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Nucleotides in the Polyomavirus Enhancer That Control Viral Transcription and DNA Replication

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The polyomavirus enhancer is required in *cis* for high-level expression of the viral early region and for replication of the viral genome. We introduced multiple mutations in the enhancer which reduced transcription and DNA replication. Polyomaviruses with these mutant enhancers formed very small plaques in whole mouse embryo cells. Revertants of the viral mutants were isolated and characterized. Reversion occured by any of the following events: (i) restoration of guanosines at nucleotide (nt) 5134 and nt 5140 within the adenovirus 5 E1A enhancer core AGGAAGTGACT; (ii) acquisition of an A→G mutation at nt 5258, which is the same mutation that enables polyomavirus to grow in embryonal carcinoma F9 cells; (iii) duplication of mutated sequences between nt 5146 and 5292 (including sequences homologous with immunoglobulin G, simian virus 40, and bovine papillomavirus enhancer elements). Reversion restored both the replicative and transcriptional functions of the viruses. Revertants that acquired the F9 mutation at nt 5258 grew at least 20-fold better than the original mutant in whole mouse embryo cells, but replicated only marginally better than the original mutant in 3T6 cells. Viruses with a reversion of the mutation at nt 5140 replicated equally well in both types of cells. Since individual nucleotides in the polyomavirus enhancer simultaneously altered DNA replication and transcription in specific cell types, it is likely that these processes rely upon a common element, such as an enhancer-binding protein.

Transcriptional enhancers are *cis*-acting DNA elements that stimulate gene expression. The distinguishing feature of enhancers is their ability to activate RNA polymerase II transcription of linked genes in a relatively orientation- and distance-independent fashion (2, 3, 49). Transcriptional enhancers were first detected in simian virus 40 (SV40) and polyomavirus (2, 3, 15, 28, 49), and they have since been found in the genomes of numerous other DNA and RNA tumor viruses, as well as in cellular genes (reviewed in references 57 and 65). Some yeast genes have upstream activator sequences whose function resembles that of enhancers (29). Enhancer activity is often restricted to particular species and tissues (14, 24, 34, 41, 42, 64). This ubiquity and specificity of action indicates that enhancers play a central role in the control of eucaryotic gene expression. However, the mechanism of enhancer action is unknown.

The polyomavirus enhancer region is required in *cis* both for early gene expression (23, 41, 50, 73, 75) and for DNA replication (16, 24, 46, 51, 75). Deletion analyses indicate that the enhancer contains sequences which are functionally redundant in fibroblasts (30, 73, 75). One enhancer fragment, which is delimited by the *Bcl*I and *Pvu*II sites (nucleotide [nt] 5046 to nt 5152; numbered as in reference 13), activates the α-collagen promoter in established mouse fibroblasts (34) and the β-globin promoter in HeLa cells (73). Within this fragment occurs a sequence homologous to the adenovirus 5 E1A enhancer and to other viral cellular enhancers (31). Tandemly repeated copies of a synthetic DNA containing this sequence element activate polyomavirus transcription and DNA replication in fibroblasts (75). Thus several lines of evidence suggest that the replicative and transcriptional functions of the enhancer are linked.

Other functionally important segments of the polyomavirus enhancer lie within the *Pvu*II-*Pvu*II fragment (nt 5152 to 5289, termed *Pvu*II-4) adjoining the origin of DNA replication. Short sequence motifs within this fragment are also present in the immunoglobulin G (IgG) enhancer (1), the SV40 enhancer (79), and the bovine papillomavirus type 1 enhancer (78). Deletion of part or all of this segment of DNA alters the polyomavirus host range, suggesting that elements within it can act in a negative as well as in a positive manner (10, 71).

Polyomavirus variants selected to grow in embryonal carcinoma F9 cells acquire a single A-G transition at nt 5258 (23, 41, 64). An enhancer bearing this base change activates transcription of the polyomavirus early promoter as well as of heterologous promoters in embryonal carcinoma cells (6, 34, 45). This mutation may generate an additional enhancer motif for the action of positive regulatory factors (34, 48), or alternatively, it may render ineffective a target for *trans*-acting factors which negatively control enhancer elements (33).

Our study has focused on two questions: which nucleotides are essential for polyomavirus enhancer function in fibroblasts, and do mutations that inactivate replication have a parallel deleterious effect on transcription? We have identified specific nucleotides in the enhancer that are essential for both replication and transcription in fibroblasts. Furthermore, reduction of replication is accompanied by a concomitant reduction in transcription, and restoration of replication accompanies a restoration of transcription. This provides strong evidence that these processes are functionally linked through the enhancer. In addition, we have found that mutation of nt 5258 will activate an otherwise nonfunctional enhancer in whole mouse embryo (WME) cells, but has little effect upon enhancer function in differentiated cell lines.

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MATERIALS AND METHODS

Materials. Enzymes were purchased from Bethesda Research laboratory or New England Biolabs and were used as specified by the suppliers. α^{32}P-labeled deoxyribonucleotides were purchased from New England Nuclear Corp. Oligonucleotide primers used for DNA sequencing and primer extension analyses were prepared on an Applied Biosystems 380A DNA synthesizer and purified through 20% polyacrylamide denaturing gels.

Cell cultures. WME cells were obtained from 12- to 14-day-pregnant CD-1 mice. Swiss mouse 3T6 cells were propagated in Eagle medium containing 5% calf serum. WOP 3027-3 cells (which constitutively express large T-antigen; 11) were grown in Dulbecco modified essential medium supplemented with 10% calf serum. The mouse L-cell line DAP-3 (9) was grown in Dulbecco modified essential medium containing 10% fetal calf serum.

Mutagenesis of the polyomavirus enhancer. An 1,800-base-pair (bp) fragment of polyomavirus DNA, extending from the *Bcl*I site (nt 5046; polyomavirus A3 strain) to the *Eco*RI site (nt 1575), was cloned into the *Bam*HI and *Eco*RI sites of M13mp8. This construction is designated mp8Py1 (see Fig. 1A). Sodium bisulfite mutagenesis of single-stranded mp8Py1 DNA was performed as previously described (19, 67). A 5- to 10-fold molar excess of protecting fragments homologous to nontarget sequences was mixed with single-stranded mp8Py1 DNA in 50 mM sodium phosphate (pH 6.8), boiled for 3 min, and cooled to 60°C for 10 min. The DNA was treated with sodium bisulfite for 3 h at 37°C and then was desalted on BioGel P-10. The sulfite adducts were removed by treatment with Tris base, and the single-stranded gaps were repaired by using the Klenow fragment of DNA polymerase I. After transfection into *Escherichia coli* JM103, single plaques were picked, and base-pair substitutions were identified by sequencing.

Three hours of exposure to sodium bisulfite generated mutants with an average of 14 transitions per enhancer region. The distribution of these mutations was decidedly nonrandom. Sixteen target sites within the enhancer were relatively resistant to bisulfite, whereas five sites were hypersensitive. Although not all the resistant and hypersensitive sites could be fitted into easily recognizable secondary structures, it is noteworthy that the single-stranded DNA between nt 5195 and nt 5239 probably assumes a stem-loop structure similar to that suggested by Soeda et al. (68), since cytosines in the putative stem regions were protected from bisulfite and cytosines in the putative loops were hypersensitive.

Construction of pAdPyE$^-$ expression vector. Plasmid pAdPyE$^-$ (see Fig. 1B) was used as a cloning vector for the analysis of transcription of the mutated enhancer regions. This vector preserves the normal enhancer-promoter combination present in polyomavirus, in the event that other promoters might not respond to the polyomavirus enhancer or to mutations within it (4, 59). pAdPyE$^-$ contains: (i) a fragment of polyomavirus DNA from *Pvu*II (nt 5295) to *Eco*RI (nt 1575), joined to an *Mbo*I fragment (nt 2784 to nt 3406) containing the early region polyadenylation sequence; (ii) the adenovirus VA genes within a *Sal*I-*Hin*dIII DNA fragment (21); and (iii) a short DNA fragment from the pUC18 polylinker containing *Hin*dIII, *Sph*I, and *Pst*I restriction endonuclease sites. Deletion of the polyomavirus large T-antigen sequences between nt 1575 and nt 2785 prevents activation of polyomavirus DNA replication and autoregulation of early mRNA transcription. pAdPyE$^-$ DNA contains the adenovirus VA genes to provide an internal control for variations in transfection efficiency. Transcription of the VA genes from this DNA has been measured in the presence and absence of the polyomavirus early genes and the polyomavirus enhancer. Expression of the early genes has no effect upon transcription of the VA genes (S. L. Berger, Ph.D. thesis, University of Michigan, Ann Arbor, 1986), nor does the polyomavirus enhancer affect VA gene expression (5). This pAdPyE$^-$ vector differs from the pAdPyE$^-$ construction described previously (5) by the substitution of *Pst*I, *Sph*I, and *Hin*dIII sites for *Stu*I-*Pvu*II polyomavirus sequences upstream of the polyomavirus origin of replication.

Enhancer sequences from M13 mp8Py1 were inserted into pAdPyE$^-$ after the vector was opened with *Hin*dIII and *Pst*I and the *Pst*I site was blunted with T4 DNA polymerase. Wild-type or mutated mp8Py1 DNA was digested with *Hin*dIII and *Pvu*II, and the fragment containing the enhancer region was ligated to pAdPyE$^-$ and introduced into *E. coli*. The sequences of the cloned enhancer regions were verified by sequencing. To introduce enhancer sequences from the B1 revertant viral genomes into pAdPyE$^-$, the polyomavirus *Bcl*I-*Eco*RI fragments were isolated and first cloned into pUC18. These recombinants were subsequently digested with *Hin*dIII and *Pvu*II, and the 264-bp fragment containing the revertant enhancer region was ligated to pAdPyE$^-$.

Construction of polyomavirus genomes containing the enhancers of mutants B1 and B122. To introduce the mutant B1 enhancer into a full-length polyomavirus genome, a 360-bp fragment extending from a *Sau*3A site (nt 5046) to the *Bgl*I site (nt 102) was purified from M13-B1 polyomavirus DNA. For the mutant B122 enhancer, a 366-bp fragment extending from the *Sal*I site of the M13 polylinker (adjacent to the polyomavirus *Bcl*I site) to the *Bgl*I site was purified from the M13-B122 recombinant. The *Sal*I and *Bcl*I ends to be ligated together were first blunted with Klenow polymerase. A plasmid containing polyomavirus A2 DNA, pG43-70 (73), was digested with *Bcl*I and *Bgl*I, and the two fragments lacking enhancer sequences were purified. These three fragments were ligated to the enhancer-containing fragment overnight at 4°C at high DNA concentrations in the presence of 0.1 mM spermidine. This DNA mixture was then digested with *Xho*I to remove DNAs with residual contaminants of the pG43-70 enhancer region, followed by transfection into *E. coli* HB101. *Xho*I-resistant recombinants were isolated, and their sequences were confirmed by DNA sequencing.

Mutagenesis and isolation of revertants. Plasmids containing the B1 and B122 gemones were transformed into the *E. coli* mutator strain mut D5 k1617 and grown in L-broth with 10 μg of thymidine per ml (20). Plasmid DNAs were isolated after either 10 or 40 cell generations, sufficient to introduce one or two mutations per genome, and were digested with *Eco*RI. Polyomavirus DNAs were transfected into WME cells with DEAE-dextran (47), and single plaques that were significantly larger than those of the unmutated B1 control were picked and amplified as viral stocks in WME cells. DNAs that were not passed through the *E. coli* mutator strain produced few or no large plaques. To prepare viral DNA, each stock was used to infect 3T6 cells at approximately 2 PFU per cell. At 44 h postinfection, viral DNA was extracted (38) and purified by CsCl gradient centrifugation. Viral DNA was cloned into pBR322 digested with *Bam*HI, and the enhancer-origin regions of recombinants were sequenced.

DNA replication assays. Samples of 1.5×10^6 3T6 cells in 60-mm culture dishes were transfected with a mixture of 0.5 μg of double-stranded mp8Pyl DNAs containing mutated

enhancers, 0.5 μg of *Bam*HI-digested polyomavirus pBR322 recombinant DNAs, and 11.5 μg of salmon sperm DNA. Alternatively, cells were transfected with 1 μg of linearized polyomavirus DNA plus 12 μg of salmon sperm DNA. At 4 to 5 h after the calcium phosphate coprecipitate was added to the cells, the medium was replaced with 1 ml of 20% (vol/vol) glycerol 1 in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline (for 3 min) or in Eagle medium (for 1 min) (55). The cells were rinsed and then covered with medium and incubated at 37°C. In some cases (noted in the text), cells were transfected with 0.1 μg of linearized full-length polyomavirus DNA with or without helper DNA, using DEAE-dextran (47). Two days after transfection, low-molecular-weight DNA was isolated (38) and digested with pancreatic RNase and pronase before phenol extraction and ethanol precipitation. The purified DNAs were digested either with *Mbo*I or with *Dpn*I plus the restriction enzyme originally used for cloning, either *Bam*HI or *Eco*RI, to linearize *Dpn*I-resistant DNA. The digested DNAs were fractionated through agarose gels, transferred to nitrocellulose, and hybridized to radiolabeled polyomavirus DNA.

Transcription assays. Plasmid DNAs were purified by two rounds of banding in CsCl density gradients containing ethidium bromide. Cells at 50% confluence in 100-mm-diameter plates were transfected with 20 μg of plasmid DNA per ml of precipitate by calcium phosphate coprecipitation (55, 74). At 48 h after transfection, total cellular RNA was isolated by extraction with guanidium isothiocyanate and hot phenol, followed by treatment with DNase I, digestion with proteinase K, and ethanol precipitation. Approximately 100 μg of whole-cell RNA was isolated from each transfection.

Adenovirus VAI RNA was detected by quantitative S1 nuclease analysis (77) using an adenovirus 370-bp *Taq*I fragment (map unit 28.23 to 29.24). Hybridization of 5 μg of whole-cell RNA and 0.02 pmol of probe (specific activity, 10^7 cpm/μg) and subsequent S1 nuclease digestion were performed as previously described (5, 17). Digestion products were fractionated by electrophoresis through 12% acrylamide–8 M urea gels. Autoradiographic exposure of 5 to 18 h was sufficient for visualization of bands.

Polyomavirus early mRNAs were analyzed by primer extension (25) using an end-labeled synthetic oligonucleotide primer (nt 323 to nt 337). The radiolabeled primer (0.04 pmol) was annealed to RNA and extended using mouse mammary tumor virus reverse transcriptase. The major extension product was 172 nt. Autoradiographic exposure of 12 to 36 h was required for visualization of bands.

RESULTS

Mutagenesis of the polyomavirus enhancer region. The polyomavirus enhancer is functionally redundant for both transcription and DNA replication (32, 46, 68), To inactivate each of the redundant elements, we introduced multiple point mutations in the enhancer. An 1,800-bp DNA fragment containing the enhancer region, the origin of replication, and part of the T-antigen coding sequences was cloned into the single-stranded phage vector M13mp8, generating mp8Pyl (Fig. 1A). G-C to A-T transitions were introduced in the enhancer by treating mp8Pyl DNA with sodium bisulfite. Seventy-five mutants with single or multiple alterations were identified by sequencing. Greater than 90% of the susceptible sites within the enhancer region were modified at least once. Nucleotide changes in the enhancers of a small group of these mutants are shown in Fig. 2. None of the mutants (except B120) has other mutations in the origin-promoter region. Mutant B120 contains several mutations at the origin; consequently, we have not been able to assess the effect of its enhancer mutations on replication function.

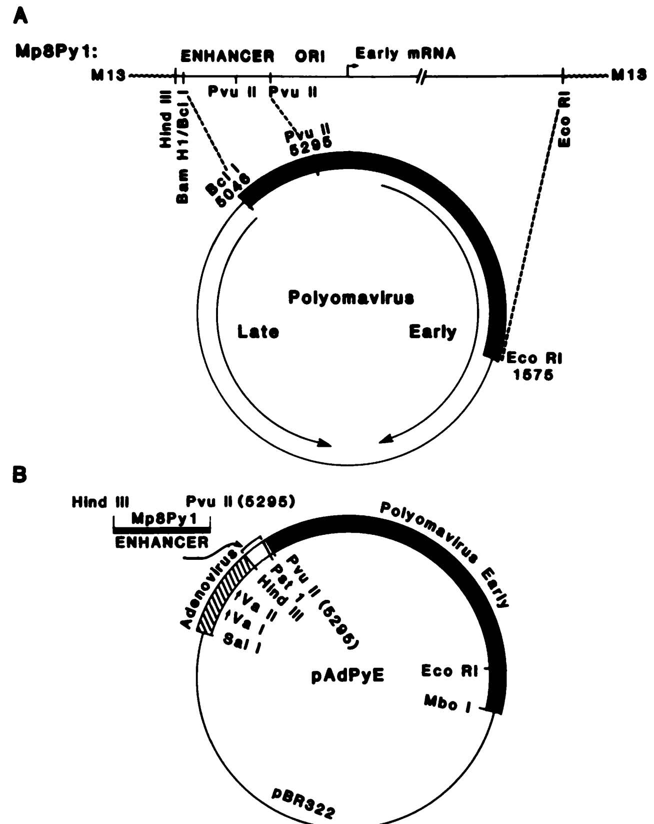

FIG. 1. Polyomavirus intergenic control region and early gene coding sequences present in mp8Pyl and pAdPyE. (A) mp8Pyl contains the *Bcl*I-*Eco*RI fragment of polyomavirus DNA ligated to the *Bam*HI and *Eco*RI sites of M13mp8. Approximate locations of the enhancer region and origin of replication are shown. The M13 *Hin*dIII site and polyomavirus *Pvu*II site (nt 5295) were used to excise mutated enhancer regions for cloning into pAdPyE⁻. (B) pAdPyE⁻ contains the adenovirus VA genes, a portion of the polyomavirus early gene coding sequences, and the early gene polyadenylation site within pBR322. Mutated enhancer regions were cloned into pAdPyE⁻ from mp8Pyl as *Hin*dIII-*Pvu*II fragments.

Effect of enhancer point mutations on polyomavirus replication. Replication of selected mutants containing multiple nucleotide changes in the enhancer was measured after transfecting double-stranded phage DNA into mouse 3T6 cells. Large T-antigen was supplied in *trans* by cotransfected polyomavirus DNA cleaved from its plasmid vector. (This DNA also may compete for limiting replication factors.) Low-molecular-weight DNAs were isolated 48 h after transfection and digested with *Mbo*I, to cleave unmethylated DNA which has replicated in mammalian cells (56). The digests were fractionated by agarose gel electrophoresis, blotted onto nitrocellulose, and probed with nick-translated mp8Pyl DNA.

As expected, the wild-type enhancer in mp8Pyl efficiently acted in *cis* to activate replication (Fig. 3, lanes 1 and 6). DNAs containing mutated enhancer regions are shown in Fig. 3, lanes 2 through 5, 7, and 8. Mutant B4 replicated efficiently, indicating that the mutations in its enhancer have little effect upon replication. Mutant B5 exhibited somewhat

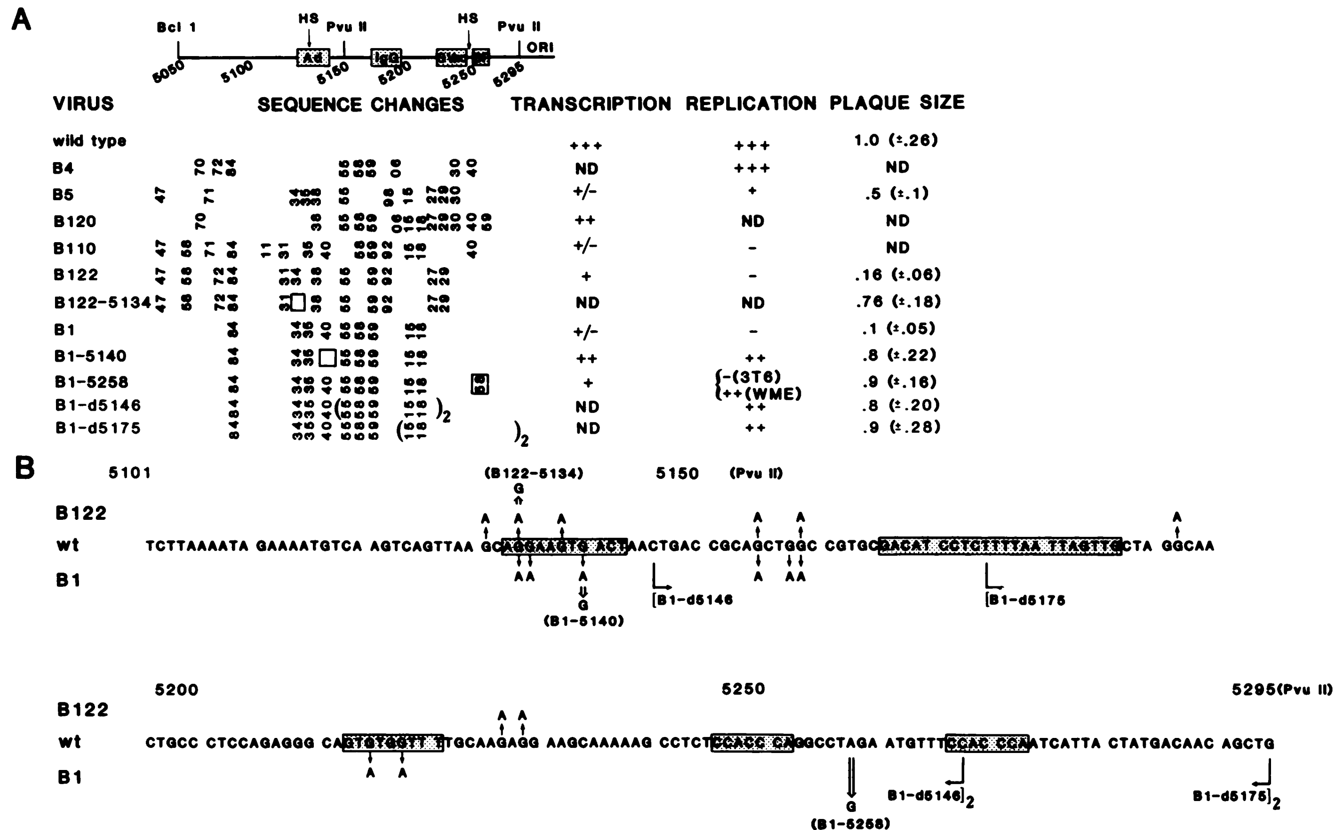

FIG. 2. Schematic diagram of polyomavirus DNAs containing mutations in the enhancer region. (A) The line drawing (top) is a diagram of the enhancer region. Regions of homology to sequences found in adenovirus (Ad) (31), IgG (1), SV40 (SV) (79), and bovine papillomavirus 1 (BP) enhancers (78) (boxes) and the locations of two DNase I-hypersensitive sites (8, 35) (HS) are indicated. Sites of base substitutions in each point mutant are designated by the last two digits of the nucleotide number. The single nucleotide reversions in B1-5140 and B122-5134 are marked by open boxes. The pseudoreversion in B1-5258 is boxed. The ability of the mutated enhancer regions to support replication (in 3T6 cells) or to activate transcription (in DAP-3 cells) is indicated (+++, wild type; ++, 50 to 80%; +, 10 to 50%; −, 0 to 10%). Plaque sizes were measured from assays in WME cells. (B) Nucleotide sequence of relevant enhancer region of wild type polyomavirus (strain A3), mutants B1 and B122, and the revertants B1-5140, B1-5258, B1-d5148, B1-d5175, and B122-5134. Boxed sequences indicate core homologies with adenovirus, IgG, SV40, and bovine papillomavirus enhancers, as depicted in panel A.

reduced replication efficiency. The replication of mutants, B1, B110, and B122 was virtually eliminated, indicating that important nucleotides in these enhancer regions have been altered. Since these mutants share many sequence changes in the enhancer region with mutants B4 and B5 (Fig. 2), most of the altered nucleotides in mutants B1, B110, and B122 are unlikely to cause their defects in replication.

Effect of enhancer region mutations on polyomavirus early gene transcription. To assess the ability of the mutant enhancers to potentiate transcription from the polyomavirus early promoter, DNA fragments including the enhancers were recloned into a plasmid vector containing part of the large T-antigen coding sequences and, to provide an internal control for transfection efficiency, the adenovirus VA genes (Fig. 1). pAdPyE DNAs containing the altered enhancers were transfected into mouse DAP-3 cells, and total cellular RNA was prepared 48 h later. The level of polyomavirus early gene transcription was analyzed by primer extension of the RNAs.

The wild-type polyomavirus enhancer region stimulated transcription of the early gene region (Fig. 4A, lanes 9 through 11). Transcription of the polyomavirus early genes potentiated by mutated enhancer regions as compared with the wild-type enhancer is shown in Fig. 4A, lanes 1 through 8. The enhancer of mutant B120 potentiated transcription nearly as efficiently as the wild-type enhancer. The enhancers of mutants B5, B110, B122, and B1 did not potentiate transcription above that observed with a plasmid lacking the entire enhancer domain, pAdPyE. Similar results were obtained in two other independent transfections (data not shown). The level of adenovirus VAI RNA was comparable among all samples, indicating similar transfection efficiencies and RNA recoveries (Fig. 4B). Thus, as with the analyses of the replicative capacity of these mutants, with these mutants it is possible to focus on specific nucleotides important for transcriptional activation.

All of the mutated enhancers cloned into pAdPyE$^-$ were tested for their *cis*-acting effect on replication in mouse 3T6 cells. The effects were qualitatively similar to those measured for the mp8Py1 mutant constructs described in the previous section (data not shown).

Mutations in the B1 and B122 enhancers reduce polyomavirus viability. To determine how these mutations affected the growth of polyomavirus, we reconstructed viral genomes containing the B1 and B122 enhancer regions. When DNA replication and transcription were measured after transfection of the reconstructed B1 DNA into WME cells, the results were consistent with the transient expression assays

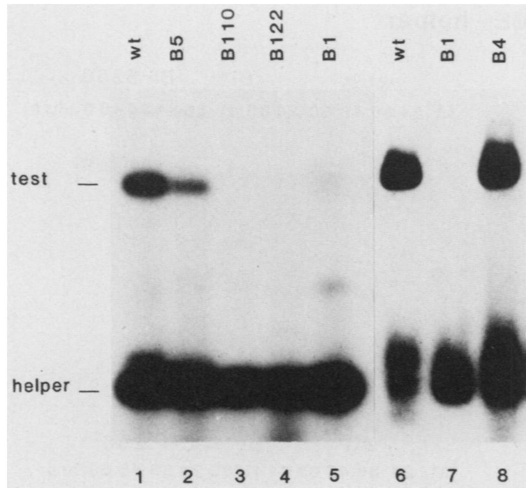

FIG. 3. Analysis of replication of M13 DNAs containing wild-type and mutated enhancer regions. 3T6 cells were transfected with the indicated mp8Py1 test DNAs. Low-molecular-weight DNAs isolated 48 h after transfection were digested with *Mbo*I and fractionated through 0.8% agarose gels. Digestion products were blotted onto nitrocellulose and hybridized to radiolabeled mp8Py1 DNA. Lanes 1 through 5 and 6 through 8 are from the two separate experiments and show characteristic fragments produced after digestion of test and helper DNAs.

in 3T6 cells. DNA replication was low regardless of whether large T-antigen was provided in *trans*. By 60 h, only low amounts of *Dpn*I-resistant DNA were detected (Fig. 5A), but in similar experiments, no *Bcl*I-sensitive DNA (which distinguishes between input and replicated DNA, as does *Dpn*I) was observed (data not shown). One round of replication is sufficient to render DNA resistant to *Dpn*I, but at least two rounds of replication are required for methylated DNA to become *Bcl*I sensitive. Thus, in 60 h the mutated enhancer

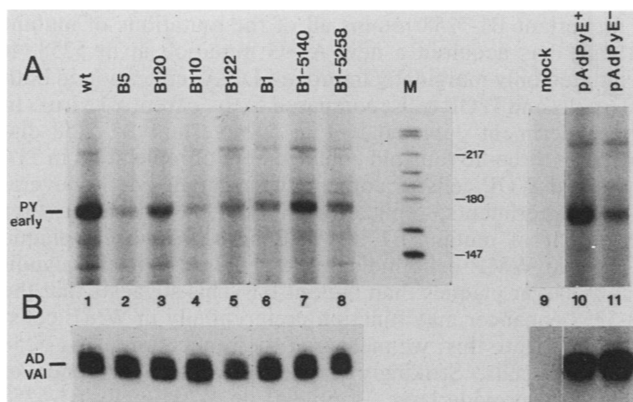

FIG. 4. Expression of polyomavirus early region and adenovirus VAI genes from pAdPyE DNAs. (A) Primer extension analysis of polyomavirus early gene transcription in DAP-3 cells. The level of early region RNA synthesized from transfected pAdPyE+ DNA, containing an unmutated enhancer region, is shown in the lane marked wt. Other lanes are assays of RNAs isolated from cells transfected with the mutant DNAs indicated above each lane. M, End-labeled *Hpa*II-digested pBR322 DNA fragments in nucleotides. Arrows indicate expected size of reaction products. (B) S1 nuclease analysis of adenovirus VAI gene transcription in DAP-3 cells. Lanes are marked as in panel A.

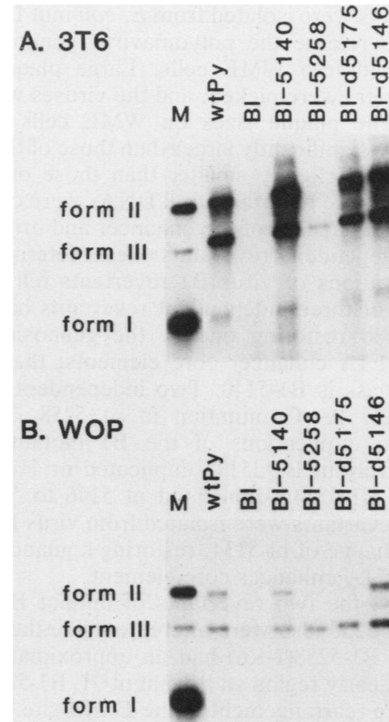

FIG. 5. DNA replication assay of mutant B1 and its revertants in (A) 3T6 cells and (B) WOP cells. Cells were transfected with linearized polyomavirus DNA (either *Bam*HI or *Eco*RI, depending upon the site originally used for cloning into pBR322). Low-molecular-weight DNA was isolated 60 h posttransfection, repetitively digested with *Dpn*I and the restriction enzyme originally used for cloning, fractionated on an agarose gel, blotted and hybridized to radiolabeled pBR322-polyomavirus DNA. The bands in the leftmost lanes (M) in both panels represent the three conformers of polyomavirus DNA. Circular DNAs (form I and form II) remaining after *Bam*HI or *Eco*RI digestion have lost sites for these enzymes after transfection into cells (76), as those DNA samples were repetitively digested with each enzyme. In each lane, the *Dpn*I digestion products at the bottom of the gel (not shown) were of comparable intensity, indicating that equal amounts of DNA were applied to the cells

was incapable of activating two or more rounds of replication.

We were surprised, therefore, to observe that mutants B1 and B122 were capable of forming plaques in WME cells. Although these plaques are substantially smaller than those of the wild type (Fig. 2), their appearance indicates these viruses go through multiple rounds of replication and reinfection. The transient expression assays of DNA replication apparently overstate the magnitude of the defect caused by the mutant enhancers. Nevertheless, it was possible to use mutants B1 and B122 to isolate revertants which displayed normal growth, detected by the formation of large plaques.

Isolation of polyomavirus revertants with functional enhancers. We used an *E. coli* mutator strain to generate revertants of the mutant viral genomes, as it induces a wide variety of transitions, transversions, and frameshift mutations (20). Furthermore, the frequency of mutations can be controlled. Under the conditions that were employed, only one to two mutations should have been introduced per genome, making it unlikely that multiple mutations would occur in functionally related genes.

Plasmid DNAs were isolated from *E. coli* mut D5 digested with *Eco*RI to release the polyomavirus genomes, which were transfected into WME cells. Large plaques in the WME monolayers were picked, and the viruses were grown into stocks. The plaque sizes on WME cells of all the revertants were significantly larger than those of the parental viruses, but were slightly smaller than those of wild-type virus (Fig. 2). These revertant viral DNAs were cloned, and the nucleotide sequences of the enhancer and origin regions of several independent revertants were determined. The sequence alterations of virus B1 revertants fell into three classes. Each of three independent revertants had a reversion at nt 5140 restoring one of the guanosines in the adenovirus 5 E1A enhancer core elements; the prototype virus in this class is B1-5140. Two independent revertants acquired a new A→G mutation at nt 5258. Two other revertants had duplications of the B1 mutant enhancer sequences: revertant B1-d5175 duplicated nt 5175 to 5292, and revertant B1-d5146 duplicated nt 5146 to 5268. Eight independent revertants were isolated from virus B122. Each had a single change of nt 5134, restoring a guanosine in the adenovirus 5 E1A enhancer core element.

Interestingly, the two revertants of mutant B1 with the mutation at nt 5258 also were altered outside the enhancer-origin region. B1-5258(1-R6) had an approximately 300-bp deletion of the early region starting at nt 91. B1-5258(1-R5-4) had a sequence rearrangement at the *Eco*RI site. Both were incapable of making functional large T-antigen, and consequently, each must have been present in mixed plaques during their isolation. It is likely these sequence rearrangements occurred during the production of virus stocks. To measure the effect of the mutation at nt 5258 upon B1 enhancer function, the *Bcl*I-*Bgl*I fragment of one revertant, B1-5258(1-R5-4), was reconstructed into a wild-type polyomavirus genome.

Revertant enhancers restore polyomavirus DNA replication. We measured the replication of four revertants of mutant B1 (representing the three B1 revertant classes) by transfecting DNAs into mouse 3T6 cells and into WOP cells (the latter supply polyomavirus large T-antigen in *trans*) (Fig. 5). Revertants B1-5140 and B1-d5146 replicated nearly as well as wild-type polyomavirus both in 3T6 cells and in WOP cells. Measurement of the kinetics of DNA replication in 3T6 cells and in WME cells indicated that replication of wild-type polyomavirus peaks around 40 h posttransfection, whereas B1-5140 required 60 h to reach similar DNA levels (data not shown). DNA replication of revertant B1-d5175 was enhanced relative to that of mutant B1, but was not as efficient as that of B1-5140 and B1-d5146.

Revertant enhancers restore polyomavirus transcription. Comparison of the replication of these DNAs in WOP and 3T6 cells allows us to distinguish the role of the enhancer in activating DNA replication versus its role in activating both DNA replication and early gene expression. (We recognize that this assertion is justified, however, only if WOP cells and 3T6 cells differ primarily by the presence of functional large T-antigen.) In WOP cells, replication of B1-5140, B1-d5146, and B1-d5175 DNA was considerably greater than that of mutant B1. This indicates that the enhancers of these three revertants activate DNA replication better than does the B1 enhancer. For these revertants to replicate efficiently in 3T6 cells (and to form large plaques in WME cells), efficient transcription of the viral early genes is also required, which suggests that reversion is accompanied by restored transcriptional efficiency. We directly measured transcription from the early promoter in fibroblasts

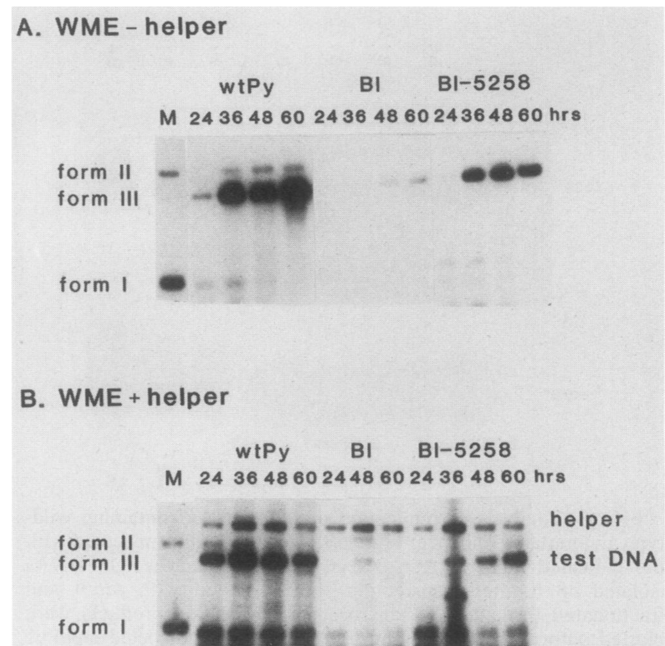

FIG. 6. DNA replication assay of revertant B1-5258 in whole mouse embryo cells. WME cells were transfected with linearized polyomavirus DNA alone (A) or together with polyomavirus-pBR322 DNA as helper (B). Low-molecular-weight DNA was isolated at 24, 36, 48, or 60 h posttransfection, digested with *Dpn*I and *Eco*RI, fractionated on agarose gel, blotted, and hybridized to radiolabeled pBR322-polyomavirus DNA.

transfected by revertants B1-5140 and B1-5258. Potentiation of transcription by the B1-5140 enhancer was considerably greater than that of the B1 mutant and nearly as efficient as that of the wild-type enhancer (Fig. 4). Thus, in this revertant, a single nucleotide change restored both transcription and DNA replication. In contrast, transcription driven by the B1-5258 enhancer was only slightly greater than that of the B1 parent, as was DNA replication in established cell lines (Fig. 4 and 5).

Revertant B1-5258 retains all of the mutations of mutant B1 and has acquired a new A→G mutation at nt 5258. It exhibited only marginally improved DNA replication in both 3T6 cells and WOP cells, compared to the parent B1 virus. In the experiment depicted in Fig. 5, revertant B1-5158 displayed a three- to fourfold enhancement of replication in 3T6 cells and WOP cells as compared to mutant B1. In several other experiments, replication of B1-5258 was no greater than that of mutant B1 in WOP cells. However, plaque assays in WME cells indicated that B1-5258 forms significantly larger plaques than mutant B1. This suggests that the B-5258 enhancer may function preferentially in WME cells. To investigate this, we measured the replication of B1-5258 in WME cells. Strikingly, regardless of the presence of helper (to provide large T-antigen), in WME cells B1-5258 DNA was replicated at least 20-fold better than that of mutant B1 (Fig. 6). Thus, the B1-5258 enhancer is capable of activating DNA replication in WME cells, but is much less effective in established cell lines such as 3T6 or WOP cells.

DISCUSSION

Nucleotides in the polyomavirus enhancer essential for replication. The polyomavirus enhancer contains one or more sequence elements important for DNA replication in

fibroblasts, as delimited by several analyses of deletion mutants (30, 51, 73, 75). Sequences within the *Bcl*I-*Pvu*II fragment which are important for DNA replication in fibroblasts occur between nt 5132 and 5155. This element is homologous to sequences in the transcriptional enhancers of the adenovirus 5 E1A gene (31) and the human β-interferon gene (26). Furthermore, it is duplicated in various laboratory isolates of polyomavirus (60, 61) and is responsible for DNase I hypersensitivity of polyomavirus chromatin (8).

Analysis of the nucleotide substitutions between the *Bcl*I and the central *Pvu*II sites in the point mutants we have isolated suggests that base changes at nt 5134, 5135, 5138, and 5140 might be responsible for inactivating the replicative function of this enhancer region. All other changes between the *Bcl*I-*Pvu*II sites in these mutants are outside the important sequence element described above. The significance of the adenovirus 5 E1A enhancer core sequence is underscored by the isolation of revertant B1-5140, whose replication and transcription are restored by the single base substitution at nt 5140. The functional defect of mutant B110 might also be explained by the change at nt 5140. The importance of nucleotide 5134 in the adenovirus core homology sequence is shown by the isolation of a B122 revertant with a change at this site. Mutant B122 also shares changes at nt 5138 with mutant B5, which is marginally competent for replication. Conceivably, the combination of these changes with the mutation at 5131 renders mutant B122 defective.

The failure of mutants B1, B110, and B122, to replicate indicates that the additional (redundant) replication activator elements within the *Pvu*II-4 fragment must have been inactivated. Although the limits of these elements have not been well defined by deletion analysis, one boundary is approximately nt 5176 to 5229 (30, 73, 75). This region is bound by proteins isolated from several fibroblast cell lines which support polyomavirus replication and gene expression (7, 22, 54, 58), and it contains sequence homologies to the IgG and SV40 enhancers. Point mutations in mutants B1, B110, and B122 occur within this region at nt 5192, 5215, 5218, and 5227. As mutant B1 contains changes at only nt 5215 and nt 5218, it is likely that one or both are inactivating events. These two nucleotides in the SV40 enhancer are highly conserved and are important in its function (79, 82). Mutant B122 has changes at nt 5192, 5227, and 5229. Either or all of these may be inactivating events. Construction of single point mutants or isolation of additional revertants will be necessary to confirm their importance.

Nucleotides in the polyomavirus enhancer essential for transcription. Qualitative measurements of expression from the polyomavirus early promoter in viruses with enhancer deletions indicated that the *Bcl*I-*Pvu*II and the *Pvu*II-4 fragments were important, but attempts to delimit essential sequences were unsuccessful (75). The demonstration that four or more tandem copies of an oligonucleotide with a sequence from nt 5132 to 5155 is capable of activating expression of the β-globin gene in HeLa cells indicates this region may be part of the *Bcl*I-*Pvu*II transcriptional enhancer element. In addition, an analysis of the enhancer region of the polyomavirus F9-1 mutant suggests that one important transcriptional element in the *Pvu*II-4 fragment has approximately the same borders as those described above for the DNA replication element (6). Herbomel et al. (34) and Linney and Donerly (45) showed that the *Bcl*I-*Pvu*II fragment and the *Pvu*II-4 fragment are independently capable of activating transcription of heterologous promoters in mouse fibroblasts. In many of these studies, the extent of transcriptional activation was rather limited, and the use of heterologous promoters or nonmurine cells leaves open to question the significance of these elements for polyomavirus gene expression.

We examined the capacity of seven mutant enhancers to stimulate early gene expression in mouse DAP-3 cells. Mutants B1, B5, B110, and B122 were reduced in transcription. The wild-type enhancer and the enhancers of mutant B120 and revertant B1-5140 functioned efficiently. Measurement of differences in transcriptional activation by these enhancers is difficult because of the basal level of expression that occurs even in the absence of a functional enhancer. However, it is apparent from the transient expression assays, as well as from the replication assays in 3T6 cells and the growth of the viruses in WME cells, that reversion of nt 5140 restores efficient transcription. Similarly, the more efficient replication of B1-d5146 and B1-d5175 in 3T6 cells, where large T-antigen expression is required for DNA replication, suggests that their enhancers also potentiate transcription better than the mutant B1.

Most of the mutant enhancers we have characterized are reduced in their ability to activate both transcription and DNA replication. This suggests that these disparate processes require a common element. Proof of such a common element is provided by the observation that a single nucleotide reversion in the enhancer of mutant B1 at nt 5140 restores both polyomavirus transcription and DNA replication. Previously, this region was found to be capable of activating replication of polyomavirus DNA in mouse cells, when present in two or more copies, and transcription from the β-globin promoter in HeLa cells, when present in four or more copies (75).

Elements in the *Pvu*II-4 fragment required for enhancer function. The *Pvu*II-4 fragment does not appear to have as important a role as a transcriptional enhancer in mouse fibroblasts as the *Bcl*I-*Pvu*II fragment. Transcription of the early genes of mutant B120 proceeds efficiently, even though its enhancer has numerous mutations in the *Pvu*II-4 fragment. Little expression of mutant B122 was detected, although it has few mutations in this element, notably where sequences homologous to the SV40 core enhancer element are unchanged. Thus, the function of this region is dependent upon more than the SV40 enhancer core sequences, a conclusion consistent with recent deletion analyses (6, 30, 75).

The structures of the revertants containing duplications in the enhancer provide additional information about essential enhancer sequences. Both revertants have in common tandem duplications from nt 5175 to 5265, which contain sequence elements that bind host nuclear proteins (7, 22, 54, 58). The B1-d5146 enhancer also includes a duplication of the IgG homologous element; this revertant potentiated replication better than the B1-d5175 enhancer, which has only one complete copy of the IgG element. Thus, the region between nt 5146 and 5175 must help potentiate enhancer function in vivo.

While our studies were in progress, several reports appeared describing reversion analyses of SV40 mutants having defective enhancers. All of the SV40 revertants were restored in viability as a result of duplications of mutant enhancer elements (36, 37). These SV40 revertants are similar to the polyomavirus duplication revertants we isolated, since multiple copies of a defective enhancer compensates for a loss of function in the single-copy enhancer.

An A-to-G mutation at nt 5258 generates a dominant, cell-type-specific enhancer sequence. Wild-type polyomavirus does not productively infect murine teratocarcinoma stem

cells (70). PyEC (F9) host range mutants, which productively infect undifferentiated EC cells, contain an A:T to G:C transition at nt 5258, often accompanied by tandem duplications of sequences encompassing the point mutation (23, 41, 64). This mutation not only activates polyomavirus replication in EC cells (24) but also activates heterologous gene expression, as measured by transient expression assays in EC cells (6, 34, 45). Measurement of the effect of changes at nt 5258 on virus growth in fibroblasts has not been possible, since the other components of the polyomavirus enhancer function in such cells. However, the function of these redundant elements is reduced in mutant B1; consequently, the reversion at nt 5258 permits us to selectively measure the role of this element in fibroblasts. Revertant B1-5258 does not function well in DAP 3 or 3T6 cells, but functions efficiently in WME cells. Although 3T6 cells are derived from mouse embryo cells, they are a more homogeneous cell population and appear to be more differentiated, based on measurements of collagen and hyaluronic acid synthesis (27). In contrast, WME cells are heterogeneous and contain some cell types able to provide a suitable environment for the function of the B1-5258 enhancer sequences.

Since both positive and negative regulatory factors are capable of altering the function of enhancers, the change at nt 5258 in PyEC mutants may be modifying enhancer function in several ways. It might alter the binding of negative regulatory factors, or it might create a new site for positive-acting factors. The failure to observe activation of enhancer function upon deletion of the sequences surrounding nt 5258 (75) and the functional restoration of the mutant B1 enhancer by a single mutation at nt 5258 argue strongly that this mutation generates a functional positive-acting enhancer element.

It is noteworthy that an A→G transition at nt 5258 creates a better TGGCA consensus sequence [5'-TAGAA(N_3)TTCCA-3' to 5'-TGGAA(N_3)TTCCA-3']. The TGGCA consensus sequence is involved in transcriptional activation of numerous genes in transient expression assays (52, 53, 72). The TGGCA protein, which is present in many cell types, specifically interacts with these sequences. Strikingly, the TGGCA protein and nuclear factor 1 (from HeLa cell nuclei) both enhance initiation and elongation activity of adenovirus replication in vitro and bind to TGGCA consensus sequences (43). Whether the function of the new polyomavirus enhancer region generated by a mutation at nt 5258 is mediated by the TGGCA protein or nuclear factor 1 is presently unclear.

***cis*-Acting effects of the polyomavirus enhancer on transcription and replication are related functions.** Most of the mutant enhancers we have tested are reduced in their ability to activate both transcription and DNA replication. Previous studies have suggested that transcription and DNA replication are both mediated through the enhancer (16, 24, 75). Proof of a common element is provided by the observation that a single nucleotide reversion in the enhancer of mutant B1 at nt 5140 restores both polyomavirus transcription and replication. This change occurs in a sequence highly homologous to the adenovirus 5 E1A enhancer. It is noteworthy that this element does not appear to have a role in adenovirus DNA replication (32).

Among the multiple ways that enhancer elements might activate both transcription and DNA replication, we favor the possibility that proteins binding to the enhancer directly influence the formation of transcriptional and replicative preinitiation complexes. That such complexes occur is well documented (12, 18, 63, 81). Their formation is likely to be affected in several ways by enhancer-binding proteins. Occlusion of the core origin sequences and the early promoter ATA homology by nucleosomes is prevented by enhancer sequences (39, 40, 62) and, although not yet proven, the formation of preinitiation complexes may be positively regulated by such proteins. In vitro replication and transcription systems are either not dependent or only moderately dependent upon enhancer sequences (44, 66, 69, 80; C. Prives, Y. Murakami, F. Kern, W. Folk, C. Basilico, and J. Hurwitz, unpublished data), presumably because the template configuration and factor concentrations in vitro are different from those occurring in vivo.

The distinctive features of polyomavirus chromatin, such as DNase 1 hypersensitivity (8, 35), undoubtedly reflect binding of proteins in vivo to the enhancer. Analyses of the chromatin structure of the polyomavirus enhancer, in conjunction with genetic analyses and the development of in vitro systems which utilize chromatin-organized templates, should define the mechanisms by which enhancer-binding proteins regulate both transcription and DNA replication.

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