JOURNAL OF VIROLOGY, Feb. 1990, p. 476–485
0022-538X/90/020476-10$02.00/0

Vol. 64, No. 2

# Genetic Analysis of the Enhancer Requirements for Polyomavirus DNA Replication in Mice

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Received 15 June 1989/Accepted 9 October 1989

**In this report, we describe the first systematic analysis of the genetic requirements for polyomavirus (Py) enhancer-activated viral DNA replication during the acute phase of infection in mice. Four mutants were made which substituted *XhoI* sites for conserved enhancer consensus sequences (adenovirus type 5 E1A, c-*fos*, simian virus 40, and a glucocorticoidlike consensus sequence). Viral DNA replication in infected mouse organs was measured by DNA blot analysis. Only the loss of the glucocorticoidlike consensus sequence element significantly reduced Py DNA replication in the kidneys, the primary target organ for viral replication. The loss of the c-*fos*, adenovirus type 5 E1A, or simian virus 40 consensus sequences, however, expanded organ-specific viral DNA replication, relative to wild-type Py, by allowing high-level replication in the pancreas or heart or both. Analysis of Py variants selected for replication in undifferentiated embryonal carcinoma cell lines (PyF441, PyF111) showed that there was little change in levels of viral DNA replication in kidneys and other organs as compared with those in the wild-type virus. If the entire B enhancer is deleted, only low overall levels of viral replication are observed. Wild-type levels of replication in the kidneys can be reconstituted by addition of a single domain from within the A enhancer (nucleotides 5094 to 5132) to the B enhancer deletion virus, suggesting that a single domain from the A enhancer can functionally substitute for the entire B enhancer. This also indicates that the determinants for kidney-specific replication are not found in the B enhancer.**

The polyomavirus (Py) enhancer is composed of two adjacent but distinct enhancers, A and B, conveniently defined by the restriction fragments *BclI-PvuII* (nucleotides nt 5021 to 5131) and *PvuII-PvuII* (nt 5132 to 5265), respectively (14). Extensive deletion analysis has identified several functional domains within the Py enhancer which correspond closely to binding sites for nuclear factors (25–28, 39). The function of the Py enhancer as a determinant of viral cell-type-specific replication and transcription was first observed in mutants that allowed for growth of Py in undifferentiated embryonal carcinoma (EC) cells (12, 18, 33). These mutants contained sequence changes within their enhancer DNA that ranged from a single point mutation to duplications that encompassed the point mutation. Other Py host range mutants have also been selected that allow replication in the normally nonpermissive Friend erythroleukemia cells and neuroblastoma cells (6–8). These mutants were similarly found to have duplications, transpositions, and deletions within the enhancer region which confers a cell-specific *cis* advantage for DNA replication over the parental viral strain (7, 8). In addition, simple transposition of the A and B enhancers can alter the cell specific phenotype, showing that the relative enhancer position can affect cell type specificity (2).

In all these experiments, however, permanent cell lines have been used to determine how the enhancer controls viral growth, replication, or transcription. These cell lines are, at least, immortalized and thus have altered growth control relative to normal tissues. These experiments have led to the view that the A and B enhancers appear to be functionally redundant for DNA replication in permissive cells (28). In the work described in this report we examined the enhancer requirements for viral DNA replication in the context of whole virus in the organs of its natural host, the mouse.

Py replicates in three phases following intraperitoneal inoculation of newborn mice: an initial acute and systemic phase in which viral replication peaks around 6 to 8 days postinfection (p.i.), followed by a phase of viral clearance from most organs except the kidneys, and, finally, a third phase in which viral DNA and virus production persist at low levels in the kidneys (10). The kidneys appear to be the primary target organs for murine infections, although Py is capable of infecting a diverse number of tissue types of both mesenchymal and epithelial origin (4, 5). We have clearly shown that the organ specificity of acute infections in mice can be altered by changes within the Py enhancer when we observed that the substitution of the Moloney murine leukemia virus enhancer for the Py B enhancer yields a virus which has in vivo specificity for the pancreas but not the kidneys (31).

We have focused our studies on determining some of the genetic requirements for Py enhancer-driven DNA replication during in vivo infection of newborn mice. In addition, we have examined whether the recombinant viruses derived in these experiments alter the organ specificity of Py replication. Three sets of mutants were analyzed. The first set of mutants contain deletions of binding sites for specific, highly conserved consensus sequences which are thought to bind various nuclear factors. The second set of mutants are variants which were selected for growth in undifferentiated embryonal carcinoma cells and therefore have altered in vitro specificity. In the third set of mutants, the entire B enhancer is deleted and is replaced with various subenhancer domains. We observed striking and unexpected alterations in both the level and organ specificity of viral DNA replication with some but not all of these mutants. It is proposed that small changes within the enhancer (deletion substitution of consensus sequences) can alter the organ specificity of the virus but that gross deletion or mutation in

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several sites within the enhancer is detrimental to virus replication, suggesting that at least two functional enhancer domains are required for efficient in vivo kidney replication.

## MATERIALS AND METHODS

**Cell lines and viruses.** Mouse 3T6 fibroblasts were grown in Dulbecco modified Eagle medium as described previously (10). The PyA2 strain was used as the wild-type strain. The PyA3, PyF441, PyBstEII, and PyNruI strains were obtained from F. Fujimura (38). Virus infection was done as previously described (10). Briefly, 3T6 cells, at 70% confluencey, were infected at a multiplicity of infection of 0.01 and were maintained on 2% fetal calf serum in Dulbecco modified Eagle medium. The medium was changed every 3 to 4 days until 70 to 90% of the cells displayed a cytopathic effect. Cells were harvested and lysed by sonication. After removal of cellular debris, virus titers were determined by the plaque assay as previously described (10). To generate recombinant virus stocks, we transfected 3T6 cells with recombinant Py DNA, maintained them until a cytopathic effect was observed, and harvested them as described above.

**Construction of Py recombinants.** The *Bam*HI (nt 4634)-*Pst*I (nt 490) restriction fragment of wild-type Py was subcloned into pIBI31 by standard techniques. This plasmid was used as the template for subsequent in vitro mutagenesis. Four oligonucleotides were synthesized by using a Biosearch 8600 DNA synthesizer and purified on 12% polyacrylamide–urea gels. These oligonucleotides contain an internal *Xho*I site (underlined), which replaces the consensus sequence indicated (see Fig. 1). They are designated as follows: PyGRE, 5′-AAAATGTCACTCGAGCAGGAAGT-3′; PyAdE1A, 5′-TCAGTTAAGCCTCGAGTAACTGACC-3′; Pyc-fos, 5′-CTGACCGCAGCTCGAGCTTTTAATTA-3′; PySV40, 5′-CAGAGGGCAGCTCGAGCCAAGAGGAA-3. In vitro mutagenesis was performed by using the protocol provided by the manufacturer (International Biotechnologies, Inc.). To make full-length viral DNA, we cloned the *Bam*HI (nt 4634)-*Bgl*I (nt 95) fragment of the mutants into a plasmid containing wild-type Py DNA (cloned at the *Eco*RI site) deleted of the *Bam*HI-*Bgl*I fragment. Construction of Py genomes containing double-stranded synthetic oligonucleotides to replace the B enhancer was previously described (3). Nucleotide numbering is according to Soeda et al. (34).

**DNA transfection.** Before transfection, bacterially cloned Py genomes were excised from plasmid sequences by digestion with *Eco*RI, purified by electrophoresis in SeaPlaque (FMC Corp., Marine Colloids Div.) agarose gels, and ligated into circular forms with T4 DNA ligase. DNA (2 μg) was transfected by the DEAE-dextran–chloroquine method as described by Luthman and Magnusson (20).

**Extraction of viral DNA and replication analysis.** Viral nuclear DNA was extracted 48 h after transfection by the method of Hirt, as described previously (15). A 1-μg sample of DNA was left undigested or was digested with *Mbo*I, electrophoresed on 1% agarose gels, and transferred to nitrocellulose membranes by the method of Southern (35). Py-specific sequences were detected by hybridization to plasmid containing the Py genome which was labeled with [$^{32}$P]dCTP by nick translation (30).

**Mice and infection.** BALB/c mice (Bailey strain) were obtained from Charles River Breeding Laboratories and bred in the University of California, Irvine, facilities. Newborn mice were infected intraperitoneally with $1 \times 10^7$ to $5 \times 10^7$ PFU/ml when less than 24 h old. At 6 days p.i., mice were sacrificed and were used for DNA tissue extraction (10). A minimum of two separate litters with four mice per litter were used for analyzing each recombinant. DNA was extracted from organs by sodium dodecyl sulfate lysis of nuclei as previously described (10). A 10-μg sample of organ DNA was digested with *Eco*RI or *Xho*I (where noted), electrophoresed on a 1% agarose gel, and transferred to nitrocellulose by Southern transfer (35). Replication levels were determined from quantitative densitometer analysis of the Southern autoradiograms which had been exposed to yield linear grain densities with respect to isotope quantity. Copy levels of viral DNA were determined by comparison with reconstructed control tracks containing known quantities of viral DNA. Viral DNA levels from at least two different experiments were measured.

## RESULTS

**Deletion of single enhancer cores can alter the level and organ pattern of Py replication in mice.** To examine how the loss of a single well-conserved enhancer consensus sequence would affect Py replication during the acute phase of infection in mice, we deleted selected consensus sites within the Py enhancer and substituted an *Xho*I linker by using site-directed mutagenesis. The structures of the recombinant genomes are shown in Fig. 1. Also shown are the factor-binding sites in both the parental and the mutant enhancers. Four different deletions were made, deleting sequences corresponding to the adenovirus type 5 E1A (AdE1A) consensus (13), the c-*fos* consensus (37), the simian virus 40 (SV40) core consensus sequences (40), and a previously unrecognized sequence which is similar to half of the glucocorticoid response element (GRE) (24). The AdE1A core has been shown genetically to be important as a 12-*O*-tetradecanoylphorbol-13-acetate-responsive element (41) and is also believed to be important for Py DNA replication, as shown by reversion analysis (36). The AdE1A core also contains the binding site for the nuclear transcription factor PEA1 and part of the binding site for PEA2 (29). The SV40 core has been shown to be able to bind two nuclear factors: PEB2, which binds between nt 5184 and 5194 (22), and EBP20, which covers the entire sequence and binds from nt 5185 to 5201 (16). The GRE core has not previously been described within the Py enhancer, and no proteins have yet been identified that bind to this region of the Py genome. The *Pvu*II site is contained within part of the c-*fos* core and is homologous to the AP4-binding site, but binding has not been demonstrated (17). However, PEB1 has been shown to bind to part of the c-*fos* core. Thus, there is substantial biochemical evidence that these sequences may be important for virus replication.

The replication analysis of these enhancer consensus deletion viruses in 3T6 cells is shown in Fig. 2. Replication of nuclear DNA which was left uncut is apparent by the supercoiled (S) band of DNA. DNA digested with *Mbo*I will cleave replicated (unmethylated) DNA (1). Quantitation of the autoradiograms indicated that PydlAdE1A and PydlSV40 DNA replicated to wild-type levels. PydlGRE and Pydlc-fos both replicated slightly better (twofold) than wild-type DNA. Thus, these mutants all replicate as well as or better than wild-type virus in 3T6 cell culture. This is consistent with previous results of others that no single enhancer core consensus sequence appears to be essential for Py replication in 3T6 cells (36, 39).

The effects of these deletions on Py organ-specific replication during the acute phase of infection (6 days p.i.) are shown in Fig. 3. All core deletion viruses except one were

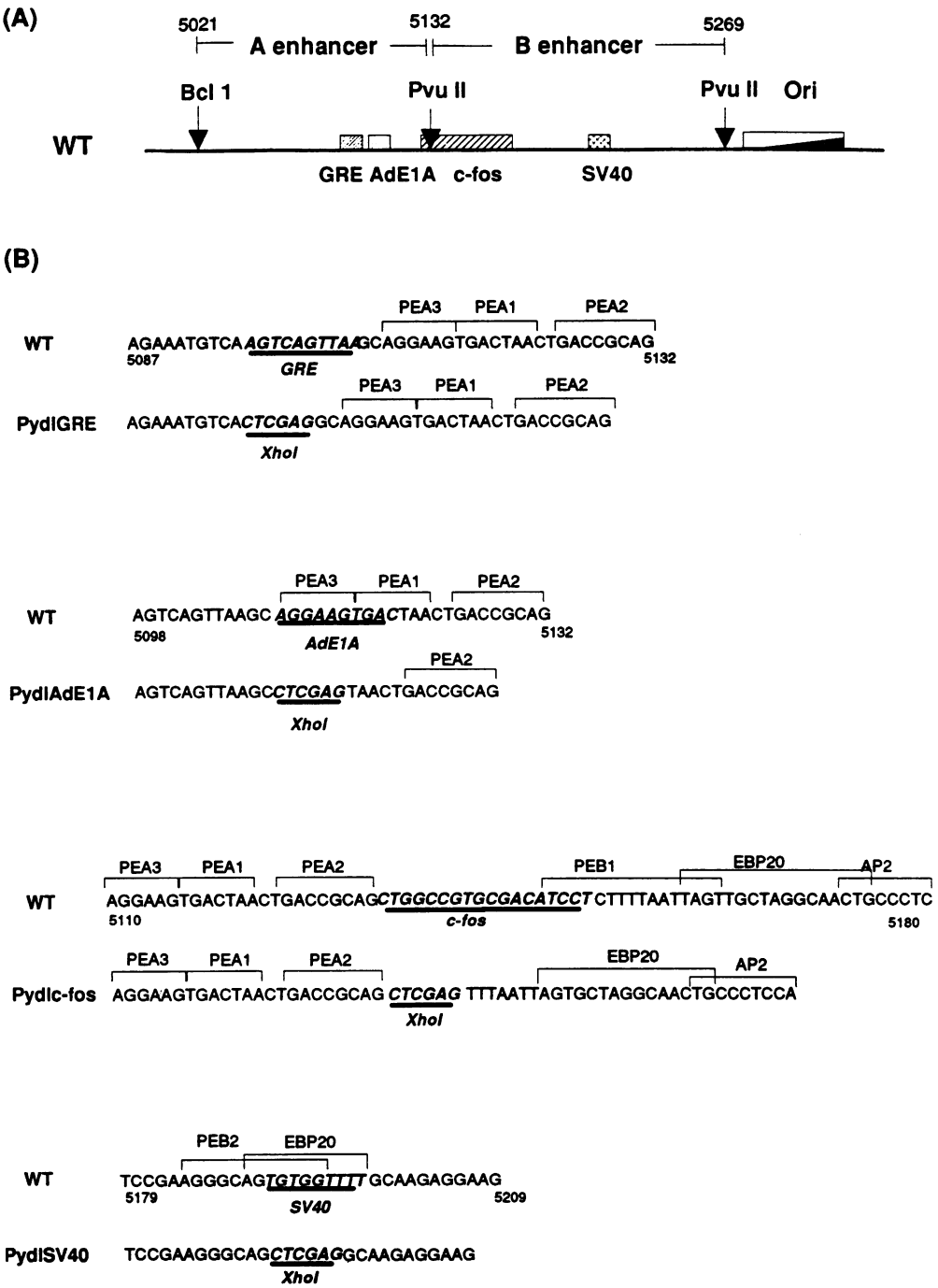


FIG. 1. Genetic structure of the Py enhancer core consensus deletions. (A) Schematic diagram indicating the location of consensus sequences within the Py enhancer. Shown is the wild-type enhancer region and the origin of replication (Ori). (B) Nucleotide sequence of consensus deletions and comparison with wild-type (WT) sequences. PydlGRE deletes a putative GRE consensus sequence (24) in the Py enhancer from nt 5098 to 5106. PydlAdE1A deletes the Ad5 E1A consensus sequence (13) from nt 5110 to 5119. Pydlc-fos deletes the c-*fos* consensus sequence (37) from nt 5133 to 5149. PydlSV40 deletes the SV40 consensus sequence (40) from nt 5190 to 5200. All deletions are replaced with an *Xho*I restriction site. Also shown are the binding sites for nuclear factors. Three murine factors have been identified which bind within the A enhancer (PEA3, PEA1, and PEA2) (22). Several factors bind within the B enhancer, including PEB1 and PEB2 (29) and EBP20 (16). The AP2-binding site is based on homology to a known binding site within the SV40 enhancer (17).

active for Py DNA replication in the kidneys to levels equivalent to the wild-type virus. PydlGRE replication, however, was reduced 10-fold in comparison with that of the wild-type virus, suggesting that this previously unrecognized element is important for fully activating Py replication in the kidneys. Other deletions, while not affecting levels of viral replication in the kidneys, were shown to affect the organ specificity. The wild-type virus does not replicate efficiently in the pancreas. However, Pydlc-fos replicates to almost 500 copies per cell in the pancreas. This very high level of

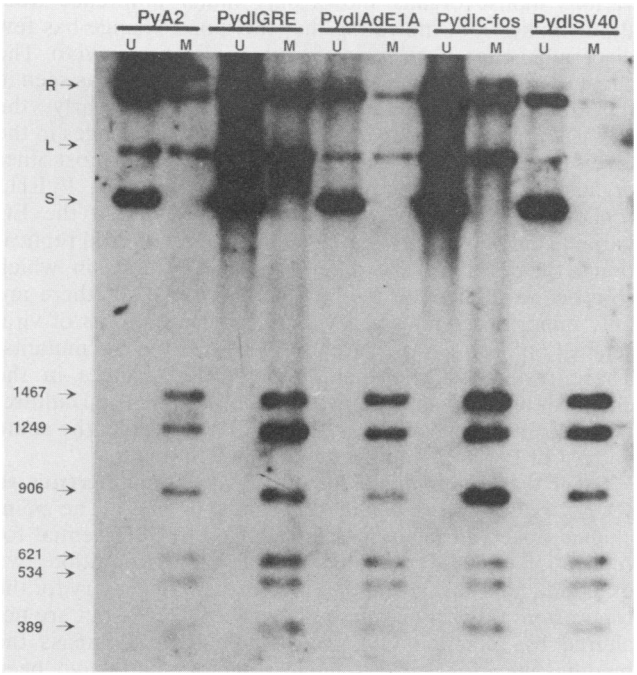


FIG. 2. Replication of Py enhancer core consensus deletions in 3T6 cells. Nuclear DNA was either undigested (U) or digested with *Mbo*I (M) and analyzed by DNA blot hybridization. Linear (L), relaxed circular (R), and supercoiled (S) DNAs are indicated, as are the sizes of the restriction fragments generated from the *Mbo*I digest. Only 1 μg of nuclear DNA was analyzed per track. Autoradiograms were exposed for 48 h.

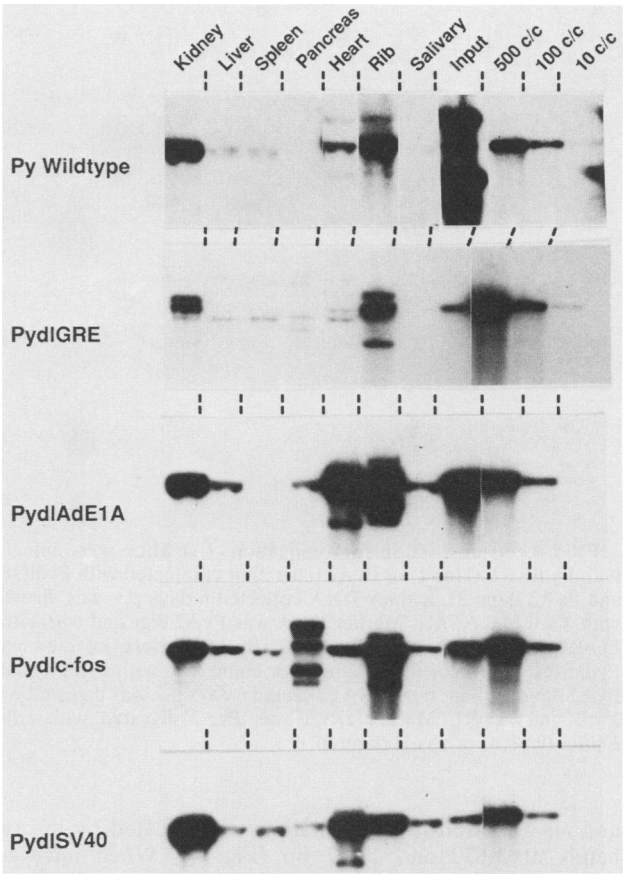


FIG. 3. Analysis of in vivo DNA replication of Py core consensus deletions in mouse organs. Organ DNA was extracted and analyzed from mice infected with the indicated mutant Py. Organs are labeled as indicated. DNAs were digested with *Xho*I, except for wild-type DNA (PyA2), which was digested with *Eco*RI. The track labeled "Input" corresponds to viral DNA obtained after infection of 3T6 cells to generate virus stocks for infection of mice. Input DNA was digested with *Xho*I, except for the wild-type input DNA, which was undigested. Copy-per-cell (c/c) control lanes are also included.

organ-specific viral DNA replication almost equals the level of wild-type virus replication in the most permissive organs, the kidneys. This result suggests that the deletion of this core sequence has expanded the organ specificity. After infection with PydlAdE1A and PydlSV40, high levels of replication were observed in the heart, an organ in which the wild-type DNA replicates poorly. This again suggests that deletion of a single core sequence can expand the organ specificity of virus replication. PydlGRE replicated poorly in most organs, and no expansion of organ specificity was seen, suggesting that this element is generally important for replication in the animal. It is also noted that additional, unexplained, subunit-length bands can be seen in the Southern analysis of some of the mutants (PydlGRE, Pydlc-fos, and PydlAdE1A) but that these bands occur mainly in some specific tissues and organs (i.e., the ribs with PydlGRE and PyAdE1A, the pancreas with Pydlc-fos, and the kidneys with PydlGRE). Since these extra bands cannot be detected in the DNA from parental viral stock used to infect these mice (Fig. 3, lane "input"), it appears that DNA alterations were generated de novo during passage of the viral stock in mice (possibly defective virus). In addition, other viral stocks with enhancer mutations do not show this genetic variability, indicating that this is not a general observation. Further analysis is required to determine the nature of these extraneous DNA bands.

**Py replication is restricted in *cis* for replication.** Our analysis has measured levels of viral DNA replication. However, it appears possible that changes in the enhancer also affect transcription of T antigen and thus indirectly affect levels of replication. To examine whether the observed altered levels of viral DNA replication are restricted in *cis* or whether replication can be activated by production of greater T antigen levels, mice were infected with a high titer of mixed virus to see whether complementation of viral DNA replication would occur. Coinfections with PyA2 and Pydlc-fos or PyA2 and PydlGRE were chosen to examine examples of expanded organ specificity (e.g., pancreas replication of Pydlc-fos but not PyA2) and restricted specificity (e.g., decreased kidney replication of PydlGRE relative to PyA2) of viral replication. The pancreas or kidney DNA was extracted at 6 days p.i. and analyzed for levels of viral replication (see Fig. 5). To distinguish the PyA2 viral DNA from mutant DNA, we digested organ DNA with *Eco*RI and *Xho*I. Since PyA2 has no *Xho*I site, it should be linearized by *Eco*RI, but PydlGRE and Pydlc-fos will yield two bands (3,670 and 1,627 base pairs [bp]). The experiment is somewhat complicated by the observation (Fig. 3) that these mutant DNAs are rearranging in these tissues. Consistent with this point, after digestion and Southern analysis of the organ DNA from Pydlc-fos- or PydlGRE-infected mice two separate populations were observed: an *Xho*I-resistant population, as indicated by the linear band migrating at 5,297 bp,

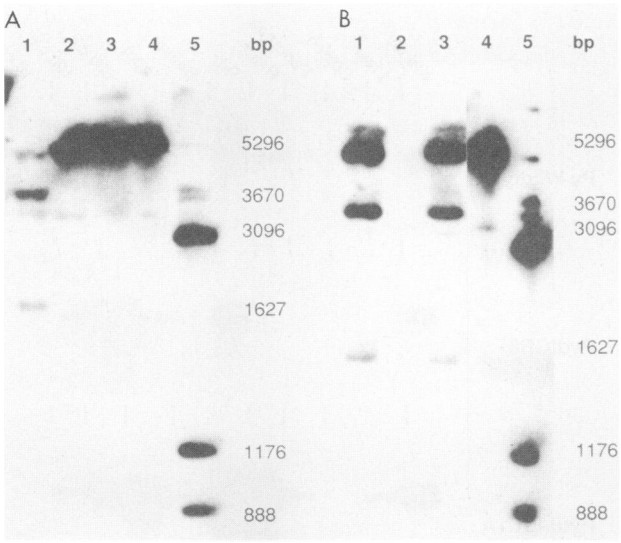


FIG. 4. *cis* restriction for replication. (A) Mice were infected with PydlGRE (lane 1) or PyA2 (lane 2) or coinfected with PydlGRE and PyA2 (lane 3). Kidney DNA collected 6 days p.i. was digested with *Xho*I and *Eco*RI. Marker DNA was PyA2 digested with either *Eco*RI (lane 4) or *Pvu*II (lane 5). (B) Mice were infected with Pydlc-fos (lane 1) or PyA2 (lane 2) or coinfected with Pydlc-fos and PyA2 (lane 3). Pancreas DNA collected 6 days p.i. was digested with *Xho*I and *Eco*RI. Marker DNA was PyA2 digested with either *Eco*RI (lane 4) or *Pvu*II (lane 5).

and an *Xho*I-sensitive population, as indicated by the two bands at 3,670 and 1,627 bp (Fig. 4). When mice are coinfected with PyA2 and Pydlc-fos, no relative increase in total levels of the 5,297-bp band are seen in the pancreas. This indicated that wild-type virus was not detectably complemented to replicate in the pancreas. The lack of complementation should be more obvious in the kidneys of mice coinfected with PyA2 and PydlGRE. An increase in both the 3,670-bp band and the 1,627-bp band should be unambiguous in the background of high-level wild-type replication (5,296 bp). The results of the experiment show that the both the mutant bands actually decreased in the mixed infection, indicating that PydlGRE not only was not complemented by wild-type virus but also may have been competitively inhibited by wild-type replication. Thus, the decreased replication of PydlGRE is restricted in *cis* in the kidneys.

**F9 EC-selected Py variants do not alter in vivo patterns of replication.** The first isolated cell-type-specific variants of Py were selected for replication in undifferentiated F9 EC cells (11, 12, 18, 33). Although these viruses have clearly altered specificity in cell culture, it was unknown how these changes would affect the in vivo replication of the virus. To examine this, we infected newborn mice with two EC variants, PyF441 and PyF111, and analyzed viral organ-specific replication during the acute phase of infection. PyF441 has an A · T-to-G · C transition at nt 5235 that is required for replication in EC cells (to facilitate comparison, mutants are numbered relative to the PyA2 strain). PyF111 duplicates nt 5211 to 5241, which encompasses the PyF441 point change (Fig. 5A). Because the parental strain of these F9 EC mutants was the PyA3 wild-type strain rather than the PyA2 strain used in our other analysis, we also examined the in vivo replication of the PyA3 strain as a control. PyA3 replicates to high levels in the kidneys (Fig. 5B), similar to PyA2 (Fig. 3). Analysis of PyF441 DNA replication in various mouse organs shows only minor difference from PyA3 replication, indicating that this point change has few discernible effects on levels of acute replication in vivo. The slight increase in the level of replication in the ribs as seen in this autoradiogram was not reproducible. Similarly, the PyF111 variant also demonstrated only small changes in the levels of acute viral replication in the kidneys and most other organs in vivo. Some decrease in the level of PyF111 replication in the ribs was apparent. PyA3 and the EC variants did show a low but detectable level of viral replication in the pancreas. This differs from the PyA2 strain, which showed no replication in the pancreas. Overall, there are only minor differences in levels and organ patterns of viral replication between the parental PyA3 and the EC mutants. These results suggest that although the changes in the enhancer alter the specificity of Py replication in cell culture, they do not necessarily correlate with a change in the acute pattern of Py DNA replication in newborn mice.

**Other domains required for replication in undifferentiated F9 EC cells affect levels of replication in mice.** The point change observed in PyF441 is reported to be essential for replication in undifferentiated EC cells (12). In addition, two other domains have been identified that are necessary for the replication of Py in undifferentiated F9 EC cells but are not altered by selection (38). These domains encompass the binding sites for various nuclear factors and have been mutated in the PyBstEII and PyNruI virus isolates by Tseng and Fujimura (38). These mutants fail to replicate in undifferentiated F9 cells but replicate efficiently in differentiated F9 cells and in 3T6 cells (38). Representations of their genomes are shown in Fig. 5A. Analysis of these mutants in vivo shows that after intraperitoneal infection, they both reproducibly have 50% lower levels of replication in the kidneys than the wild type does (Fig. 5B). In addition, there is a decrease in the levels of overall viral replication in the other organs analyzed. These domains appear, therefore, to contribute to in vivo replication activity.

**More than one enhancer is required for efficient in vivo replication.** Because it has previously been proposed that the A and B enhancers are redundant in permissive cell culture (14, 39), it was of interest to determine whether both enhancers are required for viral replication or whether a single enhancer is sufficient for replication in vivo. Wild-type virus (PyA2) replicates to high levels in the kidneys at 6 days p.i. (Fig. 6B). In contrast, when the B enhancer is deleted (Fig. 6A, PyA/Δ), DNA replication is reduced approximately 20-fold relative to the wild type and indicates that the A enhancer alone is not sufficient for high-level Py replication in the kidneys.

**An A enhancer domain can functionally substitute for the complete B enhancer.** To determine whether there were identifiable subenhancer domains within either the Py A or B enhancer that could functionally substitute for the B enhancer by complementing the ability of the B enhancer deletion virus (PyA/Δ) to replicate in the kidneys, we used recombinant viruses that contained one of four genetically defined Py subenhancer domains (3) in place of the B enhancer (nt 5132 to 5265): one domain from within the A enhancer (A domain) and three domains found within the B enhancer (Pal, B, and Bpv domains) (Fig. 6). These four subenhancer domains are about 30 to 40 nucleotides in length and encompass several adjacent consensus or factor-binding sites. At least three of these subenhancer domains have previously been shown to be functional and to have distinct effects on the activation of replication and transcription in various lymphoid and fibroblast cells in culture (3).

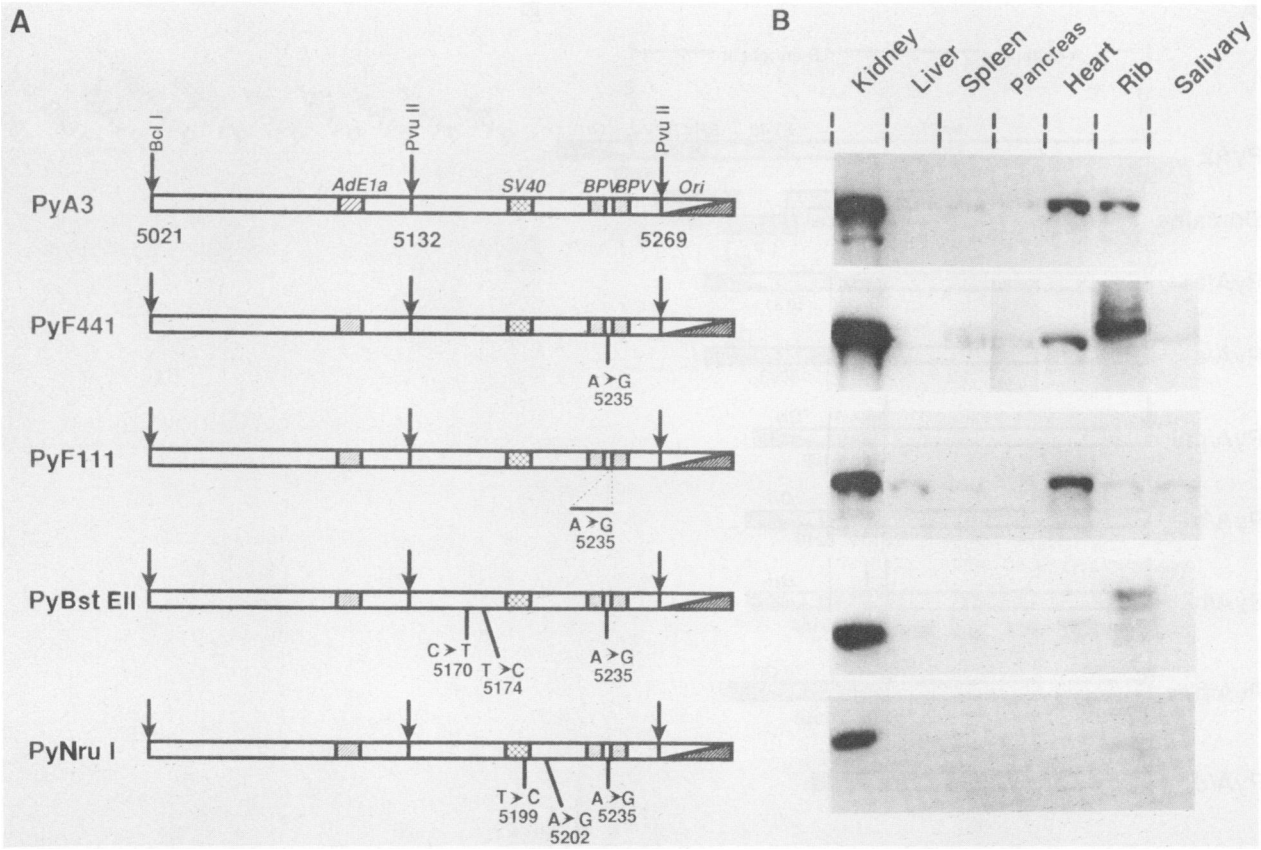


FIG. 5. Schematic diagram and replication analysis of EC host range variants. (A) Diagram of the Py EC host range variants. The numbering shown is relative to the PyA2 strain and not the PyA3 strain, to facilitate comparison of sequences and sequence elements within the enhancer region. PyA2 and PyA3 have identical sequences in the enhancer region. PyF441 contains an A-to-G transition at nt 5235. PyF111 contains a duplication from nt 5211 to 5241 and contains the point change at nt 5235. PyBstEII and PyNruI contain point changes in addition to the point change at nt 5235, as shown, and their genetic construction has been previously described (38). (B) Replication of Py enhancer recombinants in mice. DNA blot hybridization with 10 μg of organ DNA from Py-infected mice sacrificed 6 days p.i. DNA was digested with *Eco*RI. Autoradiograms were exposed for 16 h.

These viruses were used to infect newborn mice, and the levels of activation of viral replication in the kidneys (6 days p.i.) relative to both the wild-type virus and the PyA/Δ enhancer deletion virus are shown in Table 1. The B subenhancer domain, in either orientation (PyA/b+ or PyA/b−), did not activate Py DNA replication in kidneys above levels seen for the parental deletion (PyA/Δ) (Fig. 6B). The Bpv domain appeared to actually decrease DNA replication, since no detectable DNA replication was observed in the kidneys, not even the low level of replication seen with the parental deletion. PyA/bpv also contains the point mutation found in the EC PyF441 mutant (an A-to-G transition at nt 5235). The inability to detect any replication in the kidneys, even though infectious virus can be obtained from cell culture, suggests that this element might repress the residual Py replication in the kidneys. PyA/pal+, which contains the Pal domain, was able to activate Py replication in the kidneys fivefold above the activation by the parental deletion. Therefore, this Pal subenhancer domain has clear, albeit reduced, activity for Py replication in the kidneys. With the 40-bp A domain in a positive orientation (PyA/a+), however, replication was activated to levels similar to those seen after infection with wild-type virus. These results suggest that components of the B enhancer are not needed for full replication in the kidneys but also that the remaining A enhancer is, by itself, not sufficient for high-level replication in the kidneys. It appears that at least two enhancer domains are necessary for high-level DNA replication in the kidneys and that either two A enhancers or an A enhancer plus an essentially complete B enhancer can functionally substitute for the wild-type enhancer. In addition, this activation is dependent on the orientation of the A domain, since the positive orientation (PyA/a+) led to 10-fold-higher viral DNA replication than the negative orientation did (PyA/a−); this suggests that, at least for some elements, there appears to be a requirement for a specific orientation relative to the replication origin. Viral replication in other organs was analyzed. With the exception of PyA/a+, low-level replication in the kidneys correlated with an overall decrease in the levels of replication, and no change in tissue specificity was observed (data not shown).

A possibility was that PyA/Δ, PyA/b, PyA/pal, and PyA/bpv could have less efficient enhancers and therefore might have a time lag in replication relative to the wild-type virus. Mice infected with these viral recombinants were sacrificed at 8 days p.i., and the levels of viral replication in the kidneys were compared with those at 6 days p.i. An 8-day time point was chosen because the immune system begins to clear the virus infection from mice at 10 to 12 days p.i. No

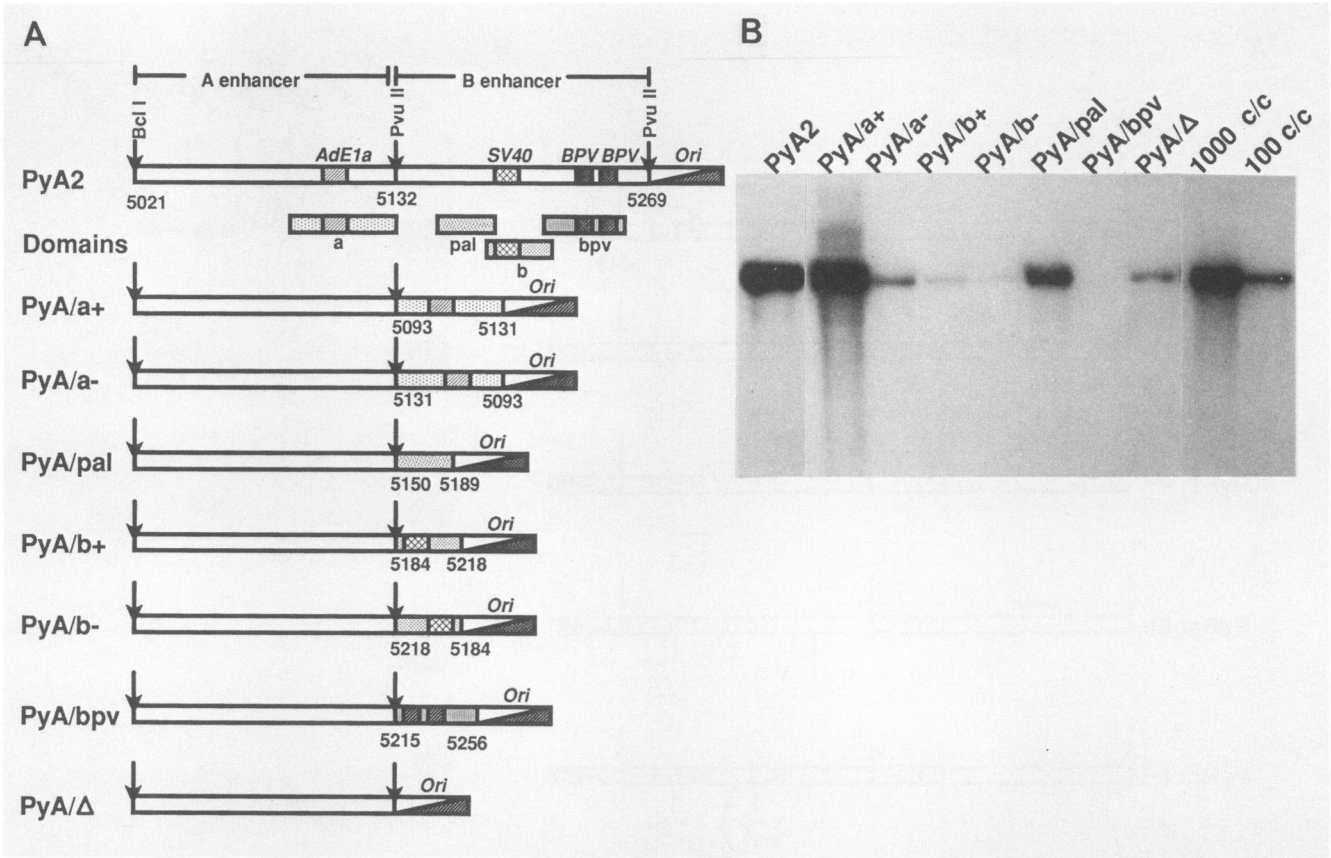


FIG. 6. (A) Schematic diagram of the enhancer region of B enhancer deletion recombinants. The structures are aligned relative to the wild-type enhancer at the *BclI* site at nt 5021. Regions with homology to the adenovirus type 5 E1a (AdE1a) (13), SV40 B core (SV40) (40), and bovine papillomavirus consensus sequence (BPV) (19) are shown. All of the recombinants delete the B enhancer from nt 5132 to 5269. PyA/a+ contains the A domain from nt 5093 to 5132 in the positive orientation relative to the origin of replication (Ori). PyA/a− contains the A domain inverted relative to the Ori. PyA/pal contains the Pal domain (nt 5150 to 5189) in a positive orientation relative to the Ori. PyA/b+ and PyA/b− contain the B domain (nt 5184 to 5218) in a positive and a negative orientation relative to the Ori, respectively. PyA/bpv contains the Bpv domain (nt 5215 to 5256) in a negative orientation relative to the Ori. It also contains a point mutation at nt 5235 that is found in the PyF441 mutant (Fig. 4). PyA/Δ deletes the B enhancer. (B) Replication of Py enhancer recombinants in kidneys. DNA blot hybridization was done with 10 μg of kidney DNA from Py-infected mice sacrificed 6 days p.i. DNA was digested with *Eco*RI. Also included are copy-per-cell (c/c) reconstruction lanes containing Py DNA linearized with *Eco*RI. Kidneys are from mice infected with the Py recombinant as labeled. Exposure was for 16 h.

increase in viral DNA replication was observed, suggesting that the decrease in the levels of viral DNA was due to the inability to activate replication rather than to a lag in replication time (data not shown).

TABLE 1. Recombinant Py DNA replication in kidneys of infected mice

| Virus | c/c[a] | c/c normalized to[b]: | |
|---|---|---|---|
| | | PyA2 | PyA/Δ |
| PyA2 | 1,030 | 1.0 | 22 |
| PyA/a+ | 1,120 | 1.1 | 25 |
| PyA/a− | 100 | 0.1 | 2.0 |
| PyA/b+ | 30 | 0.02 | 0.6 |
| PyA/b− | 10 | 0.001 | 0.2 |
| PyA/pal | 210 | 0.2 | 5.0 |
| PyA/bpv | <5 | | |
| PyA/Δ | 50 | 0.04 | 1.0 |

[a] The copies per cell (c/c) of viral DNA in the kidneys after infection with the recombinant viruses.

[b] The copies per cell are normalized to the level of the wild-type replication (PyA2) in the kidneys and also to levels after infection with the B enhancer deletion virus, PyA/Δ.

## DISCUSSION

We have examined how alterations in the enhancer sequences can affect Py DNA replication in the kidneys and other organs following i.p. infection of newborn mice. The mutants which altered only a specific consensus sequence of the enhancer allowed us to examine the requirement for replication of these conserved, and presumably important, elements within both the A and B enhancers. We observed that the deletion of a single consensus sequence in PydlAdE1a, PydlSV40, and Pydlc-fos strongly elevated the levels of Py replication in some organs but did not significantly affect the levels of Py replication in the kidneys. The expansion of high-level organ-specific DNA replication to new organs with high-level replication maintained in the kidneys contrasts with our previous results that a virus containing a heterologous enhancer was restricted for replication to only the pancreas (31). This could reflect the nature of the genetic changes, since in one case (core consensus deletions) we have deleted subsets of the Py enhancer and in the other (heterologous enhancer) we have exchanged the entire Py B enhancer for the Moloney murine leukemia virus enhancer. The usual view of the consensus sequences within

the enhancer is that they represent binding sites for various nuclear factors and that it is the binding of these factors which can activate transcription and DNA replication by unknown mechanisms. The implication from our results is that these factors appear to be functioning as organ-specific repressors rather than activators of viral replication. It is also possible that the passage of Py in culture has selected for consensus sequences which are functioning in permanent cell lines but which restrict activity in mouse organs. The deletion of the consensus sequences not only deleted factor-binding sites but also changed their relative spacing (Fig. 1). A striking expansion of organ-specific replication was not expected, since most of these factors are believed to be positive activators of transcription (17, 21). It is relevant that both the PyBstEII mutant and the PyNruI mutants, which encompass point changes within binding sites rather than deletion of factor-binding sites, do not expand the organ specificity, even though they have been shown by others (38) to eliminate factor binding. This suggests that the change in spacing or juxtapositioning of the factor-binding sites may contribute to the altered phenotype of Py organ-specific replication. However, our current results do not allow us to distinguish between these and other models.

Our experiments have analyzed levels of viral DNA replication as a function of changes in the enhancer. Although enhancers are thought of as *cis* restricted for transcription, we have shown previously that enhancer changes restrict cell-specific replication in *cis* after in vivo infection (31). This has also been observed for mutants selected for growth in undifferentiated EC cells (12, 23, 33, 38) and also in neuroblastoma and Friend erythroleukemia cells (6–8) and various lymphoid cells (2, 3, 9). In fact, in all the numerous studies to date which have examined this issue, cell-specific replication of Py DNA is restricted in *cis*, and it is not restricted because early gene transcription is indirectly limiting replication. Our current results also support this view. Coinfection by mutants with expanded pancreas specificity and restricted kidney specificity (Pydlc-fos and PydlGRE, respectively) and PyA2 established that organ-specific replication of these mutants also appears to be *cis* restricted at the level of DNA replication.

The AdE1A consensus in the enhancer is not essential for Py DNA replication, as seen by high-level replication both in 3T6 cells and in kidneys. These results might appear to be in conflict with the results of Tang et al. (36), who showed by reversion analysis that Py mutants which contain point changes that inactivate this AdE1A site can regain full activity by a single reversion of A to G at nt 5115. This, they proposed, demonstrated the importance of the AdE1A consensus sequence for Py replication. It must be noted, however, that additional B enhancer mutations were present in the PyB1 mutant used by Tang et al. (36). It is probable that these additional changes contributed to the observed dependence on a functional AdE1A consensus sequence. This implies that only in the absence of a functional B enhancer is the AdE1A consensus sequence crucial for viral DNA replication. This view is not inconsistent with our results obtained after infection with PyNruI and PydlSV40. Both of these viruses affect the SV40 core consensus site; however, infection with PydlSV40 does not affect virus replication in the kidneys relative to wild-type virus replication, whereas the PyNruI decreases replication. This results suggests that replication of the PyNruI virus is also affected by the PyF441 point mutation.

Mutational loss of the sequences between nt 5098 and 5110 (PydlGRE) decreased the levels of viral DNA replication in kidneys. This is an unexpected result, since no known nuclear factors have yet been shown to bind to this region (17), nor have previous deletion analyses demonstrated an activity for this region in cell culture (27, 39). We observed that PydlGRE replicated to a twofold-higher level than wild-type levels in 3T6 cells, which also indicates that it is not an essential element for replication in cell culture. PydlGRE could be affecting the start sites of late transcription (nt 5021 to 5075), although that seems unlikely, since it is capable of replicating to equal efficiency as the wild type in 3T6 cells. These results suggest that there can be striking differences between cell culture systems and the organs of the mouse with respect to their genetic requirements for DNA replication. It is possible that established cell lines with their altered replication control are less restrictive for viral DNA replication. Other experiments (R. Rochford and L. P. Villarreal, manuscript in preparation) also show major differences between the ability of a Py with a Moloney murine leukemia virus enhancer to replicate in the pancreas versus pancreas cell line in culture. It appears that caution is necessary in extrapolating the results from cell culture experiments to in vivo experiments.

The mutants which were selected for growth in undifferentiated EC cells (PyF441 and PyF111) had little effect on acute viral replication. Both the levels of viral DNA replication and the specificity of organs infected remained relatively unchanged from those for wild-type virus infection. These results appear to offer little insight into how the undifferentiated EC cells relate to the cells to a newborn mouse which are available for Py infection. Because EC cells are believed to be representative of early cells in development, it is possible that early mouse embryos will have to be used to examine the in vivo significance of the changes in regulatory DNA which occur in PyF441 and PyF111.

Our results indicate that one copy of the A enhancer is insufficient to fully activate Py DNA replication in kidneys. Replication of the B enhancer deletion virus (PyA/Δ) was reduced 20-fold in kidneys relative to that of the wild-type virus. Substitution of various subenhancer domains in place of the B enhancer identified regions which could either activate viral DNA replication (PyA/a+, PyA/pal), have little effect on replication (PyA/b+), or repress viral DNA replication (PyA/bpv) in kidneys. Surprisingly, the three domains from within the B enhancer either were not active or were less active than the A subenhancer domain. The repressive effect of the Bpv domain on viral DNA replication has also been observed in various cells in culture and appears to be a consistent phenotype of this recombinant enhancer (3). This Bpv domain, in addition, contained the point mutation characteristic of EC-selected variants (nt 5233). Since the PyF441 and PyF111 variants replicated well in kidneys, it appears that other elements of the B enhancer must be counteracting the repressive effects of this Bpv domain when it alone replaces the B enhancer. Although such behavior of the Bpv domain would be consistent with a role in the negative regulation of viral DNA replication, there is little other evidence to support this view. It is possible that the boundaries made during the construction of this domain have artificially made it a negative element for unknown reasons.

Most interesting was the ability of the A domain (PyA/a+) to fully substitute for the function of the B enhancer in viral DNA replication in the kidney. This domain includes the putative GRE-like element, the AdE1A consensus sequence, and the contiguous binding sites for the nuclear factors

PEA1, PEA2, and PEA3 (22). This establishes that the B enhancer is not needed for high-level replication of viral DNA in mice. The orientation dependence of this second A enhancer for viral DNA replication has also been previously observed in cell culture experiments in both a macrophage cell line (P388) and a T cell line (AKR1) (3). This may implicate different (polar) sequence requirement with respect to enhancer-driven DNA replication versus transcription. In a functional sense, it appears that the A and B enhancers displayed the same organ (kidney) specificity. Indeed, in our analysis, specificity changes were often better correlated to the loss and possible altered spacing of sequence elements rather than to their presence. However, this does implicate a redundant function between the A and B enhancer with respect to kidney-specific replication. This is similar to the proposals that A and B enhancers are functionally redundant in permissive cell culture experiments (39), but difficult to reconcile with experiments which show that the same A and B enhancers have different tissue specificities (14).

Enhancers which duplicate the A domain are commonly seen in both natural isolates (32) and tissue-specific variants (6–8, 18) of Py, and these same variants were, in fact, the genetic basis we used to choose the boundaries of the A domain (3). However, these variants (especially the natural isolates) typically maintain most of the B enhancer. What, then, is the necessity of the B enhancer if a duplicated A enhancer is fully active for mouse kidney replication? We suspect that the B enhancer does have an important biological function and that this function may be related to the ability of Py to persistently infect mouse kidneys (Rochford and Villarreal, in preparation). Studies are in progress to identify how the enhancer region can determine and/or affect Py replication during persistent infection.

## ACKNOWLEDGMENTS

We thank Frank Fujimura, Nicholls Institute, for providing the PyBstEII and PyNruI virus recombinants. We also acknowledge C. T. Davis for critical reading of the manuscript, J. P. Moreno for technical assistance, and N. DePolo for assistance with the site-directed mutagenesis.

This work was supported by Public Health Service grant GM36605 from the National Institutes of Health, by the Focused Research Program on the Molecular Biology of Animal Viruses at the University of California at Irvine, and by the University of California Cancer Research Institute.

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