MOLECULAR AND CELLULAR BIOLOGY, Nov. 1988, p. 5000–5015
0270-7306/88/115000-16$02.00/0

Vol. 8, No. 11

Multiple Subelements within the Polyomavirus Enhancer Function Synergistically To Activate DNA Replication

WILLIAM J. MULLER,† DANIEL DUFORT,‡ AND JOHN A. HASSELL‡*

Department of Microbiology and Immunology, McGill University, 3775 University Street, Montreal, Quebec, Canada H3A 2B4

Received 13 May 1988/Accepted 23 August 1988

The polyomavirus origin for DNA replication comprises at least two essential, but functionally distinct, *cis*-acting components. One of these, the origin core, is required only for DNA replication. It includes binding sites for large T antigen and the origin of bidirectional DNA replication. The other component is required for both transcription and DNA replication and is represented by two functionally redundant regions, α and β, which are elements of the polyomavirus enhancer. Whereas either enhancer element will activate DNA replication, both enhancer elements are required to constitute a functional enhancer of transcription. To identify the sequences that make up each enhancer element, we have subjected them separately to in vitro mutagenesis and measured their capacity to activate replication in *cis* of the origin core in MOP-8 cells, which provide all *trans*-acting replicative functions including large T antigen. The results reveal that the β enhancer element is composed of three subelements, two auxiliary subelements, and a core subelement. The core subelement independently activated DNA replication, albeit poorly. The auxiliary subelements, which were inactive on their own, acted synergistically with the core subelement to increase its activity. Interestingly, dimers of the β core subelement functioned as well as the combination of a β auxiliary subelement and a core subelement, suggesting that the subelements are functionally equivalent. The α enhancer element is organized similarly; it too comprises an auxiliary subelement and a core subelement. These results lead us to suggest that the polyomavirus enhancer comprises two levels of organization; two or more enhancer elements form an enhancer, and two or more subelements make up an enhancer element. The subelements share few sequences and serve as binding sites for distinct cellular factors. It appears, therefore, that a number of different cellular proteins function cooperatively to activate polyomavirus DNA replication by a common mechanism.

Replication of polyomavirus (Py) requires the interplay of a virally encoded protein, large T antigen, cellular proteins including permissive factors, and *cis*-acting sequences defining the functional replication origin (3). Large T antigen is required to initiate viral DNA replication (5) and acts by binding to sequences within and adjacent to the replication origin (7, 9, 34). Once bound to DNA, Py large T antigen, by analogy to that of simian virus 40 (SV40), unwinds DNA in its vicinity, thereby permitting the action of cellular enzymes including permissive factors (43). The latter is likely DNA primase-DNA polymerase α complex or an associated cellular protein (25, 36). The formation of an initiation complex is followed by RNA-primed DNA synthesis which occurs initially within the origin at several sites on the template strand encoding the early proteins. Subsequently, initiation of RNA-primed DNA synthesis occurs on the opposite strand of replication forks (11).

The replication origin of Py comprises two functional components, an auxiliary component and a core component (23, 41). The origin core is the site of action of large T antigen and comprises no more than 67 base pairs (bp) (14, 23). It is composed of three regions including an AT-rich stretch at its late border, a central GC-rich palindrome (which includes two repeats of the large T antigen binding site, 5′-GAGGC-3′, on each strand [7, 32, 33]), and an imperfect repeat defining its early border. Mutations in each of these sequence features impair the capacity of the origin core to function (18, 40).

The auxiliary component of the Py origin is positioned at its late border and comprises two functionally redundant elements which make up part of the viral enhancer (23). These elements have been named alpha (α) and beta (β) (23) and correspond to enhancer elements 2 and 3 (22). (The names α and β were applied to the replication activators at a time when their identity to the transcription enhancer elements was not known.) A functional Py origin comprises either one of the enhancer elements, α or β, and the origin core. The Py origin core cannot function on its own as an autonomous origin in permissive mouse cells expressing large T antigen (23, 41, 42).

How the enhancer elements function to exert their effect on replication or transcription is not understood. To better understand this process, we have attempted to identify the viral sequences in the enhancer elements which are required for replication activation. Because only one element is sufficient to activate Py DNA replication, whereas a minimum of two is required to enhance transcription, we used the replication assay to assess the activity of mutant forms of each enhancer element. To accomplish this objective, we mapped the borders of the α and β elements by deletion mutagenesis and identified the critical sequences within their borders by linker-scanning (LS) mutagenesis. The results of our analyses revealed that each enhancer element is composed of the following subelements: auxiliary sequences, whose mutation reduced function, and core sequences, whose mutation abolished function. The auxiliary subelement and core subelement acted synergistically to activate

* Corresponding author.
† Present address: Department of Genetics, Harvard Medical School, Boston, MA 02115.
‡ Present address: Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, Ontario, Canada L8S 4K1.

DNA replication. The subelements share few sequences and interact in vitro with different DNA-binding proteins (2, 6, 13, 28, 30–32). These results suggest that a number of different enhancer-binding proteins can activate Py DNA replication, presumably by a common mechanism.

MATERIALS AND METHODS

Cells. MOP-8 cells (24) were grown on plastic petri dishes with Dulbecco modified Eagle medium supplemented with 10% (vol/vol) calf serum and antibiotics and maintained at 37°C in a humidified CO_2 atmosphere. Cells were passaged by trypsinization after reaching confluence and were replated at approximately 10^4 cells per cm^2.

Preparation of DNA and its modification. Recombinant plasmid DNAs were isolated from *Escherichia coli* DH1 and purified by CsCl density gradient centrifugation (8). Digestions of DNA with restriction endonucleases were performed in accordance with conditions specified by their manufacturers. Modification of DNA by treatment with the Klenow fragment of DNA polymerase I, T4 DNA ligase, and *Bal* 31 nuclease was carried out as described previously (19).

Construction of recombinant plasmids. The recombinant plasmids that served as substrates for mutagenesis or were used in replication assays were derived from molecules whose construction has been described previously (23). The various recombinant plasmids were named according to their composition whenever possible. The abbreviation pd (plasmid deleted) refers to pML2, P refers to Py, and the remaining letters or numerals refer to restriction endonuclease cleavage sites and nucleotide (nt) numbers in Py DNA.

To facilitate the isolation of deletion mutants and LS mutants, we modified pML2 DNA as follows. The *Bam*HI site was destroyed by cleavage with *Bam*HI, the ends were digested with *Bal* 31 nuclease, and an *Xho*I linker was ligated to them. After cleavage with an excess of *Xho*I, ligation, and transformation of *E. coli*, a plasmid which had lost nt sequences 325 through 465 and acquired an *Xho*I linker in their place was recovered and sequenced. This plasmid, pMLX, was further modified by removal of the *Sal*I site at nt 651 and the replacement of this with a *Bam*HI site. This was achieved by cleavage of pMLX with *Sal*I, backfilling the 3′-recessed ends with the Klenow fragment of DNA polymerase I, and ligation of *Bam*HI linkers. Cleavage of this DNA with an excess of *Bam*HI followed by circularization with T4 ligase and transformation of *E. coli* led to the recovery of the plasmid, pMLXB, which contained a *Bam*HI site in place of the *Sal*I site. pMLXB served as a vector for the Py origin and its mutated derivatives.

Isolation of mutant recombinant plasmids. A series of late-side (ΔLB) and early-side (ΔEB) unidirectional deletion mutants of the β enhancer element was constructed by use of the plasmid pdPP1(B)Bg(H) as displayed in Fig. 1. The latter plasmid contains Py sequences from nt 5130 through the numbering origin to nt 90 as a *Bam*HI-to-*Hin*dIII fragment cloned between the same sites in pML2 (23). The numbering system of Soeda et al. (37) is used here. Late-side unidirectional deletion mutants were isolated by cleavage of this plasmid with *Bam*HI, erosion of the ends for various periods with *Bal* 31 nuclease, blunting of the ends with the Klenow fragment of DNA polymerase I, and ligation of *Xho*I linkers (5′-CCTGCAGG-3′). The DNA was then digested to completion with *Hin*dIII and *Xho*I, and the Py sequences were cloned between the *Hin*dIII and *Xho*I sites of pMLXB.

Early-side unidirectional deletion mutants of the β element were isolated similarly by digestion with *Pvu*II, which cleaves pdPP1(B)Bg(H) once within Py sequences at nt 5264, followed by digestion with *Bal* 31, treatment with the Klenow fragment of DNA polymerase I, and ligation of *Xho*I linkers. The Py sequences corresponding to the β element were excised by cleavage with *Bam*HI and *Xho*I and were cloned into pMLXB carrying the Py origin core (nt 5265 to 90) as an *Xho*I (nt 5265)-to-*Hin*dIII (nt 90) fragment. A positive-control plasmid, pdPP1(B)P2(X)Bg(H), for the early β deletion mutants was constructed by replacing the *Pvu*II site at nt 5264 with an *Xho*I linker; the β element was then cloned in pMLXB carrying the origin core. Two mutants in this series, ΔEB5209 and ΔEB5202, are larger than the others (ΔEB5240, ΔEB5223, and EB5170), because their plasmid DNA comprises pML2 rather than pMLXB sequences. pML2 is 140 bp larger than pMLXB.

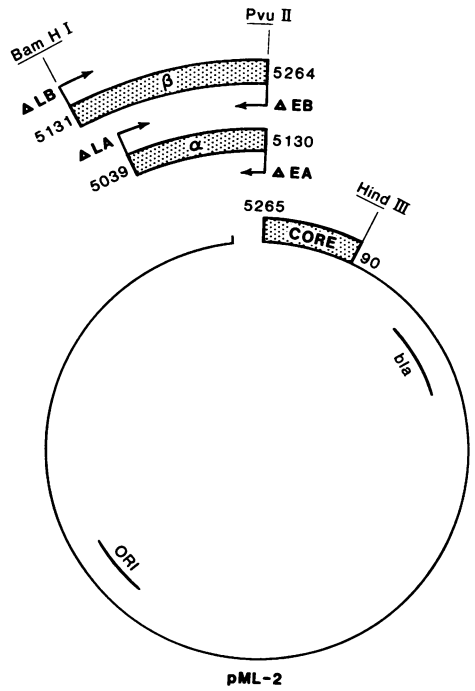


FIG. 1. Schematic representation of the plasmids bearing different configurations of the Py origin that were used as substrates for mutagenesis. The Py origin core with either the α or β element at its late border was cleaved with *Bam*HI to generate a set of late β (ΔLB) or late α (ΔLA) unidirectional deletion mutants and with *Pvu*II to derive a set of early β (ΔEB) or early α (ΔEA) unidirectional deletion mutants as described in Materials and Methods. The plasmids also contain the pML2 origin for DNA replication (ORI) and the ampicillin resistance gene (bla). Numbers indicate nt positions.

Late-side unidirectional deletion mutants of the α element (ΔLA) were isolated by cleavage of pdPB503dl300 (23) with *Bam*HI (Fig. 1). This plasmid contains Py sequences from nt 5039 to 5130 linked to the Py origin core (nt 5265 to 90) and lacks the β element (21). The *Bam*HI site occurs in a linker juxtaposed next to nt 5039 (21). The linearized DNA was treated with *Bal* 31 as described earlier, and the Py sequences were cloned between the *Hin*dIII and *Xho*I sites of pMLXB.

Early-side unidirectional deletion mutants of the α element (ΔEA) were also derived from pdPB503dl300 (Fig. 1). Its DNA was digested with *Pvu*II, which cleaves once at nt 5130 in Py sequences, and the linearized DNA was treated as described previously. The deleted α elements were removed

by cleavage with *Bam*HI and *Xho*I and were cloned between these same sites in pMLXB DNA bearing the Py origin core.

LS mutants were constructed by joining appropriate pairs of late- and early-side deletion mutants across their *Xho*I ends. Because our collection of deletion mutants was not large, we were unable, in several cases, to generate perfect LS mutants in which 8 bp of *Xho*I linker DNA replaced 8 bp of Py DNA. To expand the number of LS mutants, three (LS5151/5166, LS5109/5113, and LS5092/5108) were created by joining the ends of deletion mutants from two libraries that terminated in different linkers (viz., *Bam*HI and *Xho*I linkers). Finally, one LS mutant, LS5173/5188, contains an insertion of 6 bp (5′-TCTCGA-3′) of unknown origin as well as a complete 8-bp *Xho*I linker replacing viral sequences.

DNA transfection and measurement of DNA replication. CsCl gradient-purified, supercoiled plasmid DNAs were transfected into MOP-8 cells by a modification of the DEAE-dextran technique (23). Generally, 5×10^5 cells on a 60-mm dish were transfected with 1.0 μg of DNA, and cell lysates were harvested 48 h posttransfection by the Hirt procedure. Quantitative replication assays were performed by transfecting simultaneously with 0.5 μg each of test DNA and wild-type (wt) competitor DNA.

The replication of the recombinant plasmid DNA in MOP-8 cells was assessed by the assay devised by Peden et al. (29). In outline, the low-molecular-weight DNA recovered from transfected MOP-8 cells was digested with *Dpn*I and a one-cut restriction endonuclease for the transfected DNA (generally *Bam*HI). The products were separated by electrophoresis through a 1.0% (wt/vol) agarose gel, transferred to nitrocellulose, and visualized by hybridization to ^{32}P-labeled, nick-translated pdPBR2 DNA (23). pdPBR2 DNA is composed of pML-2 DNA and the small *Bam*HI-to-*Eco*RI fragment of Py DNA, which contains the viral replication origin. After the nitrocellulose sheet was dried, it was exposed to XAR-5 film (Eastman Kodak Co.) and Cronex Lightning-Plus intensifying screens (E. I. du Pont de Nemours & Co., Inc.) for 3 to 12 h. After autoradiography, the bands representing replicated DNA were quantitated by laser densitometric scanning of autoradiograms exposed for various periods to ensure that the band intensity was directly proportional to the number of labeled DNA molecules in each band. The replication assays were repeated at least four times with a minimum of two different preparations of wt and mutant plasmids. In separate experiments, the extents of replication of the same DNAs were within 10% or less of each other.

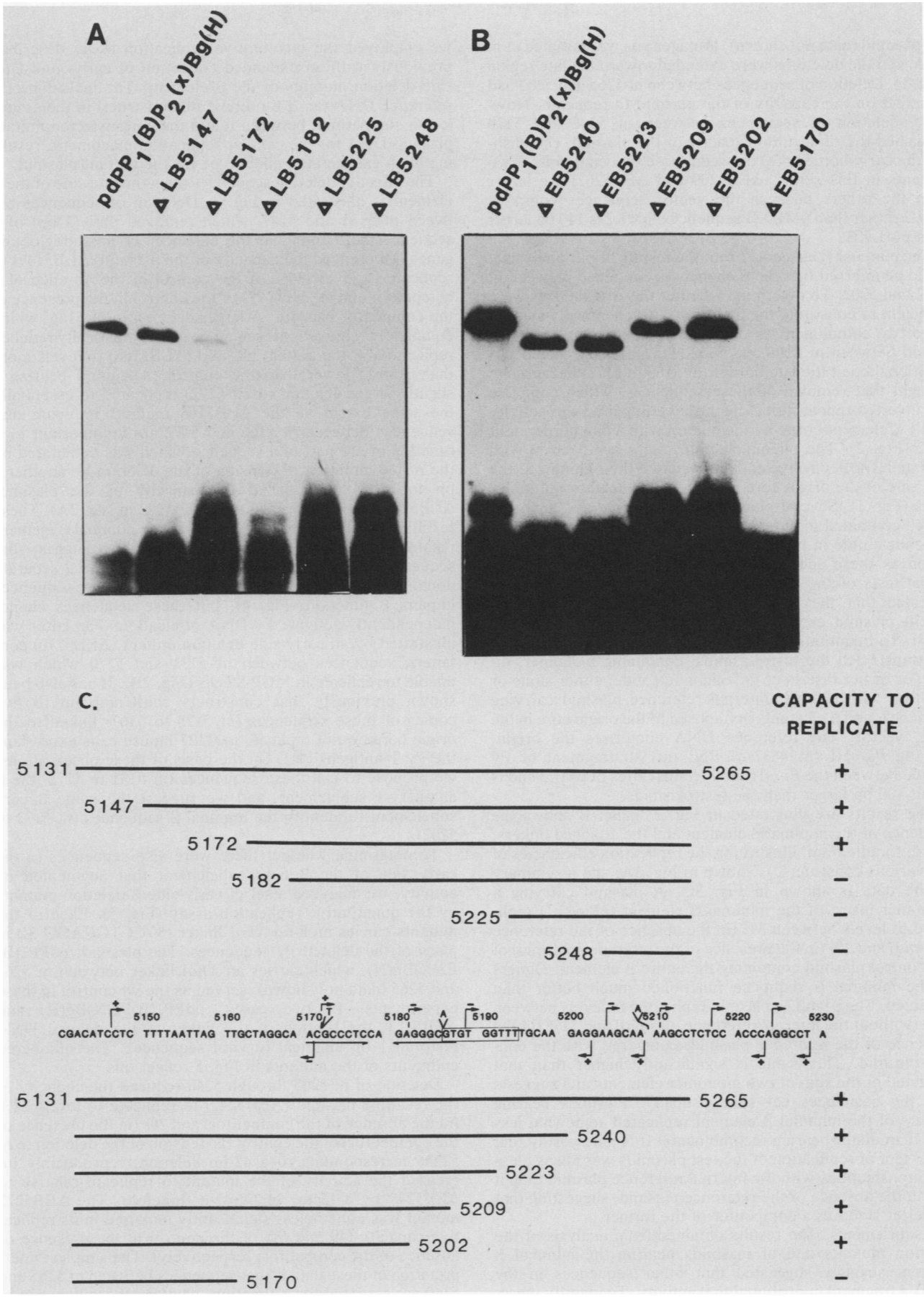
RESULTS

To define the sequence requirements for function of the α and β elements, we employed in vitro mutagenesis techniques to recover deletion and LS mutants whose replicative capacity was tested relative to an appropriate control plasmid in permissive 3T3 cells expressing the Py T antigens (MOP-8 cells). Analysis of the deletion mutants allowed us to define the borders of the elements, whereas analysis of the LS mutants permitted identification of functional sequences within their borders. Py sequences bearing mutations in either the α or β elements were cloned in their natural orientation at the late border of the origin core in a common genetic background (Fig. 1). The replicative capacity of the mutant origins was assessed after transfection of MOP-8 cells by recovering low-molecular-weight DNA and cleaving it with *Dpn*I and a one-cut restriction endonuclease for the transfected plasmid. The DNA replication assay is based on the fact that *Dpn*I cleaves methylated, unreplicated DNA but not unmethylated DNA that has replicated at least once in mammalian cells (29). The one-cut restriction endonuclease, generally *Bam*HI, served to collect the replicated DNA as a single species visible in autoradiograms of Southern blots. The *Dpn*I cleavage products of unreplicated DNA appear as a smear of fragments at the bottom of such autoradiograms, because there are 16 or more *Dpn*I cleavage sites in the transfected plasmids. Application of this method allowed us to determine the borders of the enhancer elements and to identify the active sequences within them.

Borders of the β enhancer element. We showed previously that the β element is contained within the *Pvu*II-4 fragment of Py between nt 5131 and 5264 (23). To more precisely define its limits, we constructed a series of late (ΔLB) and early (ΔEB) unidirectional deletion mutants and measured their capacities to replicate in MOP-8 cells (Fig. 1). To map the late border of the β element, we began deletion mutagenesis at nt 5131 and extended the deletions toward the early region to various positions within the *Pvu*II-4 fragment (Fig. 2A and C). The removal of sequences between nt 5131 and 5147 had no effect on the replication capacity of the plasmid. By contrast, deletion of sequences between nt 5147 and 5172 impaired but did not abolish the capacity of the plasmid to replicate. The ΔLB5172 mutant lacked 2 bp of a 9-bp, GC-rich inverted repeat (5′-ACTGCCCTC-3′). The latter is situated adjacent to a sequence (5′-GTGTGGTTT-3′) that shares sequence identity with the SV40 enhancer core motif, 5′-G(C)G(C)T(C)GTGGA(T)A(T)A(T)G-3′ (15), which is thought to serve as a binding site for the cellular proteins AP3 (20), PEB1 (30), and EBP20 (13). Further removal of 10 bp between nt 5172 and 5182 completely abolished the capacity of the plasmid to replicate. The replication-defective ΔLB5182 mutant lacks the inverted repeat motif but retains the SV40 enhancer core sequence. These results suggest that the late border of the β element is between nt 5172 and 5182 and that sequences between nt 5147 and 5172 augment its activity.

The early border of β was determined by analyzing the replication of a set of early unidirectional deletion (ΔEB) mutants (Fig. 2B and C). A plasmid, pdPP1(B)P2(X)Bg(H), was constructed that contained an *Xho*I linker between nt 5264 and 5265 to serve as a positive control. The insertion of the 8-bp linker, 5′-CCTGCAGG-3′, restored nt 5265 to the β element. This linker did not alter the replicative capacity of

FIG. 2. Replication capacity of a set of late and early unidirectional deletion mutants of the β element. (A) Replication of late β deletion mutants. (B) Replication of early β deletion mutants. The autoradiograms shown in panels A and B were derived from the same experiment, but were processed separately and exposed for different periods. (C) Schematic illustration of the structures of the β deletion mutants relative to the Py nt sequence. The sequence has the same polarity as early mRNA and is numbered according to Soeda et al. (37). The changes to this sequence are depicted above it. These include insertion of a T between nt 5172 and 5173, insertion of an A between nt 5185 and 5186, and insertion of an A between nt 5208 and 5209. The boxed sequences are homologous to the SV40 enhancer core motif. The arrows underline perfect inverted repeats. The angled arrows above and below the sequence depict deletion endpoints, and the plus and minus indicate whether mutants with these endpoints replicated (+) or not (−). The extent of replication of each DNA was not quantitated and consequently the replicational phenotypes are designated as either + or −.


A
pdPP1(B)P2(x)Bg(H)
Δ LB5147
Δ LB5172
Δ LB5182
Δ LB5225
Δ LB5248
B
pdPP1(B)P2(x)Bg(H)
Δ EB5240
Δ EB5223
Δ EB5209
Δ EB5202
Δ EB5170
C.
CAPACITY TO REPLICATE
5131
5265
+
5147
+
5172
+
5182
−
5225
−
5248
−
5150
5160
5170
5180
5190
5200
5210
5220
5230
CGACATCCTC TTTTAATTAG TTGCTAGGCA ACGCCCTCCA GAGGGCGTGT GGTTTTGCAA GAGGAAGCAA AAGCCTCTCC ACCCAGGCCT A
5131
5265
+
5240
+
5223
+
5209
+
5202
+
5170
−

the plasmid (data not shown). Mutagenesis was initiated at nt 5265, and the deletions were extended toward the late region (Fig. 1). Deletion of sequences between nt 5265 and 5202 had no effect on the capacity of the plasmid to replicate. However, deletion of sequences between nt 5202 and 5170 abolished the replication capacity of the plasmid, establishing the early border of β between these two endpoints. Two mutants in this series (ΔEB5209 and ΔEB5202) are larger than the others because the vector sequences comprise pML2 rather than pMLXB sequences. pML2 is 140 bp larger than pMLXB.

The minimal β element. From the results of our analysis it could be inferred that the β element is contained between nt 5172 and 5202. To determine whether these sequences were sufficient to constitute the β element, we chemically synthesized two complimentary oligonucleotides that spanned the region between nt 5168 and 5202 (Fig. 3B). We included 4 additional bp at the late border (nt 5168 to 5172) because we thought that removal of these sequences, which comprise the inverted-repeat motif, might have impaired the activity of the β element borne by the mutant with a late border at nt 5172 (Fig. 2). The oligonucleotides were synthesized with *Sal*I and *Xho*I cohesive ends to facilitate their cloning at the late side of the origin core in pMLXB as head-to-tail multimers (Fig. 1). Several plasmids were isolated and analyzed which contained monomer, dimer, and trimer inserts of the oligonucleotide in head-to-tail array and in the same orientation as these sequences normally are positioned in Py. Initial tests of the replicative capacity of these constructs revealed that they replicated poorly compared with the parent plasmid carrying the entire β element (nt 5130 to 5264). To quantitatively measure their capacity to replicate we transfected the test plasmids, containing monomer, dimer, or trimer inserts of the oligonucleotide, either alone or together with a larger internal reference plasmid carrying both the α and β elements juxtaposed to the origin core in the pML vector. The reference DNA comprises the origin-bearing *Bam*HI (nt 4632)-to-*Bgl*I (nt 90) fragment of Py cloned between the *Bam*HI and *Hin*dIII sites of pML2 and is about 400 bp larger than the test plasmids.

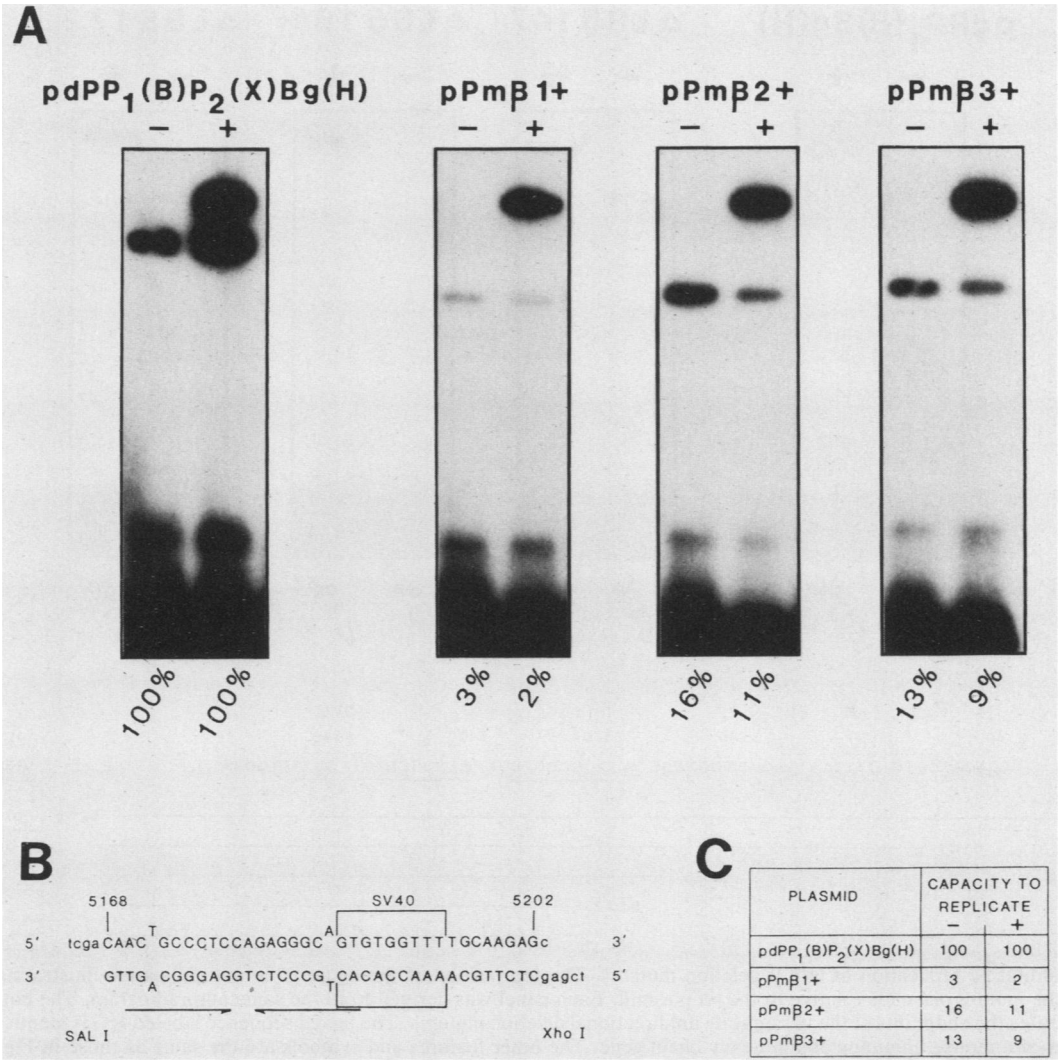
The results are illustrated in Fig. 3; panel B shows the sequence of the minimal β element and the attached linkers. The autoradiogram, illustrating the replication efficiencies of the various constructs, is shown in Fig. 3A, and a summary of the data is shown in Fig. 3C. A plasmid carrying a monomer insert of the minimal β element (pPmβ1$^+$) replicated to levels between 3% (in the absence of the reference plasmid) and 2% (in the presence of the reference plasmid) of the control plasmid containing the entire β element. Dimers of the minimal β sequence functioned much better than expected. The pPmβ2$^+$ plasmid replicated to levels between 16% (without the internal reference plasmid) and 11% (in the presence of the reference plasmid) compared with the control plasmid. This value is significantly higher than that expected of the sum of two monomer elements and suggests that the sequences act synergistically. Plasmids bearing trimers of the minimal β element replicated somewhat less well than dimer-bearing recombinants. It is noteworthy that the extent of replication of the test plasmids was always less in cotransfections with the internal reference plasmid than it was in the absence of the reference plasmid, suggesting that the latter acted as a competitor of the former.

β subelements. The results obtained from analysis of the deletion mutants and of plasmids bearing the minimal β element strongly suggested that other sequences in the *Pvu*II-4 fragment contributed to β activity. To identify these, we employed the quantitative replication assay described previously with an expanded collection of early- and late-side deletion mutants of the β element. The inclusion of a reference DNA (an α-β-origin core construct) in the assays led to competition between it and the cotransfected mutant plasmid DNA for the cellular replication machinery, resulting in an exaggerated phenotype for the mutant plasmid.

The effect of deleting sequences from the late side of the β element is illustrated in Fig. 4. Deletion of sequences between nt 5131 and 5147, which removed part (3 bp) of a sequence motif found in the enhancer of immunoglobulin genes (1), reduced the capacity of the plasmid (ΔLB5147) to replicate to levels 34% of the control in the absence of a competitor and to levels 7% of the control in the presence of the competitor plasmid. A larger deletion to nt 5166, which completely removed this sequence motif, drastically reduced replication of the mutant plasmid (ΔLB5166) to levels more than 30-fold lower than the control. A mutant bearing a slightly larger deletion (to nt 5172) replicated to essentially the same extent as the ΔLB5166 mutant, implying that sequences between nt 5166 and 5172 are unimportant to β function or are part of a sequence which was debilitated by the Δ5166 mutation. Extension of the deletion by another 8 bp to nt 5180 abolished the capacity of the plasmid, ΔLB5180, to replicate (also see ΔLB5182 in Fig. 2A). These results confirm that the late border of the minimal β element resides between nt 5172 and 5180 and also suggest that sequences between nt 5131 and 5172 constitute a separate domain of the β element. Deletion of these sequences impairs β function (Fig. 4), but these sequences cannot independently activate Py DNA replication. The latter was illustrated by an early-side deletion mutant (ΔEB5170) containing sequences between nt 5131 and 5170 which was unable to replicate in MOP-8 cells (Fig. 2B). It has also been shown previously that constructs containing up to five copies of these sequences (nt 5128 to 5167) linked to the origin core do not replicate in C127 mouse cells expressing the Py T antigens (41). On the basis of these observations, we propose to call these sequences (nt 5131 to 5172) the β auxiliary-1 subelement, and we suggest the name β core subelement to identify the minimal β sequences (nt 5172 to 5202).

To determine whether there were also sequences to the early side of the β core subelement that augmented its activity, we analyzed a set of early-side β deletion mutants by the quantitative replication assay (Fig. 5). Each of the mutants carries an 8-bp *Xho*I linker (5′-CCTGCAGG-3′) in place of the deleted Py sequences. The plasmid, pdPP1(B) P2(X)Bg(H), which carries an *Xho*I linker between nt 5264 and 5265 (data not shown), served as the wt control in these experiments. In two cases, pdPP1(B)P2(X)Bg(H) and ΔEB5223, the ligation of the linker to the deleted DNA restored 1 bp identical to viral sequences. The numbered endpoints of the mutants in Fig. 5 reflect this.

Deletion of nt 5265 through 5240 reduced the capacity of the resulting plasmid (AEB5240) to replicate to levels 22% (in the absence of the competitor) and 2% (in the presence of the competitor) of the control. Extension of the deletion to nt 5223, corresponding to a 42-bp deletion, reproducibly increased the activity of the mutant to replicate relative to ΔEB5240 by a factor of two- to threefold. The ΔEB5223 mutant was nonetheless significantly impaired in its replicative capacity (49 and 6% of the control in the presence or absence of the competitor, respectively). The simplest interpretation of these data is that sequences between nt 5265 and 5240 are essential for β function, whereas those between nt



PLASMID	CAPACITY TO REPLICATE –	+
pdPP1(B)P2(X)Bg(H)	100	100
pPmβ1+	3	2
pPmβ2+	16	11
pPmβ3+	13	9

FIG. 3. Quantitative replication of plasmids bearing monomer, dimer, or trimer inserts of the minimal β element. (A) Autoradiogram of the extent of replication of the various plasmids. Each pair of lanes was originally derived from the same autoradiogram. The + and – symbols above each panel refer to the presence (+) or absence (–) of the internal control plasmid in each lane. The internal control plasmid is the slowest-migrating species in these lanes. The numbers below the lanes represent the extent of replication of plasmids relative to that of the wt plasmid, pdPP1(B)P2(X)Bg(H), which was arbitrarily set at 100%. The efficiency of replication of the experimental plasmids bearing the minimal β element was calculated relative to the wt plasmid in the lanes without the internal control. For those lanes containing the internal control, the ratio of replication of the minimal β element-bearing plasmids relative to the internal control was calculated, and this ratio was then expressed as a percentage of the corresponding ratio for the wt plasmid, pdPP1(B)P2(X)Bg(H). pPmβ1$^+$ carries a single insert of the minimal β element, pPmβ2$^+$ carries two head-to-tail tandem inserts of the minimal β element, and pPmβ3$^+$ carries three head-to-tail tandem inserts of the minimal β element. (B) Sequence of the minimal β element. The nt bases represented by lowercase letters are not found in Py. They correspond to restriction endonuclease cleavage sites. The arrows underlining the sequences are perfect inverted repeats, and the boxed sequences are homologous to the SV40 enhancer core motif. The numbers refer to nt positions in Py DNA. (C) Summary of the extent of replication of the experimental plasmids as a percentage of that of the wt plasmid.

5240 and 5223 are not. The increased activity of the ΔEB5223 mutant relative to the ΔEB5240 mutant could have resulted from moving the remaining β subelements closer to the origin core. However, other interpretations are also possible (see below). An even larger deletion mutant (ΔEB5209) with an endpoint at nt 5209 replicated to levels significantly lower than the preceding mutants (ΔEB5240 and EB5223) in this series, suggesting that sequences between nt 5223 and 5209 are also essential to β function. The remaining mutant in this set, ΔEB5202, replicated to levels about twofold higher than ΔEB5209, which bears a smaller deletion. Therefore, the sequences between nt 5202 and 5209 are probably unessential for β function. It is noteworthy that this mutant, ΔEB5202, which retains the β auxiliary-1 subelement and the β core subelement, replicated better under noncompetitive conditions (18% of the control) than the pmB1$^+$ plasmid (3% of the control), which contains only the β core subelement (compare Fig. 3 with Fig. 5). This observation substantiates our contention that the β auxiliary-1 subelement augments the activity of the β core subelement. It is also noteworthy that the replicative capacity of the ΔEB5202 mutant was approximately the same in noncompetitive conditions as that of the plasmid carrying a dimer insert of the β core subelement (Fig. 3), suggesting that the auxiliary subelement and core subelement are functionally redundant.

The results of the analysis of the early-side β deletion

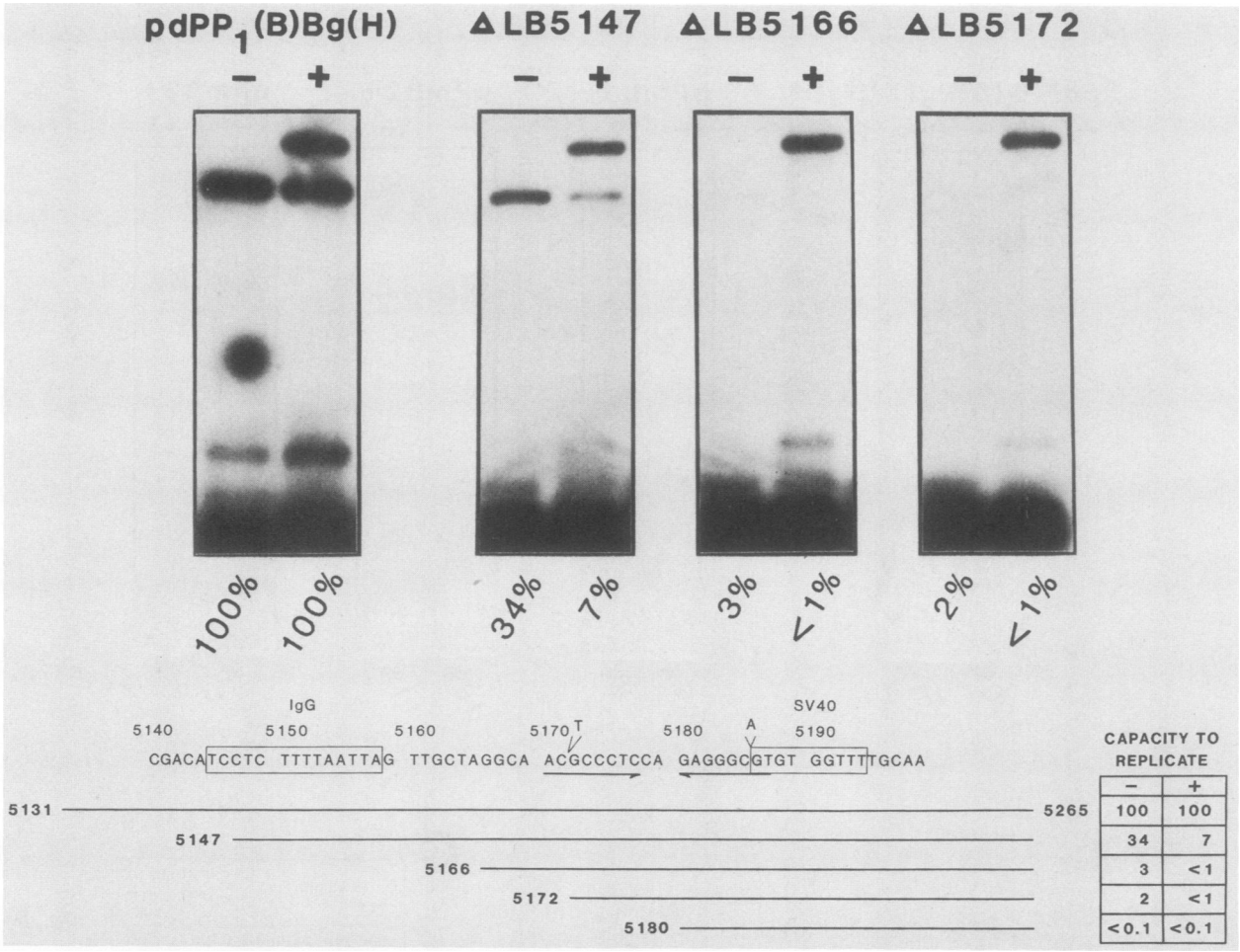


FIG. 4. Quantitative replication of late β deletion mutants. The top portion of the figure is an autoradiogram illustrating the extent of replication of the mutant plasmids relative to the wt plasmid. Each panel was derived from the same autoradiogram. The bottom portion of the figure illustrates the endpoints of the various late unidirectional deletion mutants. The boxed sequence labeled IgG is identical to one found in the enhancer of a mouse immunoglobulin heavy chain gene. The other features and symbols are the same as those in Fig. 3.

mutants could be interpreted to mean that two auxiliary subelements exist on the early side of the β core subelement. One of these could be located between nt 5209 and 5223, whereas the other could be situated between nt 5240 and 5265. This interpretation ignores the possible effect on replication capacity of altering the spacing between sequences in the β element and those in the origin core. That this parameter plays a role is supported by the observation that some mutants in this series replicated better than others bearing smaller deletions. For example, ΔEB5223 replicated better than ΔEB5240, and ΔEB5202 replicated better than ΔEB5209 (Fig. 5). Interestingly, the deletions suffered by ΔEB5223 and ΔEB5202 are close to even multiples of one-half of a DNA turn (3.9 and 5.9 helical turns, respectively), whereas those of ΔEB5240 and ΔEB5209 are nearly odd multiples of half a DNA turn (2.3 and 5.2 helical turns, respectively). This suggests that the phenotype of each mutant could have resulted from an effect of spacing or deletion (or both) of essential sequences. Whatever the effect of spacing, it seems unlikely that the phenotype of the early-side deletion mutants is due solely to variation of this parameter, because each of the mutants, including those deleted of even multiples of half a DNA turn, were debilitated relative to the control and the severity of the defect in each mutant generally correlated with deletion size. Nonetheless, to obtain confirmation of the proposed structure of the β element, we recombined early- and late-side β deletion mutants to generate LS mutants, whose lesions do not alter the spacing between the β element and the origin core.

Five LS mutants were isolated (Fig. 6). Three of these, LS5151/5166, LS5173/5188, and LS5202/5211, are imperfect and lack 1 bp relative to the wt sequence. LS5209/5218 is a perfect LS mutant, and LS5240/5248 contains a 1-bp insertion relative to wt. With one exception, LS5240/5248, all of the mutants were debilitated in their capacity to replicate. Interestingly, the LS5240/5248 mutation occupies 8 of the 25 bp identified previously (Fig. 5, ΔEB5240) to be important for β element function. Therefore, if the phenotype of ΔEB5240 is due only to the loss of functional sequences and not to an effect of spacing, then the important sequences must be located between nt 5248 and 5265. There are no known conserved enhancer or promoter motifs in this region.

Two LS mutants, LS5209/5218 and LS5202/5211, which map to the early side of the β core subelement, were unable to replicate efficiently. Interestingly, one of these mutants (LS5202/5211), whose mutation lies closest to the β core subelement, replicated better than the other (LS5209/5218), whose mutation maps farther away. The mutations overlap

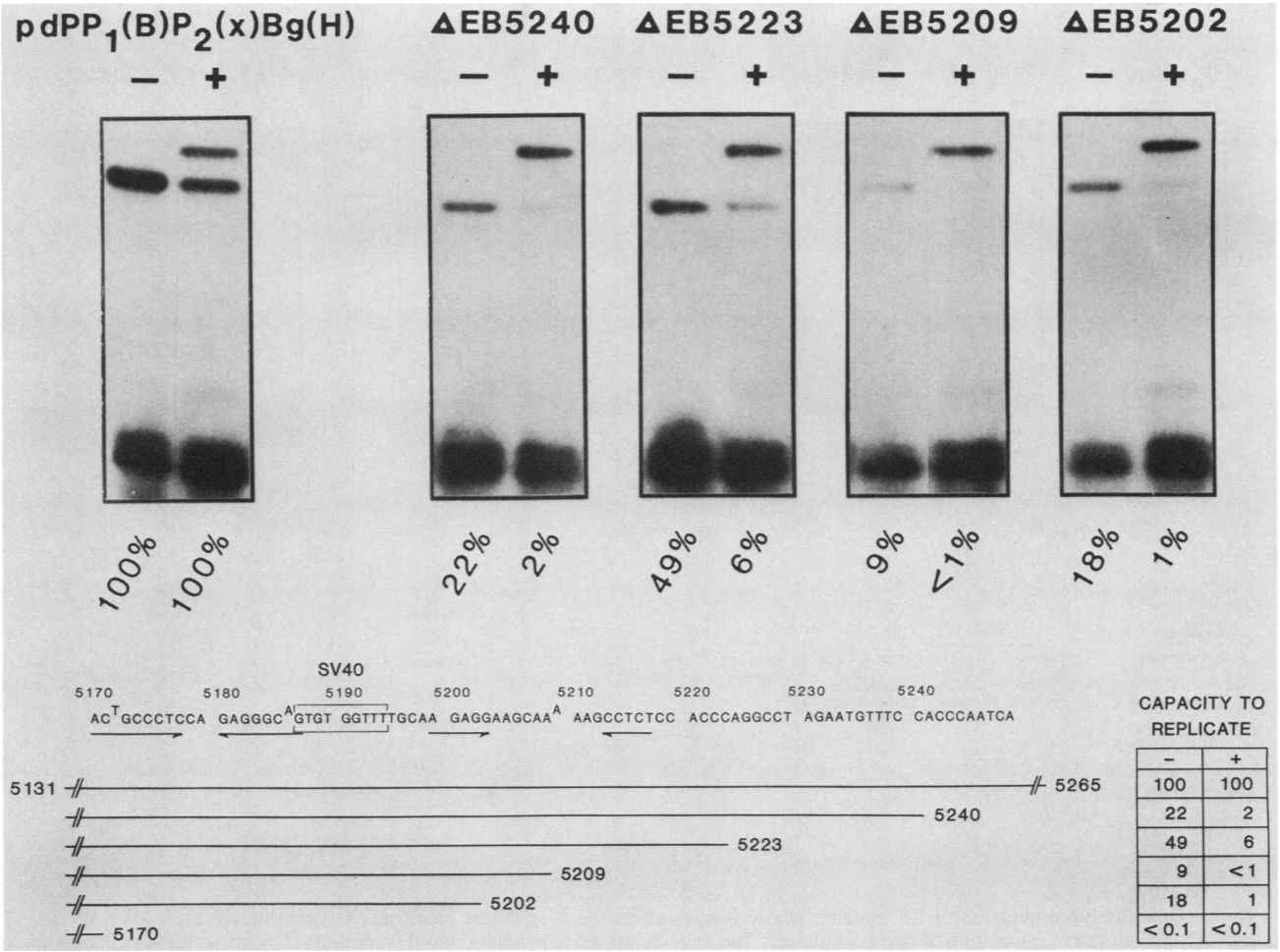


FIG. 5. Quantitative replication of early β deletion mutants. Each panel at the top was derived from the same autoradiogram. The other features and symbols are the same as those in Fig. 3.

by 1 bp and may differentially impair the function of the same sequence element. Recall that deletion of sequences between nt 5223 and 5209 resulted in a five- to sixfold drop in the replicative capacity of the ΔEB5209 plasmid relative to the ΔEB5223 mutant (Fig. 5), and deletion of another 7 bp from the ΔEB5209 mutant to yield the ΔEB5202 plasmid did not exacerbate the phenotype of the ΔEB5209 mutant. The region spanned by the two LS mutants maps between nt 5203 and 5218. Taken together, these observations suggest that a β auxiliary subelement (β auxiliary-2) exists between nt 5209 and 5218. This region contains an SV40 enhancer core motif [5′-G(C)G(C)T(C)GTGGA(T)A(T)A(T)G-3′] on the strand opposite to that shown in Fig. 6, which is 77% homologous to the consensus. At high protein-to-DNA ratios, EBP20 protects sequences between nt 5215 and 5228 from DNase I digestion (13).

An LS mutation within the β core subelement drastically reduced the replicative capacity of the LS5173/5188 mutant (Fig. 6). The mutation, however, did not abolish the replicative capacity of the mutant. This LS mutant replicated to the same extent as did a plasmid bearing only the β core subelement (compare Fig. 3 and Fig. 6). The mutation may not have completely inactivated the β core subelement, or the activity of the remaining auxiliary subelements may have compensated for the loss of β core activity.

Finally, the LS5151/5166 mutant, bearing a mutation in the β auxiliary-1 subelement defined by deletion mutagenesis, replicated very poorly by comparison with the wt control plasmid. The phenotype of the mutant confirms our contention that sequences to the late side of the β core subelement augment its activity. The mutations borne by the LS mutant (LS5151/5166) and the late β deletion mutant (ΔLB5147), which define this auxiliary subelement, do not overlap. These mutations may independently affect different parts of the same sequence (i.e., the conserved immunoglobulin enhancer motif), or they may separately affect two different sequence motifs, for example, one between nt 5131 and 5147 and another between nt 5151 and 5166.

Analyses of the early and late β deletion mutants and the LS mutants strongly suggest that the β enhancer element comprises multiple functional subelements whose activities differ in MOP-8 cells. Individual auxiliary subelements did not have any intrinsic activity, and deletion of these sequences from the β enhancer element impaired but did not abolish its activity. Unlike the auxiliary subelement, the β core subelement was able to function as an enhancer element, albeit poorly, and supplementing this subelement with the auxiliary subelements radically improved its activity. Moreover, dimers of the β core subelement functioned much better than a monomer of the same element to levels about the same as a plasmid containing the β auxiliary-1 and core subelements. Additionally, the phenotype of the LS5173/

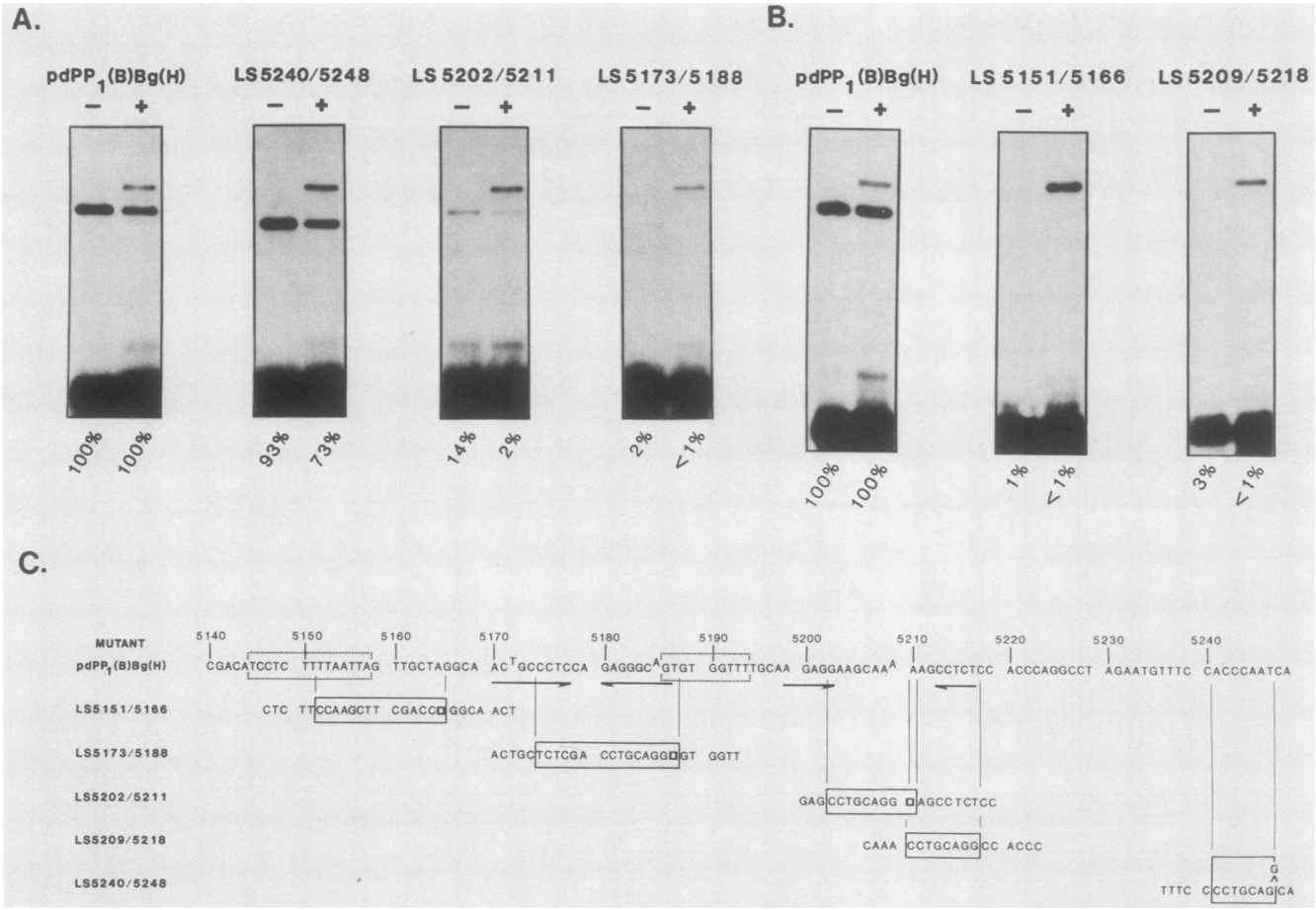


FIG. 6. Quantitative replication of LS mutants whose lesions affect the β enhancer element. (A) Quantitative replication of three LS mutants. (B) Quantitative replication of two LS mutants. The data illustrated in panels A and B were derived from separate experiments. (C) Sequence of the β element spanning the region affected by the LS mutations. The sequence affected by each mutation is boxed. The open square in each of the first three boxes (LS5151/5166, LS5173/5188, and LS5202/5211) denotes a 1-bp deletion. The sequences flanking the box are the same as the wt sequence, which is depicted above. LS5240/5248 carries a 1-bp insertion relative to the wt sequence. The other symbols are the same as those in Fig. 3.

5188 mutant, whose mutation resides in the center of the β core subelement, could be interpreted to mean that two auxiliary subelements can partially compensate for the loss of the β core subelement. These results imply that the various subelements in β are functionally redundant and that they act synergistically to activate Py DNA replication.

Borders of the α enhancer element. We showed previously that sequences between nt 5039 and 5130, which define the α enhancer element, could functionally substitute for the β element (23). To define the borders of the α element more precisely, we constructed a series of late (ΔLA) and early (ΔEA) unidirectional deletion mutants and measured their capacities to replicate in MOP-8 cells. To map the late border of the α element, we began mutagenesis at nt 5039 and extended the deletions toward the early region (Fig. 1). Deletion mutants with endpoints at nt 5063, 5066, 5073, and 5097 replicated nearly as well as the control plasmid, pdPB503Bg(H)dl300, comprising sequences from nt 5039 to 5130 (α) linked to those from nt 5265 to 90 (the origin core) (Fig. 7A and C). Extension of the deletion from nt 5097 to 5120 resulted in a mutant plasmid, ΔLA5120, which was unable to replicate. This established the late border of the α element between nt 5097 and 5120. The 23 bp between these endpoints contains part of an inverted repeat and a sequence perfectly homologous to the adenovirus E1A enhancer core motif, 5′-A(C)GGAAGTGAA(C)-3′. Three nuclear factors from 3T6 cells, PEA1, PEA2, and PEA3, protect sequences within this stretch from digestion by DNase I (32; M. E. Martin, J. Piette, M. Yaniv, W.-J. Tang, and W. R. Folk, personal communication). PEA1 apparently recognizes the same sequence as do the HeLa cell factor AP1 (16, 17) and the yeast factor GCN4 (12).

To establish the early border of the α element, we began mutagenesis at nt 5130 and extended the deletions toward the late region (Fig. 1). Deletion of sequences between nt 5130 and 5120 abolished the replicative capacity of the plasmid ΔEA5120 (Fig. 7B and C). Four other mutants in this series also failed to replicate. These observations allow us to map the early α border between nt 5130 and 5120. This region contains the other part of the inverted repeat referred to earlier and a PEA2-binding site (Fig. 7C). Therefore, the minimal α element is no more than 33 bp long and must be contained between nt 5097 and 5130.

α subelements. To determine whether the α element, like the β element, was composed of a core subelement and auxiliary subelements, we measured the replicative capacity of the ΔLA mutants using a quantitative assay. In addition, we isolated one more mutant (ΔLA5108) with a late endpoint at nt 5108. The results of this analysis are shown in Fig. 8. The wt control plasmid, pdPB503Bg(H)dl300, replicated

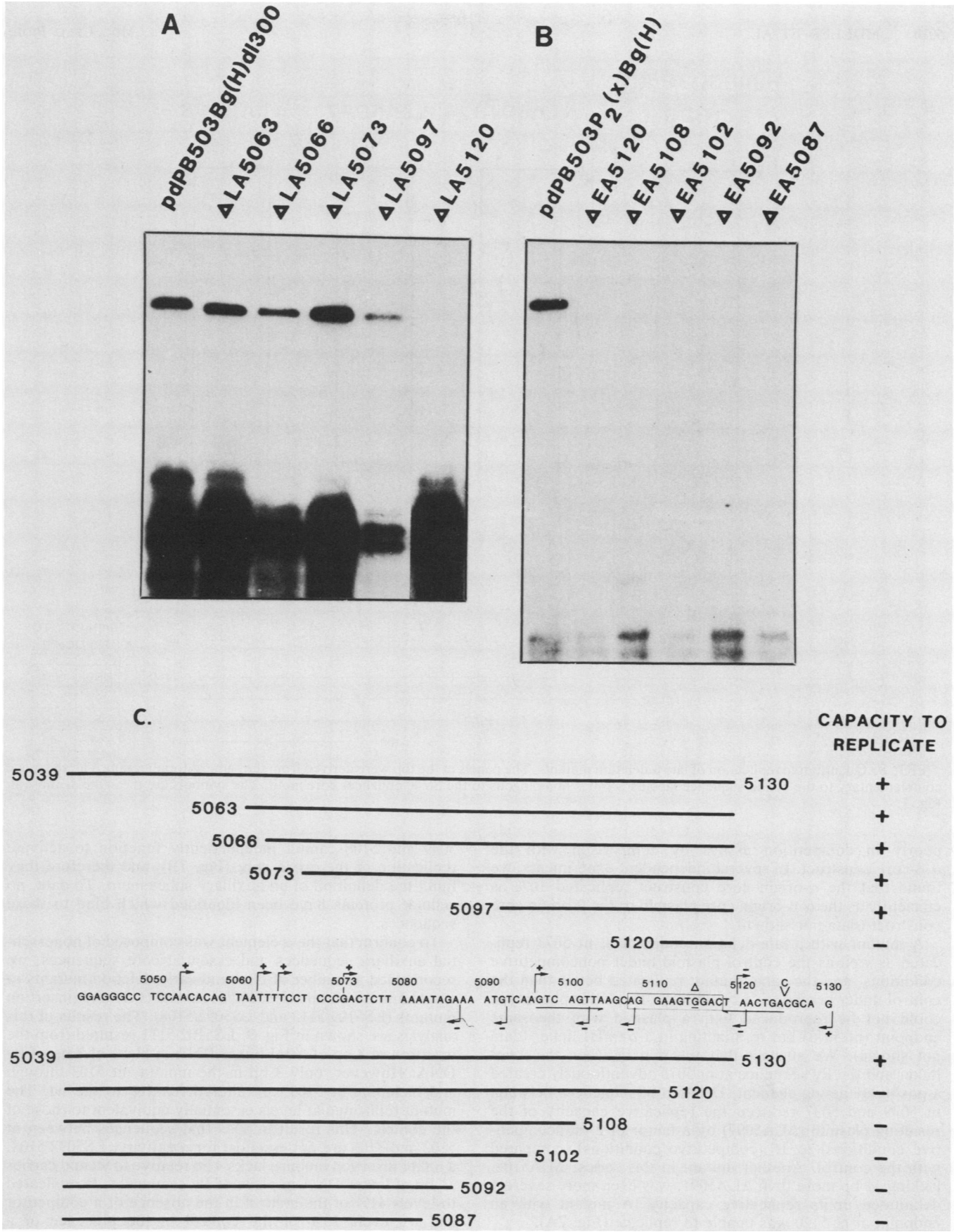


FIG. 7. Replication capacity of a set of late- and early- unidirectional deletion mutants of the α element. (A) Autoradiogram illustrating the replication of the late-deletion mutants. (B) Autoradiogram of the replication of the early-deletion mutants. (C) Physical maps and qualitative replication phenotypes of the deletion mutants. The sequence of Py spanning the α enhancer element is shown. The boxed sequence is a DNA stretch perfectly homologous to the adenovirus E1A enhancer core motif. The triangle above the G at nt 5116 indicates that this nt is not present in our strain of Py. The nt letter C above the sequence represents an insertion found in our strain. The symbols are the same as those in Fig. 3.

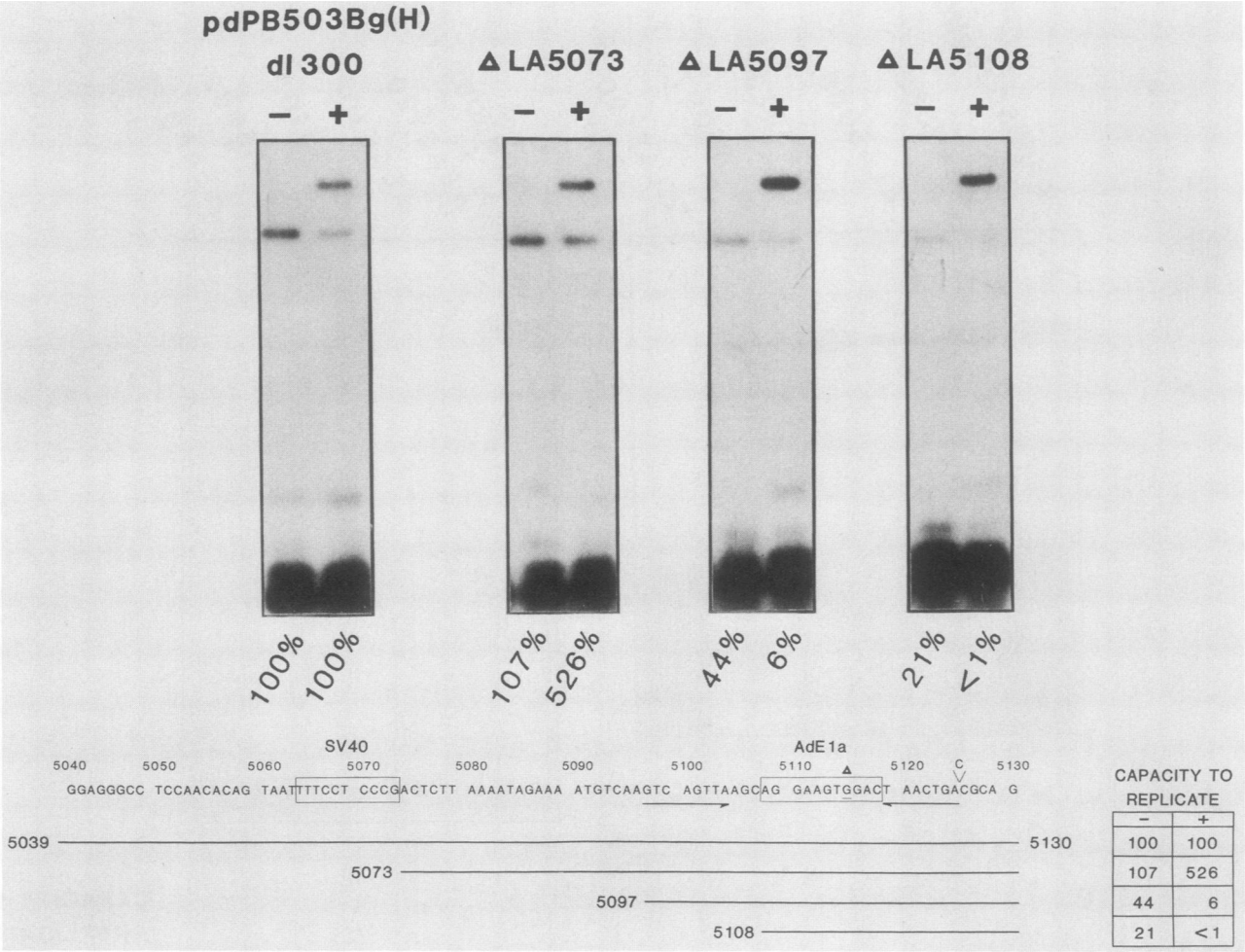


FIG. 8. Quantitative replication of late α deletion mutants. The panels at the top were derived from the same autoradiogram. The sequence complementary to the boxed sequence labeled SV40 is homologous to the SV40 enhancer core motif. The symbols are the same as those in Fig. 3.

poorly in competition assays by comparison with the α-β-core construct. In several independent experiments, we found that the α-origin core construct replicated 10% as efficiently as the α-β-origin core plasmid or the β-origin core construct (data not shown).

A mutant with a late-deletion endpoint at nt 5073 replicated as well as the control plasmid under noncompetitive conditions, and the same mutant replicated better than the control under competitive conditions. The latter phenotype could not be reproduced with a plasmid with the same endpoint (nt 5073) but terminating in a *Bam*HI linker (data not shown). We suspect that the join between the *Xho*I linker and the Py sequence at nt 5073 adventitiously created a positively acting element. Deletion of sequences between nt 5039 and 5097 reduced the replicative capacity of the resulting plasmid (ΔLA5097) by a factor of 2 (noncompetitive conditions) to 16 (competitive conditions), compared with the control. Another mutant in this series, ΔLA5108, lacking 11 bp more than ΔLA5097, was even more severely debilitated in its replicative capacity. A mutant with an endpoint at nt 5120 was unable to replicate (Fig. 7A).

These results define the late border of the α element between nt 5073 and 5097 and suggest that sequences between nt 5073 and 5108 augment the activity of the α core subelement (nt 5108 to 5130). The sequences between nt 5039 and 5108 cannot independently function to activate replication of the origin core (Fig. 7B), and therefore they fulfill the definition of an auxiliary subelement. To date, no cellular proteins have been identified which bind to these sequences.

To confirm that the α element was composed of nonessential auxiliary sequences and essential core sequences, we recombined a number of late- and early-deletion mutants to create one LS mutant (LS5102/5111) and two linker insertion mutants (LS5109/5113 and LS5092/5108). The results of this analysis are shown in Fig. 9. LS5102/5111 resulted from the insertion of 8 bp of *Xho*I linker DNA in place of 8 bp of Py DNA. However, only 3 bp in the mutant, nt 5103 through and including nt 5105, are altered relative to the wt. The mutant replicated at levels essentially equivalent to those of the control. This result implies that sequences between nt 5102 and 5106 are not essential for α activity. LS5092/5108, a linker insertion mutant, lacks 4 bp relative to wt and carries 11 bp of linker DNA in place of Py sequences. It replicated to levels 41% of the control in the absence of a competitor and to levels 13% of the control in the presence of a competitor. The phenotype of this mutant is essentially the same as that of ΔLA5097, which lacks sequences between nt 5039 and 5097 (compare Fig. 8 ΔLA5097 with Fig. 9 LS5092/5108), implying that sequences between 5073 and 5092 are

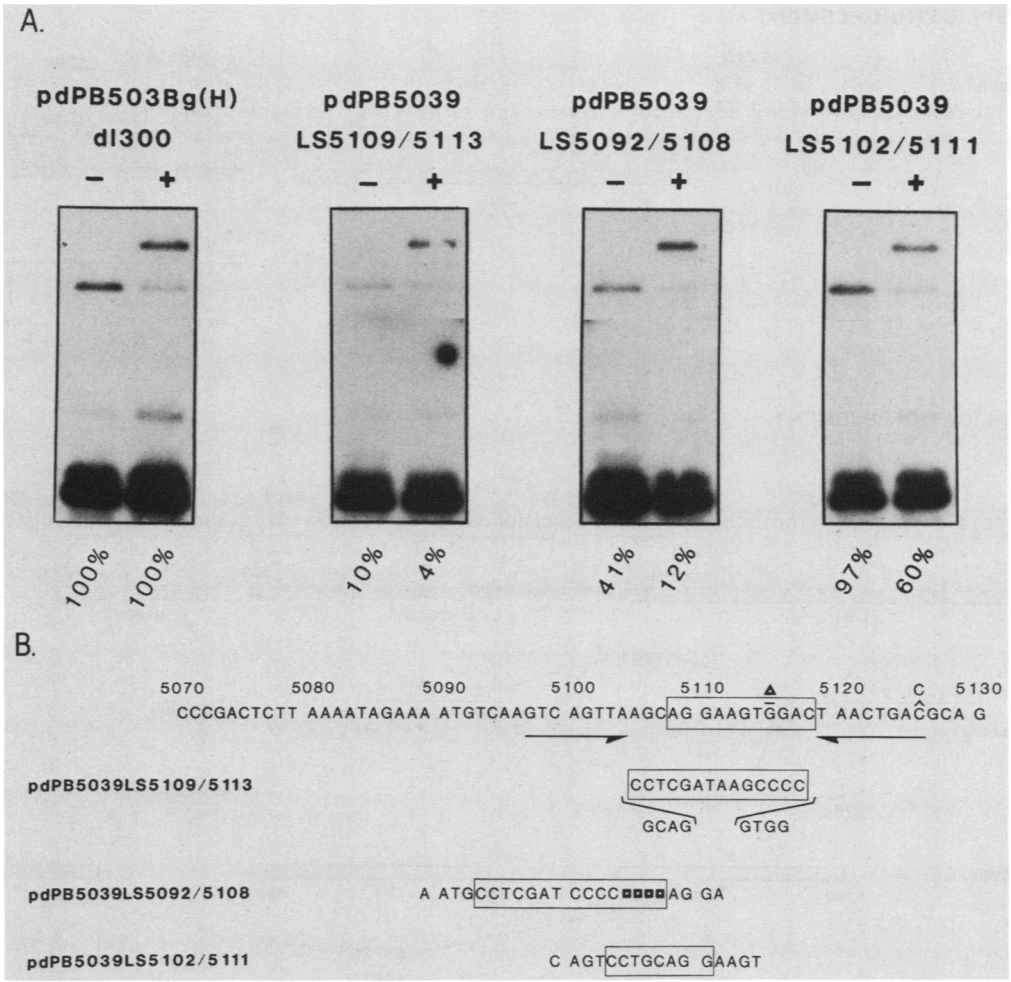


FIG. 9. Quantitative replication of LS mutants whose lesions affect the α enhancer element. (A) The panels depict autoradiograms representing different exposures of the same nitrocellulose sheet. The area between the replicated DNA and the nonreplicated DNA on the original autoradiogram was cropped from the figure to make more effective use of space. (B) Sequence of the α enhancer element and location of the α LS mutations. The dark squares with pinholes represent missing nt. These were included to allow alignment of the flanking sequences in the mutant with the wt sequence.

not essential to α activity and suggesting that the late α auxiliary subelement border is between nt 5092 and 5097. Because the effect of spacing between subelements is not known, this interpretation remains tentative. LS5109/5113 lacks 3 bp of Py DNA (nt 5110 through and including nt 5112) and contains 14 bp of linker DNA in their place, resulting in a net insertion of 11 bp. The mutation causes the deletion of 3 bp from the 5′ extremity of the adenovirus E1A enhancer core motif and changes the spacing between the late-side inverted repeat element and the enhancer core sequence. LS5109/5113 replicated poorly compared with the control, to about the same extent as ΔLA5108, which retains α core sequences between nt 5108 and 5130 but lacks the α auxiliary subelement. The phenotype of this mutant could be due to removal of α auxiliary sequences, to alteration of the spacing between α auxiliary sequences and the α core subelement, or to partial inactivation of α core function. The fact that LS5109/5113 replicated at all suggests that the essential α core sequences lie between nt 5113 and 5130.

Mutational analyses of the α enhancer element suggest that like the β enhancer element, it too is composed of auxiliary sequences and essential core sequences. The auxiliary subelement maps between nt 5073 and 5102, or more likely between nt 5092 and 5102, and augments the activity of the core subelement. The core sequences, by comparison, are absolutely essential for the α element activity, and these map between nt 5108 and 5130. Like the β subelements, the α subelements act synergistically to potentiate replication.

DISCUSSION

We have constructed and characterized the replication efficiency in 3T3 cells of a number of deletion and LS mutants with lesions in the α and β enhancer elements. This analysis revealed that each enhancer element comprises multiple subelements. The subelements can be divided into two classes: auxiliary sequences and core sequences. Individual auxiliary subelements are not able to function as enhancer elements. Their mutation reduced but did not abolish enhancer element activity. The auxiliary subelements functioned in conjunction with the core subelements to augment their activity. The core subelements alone were able to activate DNA replication, albeit poorly, and their multimerization increased their activity significantly. Mutations that affected the core sequences generally abolished enhancer element function. The two classes of enhancer

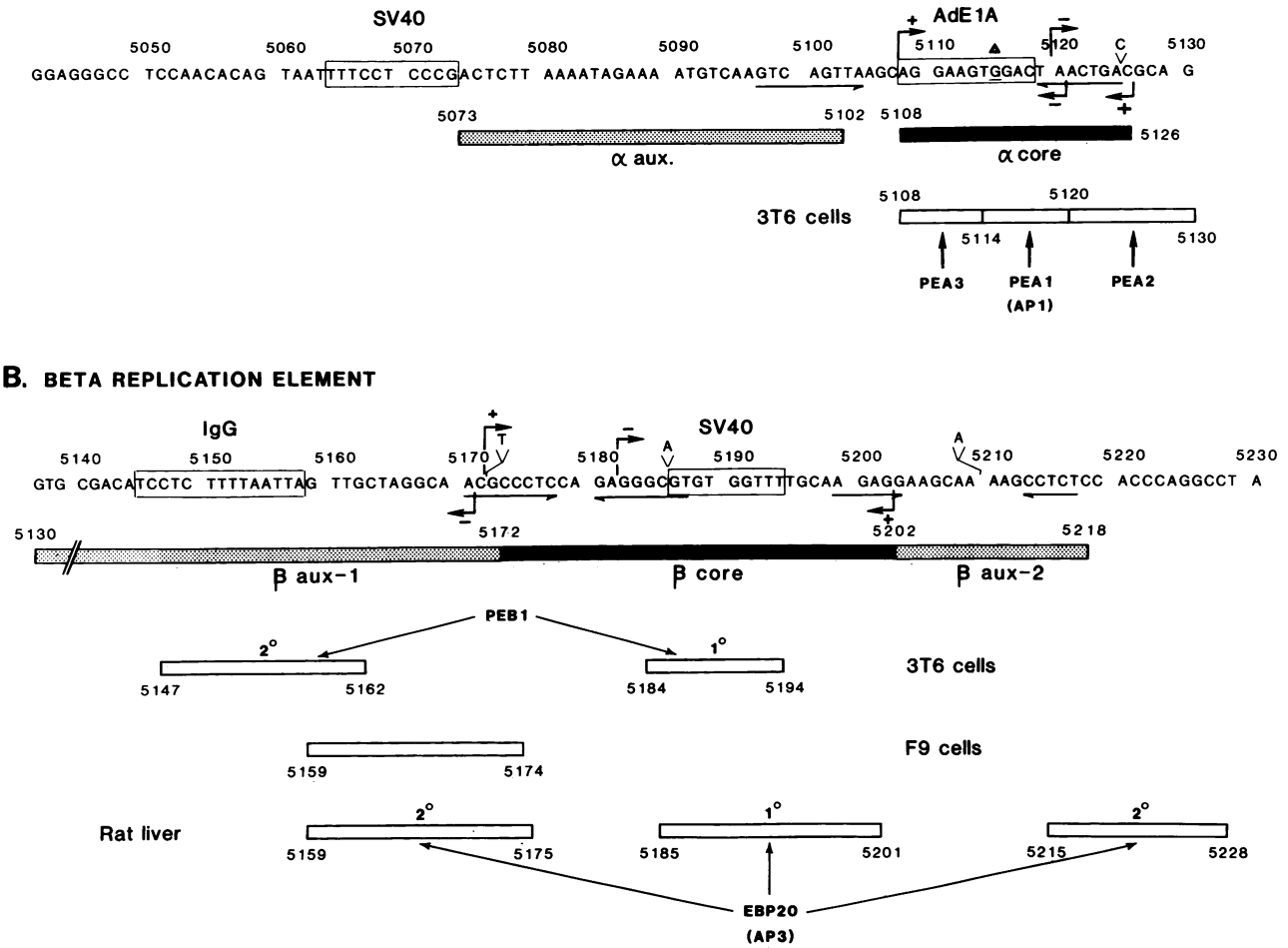


FIG. 10. Schematic features within the α and β enhancer elements. (A) Sequence of the α enhancer element. (B) Sequence of the β enhancer element. SV40, SV40 enhancer core motif. AdE1A, adenovirus enhancer core motif. IgG, conserved immunoglobulin enhancer sequence. The stippled boxes below each sequence depict the maximum length of the auxiliary subelements, whereas the dark boxes indicate the maximum size of the core subelements. The open boxes below the subelements show limits of regions protected by various nuclear factors from DNase I digestion. 1° and 2° indicate primary and secondary binding sites, respectively, for the factors. The remaining symbols are the same as those in Fig. 3.

elements acted synergistically to effect DNA replication because the activity of constructs bearing two subelements was much greater than the sum of the activity of each subelement. Similarly, dimers or trimers of enhancer core subelements functioned much better than the sum of individual core subelements, suggesting that the two classes of subelements, while possessing different activities, are nonetheless functionally equivalent.

We have tested the validity of this model for enhancer element structure by measuring the replication of all the mutants described here in a number of different mouse cell lines (D. Dufort and J. A. Hassell, manuscript in preparation). In all but one instance, the sequence requirements for replication activation were similar in the various cell lines. The exception, a mouse mammary epithelial cell line, was able to use the β auxiliary-1 subelement alone to activate origin core replication. This observation substantiates our contention that the auxiliary and core subelements are functionally equivalent. We suspect that the intrinsic activity of each subelement reflects the corresponding abundance or activity of its cognate binding factors in the cell. Different mouse cell lines likely elaborate different factors or different activities of the same spectrum of factors (for examples, see references 27, 35, and 44). Consequently, whether an enhancer subelement acts as an auxiliary sequence or core sequence will depend on the activity of the cognate factors for these sequences in the cell. There may also be enhancer subelements in α and β which we have failed to detect because their cognate factors are not active in the cell lines which we have tested.

Bearing this in mind, we summarize our results in Fig. 10, which shows the location of the subelements and the regions protected from DNase I cleavage by murine nuclear factors in vitro relative to the Py nt sequence. At least two subelements constitute the α enhancer element. One of these, the α auxiliary subelement, spans the region between nt 5073 and 5102. Deletion of these sequences from the α element reduced replication of the resulting plasmid at least 5-fold in the absence of a competitor and more than 100-fold in its presence. These sequences include an AT-rich stretch and part of an 8-bp inverted stretch. The latter is an important sequence because its mutation severely impaired the replication efficiency of the mutant plasmid (compare the replicative capacity of ΔLA5097 and ΔLA5108). Whether these

sequences serve a structural function by excluding nucleosomes from their vicinity (38) or act through a DNA-binding protein is not known. No nuclear factors have been found which bind to these sequences in vitro.

The α core subelement is located between nt 5108 and 5126. Its late border is located between nt 5108 and 5120, whereas its early border is located between nt 5130 and 5120. We have shown previously that deletion of sequences between nt 5130 and 5126 has no consequences for origin function (23). Therefore, the early border of the α core subelement is between nt 5120 and 5126. The 18 bp which define the α core subelement contain a perfect match to a conserved enhancer motif, 5′-A(C)GGAAGTGA(C)-3′, first recognized in the adenovirus E1A enhancer (10), and part of the inverted repeat motif. At least three nuclear factors have been detected which bind to these sequences. They include PEA3, which protects the sequence 5′-AGGAAG-3′ (located between nt 5108 and 5114) from DNase I digestion (M. E. Martin, J. Piette, M. Yaniv, W.-J. Tang and W. R. Folk, personal communication), and PEA1 (32), which recognizes the same sequence motif as the human factor AP1 (16, 17) and the yeast factor GCN4 (12) and binds to sequences between nt 5120 and 5130 (31). The PEA3- and PEA1-binding sites are crucial for α function, implying that one or both proteins is required for replication activation. Part of the PEA2-binding site (nt 5120 to 5126) appears to be required for replication activation, implying that PEA2 also participates in this process. Although the sequences defining both the PEA3-PEA1-binding sites and those defining the PEA2-binding sites are required for α function, neither is sufficient to activate Py DNA replication. Therefore, the function of the α core subelement requires the binding sites for at least two nuclear factors (PEA3 [or PEA1] and PEA2).

The β enhancer element also comprises multiple sequence subelements (Fig. 10). Two auxiliary subelements and one core subelement were identified. β auxiliary subelement 1 (nt 5130 to 5172) was defined by two late β deletion mutants (LB5147 and LB5166) and one LS mutant (LS5151/5166). The mutations borne by each mutant may have affected a common sequence, for example, the AT-rich sequence 5′-TCCTCTTTTAATTA-3′, which is a perfect match to one found in the enhancer of a mouse heavy-chain immunoglobulin gene (1). Alternatively, the mutations carried by these three mutants may have affected the structure of two or more nonoverlapping sequence elements; for example, the conserved AT-rich element and the binding site (nt 5159 to 5174) for a protein first found in F9 mouse embryonal carcinoma cells but subsequently found in other mouse cell lines (6, 28). The auxiliary-1 subelement was unable to activate DNA replication on its own in 3T3 cells, and sequences between nt 5128 and 5167, which include the β auxiliary-1 subelement, are unable to activate origin core replication in mouse C127 cells even when multimerized up to five times (42). Therefore, in 3T3 cells and C127 cells the β auxiliary-1 subelement cannot act as an independent enhancer element. In a mouse mammary epithelial cell line, however, sequences which constitute the β auxiliary-1 subelement activated origin core replication independently of other sequences in the β element to the same extent as did the control (D. Dufort and J. A. Hassell, manuscript in preparation). Presumably, the factor(s) which interacts with the β auxiliary-1 subelement is more active or abundant in the mammary epithelial cell line, or this cell line may express a novel factor which is not expressed in 3T3 and C127 cells.

The β core subelement is located between nt 5172 and 5202 as defined by analysis of deletion mutants, and sequences between nt 5168 and 5202 were sufficient to activate Py DNA replication. This region contains two notable sequence features: a 9-bp GC-rich inverted repeat and an SV40 enhancer core motif. The inverted repeat is essential for β core function because its deletion abolished β activity. This was revealed by examining the replication efficiency of the late β deletion mutants ΔLB5172 and ΔLB5180. The former, which possesses the inverted repeat motif, replicated, whereas the latter, which lacks it, did not. In addition, the LS mutant LS5173/5188, whose mutation principally affects the inverted repeat motif, was severely debilitated in its capacity to replicate (2% of the control in the absence of a competitor). Interestingly, this LS mutation lies entirely within the borders of the β core subelement, yet the plasmid bearing it was still able to replicate to levels equivalent to that of the minimal β subelement (3% of the control in the absence of a competitor). Perhaps the mutation did not completely inactivate β core function, or the two β auxiliary subelements functionally substituted for the β core subelement when the latter was inactivated by a mutation. The β core subelement also contains a sequence nearly identical to the SV40 enhancer motif. We do not know whether this sequence is required for β function because we did not isolate any mutants which specifically affected it. However, if it is an important sequence, then it must function with another one in the β core subelement to exert its effect, because deletion mutants which retain only the SV40 enhancer core motif and the β auxiliary-2 subelement (i.e., ΔLB5180) were replication defective.

A factor from mouse 3T6 cells, PEB1 (30), and one from rat liver, EBP20 (13), bind to the SV40 enhancer core motif in vitro. These factors may be the rodent equivalent of the human HeLa cell factor AP3 (20). PEB1 and EBP20 also bind to sequences adjacent to the primary binding site. The binding of PEB1 to the secondary site is sequence independent (31), and the binding of EPB20 to its secondary sites only occurs at high protein-to-DNA ratios (13).

The β auxiliary-2 subelement was defined by the analysis of ΔEB mutants and LS mutants. Interpretation of the replicative capacity of the ΔEB mutants is complicated by the altered spacing that results between remaining sequences in β and the origin core. We have shown that the α and β enhancer elements only function at the late border of the origin core and lose their capacity to act if moved more than 90 bp away (23) (W. J. Muller and J. A. Hassell, manuscript in preparation). Therefore, the positions of the enhancer elements are crucial to their ability to activate DNA replication. We observed that some mutants in the ΔEB series replicated better than others which have sustained smaller deletions (e.g., compare ΔEB5223 with ΔEB5240, and ΔEB5202 with ΔEB5209). Importantly, all the deletion mutants replicated less well than the control, implying that each of them was impaired relative to the wt plasmid. Nonetheless, these deletions may have affected two parameters simultaneously, and we have not relied on the quantitative data which analysis of the ΔEB mutants provided to define the β auxiliary-2 subelement. Instead, we have relied only on the analysis of two LS mutants, LS5202/5211 and LS5209/5218, whose lesions map to the early side of the β core subelement to define the β auxiliary-2 subelement. The mutations borne by these mutants span the region between nt 5202 and 5218. Another LS mutant, LS5240/5248, replicated at essentially wt levels, suggesting that sequences between nt 5240 and 5248 are not essential to β function. We cannot at this point rule out the possibility that other

sequences in this region (e.g., nt 5218 to 5240 or 5248 to 5265) contribute to β function.

No cellular factors have yet been identified which bind to sequences between nt 5202 and 5265. The rat liver nuclear factor EPB20 protects sequences between nt 5212 and 5228, which overlap with the β auxiliary-2 element (nt 5202 to 5218), from DNase I cleavage at high protein-to-DNA ratios (13), but it is not known whether this binding is sequence specific (32).

Veldman et al. (42) have previously characterized the structure of the α and β enhancer elements by replication assays in a C127 mouse cell line that expresses Py T antigens (COP-5 cells). They mapped the α element between nt 5072 and 5126 and showed that sequences between nt 5109 and 5130 would promote replication very poorly. Qualitatively, our results substantiate these conclusions. These authors did not map the borders of the β element in the absence of the α enhancer element. However, they did observe transitions in enhancer activity as sequences were deleted from the early border of an α plus β construct. These transitions occurred between nt 5214 and 5179 and between nt 5179 and 5148, corresponding to the deletion of the β auxiliary-2 subelement and the β core subelement, respectively. We have independently verified that the sequence requirements for replication activation function are the same in 3T3 cells and C127 cells (D. Dufort and J. A. Hassell, manuscript in preparation).

The sequences which make up the α and β enhancer elements correspond to Py enhancer elements 2 and 3. The Py enhancer comprises three enhancer elements, which we have named 1, 2, and 3 (22). Pairs of these enhancer elements will constitute nearly full enhancer activity (26 to 83% of the complete enhancer), but each element alone is relatively inert (from 0 to 12% of the complete enhancer). The enhancer elements, therefore, act synergistically to enhance transcription. The distance between enhancer elements is not crucial to enhancer function, because enhancer elements can be separated by up to 60 bp without deleterious consequences. Interestingly, enhancer element 1, which is totally inactive as an enhancer, is unable to activate DNA replication. Enhancer element 1 maps between nt 5039 and 5075. There is evidence in addition to the correspondence between the borders of the α and β elements and enhancer elements 2 and 3 to indicate that they comprise identical sequences. First, mutations which affect replication activation also affect enhancer activity (22, 39), and reversion of these mutations restores both functions (39). Second, foreign enhancers activate Py DNA replication (4; E. R. Bennett and J. A. Hassell, manuscript in preparation). Third, the α core subelement will enhance transcription if it is reiterated (42). Consequently, it is very likely that the Py enhancer elements and replication activators are composed of the same sequences.

In this report we show that the Py enhancer elements can be further divided into subelements which act synergistically to activate DNA replication. Therefore, the Py enhancer is organized at two levels; two or more enhancer subelements make up an enhancer element, and two or more enhancer elements form an enhancer. In many instances, the subelements correspond to protein-binding sites. The α core subelement can interact with up to three factors, whereas the β core subelement interacts with at least one factor. We are currently testing whether a single factor-binding site can constitute a subelement, whether dimers of such sites will form an enhancer element, and whether multimers of the dimers will constitute an enhancer.

The SV40 enhancer is organized similarly to that of Py (27, 35). It, too, comprises at least three enhancer elements which act synergistically to enhance transcription. At least two enhancer elements are required to form a functional enhancer of transcription. Furthermore, Ondek et al. (26) have very recently shown that each enhancer element is bipartite in structure, being composed of subelements which they term enhansons. Enhansons are the minimal unit of enhancer function and appear to be the sites of binding of individual transcription factors. A pair of enhansons constitutes an enhancer element. Unlike enhancer elements, which can be spaced apart by up to 100 bp with little effect on enhancer activity, enhansons cannot be moved apart by more than 5 bp without loss of function.

At first glance, the organization of the SV40 and Py enhancers appears remarkably similar. Each comprises three enhancer elements which in turn are composed of two or more subelements. However, we have not determined whether the Py enhancer subelements, which we have identified by their effect on replication activation, correspond to enhansons. Further experimentation will be required to address this point.

ACKNOWLEDGMENTS

We thank Monica Naujokas for excellent technical assistance and Kathy Showkenik for typing the manuscript.

This research was supported by the Medical Research Council of Canada and the National Cancer Institute of Canada. W.J.M. was supported by a graduate fellowship from the Medical Research Council of Canada, D.D. is supported by a graduate fellowship from the Natural Science and Engineering Research Council of Canada, and J.A.H. is a Terry Fox Cancer Research Scientist of the National Cancer Institute of Canada.

LITERATURE CITED

1. **Banerji, J., L. Olson, and W. Schaffner.** 1981. A lymphocyte specific cellular enhancer is located downstream of the joining region in immunoglobulin heavy chain genes. Cell **33**:729–740.
2. **Bohnlein, E., and P. Gruss.** 1986. Interaction of distinct nuclear proteins with sequences controlling the expression of polyomavirus early genes. Mol. Cell. Biol. **6**:1401–1411.
3. **DePamphilis, M. L., and M. K. Bradley.** 1986. Replication of SV40 and polyoma virus chromosomes, p. 99–246. *In* N. P. Salzman (ed.), The papovaviridae, vol. 1. Plenum Publishing Corp., New York.
4. **deVilliers, J., W. Schaffner, C. Tyndall, S. Lupton, and R. Kamen.** 1984. Polyoma virus DNA replication requires an enhancer. Nature (London) **312**:242–246.
5. **Francke, B., and W. Eckhart.** 1973. Polyoma gene function required for viral DNA synthesis. Virology **55**:127–135.
6. **Fujimura, F.** 1986. Nuclear activity from F9 embryonal carcinoma cells binding specifically to the enhancers of wild-type polyoma virus and PyEC mutant DNAs. Nucleic Acids Res. **14**: 2845–2861.
7. **Gaudray, P., C. Tyndall, R. Kamen, and F. Cuzin.** 1981. The high affinity binding site on polyoma virus DNA for the viral large T protein. Nucleic Acids Res. **9**:5697–5710.
8. **Hassell, J. A., W. C. Topp, D. B. Rifkin, and P. E. Moreau.** 1980. Transformation of rat embryo fibroblasts by cloned polyoma virus DNA fragments containing only part of the early region. Proc. Natl. Acad. Sci. USA **77**:3978–3982.
9. **Hayday, A. C., F. Chaudry, and M. Fried.** 1983. Loss of polyoma virus infectivity as a result of a single amino acid change in a region of polyoma virus large T antigen which has extensive amino acid homology with simian virus large T antigen. J. Virol. **45**:693–699.
10. **Hearing, P., and T. Shenk.** 1983. The adenovirus type 5 E1A transcriptional control region contains a duplicated enhancer element. Cell **33**:695–703.
11. **Hendrickson, E. A., C. E. Fitze, W. R. Folk, and M. L.**

DePamphilis. 1987. The origin of bidirectional DNA replication in polyoma virus. EMBO J. **6**:2011–2018.

12. **Hill, D. E., I. A. Hope, J. P. Macke, and K. Struhl.** 1986. Saturation mutagenesis of the yeast *his* 3 regulatory site: requirements for binding by GCN4 activator protein. Science **234**: 451–457.
13. **Johnson, P., W. Landschulz, B. Graves, and S. McKnight.** 1987. Identification of rat liver nuclear protein that binds to the enhancer core element of three animal viruses. Genes Dev. **1**: 133–146.
14. **Katinka, M., and M. Yaniv.** 1983. DNA replication origin of polyoma virus early proximal boundary. J. Virol. **47**:244–248.
15. **Laimins, L. A., G. Khoury, C. Gorman, B. Howard, and P. Gruss.** 1982. Host-specific activation of transcription by tandem repeats from SV40 and MSV. Proc. Natl. Acad. Sci. USA **79**: 6453–6457.
16. **Lee, W., A. Haslinger, M. Karin, and R. Tjian.** 1987. Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. Nature (London) **325**:368–372.
17. **Lee, W., P. Mitchell, and R. Tjian.** 1987. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell **325**:368–372.
18. **Luthman, H., M. G. Nilsson, and G. Magnusson.** 1982. Noncontinuous segments of the polyoma genome required in *cis* for DNA replication. J. Mol. Biol. **161**:533–550.
19. **Maniatis, T., E. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Mitchell, P. J., C. Wang, and R. Tjian.** 1987. Positive and negative regulation of transcription *in vitro*: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. Cell **50**:847–861.
21. **Mueller, C. R., A. M. Mes-Masson, M. Bouvier, and J. A. Hassell.** 1984. Location of sequences in polyomavirus DNA that are required for early gene expression in vivo and in vitro. Mol. Cell. Biol. **4**:2594–2609.
22. **Mueller, C. R., W. J. Muller, and J. A. Hassell.** 1988. The polyomavirus enhancer comprises multiple functional elements. J. Virol. **62**:1667–1678.
23. **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.
24. **Muller, W. J., M. Naujokas, and J. A. Hassell.** 1984. The isolation of transformed mouse cell lines capable of supporting the replication of polyomavirus-plasmid recombinants. Mol. Cell. Biol. **4**:2406–2412.
25. **Murakami, Y., T. Eki, M. Yamada, C. Prives, and J. Hurwitz.** 1986. Species-specific *in vitro* synthesis of DNA containing the polyoma virus origin of replication. Proc. Natl. Acad. Sci. USA **83**:6347–6351.
26. **Ondek, B., L. Gloss, and W. Herr.** 1988. The SV40 enhancer contains two distinct levels of organization. Nature (London) **333**:40–45.
27. **Ondek, B., A. Shepard, and W. Herr.** 1987. Discrete elements within the SV40 enhancer region display different cell-specific enhancer activities. EMBO J. **6**:1017–1025.
28. **Ostapchuk, P., J. Diffley, J. Bruder, B. Stillman, A. Levine, and P. Hearing.** 1986. Interaction of a nuclear factor with the polyomavirus enhancer region. Proc. Natl. Acad. Sci. USA **83**: 8550–8554.
29. **Peden, K. W. C., J. M. Pipas, S. Pearson-White, and D. Nathans.** 1980. Isolation of mutants of animal virus in bacteria. Science **209**:1392–1396.
30. **Piette, J., M.-H. Kryszke, and M. Yaniv.** 1985. Specific interaction of cellular factors with the β enhancer of polyoma virus. EMBO J. **4**:2675–2685.
31. **Piette, J., and M. Yaniv.** 1986. Molecular analysis of the interaction between an enhancer binding factor and its target DNA. Nucleic Acids Res. **14**:9595–9611.
32. **Piette, J., and M. Yaniv.** 1987. Two different factors bind to the α-domain of the polyoma virus enhancer, one of which also interacts with the SV40 and c-fos enhancers. EMBO J. **6**:1331–1337.
33. **Pomerantz, B. J., and J. A. Hassell.** 1984. Polyomavirus and simian virus 40 large T antigens bind to common DNA sequences. J. Virol. **49**:925–937.
34. **Pomerantz, B. J., C. R. Mueller, and J. A. Hassell.** 1983. Polyomavirus large T antigen binds independently to multiple unique regions on the viral genome. J. Virol. **47**:600–610.
35. **Schirm, S., J. Jiricny, and W. Schaffner.** 1987. The SV40 enhancer can be dissected into multiple segments, each with a different cell type specificity. Genes Dev. **1**:65–74.
36. **Smale, S. T., and R. Tjian.** 1986. T-antigen-DNA polymerase α complex implicated in simian virus 40 DNA replication. Mol. Cell. Biol. **6**:4077–4087.
37. **Soeda, E., J. Arrand, N. Smolar, J. Walsh, and B. Griffin.** 1980. Coding potential and regulatory signals of the polyoma virus genome. Nature (London) **283**:445–453.
38. **Struhl, K.** 1985. Naturally occurring poly (dA-dT) sequences are upstream promoter elements for constitutive transcription in yeast. Proc. Natl. Acad. Sci. USA **82**:8419–8423.
39. **Tang, W., S. Berger, S. Triezenberg, and W. Folk.** 1987. Nucleotides in the polyomavirus enhancer that control viral transcription and DNA replication. Mol. Cell. Biol. **7**:1681–1690.
40. **Triezenberg, S. J., and W. R. Folk.** 1984. Essential nucleotides in polyomavirus origin region. J. Virol. **51**:437–444.
41. **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.
42. **Veldman, G. M., S. Lupton, and R. Kamen.** 1985. Polyomavirus enhancer contains multiple redundant sequence elements that activate both DNA replication and gene expression. Mol. Cell. Biol. **5**:649–658.
43. **Wobbe, C. R., F. B. Dean, Y. Murakami, L. Weissback, and J. Hurwitz.** 1986. SV40 DNA replication *in vitro*: study of events preceding elongation of chains. Proc. Natl. Acad. Sci. USA **83**: 4612–4616.
44. **Xiao, J. J., I. Davidson, D. Ferrandon, R. Rosales, M. Vigneron, M. Macchi, F. Ruffenach, and P. Chambon.** 1987. One cell-specific and three ubiquitous nuclear proteins bind *in vitro* to overlapping motifs in the domain B1 of the SV40 enhancer. EMBO J. **6**:3005–3013.