because a 1X72 virus has not appeared in any of the other isolations of revertants and because we have been unable to isolate rearrangements of the 1X72 virus (unpublished results), it is likely that the 1X72 and 1X72 *rd*4 isolates also arose from recombination between the *dpm*16 and *dpm*2 viruses. These results show that the revertant duplications can arise by recombination between separate viral genomes. Furthermore, the paucity of 1X72 isolates from this mixed infection suggests that homologous recombination within the 41-bp region between the *dpm*6 and *dpm*2 mutations is not greatly favored over nonhomologous recombination during the isolation of revertants.

Revertant duplications are responsible for restored virus viability and enhancer function. Our analysis of the *dpm* revertants focused on the enhancer region and would not have detected additional point mutations or small rearrangements elsewhere in the revertant viral genome. Therefore, it was important to show that the rearrangements we describe were indeed responsible for restored viral growth. We accomplished this by replacing the wild-type enhancer with the revertant enhancer regions and determining whether the restored virus viability and the revertant rearrangements cosegregate. To determine whether the revertant phenotype also correlates with restored enhancer function, we assayed the ability of the rearranged mutant enhancer regions to activate transcription of the human β-globin gene during transient expression in CV-1 cells. Here, we assayed the activity of the *dpm*16 revertants *rd*56, *rd*61, *rd*71, and *rd*84, the *dpm*26 revertants *rd*43 and *rd*71, and the *dpm*2 revertant *rd*87. This selection includes revertants that define the boundaries of the common regions and also the nearly matched *dpm*16 and *dpm*26 *rd*71 revertants (see above). We also assayed the activity of the two *dpm*2/*dpm*16 recombinants 1X72 *rd*4 and *dpm*2/6 *rd*64.

The relative numbers and sizes of plaques formed by the *dpm*2, *dpm*16, and *dpm*26 mutants and revertants relative to those formed by wild-type SV40 with a 72-bp repeat (2X72)

TABLE 1. Viability and enhancer potential of *dpm*2, *dpm*16, and *dpm*26 mutants and revertants[a]

Sample	Relative infectivity	Relative plaque size	Relative level of β-globin RNA (mean ± SD)
2X72	1.0	1.0	1.0
1X72	0.5	0.6	0.2 ± 0.08
1X72 *rd*4	0.4	0.6	0.4 ± 0.01
*dpm*2/6 *rd*64	0.4	0.6	0.5 ± 0.1
*dpm*2	0.08	0.3	0.1
*dpm*2 *rd*87	0.5	0.7	0.6 ± 0.1
*dpm*16	8×10^{-3}	0.2	0.05 ± 0.01
*dpm*16 *rd*56	0.2[b]	ND[c]	0.1
*dpm*16 *rd*61	ND	ND	0.3
*dpm*16 *rd*71	0.3	0.4	0.5 ± 0.1
*dpm*16 *rd*84	0.2	0.4	0.2 ± 0.1
*dpm*26	8×10^{-4}	0.06	0.02 ± <0.01
*dpm*26 *rd*43	0.02	0.2	0.2 ± 0.06
*dpm*26 *rd*71	0.1	0.4	0.5 ± 0.1
enh$^-$			0.02 ± 0.01

[a] Relative infectivity and plaque size were assayed as described in the text. The results are the averages of two separate series of assays. The numbers and sizes of the plaques were measured 13 or 14 days posttransfection, and the results are expressed relative to those for the wild-type construct (pK1K1 2X72), which produced 1×10^4 to 2×10^4 plaques per μg, with an average diameter of 7 mm. Enhancer activity, as reflected by the relative levels of β-globin RNA after transient expression of the human β-globin gene in CV-1 cells, was determined as described in the text. The results are the averages of two independent determinations except for *dpm*2, *dpm*16 *rd*56, and *dpm*16 *rd*61, for which there was only one determination.

[b] The *dpm*16 *rd*56 revertant was tested in a separate series of transfections.

[c] ND, Not determined.

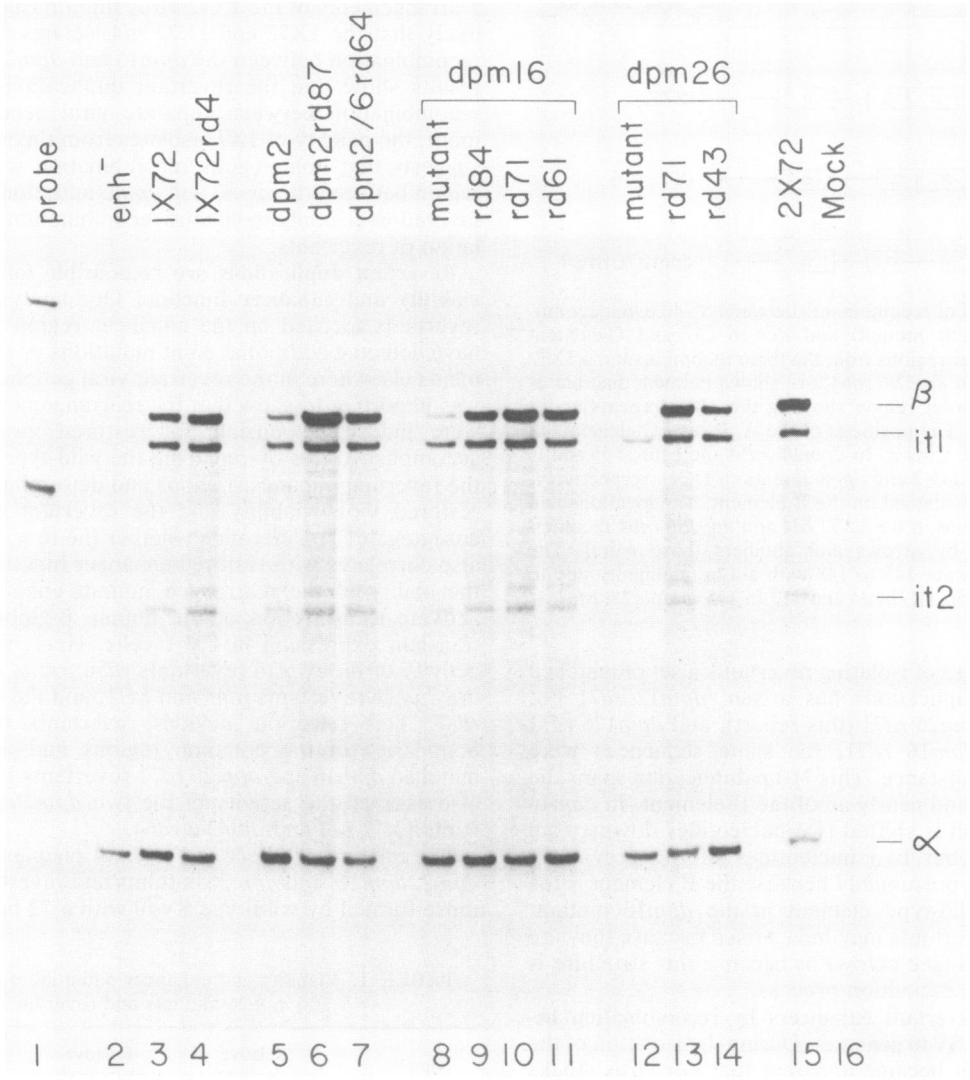

FIG. 4. Rearrangements within the enhancer region of SV40 mutants *dpm*2, *dpm*16, and *dpm*26 restore enhancer function, as assayed by transient expression of the human β-globin gene. The autoradiograph shows the relative levels of α- and β-globin probe RNAs protected from RNase digestion by cytoplasmic RNA isolated from transfected CV-1 cells. CV-1 cells (two 100-mm plates) were transfected with a total of 20 μg of plasmid DNA per plate: 4 μg of β-globin test plasmid, 2 μg of α-globin reference plasmid, and 14 μg of pUC119 carrier DNA, as described in the text. The correctly initiated α- and β-globin protected fragments are labeled α and β, respectively. Two incorrect β-globin transcripts labeled it1 and it2 probably resulted from incorrect splicing of aberrant transcripts (3). Lane 1, Undigested probe; lane 2, transfection of the enhancer deletion plasmid πSVHSβΔ128; lanes 3 through 15, transfection of the wild-type and mutant πSVHHβΔ128 plasmids as indicated; lane 16, transfection of pUC119 DNA alone.

are shown in Table 1. The *dpm*2, *dpm*16, and *dpm*26 mutants were progressively less viable than the parental wild-type virus, 1X72. Separate experiments showed that the *dpm*1, *dpm*2, and *dpm*6 mutations each affect both SV40 viability and enhancer function, with *dpm*1 having the least effect and *dpm*6 the greatest effect (data not shown). The pairwise combination of these mutations always generates less viable viruses (Table 1; 9), showing that in each instance a pair of heterologous wild-type elements is more effective for SV40 growth than are solitary A, B, or C elements. Indeed, the low level of plaque formation by the more severe mutants, such as *dpm*26, actually resulted from formation of revertants during plaque formation (e.g., *dpm*26 revertants *rd*62a, *rd*62b, and *rd*301 were directly isolated from plaques) rather than from an intrinsic ability of the mutants to produce plaques. Except for *dpm*16 *rd*84, which also contains four point mutations, assay of each of the revertant viruses showed that in each instance the revertant duplication alone can restore the viability of these viruses. For *dpm*16 *rd*84 we do not know the relative contribution of the 84-bp duplication and the point mutations (Fig. 2).

The results of an assay of enhancer function are shown in Fig. 4. Mutant and revertant enhancers were cloned into the plasmid πSVHHβΔ128, which contains the human β-globin gene. Plasmid DNAs were transfected along with an α-globin reference plasmid into CV-1 cells by calcium phosphate coprecipitation as described previously (8). The levels of α- and β-globin RNA were assayed by RNase protection of

internally labeled single-stranded RNA probes after hybridization to total cytoplasmic RNA. The results of β-globin RNA activation from two experiments are shown in Table 1. As in the virus viability assays, the revertant duplications always displayed greater levels of enhancer function than did their parental mutants. These relative increases can be quite large. For example, the simple tandem duplication in the *dpm*26 *rd*71 revertant generated an enhancer 25-fold more active than that of the parental mutant.

SV40 viability and enhancer function within each set of mutants and revertants exhibits a good correlation. Between sets, however, the correlation does not always hold true. For example, *dpm*26 *rd*71 was threefold less viable than *dpm*16 *rd*71, but both revertants were equally capable of activating β-globin gene expression (Table 1). This lack of correlation may reflect differences in how the A, B, and C elements can activate transcription in the context of the SV40 early promoter versus activation of the heterologous β-globin promoter. Alternatively, the mutations may affect other viral functions in addition to enhancer activity.

The activity of the two *dpm*2/*dpm*16 recombinants, 1X72 *rd*4 and *dpm*2/6 *rd*64, in both the plaque assays and enhancer assays was very similar to that of the 1X72 construct itself. This suggests that the spacing between the B element and the A and C elements is not critical for SV40 viability. The enhancer assay results are consistent with the results of Zenke et al. (24), which indicated that the spacing between these two sets of elements is not critical for SV40 enhancer function.

DISCUSSION

The structures of revertants of the *dpm*16 and *dpm*26 SV40 enhancer mutants, along with those of the revertants of the *dpm*12 mutant (9), show that any one of three elements within the SV40 enhancer can compensate for loss of function in the other two elements by simple duplication. These patterns of rearrangement display the plasticity of enhancers; when one or two elements are inactivated, amplification of a remaining element(s) can restore activity. A previous study showed that when all three of these elements were mutated no new elements could be identified by simple tandem duplications (8). These results suggest that in the CV-1 cell line used for the isolation of revertants the A, B, and C elements are the only enhancer elements that by simple duplication can easily compensate for loss of SV40 enhancer function.

Studies of SV40 revertants do not address the intrinsic enhancer activity of the A, B, and C elements because auxilliary elements could exist which promote enhancer function but are not capable of replacing A-, B-, or C-element function. We have tested the intrinsic activity of these elements by constructing synthetic enhancers composed of multiple tandem 17- or 21-bp oligonucleotides representing the wild-type and mutant A, B, and C elements (15). These experiments showed that each of these elements can function as an enhancer when present as multiple tandem repeats and that the *dpm*1, *dpm*2, or *dpm*6 mutations appear to completely inactivate each element. The latter results indicate that the increasingly deleterious effect of the *dpm*1, *dpm*2, and *dpm*6 mutations in the A, B, and C elements, respectively, probably reflects the relative contribution of each element to 1X72 enhancer function. Consistent with this interpretation is the finding that when the C element is mutated, the B element is more frequently duplicated than the A element to restore virus viability (8).

Zenke et al. (24) have described a detailed series of triple point mutations that scan the SV40 enhancer region and have assayed the ability of the mutants to activate the SV40 early promoter in HeLa cells. These mutagenesis experiments showed that mutations within each of the A, B, and C elements affect enhancer function. This correlation is especially gratifying because of the difference in the assays; in particular, the mutagenesis experiments assay for loss of enhancer function, whereas the revertant analyses assay for gain of function. The activity of the various triple point mutants suggested that the SV40 enhancer could be divided into two domains, A and B (24). The 30-bp A domain contains the B element identified by the SV40 revertants, whereas the 52-bp B domain encompasses both the A and C elements. Thus, the B domain of Zenke et al. (24) is composed of two separate elements, A and C. Wildeman et al. (23) have suggested that the B domain may contain two subdomains B1 and B2, but these subdomains do not match the A and C elements, since the boundary between subdomains B1 and B2 lies at the center of the A element. In our experiments, sequences on both sides of this boundary are required for A-element function (15).

The simple tandem duplications found in the *dpm*12, *dpm*16, and *dpm*26 revertants can create enhancers that are more active (as assayed by activation of β-globin gene expression) than the original wild-type 1X72 enhancer (compare for example the *rd*71 revertants of the *dpm*16 and *dpm*26 mutants [Table 1]). This contrasts with the relatively weak activity of in vitro SV40 enhancer constructs containing two copies of either the *ori*-proximal (A domain) or *ori*-distal (B domain) half of the SV40 enhancer (24). This difference is probably the result of selection of revertant duplications for function in vivo. Although these revertant duplications created effective enhancers in the β-globin expression assay, in general the revertants were less viable than the wild-type 1X72 virus. This may reflect effects on functions other than enhancer activity by the mutations or the difference in activating the SV40 early and late promoters compared with a heterologous promoter.

The sizes of the revertant duplication patterns are very heterogeneous (Fig. 2). This large variation in size and the lack of helical periodicity in the repeat sizes suggests that spacing between the elements is not critical to enhancer function. There could, however, be auxilliary elements that went undetected in our experiments and which allow for greater flexibility in the duplication patterns while still satisfying a strict spacing requirement. Although spacing between SV40 early promoter elements can be critical for promoter function (18), insertion of spacer sequences between the B and C elements do not critically affect SV40 enhancer function (24). Consistent with these results, we found that 1X72 *rd*4, which contains a 4-bp duplication (about one-half turn of the DNA helix) between the B and C elements, and *dpm*2/6 *rd*64 (Fig. 3) were as active as the 1X72 enhancer for both virus growth and enhancer function. It is not clear, however, whether this reflects a lack of stereospecific interactions among the A, B, and C elements. These changes in spacing rotate the A and C elements simultaneously with respect to the B element. The A and C elements may lie on opposite sides of the helix such that as the spacing between the B and A plus C elements is changed, the important functional interactions shift back and forth between the B and C elements and the B and A elements. Analysis of the spacing requirements of a pair of synthetic elements should resolve these different interpretations.

In general, the revertants have been isolated from pure

mutant populations, and thus it has not been possible to determine whether the rearrangements arose by an intragenomic rearrangement, e.g., strand slippage or sister chromosome exchange, or by recombination between separate genomes. The isolation of recombinants between the mutants *dpm*2 and *dpm*16 (Fig. 3) shows that the revertant duplications can arise by recombination between separate genomes. Among the three separate *dpm*2/*dpm*16 recombinants, only one appears to have arisen by homologous recombination within the 41-bp region between the *dpm*2 and *dpm*6 mutations. This result is consistent with the high rate of nonhomologous recombination observed during SV40 propagation; viruses containing genomic rearrangements due to illegitimate recombination arise rapidly during passage of SV40 at high multiplicities of infection (12, 17). Polyomavirus, a closely related papovavirus, also undergoes rearrangements which can restore activity to mutated enhancers (19) or change the host range of the viral enhancer (1, 7).

These analyses show how a high rate of nonhomologous recombination can be evolutionarily advantageous. Recent results obtained with the SV40 enhancer show that the A, B, and C elements each possess different cell-specific enhancer activities (15, 16). Thus, through amplification of particular elements by nonhomologous recombination, the cell-specific activity of the SV40 enhancer can be altered and allow for a favorable response to changes in the cellular environment.

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