MOLECULAR AND CELLULAR BIOLOGY, Apr. 1985, p. 649–658
0270-7306/85/040649-10$02.00/0

Vol. 5, No. 4

Polyomavirus Enhancer Contains Multiple Redundant Sequence Elements That Activate Both DNA Replication and Gene Expression

GEERTRUIDA M. VELDMAN, STEPHEN LUPTON,† AND ROBERT KAMEN*
Genetics Institute, Boston, Massachusetts 02115

Received 9 October 1984/Accepted 21 December 1984

Sequences that comprise the 244-base-pair polyomavirus enhancer region are also required in *cis* for viral DNA replication (Tyndall et al., Nucleic Acids Res. 9:6231–6250, 1981). We have studied the relationship between the sequences that activate replication and those that enhance transcription in two ways. One approach, recently described by de Villiers et al. (Nature [London], 312:242–246, 1984), in which the polyomavirus enhancer region was replaced with other viral or cellular transcriptional enhancers suggested that an enhancer function is required for polyomavirus DNA replication. The other approach, described in this paper, was to analyze a series of deletion mutants that functionally dissect the enhancer region and enabled us to localize four sequence elements in this region that are involved in the activation of replication. These elements, which have little sequence homology, are functionally redundant. Element A (nucleotides 5108 through 5130) was synthesized as a 26-mer with *Xho*I sticky ends, and one or more copies were introduced into a plasmid containing the origin of replication, but lacking the enhancer region. Whereas one copy of the 26-mer activated replication only to 2 to 5% of the wild-type level, two copies inserted in either orientation completely restored replication. We found that multiple copies of the 26-mer were also active as a transcriptional enhancer by measuring the β-globin mRNA levels expressed from a plasmid that contained either the polyomavirus enhancer or one or more copies of the 26-mer inserted in a site 3′ to the β-globin gene. We observed a correlation between the number of inserted 26-mers and the level of β-globin RNA expression.

Transcriptional enhancers were originally identified within the genomes of DNA tumor viruses simian virus 40 (SV40) (2, 38) and polyomavirus (8, 9) as DNA segments that stimulate the transcription of linked genes in an orientation-independent manner and that function over long distances (16, 26). Many other viral (19, 20, 22, 27–32) and several cellular (1, 14, 15, 41) enhancers have since been described. Although the general importance of enhancers in the regulation of transcription has been clearly demonstrated, numerous questions about their structure and function remain unanswered. There are striking DNA sequence homologies shared among several enhancers, but no unique consensus sequence common to all of them has emerged. Some enhancers contain direct tandem repeats of sequences about 70 base pairs (bp) in length. The repeats represent redundant copies of the same signal, because deletion of either does not alter transcriptional efficiency (2). Other enhancers lack direct repeats, but still involve elements dispersed over 100 to 300 bp that appear to be in some way functionally redundant. The polyomavirus enhancer was the first example of the latter situation (9, 46). This enhancer occurs within a 244-bp DNA fragment that also includes *cis*-acting elements essential for viral DNA replication. The sequences within the polyomavirus enhancer region that activate replication are distinct from the viral origin of replication itself, but have not been resolved from sequences with transcription enhancer activity (46).

Our laboratory has been interested in determining the relationship between the polyomavirus sequences that enhance transcription and those that activate DNA replication. This question has been addressed in two ways. One approach was to remove from the viral genome the 244-bp fragment containing both the enhancer and the replication activator and then to introduce known viral or cellular enhancer sequences. The results of this study, recently reported by de Villiers et al. (10), suggested that it is the enhancer function that is required in *cis* to activate polyomavirus DNA replication. The other approach was to construct an extensive set of deletion mutants and use these to dissect functionally the enhancer region. The results obtained are presented in this paper.

We decided to localize the sequence elements important for the activation of viral DNA replication and then to test these for enhancer activity. The mutants were tested in a quantitative assay that measured replicatory ability independent of any requirement for viral gene expression. We identified four sequence elements that are involved in the activation of replication. These elements have little sequence homology, but are functionally redundant. The presence of only one element resulted in weak or undetectable levels of DNA replication, but two or three copies of the same element, in either orientation, completely restored the replication efficiency to the wild-type level. Multiple copies of this element were also active as a transcriptional enhancer, although we observed a quantitative difference between replication activation and transcriptional enhancement.

MATERIALS AND METHODS

Cells. Mouse 3T6 fibroblast cells, COP-5 (a derivative of C127, transformed by replication-defective polyomavirus DNA; 46) cells, and human carcinoma HeLa cells were propagated in Dulbecco modified Eagle medium containing

* Corresponding author.
† Present address: Department of Microbiology, State University of New York, Stony Brook, NY 11794.

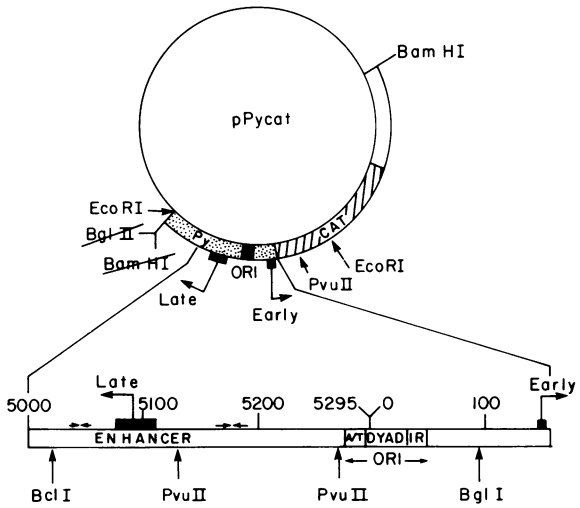

FIG. 1. Structure of pPycat. The plasmid contains the polyomavirus *Bam*HI (nt 4632)-to-*Hph*I (nt 152) fragment (comprising the late promoter, the enhancer region, the origin of replication, and the early promoter including the initiation sites) joined to the *Hin*dIII-to-*Bam*HI fragment spanning the CAT gene, intron, and polyadenylation signal from plasmid pSV2cat (17). The polyomavirus regulatory region is enlarged, demonstrating the enhancer region located between the *Bcl*I site and the origin-proximal *Pvu*II site. Small arrows indicate sequences with dyad symmetry in the enhancer. The replication origin (ORI) comprises an A+T-rich stretch (A/T), a dyad symmetry (DYAD), and an inverted repeat (IR). The major late RNA (7) and early RNA (6) start sites are indicated by black boxes and large arrows.

5% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Deletion mutants. Two sets of mutants with deletions in the polyomavirus regulatory region were initially constructed with the plasmids p73.33.1 and p84.A2.X. These plasmids contain full-length polyomavirus strain A2 DNA cloned into the *Bam*HI site of the vector pXf3 (35) and are identical except for an *Xho*I linker inserted at either the unique viral *Bgl*I site (nucleotide [nt] 93, p73.33.1) or at the *Bcl*I site (nt 5021, p84.A2.X) (Fig. 1). We use throughout the paper the nucleotide numbering system of Tyndall et al. (46). p73.33.1 and p84.A2.X DNAs were digested with *Xho*I and treated with nuclease *Bal*31 for different times. After ligation to *Xho*I linkers the DNAs were transfected into *Escherichia coli* strain HB101. Individual deletion mutants were selected, and the extents of the deletions were estimated by polyacrylamide gel electrophoresis after appropriate restriction enzyme digestion. From these bidirectional deletion mutants, as well as from certain mutants of the pdl2000 series described by Tyndall et al. (46), two sets of unidirectional deletion mutants were derived. For this purpose, we used a plasmid (pPy34BXΔ; 8) in which the entire 244-bp *Bcl*I-to-*Pvu*II enhancer fragment is deleted and replaced by an *Xho*I linker. We ligated mutant fragments, extending from the *Xho*I sites to an unique *Cla*I site within the plasmid vector, to the appropriate *Xho*I-*Cla*I fragment from pPy34BXΔ. This generated unidirectional mutants extending to various positions within the enhancer region from either the *Pvu*II site at nt 5265 or from the *Bcl*I site at nt 5021. The constructs, at this point, contained the entire polyomavirus genome except for the deleted sequences.

Plasmid pPycat was constructed by a series of steps through which the SV40 regulatory region of pSV2cat (17) was exchanged for the polyomavirus regulatory region and the pBR322 vector was replaced with a derivative that would not inhibit replication in animal cells. In a three-way ligation, we joined a *Bam*HI-to-*Pst*I fragment from plasmid pXf3(35), the *Pst*I-to*Hin*dIII fragment of plasmid pBR-BglII (pBR322 with a *Bgl*II linker in the *Eco*RI site, kindly provided by R. Kay, Genetics Institute, Inc.), and the *Hin*dIII-to-*Bam*HI chloramphenicol-acetyl transferase (CAT) gene fragment from pSVcat (16). This yielded pBglII-cat. The small *Bgl*II-to-*Hin*dIII fragment of pBglII-cat was replaced with the *Bam*HI-to-*Hph*I (nt 4632 to 152) fragment of polyomavirus DNA after blunting the *Hph*I and the *Hin*dIII sites with Klenow polymerase. The structure of the resulting recombinant, pPycat (Fig. 1), was confirmed by restriction mapping and nucleotide sequencing across the polyomavirus-to-CAT gene junction. It should be noted that this junction (GCCTCAGCTTGG) does not reconstitute the expected *Hin*dIII site, because *Hph*I cleaves one nucleotide away from its anticipated cutting site. Plasmid pPyΔcat was constructed in the same way with the *Bam*HI-to-*Hph*I fragment of pPy34BXΔ; it is therefore identical to pPycat, except for the deletion of the 244-bp *Bcl*I-to-*Pvu*II fragment.

The unidirectional deletions extending from either the *Pvu*II site or the *Bcl*I site described above were moved from the original constructs into pPycat derivatives by using three-way ligations. The *Bgl*I-to-*Bam*HI fragments (Fig. 1) of the deleted viral genome were joined with the *Bgl*II-to-*Nar*I fragment spanning the β-lactamase gene from pBglII-cat (see above) and the *Nar*I-to-*Bgl*I fragment spanning the CAT gene from pPycat. This yielded the Pvu series and the Bcl series, which differ from pPycat in the following two ways: the deletion in the polyomavirus regulatory region and the removal of the nonessential small *Nar*I fragments (791 bp) from near the replication origin in the pXf3 vector.

The structures of the final constructs were confirmed by restriction mapping, and the deletions were precisely determined by nucleotide sequencing. To facilitate the sequencing of 60 different mutants, we synthesized two different oligonucleotide primers and used alkali-denatured plasmids as templates for extension in the presence of dideoxynucleotides (P. Seeburg, personal communication). Approximately 24 mutants were selected for this detailed study.

The double deletion mutants, comprising the BcldP and PvudB series, were derived from the Bcl and Pvu series, respectively. The BcldP deletion mutants all lack the small *Pvu*II fragment (nt 5131 to 5265) and also have a deletion extending from the *Bcl*I site into the enhancer region. The PvudB series have, besides the deletion extending from the *Pvu*II site at nt 5265, a common deletion from the *Bcl*I site at nt 5021 to 5109.

To obtain the BcldP mutants, the corresponding Bcl mutants were digested with *Pvu*II. *Pvu*II cuts these plasmids at three sites, two in the enhancer region and one in the CAT coding region (Fig. 1). The large *Pvu*II fragment was then ligated to the *Pvu*II fragment spanning the polyomavirus origin of replication and extending into the CAT gene, derived from pPycat. Correct constructs were identified by hybridization with a synthetic oligomer spanning the deleted *Pvu*II fragment (nt 5131 to 5265) and by restriction enzyme mapping.

The PvudB mutants were constructed by ligating the large *Pvu*II fragment derived from the mutant B5110 to the small *Pvu*II fragment of the various Pvu mutants. *Pvu*II only cuts the Pvu mutants twice, namely, at nt 5130 and in the CAT gene (the *Pvu*II site at nt 5265 is deleted). Correct constructs were identified by restriction enzyme mapping.

Internal deletion mutants were constructed by combining the *Xho*I-*Bam*HI fragments of mutants from the Pvu and Bcl series.

Recombinants containing synthetic DNA. Synthetic oligodeoxyribonucleotides were made by J. Brown and E. Brown (Genetics Institute, Inc.) with an Applied Biosystem 380A DNA synthesizer. Two complementary 26-mers and two complementary 42-mers were prepared; on annealing these form the polyomavirus DNA sequences nt 5108 to 5130 and nt 5128 to 5167, respectively, flanked by cohesive ends corresponding to five-sixths and four-sixths of *Xho*I sites (see Fig. 6). One of the two strands of each set was phosphorylated and annealed to the second strand. The 26-mers or 42-mers were preligated for several hours to obtain multiple, tandem repeated copies, which were predominantly in a head-to-tail orientation. The synthetic oligomers were then ligated to *Xho*I-cleaved pPyΔcat (a derivative of pPycat, in which the enhancer region is deleted and replaced by an *Xho*I linker). The number and orientation of the inserted synthetic 26-mers or 42-mers was determined by restriction enzyme mapping and dideoxy sequencing with oligonucleotide primers with alkali-denatured plasmids as templates.

The 26-mers were also introduced into a plasmid containing the rabbit β-globin gene. The plasmid pβGPy enhancer (a β-globin polyomavirus enhancer recombinant that has the 244-bp polyomavirus enhancer fragment inserted at a synthetic *Xho*I site about 400 bp downstream of the β-globin-coding region; 8) was digested with *Xho*I, which excised the polyomavirus enhancer fragment, and subsequently ligated to preligated 26-mers. The number and orientation of the inserted 26-mers was determined by double digestions with *Xho*I and *Eco*RI. Since head-to-tail ligated 26-mers do not restore the *Xho*I site, the orientation of the 26-mers could be determined unambiguously. Mutants containing inverted repeated copies of the 26-mers were analyzed by Maxam and Gilbert DNA sequencing (36).

DNA replication assay. Each 90-mm culture dish containing about 2×10^6 COP-5 cells was transfected with 0.5 or 1 μg of plasmid DNA (pPycat or the enhancer deletion mutants) in a 2.5 ml of Tris-saline buffer containing 300 μg of DEAE-dextran per ml (37). Thirty minutes later, the cultures were washed with 10 ml of Tris-saline, followed by 10 ml of phosphate-buffered saline. Medium (10 ml) was then added, and the cultures were incubated at 37°C. At 24 h posttransfection, low-molecular-weight DNA was isolated by the Hirt extraction method (21). Control experiments demonstrated that the amount of replicated DNA was proportional to input in the 0.5- to 1.0-μg range at this time posttransfection. After phenol extraction and two cycles of ethanol precipitation, one-quarter of the DNA sample from each dish was digested with *Dpn*I and *Bam*HI in the presence of 20 μg of RNase A per ml (39). The digested DNAs were fractionated on 0.8% agarose gels, transferred to nitrocellulose (44), and hybridized to denatured pPycat DNA, which had been labeled with [α-^{32}P]dCTP by T4 DNA polymerase replacement synthesis after *Sau*3AI digestion (40). The band of replicated, *Dpn*I-resistant, linearized DNA was localized by autoradiography and excised for quantitation, which was accomplished by Cerenkov counting.

Analysis of β-globin mRNA levels. Each 90-mm culture dish containing about 3.6×10^6 HeLa cells was transfected with 20 μg of plasmid DNA by the calcium phosphate coprecipitation method (18). The plasmids used were pβGPy enhancer (a recombinant that has the polyomavirus enhancer inserted at an *Xho*I site downstream of the β-globin coding region; 8) or derivatives of pβGPy enhancer containing one or more copies of the synthetic 26-mer inserted in the same *Xho*I site. Cytoplasmic RNA was isolated 24 h posttransfection (11). S1 nuclease mapping (4) was done as described by Kamen et al. (23) with a single-stranded ^{32}P end-labeled DNA fragment derived from a globin gene clone lacking the first intron (2, 8) as a probe.

RESULTS

Constructs used for deletion analysis of the polyomavirus regulatory region were derived from the plasmid pPycat (Fig. 1). It contains an 815-bp segment of polyomavirus DNA spanning the enhancer, the replication origin, and both the early and late region promoters, linked to the CAT gene from pSV2cat (17). The viral DNA is orientated with the CAT coding sequences immediately downstream from the principal polyomavirus early region cap sites (6). The bacterial vector, a derivative of pXf3 (35), lacks sequences that inhibit extrachromosomal replication in animal cells (33). pPycat DNA replicates efficiently after transfection into the polyomavirus-transformed mouse cell line COP-5 (46), but since it requires a source of the polyomavirus large T antigen, it cannot by itself replicate in normal mouse cell lines such as 3T6 or C127.

The target for our deletion studies was the 244 bp of DNA previously shown to contain the polyomavirus enhancer (8, 9) and the sequences that activate viral DNA replication (34, 39, 46). This fragment extends from the *Bcl*II site in pPycat to the second *Pvu*II site (Fig. 1 and 2). The replication origin core sequence begins a few base pairs after this *Pvu*II site (24, 34, 45). Excision of the *Bcl*I-to-*Pvu*II fragment from pPycat (pPyΔcat) abolishes its ability to replicate in COP-5 cells.

Homologies between DNA sequences within the 244-bp fragment and other known viral or cellular enhancers are remarkably frequent (Fig. 2). Clusters of homologous sequences are apparent, such as those around nt 5110 or 5200. To allow for the possibility that each region of homology might represent a sequence with at least partial biological activity, we decided to construct a rather extensive series of deletion mutants. This was originally motivated by results (46) suggesting that the region was functionally repetitious with regard to both transcriptional enhancement and activation of viral DNA replication even though the important sequences included no direct repeats. Two sets of unidirectional single deletions were constructed, one extending from the *Bcl*I site toward the replication origin (the Bcl series) and the other extending from the origin proximal *Pvu*II site toward the *Bcl*I site (the Pvu series). Double deletion mutants were derived from the Bcl series by elimination of the small *Pvu*II fragment (the BcldP series) and from the Pvu series by elimination of he 89 bp immediately proximal to the *Bcl*I site (the PvudB series). All of the mutants contained synthetic *Xho*I restriction sites at the point of deletion to facilitate manipulations. The deletion mutants are numbered according to the first nucleotide present after the deletion irrespective of the fact that in some cases the *Xho*I linker restores one or more deleted nucleotides. Internal single deletions were constructed by combining members of the Bcl and Pvu series.

Replication of deletion mutants. (i) Pvu series. Mutants of the Pvu series have enhancer deletions extending from the origin-proximal *Pvu*II site (nt 5265) toward the endpoints indicated by the arrows for each deletion in Fig. 3 (e.g., in mutant P5179 the sequence between nt 5265 and 5179 is deleted). The ability of each mutant to replicate in COP-5

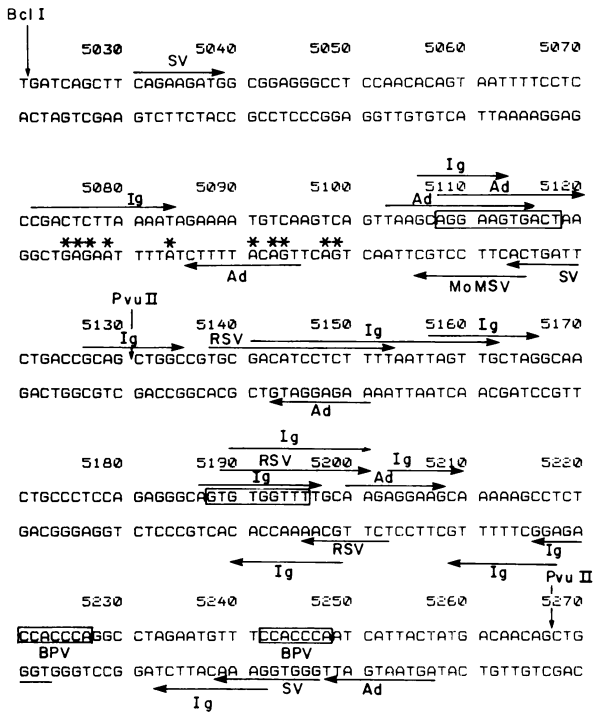

FIG. 2. Nucleotide sequence of the polyomavirus enhancer region. Arrows indicate the following homologies (80 to 100%) with sequences found in other viral or cellular enhancers: SV, SV40 72-bp repeat (2); Ad, adenovirus 5 E1A enhancer, 170 bp (19, 20); MoMSV, 72-bp tandem repeat from the Moloney murine sarcoma virus long terminal repeat (28, 29); RSV, the 143-bp enhancer region of Rous sarcoma virus (31); Ig, mouse immunoglobulin heavy-chain enhancer, 310 bp (1). Polyomavirus DNA sequences homologous to regions that were found to be crucial for enhancer activity in other enhancers are boxed. The sequence AGGAAGTGAC forms an important part of the adenovirus 5 E1A enhancer (19); the sequence GTGTGGTTT is related to the consensus sequence depicted by Weiher et al. (49) to be critical for the SV40 72-bp enhancer activity; the repeated sequence CCACCCA has homology to a similar sequence found twice in the bovine papilloma virus (BPV) enhancer in a region that is essential for enhancer function (48). The polyomavirus late transcription initiation sites (7) are marked by asterisks.

cells was measured 24 h after transfection by digesting low-molecular-weight DNA (21) with *Dpn*I and *Bam*HI. *Dpn*I cleaves only the methylated input (bacterial) DNA and will leave any newly replicated DNA molecules intact. *Bam*HI, which cuts the pPycat constructs only once (Fig. 1), generates a linear DNA fragment representing the total amount of progeny DNA in the COP-5 cells. The DNA was then fractionated on an agarose gel, blotted onto nitrocellulose, and hybridized to ^{32}P-labeled fragments of pPycat. The single band of radioactive DNA, representing progeny molecules, could be excised and counted for quantitation. Figure 3A shows the replication of the Pvu mutants. Deletion of sequences between nt 5265 and 5129 decreased the replication ability gradually to about 15% (P5129) of the wild-type level. Strikingly, the removal of 3 more bp completely abolished replication (compare mutants P5129 and P5126). We therefore identify one boundary of a minimal element involved in the activation of DNA replication between nt 5126 and 5129.

(ii) PvudB series. The progressive decline in replication efficiency observed with the Pvu series can be better appre-

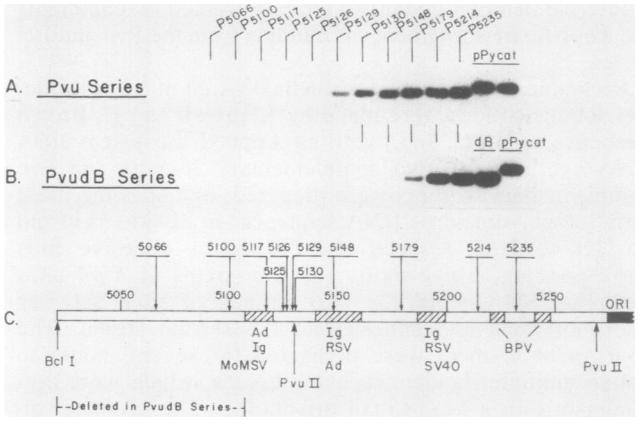

FIG. 3. DNA replication assay of enhancer region deletion mutants. COP-5 cells were transfected with 1 μg of DNA (pPycat or mutants of the Pvu and PvudB series) with DEAE-dextran. Low-molecular-weight DNA was isolated 24 h posttransfection, digested with *Dpn*I and *Bam*HI, fractionated on agarose gel, blotted, and hybridized to ^{32}P-labeled fragments of pPycat. The newly replicated DNA, linearized by *Bam*HI, was visualized by radioautography. (A) Pvu series. The deletions extend from the *Pvu*II site at nt 5265; e.g., P5214 has a deletion between nt 5214 and 5265. (B) PvudB series. The deletions are the same as those of the Pvu series plus a common deletion (dB) from the *Bcl*I site to nt 5110. Mutant dB is identical to B5110 (Fig. 4). (C) Diagram of the enhancer region. The arrows indicate the endpoints of the deletions. Sequences that have multiple homologies to other enhancers are hatched (Fig. 2). The deletion common to all PvudB mutants is indicated.

ciated from the quantitative results shown in Table 1. We suspected that important sequences involved in the activation of DNA replication were contained between the two *Pvu*II sites (nt 5130 to 5265), but that their effect could not be dramatically demonstrated because of stronger redundant element(s) between *Pvu*II and *Bcl*I (Fig. 3). We therefore introduced into the Pvu mutants a common deletion extending from the *Bcl*I site at nt 5021 to 5110 (dB deletion, Fig. 3C) and tested the double PvudB deletion series for replication in COP-5 cells. Removal of the sequence between nt 5021 and

TABLE 1. Replication efficiency of the polyomavirus enhancer deletion mutants in COP-5 cells

Pvu series			Bcl series		
Mutant	% Replication		Mutant	% Replication	
	Pvu	PvudB[a]		Bcl	BcldP[b]
pPycat	100		pPycat	100	
B5110		100	P5130		38
P5235	83	100	B5040	108	25
P5214	61	83	B5056	122	20
P5179	61	16	B5072	103	22
P5148	40	5	B5085	125	10
P5130	30	3	B5098	100	6
P5129	13		B5110	130	4
P5126	0		B5124	12	0
P5125	0		B5137	5	
P5117	0		B5166	0	
P5100	0		B5183	0	
P5066	0		B5198	0	

[a] Mutants of the PvudB series are derived from the Pvu series and have an extra deletion that extends from the *Bcl*I site toward nt 5110; the dB deletion is equivalent to B5110.

[b] The BcldP series is derived from the Bcl series, but has a common second deletion of the *Pvu*II fragment (nt 5130 to 5265); the dP deletion is equivalent to P5130.

5110 did not be itself have any effect on replication activation (mutant dB in Fig. 3B and B5110 in Table 1), but the double deletions extending from the *Pvu*II site (nt 5265) had drastically decreased replication efficiencies as may be seen by comparing the PvudB mutants and their corresponding Pvu mutants (Fig. 3A and B, Table 1). We can identify two transition points, each causing a three- to fivefold reduction in replication efficiency. One transition point lies between nt 5214 and 5179, and the other transition occurs between nt 5179 and 5148.

(iii) Bcl series. The Bcl series (Fig. 4) comprises mutants with deletions extending from the *Bcl*I site at nt 5021 toward the replication origin (e.g., mutant B5085 lacks the sequences between nt 5021 and 5085). Deletion of the 89 bp between the *Bcl*I site and nt 5110 did not alter the efficiency of replication (Fig. 4A), but a deletion up to nt 5124 markedly reduced replication to 5% of the wild-type level. Because the *Xho*I linker in B5110 in fact restores nt 5109, this identified a boundary of a sequence element involved in DNA replication between nt 5109 and 5124. Combined with the results obtained from the Pvu series presented above, we could predict that the sequence from nt 5109 to 5130 should function as a minimal replication activator.

(iv) BcldP series. The difference in replication efficiency between mutants of the Pvu and PvudB series (Fig. 3) indicated to us already that the region between the *Bcl*I site and nt 5109 (deleted in dB) in fact contains sequences which contribute to the replicatory activation, even though this was not detected in the single deletion mutants of the Bcl series. We studied this more directly with the BcldP double deletion series (Fig. 4B). These mutants have a common deletion of the *Pvu*II fragment from nt 5130 to 5265 plus variable deletions extending from the *Bcl*I site. Figure 4B shows that, in the absence of the *Pvu*II fragment, replication efficiency decreased when sequences between the *Bcl*I site and nt 5109 were deleted (Fig. 4B, Table 1). The mutant B5110dP, which contains only the sequence between nt 5109 and 5130, was able to replicate, as predicted from the

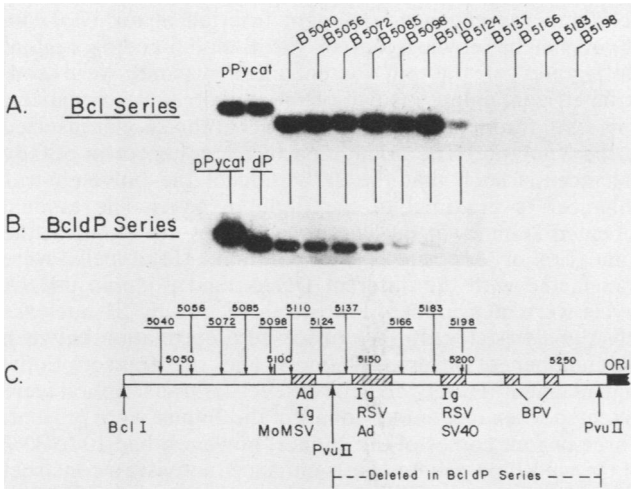

FIG. 4. DNA replication assay of enhancer deletion mutants. See the legend to Fig. 3. (A) Bcl series. The deletions extend from the *Bcl*I site; e.g., B5098 has a deletion between nt 5021 and 5098. (B) BcldP series. The double deletion mutants contain the same deletion as the corresponding Bcl mutants plus a deletion of the *Pvu*II fragment (nt 5130 to 5265). Mutant dP is identical to P5130 shown in Fig. 3. (C) See the legend to Fig. 3C. The deletion common to all BcldP mutants is indicated.

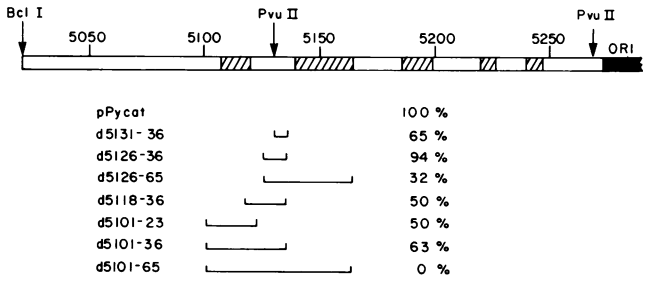

FIG. 5. DNA replication efficiency of internal deletion mutants. All mutants with internal deletions in the enhancer region contain an *Xho*I linker at the site of the deletion. The sizes of the deletions are indicated by bars and nucleotide numbers; e.g., in mutant d5131-36 the sequence from nt 5131 to 5136 is deleted. The efficiency of replication in COP-5 cells was measured by excision of the replicated DNA from the filter after hybridization followed by Cerenkov counting. The replication efficiency of pPycat was taken as 100%. Hatched regions in the enhancer represent sequences with multiple homologies to other viral or cellular enhancers (Fig. 2).

unidirectional deletion series, but in the absence of the other replication-activating elements it had an efficiency only 2 to 5% of the wild-type level.

(v) Internal deletion mutants. We can conclude from results presented thus far that the polyomavirus enhancer region contains multiple sequence elements involved in the activation of viral DNA replication. The difference in activity between single and double deletion mutants clearly showed that the elements are redundant, at least under the assay conditions we have used.

More evidence for this redundancy was provided by the study of internal deletion mutants shown in Fig. 5. The mutant lacking nt 5101 to 5136 (d5101-36) replicated at more than 50% of the wild-type level even though it has lost the entire minimal replication activation element previously located between nt 5109 and 5130. Other mutants (d5101-23 and d5118-36) that have lost part of the minimal element also had about one-half of the wild-type replication efficiency. Removal of the sequence from nt 5101 to 5165 (d5101-65), by contrast, completely abolished replication (Fig. 5). This result confirms the suggestion of Tyndall et al. (46) that there are two elements located on either side of the *Pvu*II site which are critically involved in the activation of DNA replication.

DNA replication activation by multiple copies of one element. The results presented above establish that the polyomavirus enhancer region contains multiple sequence elements that are involved in the activation of DNA replication. We wanted to test whether these elements are functionally equivalent. In other words, do multiple copies of one element restore replication to the wild-type level, or is a combination of different elements required?

The minimal element capable of activating replication was identified as the 22 bp from nt 5109 to 5130 in the experiments described above. The sequence common to the polyomavirus enhancer region and the adenovirus 5 E1A enhancer (19) is from nt 5108 to 5117. We therefore selected the viral sequence from nt 5108 to 5130 for synthesis and amplification. The double-stranded oligonucleotide shown in Fig. 6, comprising the 23 bp of viral DNA flanked by *Xho*I cohesive ends, was synthesized; one or more copies of this 26-mer were inserted into the *Xho*I site of the plasmid pPyΔcat, a pPycat derivative in which the enhancer region (nt 5022 to 5267) is deleted and replaced by an *Xho*I linker. The number and orientation of the 26-mers inserted in the

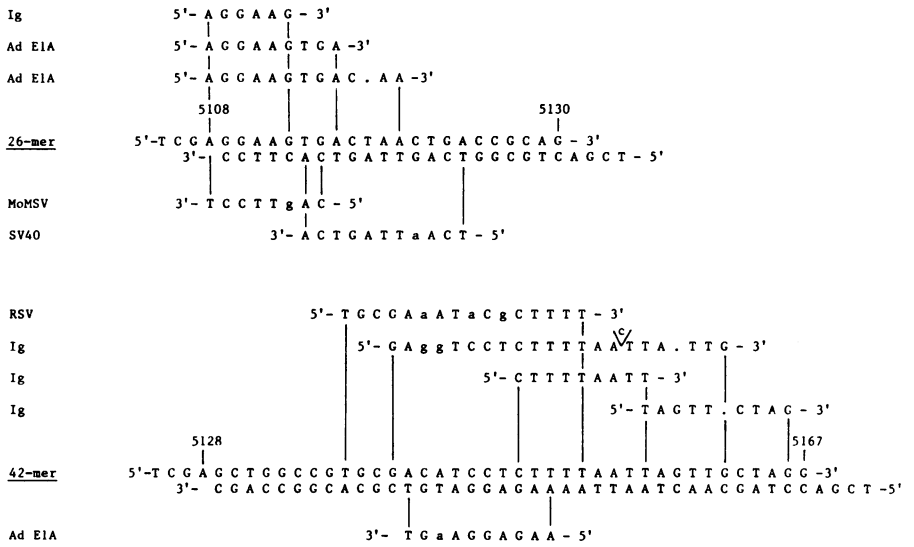

FIG. 6. Nucleotide sequence of the synthetic double-stranded 26-mer and 42-mer with *Xho*I cohesive ends. The 5′ end of each oligomer contains five-sixths of an *Xho*I site, whereas the 3′ end contains only four-sixths of an *Xho*I site. This implies that upon ligation to an *Xho*I cohesive end only the 5′ side will restore an *Xho*I cleavage site. The upper panel shows that the 26-mer contains the polyomavirus DNA sequence from nt 5108 to 5130. Homologies with sequences in the enhancers of the mouse immunoglobulin gene (Ig), adenovirus 5 E1A gene (Ad E1A), Moloney murine sarcoma virus long terminal repeat (MoMSV), and SV40 72-bp repeat are indicated. Nonhomologous nucleotides are shown in lowercase letters. The lower panel shows that the 42-mer contains the polyomavirus sequence between nt 5128 and 5167. It contains several extensive homologies to sequences present in the immunoglobulin gene (Ig), the adenovirus E1A gene, and the Rous sarcoma virus (RSV) enhancers.

plasmids were determined by analysis of restriction fragment lengths and DNA sequencing. Constructs containing one, two, or three head-to-tail tandem copies of the 26-mer inserted in either orientation were assayed for their ability to activate DNA replication. Figure 7 shows that insertion of two copies of the 26-mer was minimally sufficient to restore replication to the wild-type level. This activation was independent of the orientation of the inserted 26-mers (compare lanes 2+ and 2− in Fig. 7).

We also synthesized a double-stranded 42-mer containing the polyomavirus sequence from nt 5128 to 5167 (Fig. 2) flanked by *Xho*I sticky ends. As illustrated in Fig. 6, this 42-mer contains extensive homologies with sequences present in the mouse heavy chain immunoglobulin gene enhancer and the Rous sarcoma virus enhancer and some homology to the adenovirus 5 E1A enhancer and the 26-mer. One or more copies of the 42-mer were inserted in the *Xho*I site of the plasmid pPyΔcat. Constructs containing one, two, or five head-to-tail tandem copies of the 42-mer did not replicate at all after transfection into COP-5 cells (data not shown). This shows that the 42-mer on its own does not contain an element that is able to activate DNA replication and excludes the possibility that repeats of any sequence derived from the polyomavirus enhancer region can activate replication.

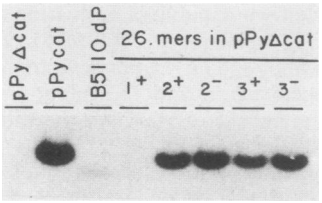

FIG. 7. DNA replication assay of constructs containing multiple copies of the synthetic 26-mer. Lanes 1, 2, and 3, respectively, contained plasmids having 1, 2, or 3 tandem repeated copies of the 26-mer inserted in the *Xho*I site of pPyΔcat (an enhancer minus derivative of pPycat) in the same orientation (+) as in the wild-type enhancer or in the opposite orientation (−).

Transcriptional enhancement by the synthetic 26-mer. The 26-mer, which contains an element that activates DNA replication, was tested for its activity as a transcriptional enhancer. We used a β-globin polyomavirus enhancer recombinant, pβGPy enhancer (8), which has the 244-bp polyomavirus enhancer fragment inserted at an *Xho*I site about 400 bp downstream of the β-globin-coding region. Derivatives of the pβGPy enhancer plasmid were constructed containing, instead of the polyomavirus enhancer, one, two, three, four, or seven copies of the 26-mer inserted in the *Xho*I site. The orientation of the enhancer in pβGPy enhancer is such that the early side of the polyomavirus enhancer is proximal to the β-globin gene. The tandem repeated 26-mers in the different mutants are either in the same (+) or opposite (−) orientation. HeLa cells were transfected with the different DNAs, and β-globin mRNA levels were measured 24 h posttransfection by S1 nuclease RNA analysis (Fig. 8). We observed a correlation between the number of inserted 26-mers and the transcriptional enhancement. Hardly detectable levels of transcription were detected when one or two copies of the 26-mer were present. Three or four copies of the 26-mer, however, had 10 to 30% of the wild-type polyomavirus enhancer activity; a construct containing seven copies of the 26-mer had a higher level of transcription than the plasmid pbGPy enhancer, which contains the wild-type polyomavirus enhancer region. The orientation of the seven copies of the 26-mer is not exclusively head to tail. The insert contains one copy in the − orientation, followed by six head-to-tail repeated copies in the + orientation. We cannot exclude the possibility that the observed enhancer activity is a result of the number of

inserted 26-mers in this construct or of the presence of an inverted repeat of two 26-mers. We obtained the same qualitative results upon transfection into mouse 3T6 cells, indicating that the 26-mer enhancer activity is similar in human and mouse cells.

The replication experiments described above have been done with polyomavirus DNA in COP-5 cells, whereas the transcription assays were performed with β-globin DNA in HeLa cells or 3T6 cells. To exclude the possibility that the difference in requirements for activation of replication (two copies of the 26-mer) and transcriptional enhancement (at least three or four copies) was caused by the different systems used, we also studied the replication efficiency of constructs related to pPy34BXΔ (8), which contain the complete polyomavirus genome (encoding large T antigen) in which the enhancer region is deleted and replaced by an *Xho*I site. One or more copies of the 26-mer were inserted in this *Xho*I site. Replication of these constructs in 3T6 cells is dependent on the ability of these DNAs to express polyomavirus large T protein from the early promoter, which requires a transcriptional enhancer. All constructs containing one to seven copies of the 26-mer were unable to replicate in 3T6 cells, although two to four copies activated replication in COP-5 cells in which large T protein is provided. These experiments clearly demonstrate that the quantitative differences observed in the sequence requirements for transcription as opposed to replication are independent of cell type and of position. The fact that the mutant containing seven copies of the 26-mer did not replicate in 3T6 cells or in COP-5 cells could be explained by the possibility that an inverted repeat of two 26-mers interferes with the structure of the origin of replication.

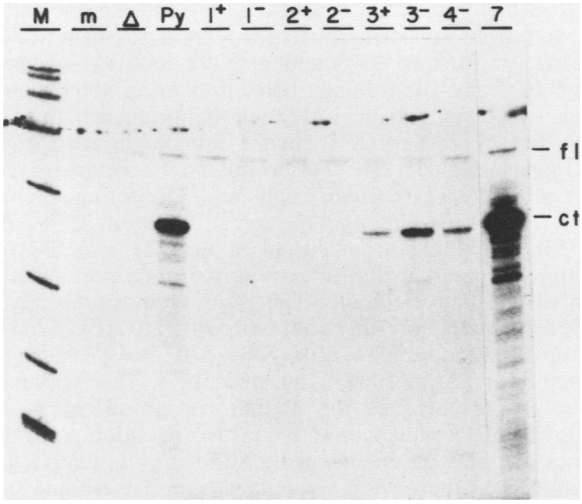

FIG. 8. S1 nuclease mapping of β-globin mRNA isolated from HeLa cells 24 h after transfection with the plasmid pβGPy enhancer (8) or derivatives lacking the polyomavirus enhancer, but containing multiple copies of the 26-mer inserted in the *Xho*I site 3′ of the β-globin coding region. Lanes: M, marker of polyomavirus DNA digested with *Dde*I; m, mock transfection; Δ, pβGPy enhancer derivative lacking the polyomavirus enhancer region; Py, pβGPy enhancer containing the polyomavirus enhancer region inserted in the *Xho*I site 3′ to the β-globin gene; 1, 2, 3, 4, and 7, pβGPy enhancer derivatives having 1, 2, 3, 4, or 7 copies of the 26-mer inserted in the *Xho*I site, respectively, in the same (+) orientation as in pβGPy enhancer or in the opposite (−) orientation. Abbreviations: fl, full-length single-stranded probe; ct, correct terminus of β-globin transcript.

DISCUSSION

To identify sequences in the polyomavirus enhancer region that are involved in the activation of viral DNA replication, we have constructed an extensive set of deletion mutants. Previous studies by Tyndall et al. (46) and Muller et al. (39) showed that the enhancer region consists of at least two sequence elements located within region A_L (or α, nt 5021 to 5130) and A_E (or β, nt 5131 to 5265), which are functionally redundant for replication activation. The individual elements within A_L and A_E also have transcriptional enhancement activity (5, 46; P. Herbomel and M. Yaniv, personal communication). Due to this redundance we had to compare numerous single and double deletion mutants for their ability to activate DNA replication to identify all sequence elements that contribute to this function. The assay conditions were chosen such that the amount of replicated DNA was proportional to the amount of input DNA at 24 h posttransfection. This enabled us to quantitate the replication activation by Cerenkov counting of the band representing the progeny DNA.

Several transition points, which indicate boundaries of functional sequence elements, were identified by a significant decrease in replication between two adjacent mutants in a deletion series. Mutants with deletions extending from the origin-proximal *Pvu*II site (Pvu or PvudB series) showed transition points between nt 5214 and 5179, nt 5179 and 5148, and nt 5129 and 5126. The sequence between nt 5214 and 5179 includes a region with multiple homologies to sequences in the enhancers of the immunoglobulin heavy-chain gene (1), Rous sarcoma virus (31), and the adenovirus 5 E1A gene (19) and contains the consensus sequence proposed by Weiher et al. (49), which is critical for the SV40 72-bp repeat enhancer activity. The next transition point between nt 5719 and 5148 includes sequences with major homologies to the immunoglobulin heavy-chain gene enhancer. Whether these two transition points indicate the boundaries of two distinct elements or are part of one element involved in replication activation cannot be deduced from these data. It is clear that the impact of the element(s) located between nt 5214 and 5148, which upon deletion cause a 15-fold decrease in replication activation, can only be observed with the PvudB deletion series, in which other elements located between the *Bcl*I site and nt 5110 are deleted. The transition point between nt 5129 and 5126 indicates a very discrete boundary of a replication-activating element because mutants P5129 and P5130 still replicate to 15 to 30% of the wild-type level, whereas mutant P5126 is completely incompetent for replication. The mutants of the Bcl series clearly mapped the other boundary of this functional element between nt 5109 and 5124. Evidence for yet another element comes from mutants of the BcldP series. Removal of sequences between the *Bcl*I site and nt 5098 in the BcldP series gave rise to a gradual decrease in replication efficiency. Although no significant transition points between adjacent mutants could be observed, it is nevertheless clear that this region also contains an element that contributes to the replication activation.

Figure 9 shows a schematic representation of the four elements described above. Because some of the elements are only identified by one transition point, the boundaries of those elements cannot be localized definitively. Herbomel and Yaniv (personal communication) have identified two sequence elements, A (including the adenovirus E1A homology) and B (including the SV40 homology), in the polyomavirus enhancer region which enhance CAT expression under

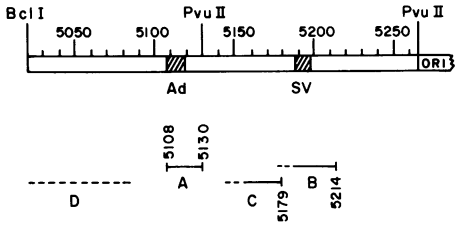

FIG. 9. Schematic representation of the polyomavirus enhancer region sequence elements A, B, C, and D, which are involved in the activation of viral DNA replication. Well-defined elements and boundaries are indicated by solid lines. Undefined boundaries are dashed. The sequences homologous to the adenovirus 5 E1A gene enhancer (Ad) and the SV40 enhancer core (SV) are indicated.

control of the α2 collagen promoter. According to their nomenclature we have named the well-defined replication activator located between nt 5108 and 5130 element A and the element identified by the transition between nt 5214 and 5179 element B. As discussed above, element C, identified by the transition between n 5179 and 5148, may be a distinct element or can be part of element B. Element D is located in the region between the *BclI* site and nt 5098. In this study we did not observe any contribution to replication activation by the sequences located between nt 5214 and 5268. We know, however, from previous studies (13, 43) that the sequence around nt 5233 plays a major role in the transcriptional enhancement as well as the replication of polyomavirus DNA in embryonal carcinoma cells.

Element A has a number of interesting features. It is homologous to a sequence that is duplicated in the adenovirus 5 E1A enhancer region and forms an important part of that enhancer (19). Ruley and Fried (42) have analyzed the DNA of several naturally occurring strains of polyomavirus and found that five strains have tandemly duplicated sequences of various sizes, but all contain the sequence between nt 5114 and 5137. Those strains, however, do not differ phenotypically from the wild-type polyomavirus strains A2 and A3. In other cases, duplications in this region can extend the host range of polyomavirus. A number of polyomavirus mutants with tandem duplications including the sequence between nt 5075 and 5135 have been isolated that are able to grow on PCC4 embryonal carcinoma cell lines (25).

The four elements that we have identified are functionally redundant. In all cases a combination of two or three elements is sufficient for efficient replication. Even element A, which has the highest activity, is not essential for high levels of replication when the other elements are present (internal deletion mutant d5101-36). In using the synthetic 26-mer comprising the polyomavirus sequence from nt 5108 to 5130, we were able to study the effect of multiple copies of one element on replication activation. Because two copies of the 26-mer in the absence of all other elements restored replication to the wild-type level, we conclude that a replication activator can comprise two identical elements or a combination of different elements that are functionally equivalent. The replication activator has one of the characteristics of an enhancer in that it can function independently of its orientation. The generation of an active transcriptional enhancer by multiplication of nonfunctional enhancer sequences has also been observed with fragments of the Rous sarcoma virus enhancer (30) and in experiments by Weber et al. (47) with an SV40 enhancer trap to identify functional enhancer sequences.

Mutants containing one or more copies of the synthetic 42-mer comprising the polyomavirus sequence from nt 5128 to 5167 did not replicate at all. The boundaries of this 42-mer were selected based upon the homology with sequences found in the enhancers of Rous sarcoma virus (31) and the immunoglobulin heavy-chain gene (1), and upon the observation that an internal deletion mutant lacking the sequence from nt 5101 to 5136 replicated to 60% of the wild-type level, whereas a mutant with a deletion from nt 5101 to 5167 did not replicate. This implied that the sequence between nt 5136 and 5167 contains an element or part of an element that is important for replication activation. Our result suggests that the 42-mer indeed does not contain a complete functional element that upon multiplication can act as replication activator.

In an experiment to study the activity of the 26-mer as a transcriptional enhancer, we observed a correlation between the number of inserted 26-mers and the level of β-globin RNA expression from the different constructs. Three or more copies of the 26-mer clearly enhanced transcription, but two copies of the 26-mer, which acted as a good replication activator, did not have significant enhancer activity. This might indicate that viral DNA replication requires an enhancer activity, but that there is a quantitative difference in the enhancer requirements for DNA replication and transcription.

We have conducted a computer-aided study of the polyomavirus enhancer region in a search for sequence features that could correspond to the four elements A through D. As pointed out previously (46), the 244-bp region includes a number of inverted repeats. The sequence in the top strand from nt 5097 to 5104 is the same as the sequence in the bottom strand from nt 5125 to 5118 (Fig. 2). We doubt whether this putative structure is important because mutant B5110, which replicated at wild-type level, lacks one half of it. Two further inverted repeats (nt 5158 to 5163 and nt 5172 to 5167; nt 5168 to 5178 and nt 5192 to 5181) occur in elements C and B. Although these may be of some significance, we cannot distinguish their importance at present from that of conserved sequence homologies that these regions also include. The 244-bp fragment contains several short stretches of repeated sequence. The tetranucleotide CAGT occurs five times (in the top strand at nt 5057, 5099, and 5186; in the bottom strand at nt 5123 and 5173) in positions suggesting that it may play an important role in each of the four elements. The same supposition can be made for the eight purine-rich tracts related to AGGAAG (in the top strand at nt 5032, 5108, 5180, 5201, and 5203; in the bottom strand at nt 5069, 5150, and 5220). This sequence, which is presented in the 26-mer, is conserved in the adenovirus E1A enhancer and is at the transition point that defines one border of element B. Although it is tempting to speculate that these short sequences comprise binding sites for enhancer factor proteins, our present data do not allow detailed interpretation.

Sequence requirements for viral DNA replication of the closely related papovavirus SV40 have been studied by several groups. Bergsma et al. (3) and Fromm and Berg (12) showed that, besides the origin of replication which comprises the palindrome and AT-rich region, sequences in the 21-bp repeats contribute to efficient replication. All of the deletion mutants used in their studies also lacked the 72-bp repeat enhancer. Recently Hertz and Mertz (personal communication) have shown that either the 72-bp repeat or the 21-bp repeat is required for the activation of SV40 DNA replication. This is similar to the situation in polyomavirus

(discussed above) in that sequences required for SV40 DNA replication are also redundant and include a transcriptional enhancer. We have also learned (M. Botchan, personal communication) that the extrachromosomal replication of plasmids containing the bovine papillomavirus plasmid maintenance sequence in bovine papillomavirus-transformed cells requires an enhancer in *cis*. It thus appears that the sequences originally identified within viral genomes as transcriptional enhancers may have more general functions, including the activation of viral DNA replication. Since the immunoglobulin heavy-chain enhancer can substitute for the polyomavirus enhancer in the activation of viral DNA replication (10), we are obviously curious to learn whether cellular enhancer sequences play analogous roles in chromosomal DNA replication.

ACKNOWLEDGMENTS

We are grateful to John Brown and Gene Brown for the preparation of synthetic oligonucleotides. We thank Jean de Villiers and Alison Cowie for valuable discussions and the gift of plasmid pβGPy-enhancer and Martha Richardson and Marybeth Erker for their help in preparing the manuscript.

LITERATURE CITED

1. **Banerji, J., L. Olson, and W. Schaffner.** 1983. A lymphocyte-specific cellular enhancer is located downstream of the joining region in immunoglobulin heavy chain genes. Cell **33**:729–740.
2. **Banerji, J., S. Rusconi, and W. Schaffner.** 1981. Expression of a β-globin gene is enhanced by remote SV40 DNA sequences. Cell **27**:299–308.
3. **Bergsma, D. J., D. M. Olive, S. W. Hartzell, and K. N. Subramanian.** 1982. Territorial limits and functional anatomy of the simian virus 40 replication origin. Proc. Natl. Acad. Sci. U.S.A. **79**:381–385.
4. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
5. **Cereghini, S., P. Herbomel, J. Jouanneau, S. Saragosti, M. Katinka, B. Bourachot, B. de Crombrugghe, and M. Yaniv.** 1983. Structure and function of the promoter-enhancer region of polyoma and SV40. Cold Spring Harbor Symp. Quant. Biol. **47**:935–944.
6. **Cowie, A., P. Jat, and R. Kamen.** 1982. Determination of sequences at the capped 5′ ends of polyoma virus early region transcripts synthesized *in vivo* and *in vitro* demonstrates an unusual microheterogeneity. J. Mol. Biol. **159**:225–255.
7. **Cowie, A., C. Tyndall, and R. Kamen.** 1981. Sequences at the capped 5′-ends of polyoma virus late region mRNAs: an example of extreme terminal heterogeneity. Nucleic Acids Res. **9**:6305–6322.
8. **de Villiers, J., L. Olson, C. Tyndall, and W. Schaffner.** 1982. Transcriptional 'enhancers' from SV40 and polyoma virus show a cell type preference. Nucleic Acids Res. **10**:7965–7976.
9. **de Villiers, J., and W. Schaffner.** 1981. A small segment of polyoma virus DNA enhances the expression of a cloned β-globin gene over a distance of 1400 base pairs. Nucleic Acids Res. **9**:6251–6264.
10. **de Villiers, J., W. Schaffner, C. Tyndall, S. Lupton, and R. Kamen.** 1984. Polyomavirus DNA replication requires an enhancer. Nature (London) **312**:242–246.
11. **Favaloro, J., R. Treisman, and R. Kamen.** 1980. Transcription maps of polyoma virus-specific RNA: analysis by two-dimensional nuclease S1 gel mapping. Methods Enzymol. **65**:718–749.
12. **Fromm, M., and P. Berg.** 1982. Deletion mapping of DNA regions required for SV40 early region promoter function *in vivo*. J. Mol. Appl. Gen. **1**:457–481.
13. **Fujimura, F. K., and E. Linney.** 1982. Polyoma mutants that productively infect F9 embryonal carcinoma cells do not rescue wild-type polyoma in F9 cells. Proc. Natl. Acad. Sci. U.S.A. **79**:1479–1483.
14. **Gillies, S. D., V. Folsom, and S. Tonegawa.** 1984. Cell type-specific enhancer element associated with a mouse MHC gene, E_β. Nature (London) **310**:594–597.
15. **Gillies, S. D., S. L. Morrison, V. T. Oi, and S. Tonegawa.** 1983. A tissue-specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene. Cell **33**:717–728.
16. **Gluzman, Y., and T. Shenk, eds.** 1983. Enhancers and eukaryotic gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Gorman, C. M., L. F. Moffat, and B. M. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
18. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
19. **Hearing, P., and T. Shenk.** 1983. The adenovirus type 5 E1A transcriptional control region contains a duplicated enhancer element. Cell **33**:695–703.
20. **Hen, R., E. Borrelli, P. Sassone-Corsi, and P. Chambon.** 1983. An enhancer element is located 340 base pairs upstream from the adenovirus-2 E1A cap site. Nucleic Acids Res. **11**:8747–8760.
21. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
22. **Jolly, D. J., A. C. Esty, S. Subramani, T. Friedmann, and I. M. Verma.** 1983. Elements in the long terminal repeat of murine retrovirus enhance stable transformation by thymidine kinase gene. Nucleic Acids Res. **11**:1855–1872.
23. **Kamen, R., P. Jat, R. Treisman, J. Favaloro, and W. R. Folk.** 1982. 5′ termini of polyoma virus early region transcripts synthesized *in vivo* by wild type virus and viable deletion mutants. J. Mol. Biol. **159**:189–224.
24. **Katinka, M., and M. Yaniv.** 1983. DNA replication origin of polyomavirus early proximal boundary. J. Virol. **47**:244–248.
25. **Katinka, M., M. Yaniv, M. Vasseur, and D. Blangy.** 1980. Expression of polyoma early functions in mouse embryonal carcinoma cells depend on sequence rearrangements in the beginning of the late region. Cell **20**:393–399.
26. **Khoury, G., and P. Gruss.** 1983. Review: enhancer elements. Cell **33**:313–314.
27. **Kriegler, M., and M. Botchan.** 1983. Enhanced transformation by a simian virus 40 recombinant virus containing a Harvey murine sarcoma virus long terminal repeat. Mol. Cell. Biol. **3**:325–339.
28. **Laimins, L. A., P. Gruss, R. Pozzatti, and G. Khoury.** 1984. Characterization of enhancer elements in the long terminal repeat of Moloney murine sarcoma virus. J. Virol. **49**:183–189.
29. **Laimins, L. A., G. Khoury, C. Gorman, B. Howard, and P. Gruss.** 1982. Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **79**:6453–6457.
30. **Laimins, L. A., P. Tsichlis, and G. Khoury.** 1984. Multiple enhancer domains in the 3′ terminus of the Prague strain of Rous sarcoma virus. Nucleic Acids Res. **12**:6427–6442.
31. **Luciw, P. A., J. M. Bishop, H. E. Varmus, and M. R. Capecchi.** 1983. Location and function of retroviral and SV40 sequences that enhance biochemical transformation after microinjection of DNA. Cell **33**:705–716.
32. **Lusky, M., L. Berg, H. Weiher, and M. Botchan.** 1983. Bovine papilloma virus contains an activator of gene expression at the distal end of the early transcription unit. Mol. Cell. Biol. **3**:1108–1122.
33. **Lusky, M., and M. Botchan.** 1981. Inhibition of SV40 replication in simian cells by specific pBR322 DNA sequences. Nature (London) **293**:79–81.
34. **Luthman, H., M. G. Nilsson, and G. Magnusson.** 1982. Non-contiguous segments of the polyoma genome required in cis for DNA replication. J. Mol. Biol. **161**:533–550.
35. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Maxam, A., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. U.S.A. **74**:560–564.
37. **McCutchan, J. H., and J. S. Pagano.** 1968. Enhancement of the

infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. J. Natl. Cancer Inst. **41**:351–357.
38. **Moreau, P., R. Hen, B. Wasylyk, R. Everett, M. P. Gaub, and P. Chambon.** 1981. The SV40 72 base pair repeat has a striking effect on gene expression both in SV40 and other chimeric recombinants. Nucleic Acids Res. **9**:6047–6068.
39. **Muller, W. J., C. R. Mueller, A.-M. Mes, and J. A. Hassell.** 1983. Polyomavirus origin for DNA replication comprises multiple genetic elements. J. Virol. **47**:586–599.
40. **O'Farrell, P.** 1981. Replacement synthesis method of labelling DNA fragments. Bethesda Research Laboratories. Focus **3**:1.
41. **Picard, D., and W. Schaffner.** 1984. A lymphocyte-specific enhancer in the mouse immunoglobulin Kappa gene. Nature (London) **307**:80–82.
42. **Ruley, H. E., and M. Fried.** 1983. Sequence repeats in a polyomavirus DNA region important for gene expression. J. Virol. **47**:233–237.
43. **Sekikawa, K., and A. J. Levine.** 1981. Isolation and characterization of polyoma host range mutants that replicate in nullipotential embryonal carcinoma cells. Proc. Natl. Acad. Sci. U.S.A. **78**:1100–1104.
44. **Southern, E. M.** 1975. Detection of specific sequences among fragments separated by agarose gel electrophoresis. J. Mol. Biol. **98**:503–515.
45. **Triezenberg, S. J., and W. R. Folk.** 1984. Essential nucleotides in polyoma virus origin region. J. Virol. **51**:437–444.
46. **Tyndall, C., G. LaMantia, C. M. Thacker, J. Favaloro, and R. Kamen.** 1981. A region of the polyoma virus genome between the replication origin and late protein coding sequences is required in cis for both early gene expression and viral DNA replication. Nucleic Acids Res. **9**:6231–6250.
47. **Weber, F., J. deVilliers, and W. Schaffner.** 1984. An SV40 "enhancer trap" incorporates exogenous enhancers or generates enhancers from its own sequences. Cell **36**:983–992.
48. **Weiher, H., and M. R. Botchan.** 1984. An enhancer sequence from bovine papilloma virus DNA consists of two essential regions. Nucleic Acids Res. **12**:2901–2916.
49. **Weiher, H., M. König, and P. Gruss.** 1983. Multiple point mutations affecting the simian virus 40 enhancer. Science **219**:626–631.