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Enhancer Dependence of Polyomavirus Persistence in Mouse Kidneys

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We previously showed that alterations in the enhancer sequence of polyomavirus DNA can alter both the level and the organ specificity of viral DNA replication during the acute phase of infection of newborn mice (R. Rochford, B. A. Campbell, and L. P. Villarreal, J. Virol. 64:476–485, 1990). In this study, we examined whether these enhancer sequence alterations can also affect polyomavirus replication during the persistent phase of infection in vivo. After infection of newborn mice with a mixture of three enhancer variants, the individual organs could select for enhancer-specific viral DNA replication during both the acute and the persistent phases of infection. Contrary to expectations, the ability of some variants to establish a high-level acute infection in some organs (e.g., the pancreas) did not necessarily lead to a persistent infection in those organs. Thus, enhancers can affect acute and persistent infections differently. In addition, some enhancer variants tended to establish a high-level persistent infection in the kidneys immediately following an acute infection; however, in all cases considerable histopathology was associated with these elevated long-term infections, and these mice were always runty. A persistent infection in the kidneys thus appears able to exist in two distinguishable states, a high-level pathological state and a low-level nonpathological state, which can be affected by the viral enhancer sequence.

The name polyomavirus (Py) is derived from observations that this virus family can induce numerous tumor types in rodents (see reference 37 for a review of the early literature). However, in nature, most Py-induced mouse tumors are rare (10) and not a component of the normal viral life cycle. With permissive in vitro cell culture infections, the Py life cycle is characterized by "runaway" viral DNA replication and cell lysis. Although some cellular lysis also appears to occur following in vivo infections, these infections generally result in a low-level persistent infection which is maintained by unknown mechanisms for the life of the animal. Thus, like that of most other DNA viruses, the in vivo Py life cycle appears to include at least two distinct phases, acute and persistent (12, 36). Following infection of newborn mice, the Py acute phase is characterized by high-level viral replication in various target organs, such as the lungs, skin, bone surfaces, salivary glands, and kidneys, with maximum levels of viral DNA occurring by 6 days postinfection (p.i.) (21). These acute organ patterns of viral replication can be experimentally modified by alterations in the *cis*-regulatory or enhancer DNA sequences (21, 30, 31). These observations have led to a proposal that the natural organ specificity of Py infections may involve ongoing organ-specific selection for *cis*-restricted viral DNA replication (4, 9, 22, 25, 30, 31). This proposal has not been directly tested. The acute phase is succeeded by the persistent phase, along with the induction of an antiviral immune response (13) which can last for the lifetime of the mouse. The persistent phase is characterized by the low-level maintenance of nondefective episomal viral DNA, most often in the kidneys, but sometimes also in the lungs (12).

Relatively little is known about the viral genetic elements which might affect persistent infections. It is clear that the configuration of the Py enhancer DNA sequence is a determinant of organ-specific replication during an acute infection, but the effects on the subsequent persistent infection are unknown. We now further examine this issue. In addition, we directly test the ability of mouse organs to select for the *cis*-restricted replication of Py DNA. Using infections with mixed virus strains, we show that organs can select for enhancer-specific viral DNA replication during both acute and persistent infections. We also examine whether viral persistence in a particular organ always results following an efficient acute infection of that target organ (12) or whether enhancer changes can affect patterns of persistent infections differently from acute infections. We show that the enhancer requirements are distinct for organ-specific acute and persistent infections and examine the histopathology of the resulting persistent infections. High-level persistent infections were seen to correlate with significant histopathology and runtiness of mice.

MATERIALS AND METHODS

Cells and viruses. The A2 strain of virus was used as the wild-type strain (PyA2, Fig. 1). The PyFl01 and PyF441 strains were obtained from F. Fujimura (Nichols Institute, San Clemente, Calif.). The generation of *dl*cfos, *dl*AdE1A, *dl*SV40, *dl*GRE, and PyMLV has been described previously (5, 32). PyACR was isolated from a heterogenous stock of virus obtained after infection of 3T6 cells (29). The virus was plaque purified twice, and the genetic structure of the recombinant viral stocks was confirmed by extensive restriction enzyme analysis (data not shown). The sequence of the enhancer region was determined by dideoxy sequencing with oligonucleotide primers complimentary to the late coding strand (beginning at nucleotide [nt] 5047) and to the early coding strand (beginning at nt 95). The structure of the enhancer region is shown in Fig. 1. The protocol for Py

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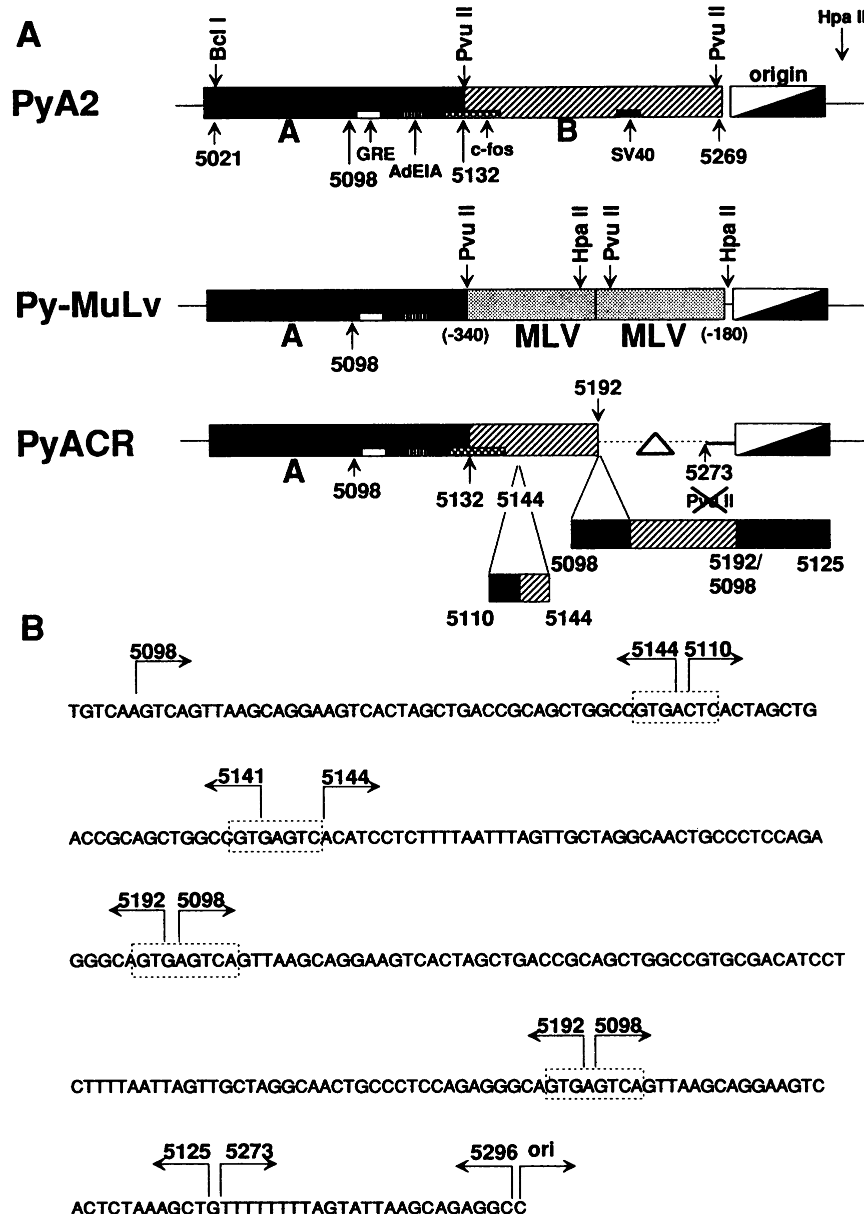

FIG. 1. Genetic structures of the enhancers of viral strains. PyA2 (wild type) contains two enhancers, the A and B enhancers; the smaller boxes indicate consensus sequences found in the *dl*GRE, *dl*AdE1A, *dl*SV40, and *dl*cfos enhancers. In PyMLV (PyMuLV), the B enhancer has been deleted and substituted with the enhancer from Moloney murine leukemia virus. PyACR has a complex enhancer. The enhancer deletes 77 bp from nts 5192 to 5269. This deletion begins at and includes the B core consensus sequence, which has homology to the *dl*SV40 core A motif. An insertion at nt 5141 contains a duplication of the sequences that span from nts 5110 to 5141. Two other duplications of sequences within the A enhancer are also present, at nts 5098 to 5192 and nts 5098 to 5125. In addition, two point changes, a G-C transversion at nt 5117 and an A-G transition at nt 5121, are also observed. (B) Sequence of the PyACR enhancer. ori, origin of replication.

propagation in mouse 3T6 cells has been described by Eckhart (14). Cells were grown in the presence of 7% fetal calf serum and nonessential amino acids in Dulbecco medium and maintained in 2% fetal calf serum during viral infection. Cells were infected with a multiplicity of infection of 0.01 PFU per cell. Titers of 10^9 to 10^{10} PFU/ml were normally obtained in the plaque assay.

Mice and infection. BALB/c mice (Bailey strain) were obtained from Charles River and bred in University of California, Irvine, animal care facilities. Newborn mice less than 24 h old were inoculated intraperitoneally with 1×10^7 to 5×10^7 PFU/ml. At 6 or 30 days p.i., mice were sacrificed and organs were removed for DNA tissue extraction or for in situ hybridization. A minimum of two separate litters with four mice per litter were used to analyze each recombinant virus. DNA was extracted from organs as previously described (12). Organ DNA was digested with the appropriate restriction enzyme, electrophoresed on a 1% agarose gel, and transferred to nitrocellulose membranes for DNA blot hybridization. Blot hybridization analysis was done with a ^{32}P-labeled genomic Py probe as previously described (4). Copy levels for viral DNA were determined by densitometer

scanning of the autoradiograms with an E-C densitometer and comparing the levels of viral DNA in organs to those in controls containing known levels.

In situ hybridization. Py DNA was chemically labeled with horseradish peroxidase by use of reagents and methods commercially available from Digene (Silver Spring, Md.). Mouse tissues were removed by dissection, fixed in phosphate-buffered Formalin (7% [vol/vol]) overnight, and transferred to 70% ethanol until paraffin embedding and sectioning to prevent overfixation. Sections (5 to 10 μm) were fixed onto silane-treated glass slides by baking at 60°C for 4 h and digested with pronase as described by Digene. Hybridization to the labeled Py probe, diaminobenzidine color development, and silver signal amplification of slides were performed in a 3-h procedure as described by Digene. Uninfected control slides and infected tissues hybridized with irrelevant probes (bacterial plasmid sequences) showed no detectable background hybridization (data not shown).

RESULTS

Organ-specific sorting of viral enhancer variants during an acute infection. Wild-type Py (PyA2) establishes a low-level persistent kidney infection in mice following a high-level acute kidney infection (11). We previously established that a Py enhancer recombinant (PyMLV) containing the Moloney murine leukemia virus enhancer substituted for the Py B enhancer (30, 33) replicates selectively in the pancreas and not in the kidneys during acute infection. In addition, we showed that various other enhancer mutants could replicate in normally nonpermissive tissues, such as the heart or pancreas, in addition to the kidneys (31). Another Py variant, PyACR, had been isolated by us during plaque purification of naturally arising variants. PyACR (containing the A enhancer core repeat) has a deletion of much of the B enhancer, substitutions of portions of the A enhancer, and various point changes (Fig. 1). PyACR was of special interest because we had observed that it would replicate in both the kidneys and the pancreas during an acute infection with high efficiency (29). Using these three variants (PyA2, PyMLV, and PyACR), we sought to establish that an organ-specific acute infection was *cis* restricted and to determine whether this organ-restricted replication was followed by an organ-specific persistent infection.

Newborn mice were infected with a mixed high-titer virus stock containing PyA2, PyMLV, and PyACR. PyACR can be easily distinguished from PyA2 and PyMLV by *Hpa*II restriction digestion, which yields a unique enhancer-spanning fragment for each of the variants (980 bp for PyACR, 885 bp for PyA2, and 742 bp for PyMLV) because of the *Hpa*II site within the enhancer. The structures of the enhancer regions are shown in Fig. 1A, and the sequence of PyACR is shown in Fig. 1B. Infected mice were sacrificed at 6 days p.i. (acute) or 30 days p.i. (persistent). Various organs were removed, and DNA was extracted and analyzed for Py-specific sequences by DNA blot hybridization. The results of this experiment are shown in Fig. 2 and 3. During the acute infections, organ-specific replication was enhancer specific and *cis* restricted with a mixed-virus inoculation (Fig. 2). PyMLV replicated efficiently only in the pancreas (note the *Hpa*II band of 742 bp with a short exposure). Similarly, PyA2 replicated preferentially in the kidneys, whereas PyACR replicated to high levels in both the pancreas and the kidneys. PyACR replication in the kidneys was somewhat higher than that of PyA2, consistent with our prior results showing that the substitution of the B enhancer

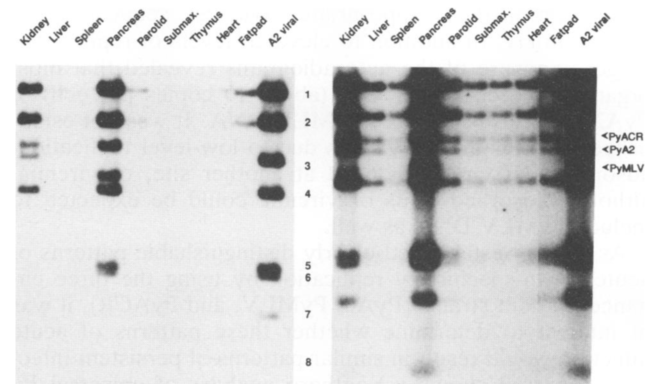

FIG. 2. Southern blot analysis of a mixed-virus infection during acute infection DNA replication. Organ DNA was extracted from mice at 6 days p.i. and analyzed by *Hpa*II digestion. PyA2 DNA from tissue cultures is included as a marker (A2 viral), and the *Hpa*II fragments are numbered. The enhancer-spanning bands (*Hpa*II-3) are indicated by the arrowheads labeled with each virus name; these *Hpa*II bands are 980 bp for PyACR, 885 bp for PyA2, and 742 bp for PyMLV. Both short (left) and long (right) exposures of the autoradiogram are shown. Submax., submaxillary gland.

with portions of the A enhancer yields a virus which replicates more efficiently in the kidneys than wild-type Py (31). These results also establish that within one animal, individual organs are able to select and replicate only those viral DNAs which have the appropriate organ-specific enhancer and further establish that the expression of a functional T antigen in these organs is not sufficient to drive viral DNA

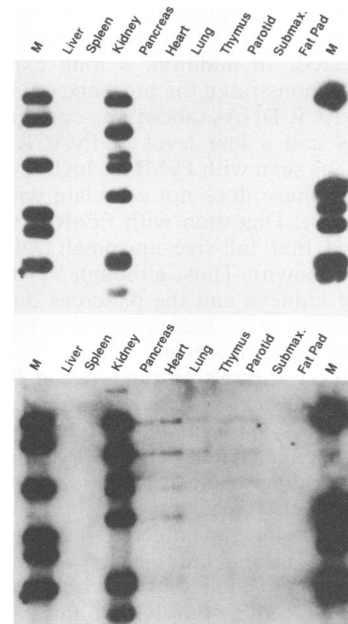

FIG. 3. Southern blot analysis of a mixed-virus infection during persistent infection DNA replication. Organ DNA was extracted from mice at 30 days p.i. and analyzed by *Hpa*II digestion. M (marker) indicates PyA2 viral DNA extracted from infected cells and digested with either *Hin*fI or *Mbo*I. Both long (bottom) and short (top) exposures of the autoradiogram are shown. Submax., submaxillary gland.

replication if the inappropriate enhancer sequences are present in *cis*. In addition to elevated levels of replication, longer exposure of the autoradiograms revealed that most organs harbored a low level (about 10 copies per cell) of PyA2 and PyACR but not PyMLV DNA. It was not established whether this DNA was due to low-level replication, adsorption of virus produced at another site, or viremia, although adsorbed virus or viremia could be expected to include PyMLV DNA as well.

As we had established clearly distinguishable patterns of acute organ-specific Py replication by using the three enhancer variant strains (PyA2, PyMLV, and PyACR), it was of interest to determine whether these patterns of acute infection would result in similar patterns of persistent infection. Figure 3 shows a Southern analysis of episomal Py DNA in various organs 30 days after infection with a mixture of the three enhancer variant strains. This time period is well into the persistent phase of infection (12, 13). The viruses were distinguished by the *Hpa*II restriction pattern. No PyMLV DNA was observed to persist in any organ examined during the mixed-virus infection. The persistence of PyMLV was also not observed when mice were infected with PyMLV alone, as analyzed by DNA blot hybridization (data not shown). Thus, despite having established an efficient acute infection in the pancreas, PyMLV was not able to establish a persistent infection in the pancreas, nor was it complemented by the presence of PyA2 for persistence in the kidneys. In contrast, PyACR DNA could be detected in the pancreas, although at very low levels (fewer than five copies per cell). Thus, it is clear that organ-specific enhancer selection during the persistent phase of viral replication is distinct from that during the acute phase.

PyACR also replicated very efficiently in the kidneys during the acute phase, and it appears that PyACR DNA was able to persist in the kidneys, albeit at levels below those of PyA2 (about 20%), but higher than those seen in the pancreas. PyA2 DNA persisted in the kidneys at significant levels, as expected. In addition, a long exposure of the autoradiogram demonstrated the presence of very low levels of PyA2 and PyACR DNAs (about one copy per cell) in the heart and lungs and a low level of PyACR DNA in the pancreas. Thus, as seen with PyMLV, high-level replication during the acute phase does not correlate with an efficient persistent infection. Digestion with *Eco*RI to linearize the genome revealed that full-size episomal DNA was maintained (data not shown). Thus, although PyACR replicated well in both the kidneys and the pancreas during an acute infection, persistence in the kidneys occurred at much higher levels than it did in the pancreas.

Effect of enhancer variants on the levels of Py DNA in the kidneys during a persistent infection. The kidneys are clearly a major target for Py persistence. As we had previously described several enhancer variants of Py which replicated efficiently in the kidneys (and in some cases other organs) during an acute infection (31), it was of interest to determine whether these variants had affected the ability of Py to specifically persist in the kidneys as well. The variants examined had selective deletions of conserved consensus sequences (*dl*AdE1A, *dl*SV40, *dl*GRE, and *dl*cfos; see Fig. 1A for the locations of consensus sequences). In addition, two B enhancer variants selected for growth in undifferentiated embryonal carcinoma F9 cell lines (PyF441 and PyF101) were also examined (16, 20). Newborn mice were infected intraperitoneally with these variants and sacrificed at 30 days p.i. The results of this analysis are presented in Fig. 4. Shown is a scatter plot of the level of Py DNA

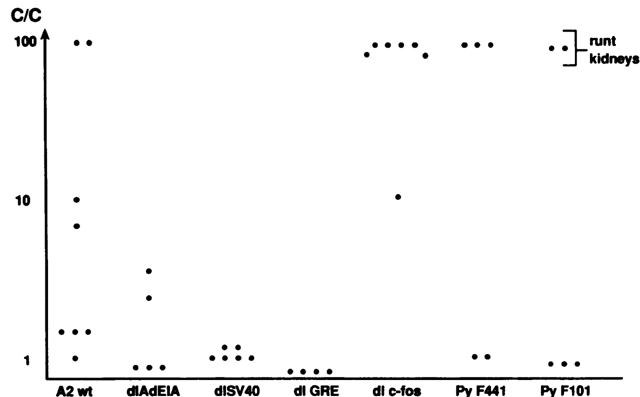

FIG. 4. Scatter plot analysis of viral DNA copy levels in persistently infected kidneys. Each point represents a value obtained from an individual mouse. Viral DNA levels were determined from Southern blot analysis of *Eco*RI-linearized DNA and by comparison with known levels of viral DNA. wt, wild type; c/c, copies of Py DNA per cell.

measured by quantitative DNA blot analysis in the kidneys of individual animals. Kidneys persistently infected with PyA2 were observed to have variable levels of Py DNA. Although some PyA2 DNA was observed in the kidneys of all animals, the levels varied and were clustered in three recognizable groups: a low-level group, with somewhat above one copy per cell; an intermediate-level group, with about 10 copies per cell; and a high-level group, with about 100 copies per cell. The high-level group usually corresponded to a minor fraction of the animals (about one in five). Although only a limited number of animals were examined in this particular experiment, our experience in examining numerous other persistently infected mice is consistent with these variable levels of Py DNA. The possibility that these variable levels reflect distinct or different states of persistence, including reactivation, is considered further below. Some of the enhancer variants examined, however, often showed less variability in the quantities of persistent Py DNA than PyA2. *dl*AdE1A, *dl*SV40, and *dl*GRE all showed low levels of DNA (five or fewer copies per cell). Mice infected with *dl*cfos, PyF441, and PyF101, however, showed different patterns of persistent infection. With these variants (especially *dl*cfos), mice often had elevated levels of Py DNA (100 or more copies per cell). In addition, all persistently infected mice with elevated levels of Py DNA were also runty and had correspondingly small kidneys. This correlation of high levels of Py DNA with runtiness of mice was also seen in mice persistently infected with PyA2, in that the 20% of animals with high levels of DNA likewise were runty. Because runtiness can rapidly follow acute infections (easily visible 7 days after infection) and because at all times p.i. these acutely infected animals have elevated levels of Py DNA (data not shown), it appears that a high-level persistent infection of these runts is not transient but is achieved and maintained soon after an acute infection.

Histological analysis and in situ hybridization of acutely and persistently infected kidneys. To determine whether the differences in the persistent pattern of replication in the kidneys resulted from changes in cell type-specific viral replication within the kidneys, we examined the cellular sites of acute and persistent replication in the kidneys by in situ

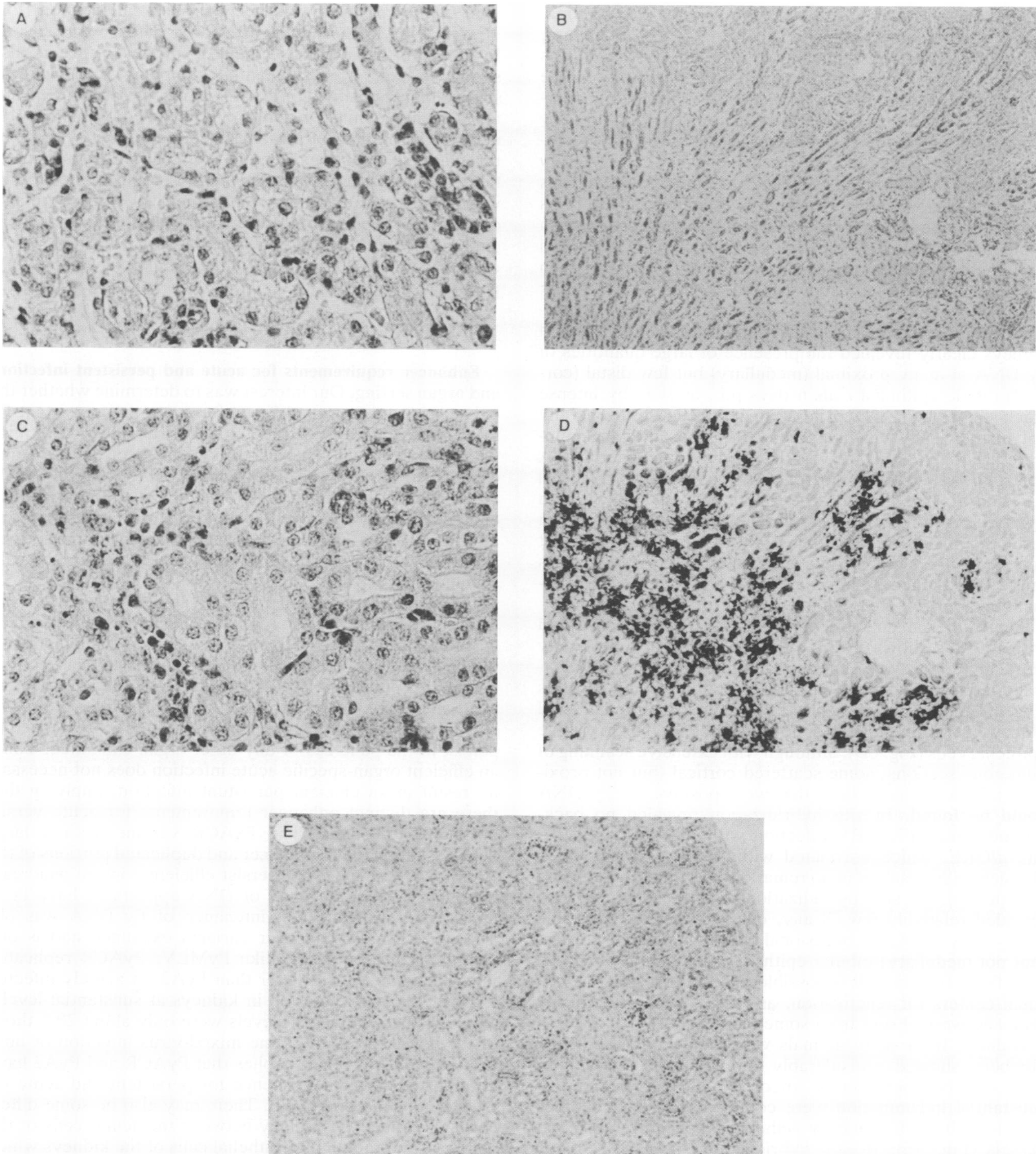

FIG. 5. Histological and in situ hybridization results for kidneys during acute infections. (A) Hematoxylin and eosin staining of a normal uninfected 6-day-old kidney (magnification, ×34). (B) In situ hybridization of the panel A section for the detection of Py DNA; neutral red counterstain (magnification, ×8.5). (C) Hematoxylin and eosin staining of a PyA2-infected 6-day-old kidney (magnification, ×34). (D) In situ hybridization for the detection of Py DNA (black areas) (magnification, ×8.5). (E) Like panel C but with a magnification of ×8.5 to show the general histology of the infected kidneys.

hybridization and also examined the resulting histopathology of Py infection. To detect Py DNA by in situ hybridization, we coupled DNase I-generated fragments of the Py genome (300 to 600 bp) directly to horseradish peroxidase. The hybridized probe was detected by the addition of an enzyme substrate (diaminobenzidine) as described in Materials and Methods. A silver amplification step was also used to allow for more sensitive detection of Py DNA.

As shown in Fig. 5A with hematoxylin and eosin staining, kidneys from a 6-day-old mouse were visibly immature, showing poorly defined tubular organization. This result was as expected, as tubules are not fully developed and tubule epithelial cells often have a large, "active" nuclear appearance. These epithelial cells are the major target of Py replication. In Py-infected kidneys, surprisingly little cytopathology was observed. Infected kidneys were generally unremarkable and looked very much like uninfected controls (compare Fig. 5A and C). In general, little inflammation was seen, although some occasional centers of inflammation could be observed. Some tubular cells with large nuclei containing irregularly condensed chromatin were also seen on occasion (data not shown), but overall little cellular immune reaction, necrosis, hyperplasia, or other cytopathology was apparent (Fig. 5E). In situ hybridization of infected kidneys clearly revealed the presence of large quantities of Py DNA in many proximal (medullary) but few distal (cortical) tubule epithelial cells 6 days p.i., as seen by intense black staining (Fig. 5D). These infected cells were generally not histologically distinguishable from the adjoining uninfected cells. No Py DNA was detected in the glomerulus. A control in situ hybridization of uninfected kidneys (Fig. 5B) revealed no background signal. Infection of kidneys with PyA2 and the other enhancer variants yielded similar in situ results, with the exception of PyMLV, which was not detected in the kidneys (data not shown). The acute phase is thus characterized by intense Py DNA replication in proximal collecting tubules, with relatively little histopathology.

A histological analysis of kidneys persistently infected with PyA2 is shown in Fig. 6. The kidneys from normally sized adult mice were generally unremarkable and pathologically indistinguishable from those from uninfected controls (Fig. 6A and C). In situ hybridization of persistently infected kidneys generally revealed no detectable signal in tubule epithelial cells (Fig. 6D), although with extensive scanning of numerous sections, some scattered cortical (but not proximal) tubule epithelial cells that were positive for Py DNA could be found. In situ hybridization revealed no background signal (Fig. 6B). Infection with *dl*AdE1A, *dl*SV40, and *dl*GRE, which correlated with low-level persistence, also generally resulted in no remarkable cytopathology (data not shown). In situ hybridization of these sections also revealed relatively few, if any, cells containing detectable levels of Py DNA. An occasional cluster of positive cortical (but not medullary) tubular epithelial cells could, however, sometimes be found. It is possible that such clusters may be small centers for reactivation, as they were not uniformly seen and were absent from some kidneys.

Kidneys from runty animals with high viral DNA copy numbers showed considerably more histopathology. In runty animals persistently infected with PyA2, areas of substantial inflammation were common, and it sometimes appeared that inflammatory cells were concentric to blood vessels (Fig. 6E). In situ hybridization of these sections revealed a significant number of foci of tubule epithelial cell nuclei which were highly positive for Py DNA (Fig. 6F). These positive in situ foci, however, often did not correlate with foci of inflammation. The histology of kidneys from runty mice infected with Py variant strains is shown in Fig. 7. For runty mice persistently infected with *dl*cfos (Fig. 7A), PyF441 (Fig. 7C), and PyF101 (Fig. 7E), inflammation was usually more severe than that seen with PyA2. The inflamed areas were often accompanied by substantial cytopathology of cortical tubule epithelial cells, including ballooning nuclei, dispersed and faint chromatin, and even loss of cells (proximal tubule epithelial) and necrosis with calcification (Fig. 7E, PyF101). In these animals, kidney damage was severe. In situ hybridization of kidneys from runty mice is also shown in Fig. 7. A substantial number of cortical cells stained highly positive for Py DNA. In contrast to the results for nonrunty animals, virtually every field had some positive nuclei. The number of positive nuclei, however, was below that in acute infections. Unlike the results for acute infections, however, few, if any, medullary tubule epithelial cells were positive. In addition, some other cell types were also occasionally seen to stain positively (muscle, fat, ganglion; data not shown). These results confirm that changes in the enhancer do not change the kidney cell type specificity of a persistent infection.

DISCUSSION

Enhancer requirements for acute and persistent infections and organ sorting. Our interest was to determine whether the enhancer requirements differed for acute and persistent Py infections of mice. We used a mixed-virus infection of mice with three different strains of Py enhancer variants and established that the organs of mice specifically replicate Py DNA and can sort the viral replicon with the compatible enhancer during both the acute and the persistent phases of a viral infection. These results further establish that organ-specific DNA replication is a major determinant of cell-specific virus replication, as previously proposed (30, 31, 33, 35). Of specific interest was the ability of PyACR and PyMLV (but not PyA2) to replicate efficiently in the neonatal mouse pancreas. Because of this acute pancreas infection, it was possible to determine whether the enhancer requirements for a persistent infection were the same as those for an acute infection. Clearly, PyMLV (with the Moloney murine leukemia virus enhancer in place of the B enhancer) failed to persist in the pancreas or any other organ examined. Thus, an efficient organ-specific acute infection does not necessarily result in an efficient persistent infection, implying that there are distinct enhancer requirements for acute versus persistent infections. The PyACR variant (with a large deletion within the B enhancer and duplicated portions of the A enhancer) also did not persist efficiently in the pancreas, although a very low level of DNA appears to have been maintained. Low-level maintenance of Py DNA was observed with most enhancer variants examined and is discussed further below. Unlike PyMLV, PyACR replicated very efficiently (even better than PyA2) in acutely infected kidneys. PyACR persisted in kidneys at substantial levels, although these persistent levels were only about 20% those of the wild type in the same mixed-virus infection of nonrunty mice. This result implies that PyACR and PyA2 have different enhancer dependence for persistent and acute infections of the same organ. There may also be some differences in cellular physiology between the acinar cells of the pancreas and the tubule epithelial cells of the kidneys which generally inhibit the establishment of a persistent infection, since none of our variants to date have established efficient persistent infections in the pancreas.

Acute infections show little pathology. The histological analysis of acutely infected kidneys revealed surprisingly little pathology in light of the relatively high levels of viral DNA replication. Little inflammation, cellular necrosis, or discernible increases in cellular mitosis were prominent. Some occasional inflammation and nuclear changes were encountered, but these changes seemed to be restricted, in that many more cells were shown by in situ hybridization to

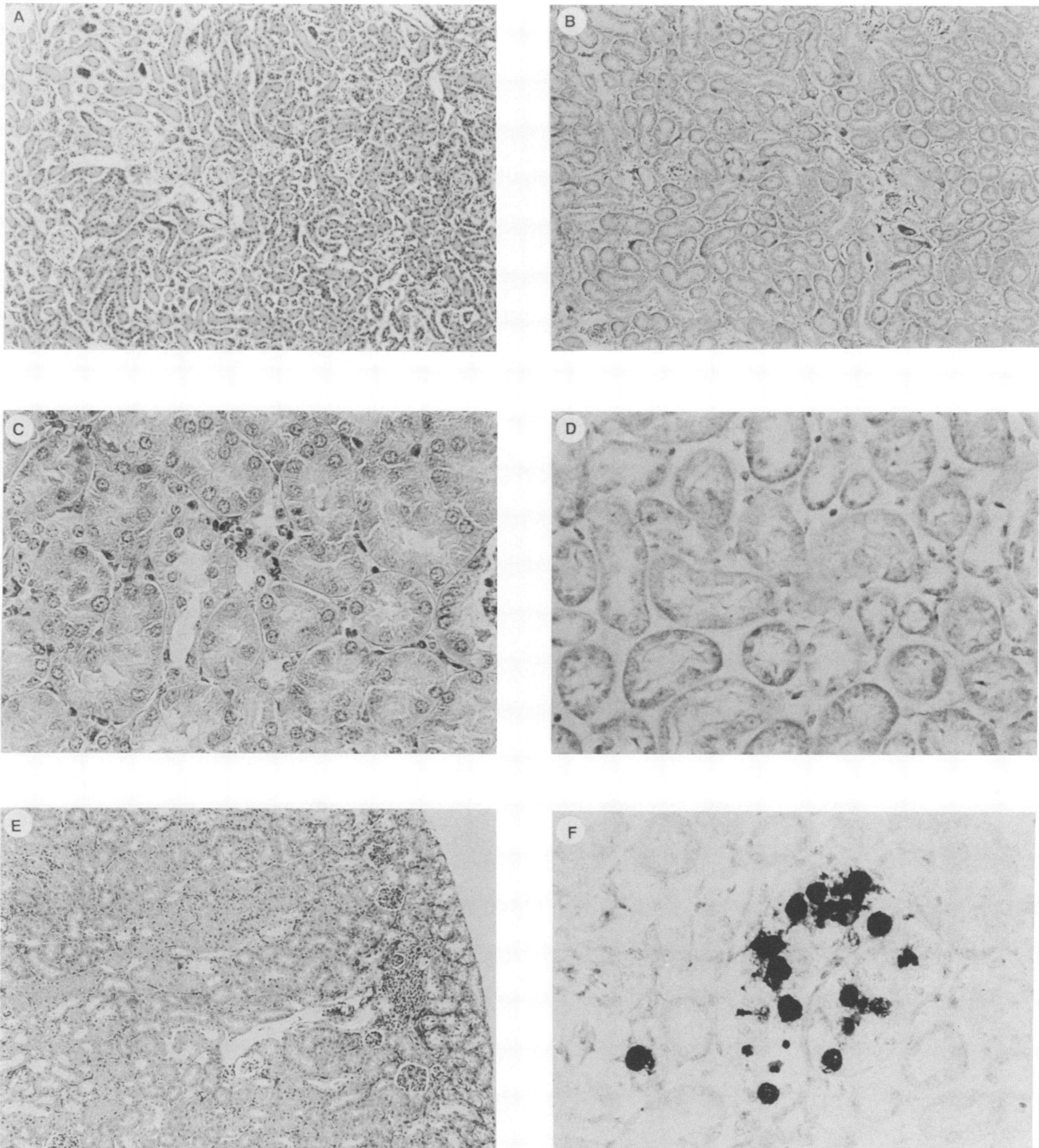

FIG. 6. Histological and in situ hybridization results for adult kidneys during persistent infections with PyA2. (A) Hematoxylin and eosin staining of a normal uninfected adult kidney (magnification, ×8.5). (B) In situ hybridization of an uninfected adult kidney (control). (C) Hematoxylin and eosin staining of a persistently infected nonrunty adult mouse kidney (magnification, ×34). (D) In situ hybridization of the section in panel C (magnification, ×34). (E) Hematoxylin and eosin staining of a kidney from a runty adult mouse persistently infected with PyA2; a magnification of ×8.5 was used to show overall pathology. (F) In situ hybridization of a kidney from a runty adult mouse persistently infected with PyA2; a magnification of ×34 was used to show the details of infected clusters of tubule epithelial cells.

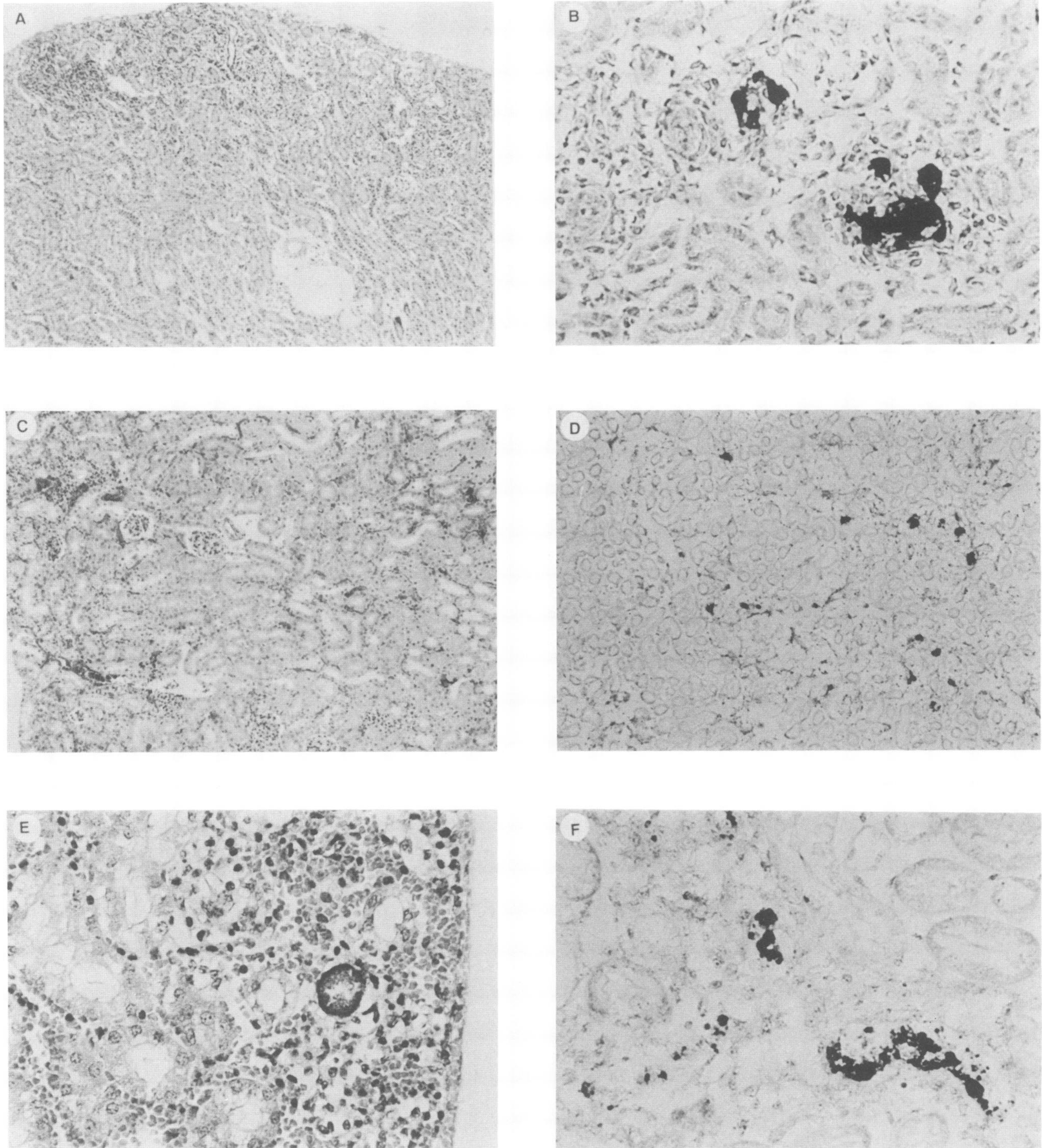

FIG. 7. Histological and in situ hybridization results for kidneys from runty adult mice persistently infected with Py variant strains. (A) Hematoxylin and eosin staining of a kidney from a runty mouse persistently infected with *dl*cfos (magnification, ×8.5); areas of inflammation are just beneath the cortex. (B) In situ hybridization of the panel A section (magnification, ×34). (C) Hematoxylin and eosin staining of a kidney from a runty adult mouse persistently infected with PyF441 (magnification, ×8.5). (D) In situ hybridization of the panel C section (magnification, ×8.5). (E) Hematoxylin and eosin staining of a kidney from a runty adult mouse persistently infected with PyF101. An extensive loss of tubule epithelial cells and an area of calcification (dark circle to the right of center) are apparent (magnification, ×34). (F) In situ hybridization of the panel E section (magnification, ×34).

replicate Py DNA. Also, it was noted that uninfected medullary tubule epithelial cells, which were the main site for Py DNA replication, had a distinctly immature appearance, consistent with reports that these cells actively terminally differentiate and synthesize DNA during the first week after birth (2, 3, 38). In normally sized healthy adult mice, persistently infected kidneys generally showed little pathology as well. An occasional tubule epithelial cell nucleus positive for Py DNA could be found in some but not in most sections. These positive cells were almost always in the cortical tubules, as opposed to the predominantly medullary location of positive cells in acute infections. This result appears to identify an interesting tubule cell type preference between wild-type persistent (i.e., medullary) and wild-type acute (i.e., cortical) infections. We suspect that these positive clusters in persistently infected kidneys may be sites of reactivation and that Py DNA is typically maintained at low levels throughout the kidneys (below the level of sensitivity of in situ hybridization, estimated at more than 10 copies per cell), because all kidneys were positive for Py DNA in Southern analysis, even when no in situ hybridization signal was seen, and cortical tubule epithelial cells are a minority of all the cell types in extracted kidneys. The relative lack of inflammation, necrosis, and cytopathology during most acute and persistent infections makes it difficult to recognize a Py-infected kidney by a simple histopathological examination.

Three distinct levels of persistent infections. The levels of persistent PyA2 DNA in kidneys vary widely and occur in what appear to be three distinct levels: low (about one copy per cell), intermediate (about 10 copies per cell), and high (about 100 copies per cell). High-level persistence correlated 100% with the occurrence of runtiness in mice. Runtiness was always observed to immediately follow acute infections, so it appears that this high-level persistence is established early on and is not due to the reactivation of latent virus. Such an early establishment of persistence may indicate that acute infections are not controlled by the immune response of the runty mice. These runty animals are moribund at 30 days p.i. and generally do not live longer than 2 months. Low-level persistence of Py DNA was observed in almost all animals and in many different tissues with most variants (except PyMLV) of Py examined, indicating that this specific state is readily achieved but may fade with the increasing age of the mice (29a). It is possible that the intermediate levels of persistence (10 copies per cell) which were seen represented authentic reactivation and could correspond to the clustered foci of Py DNA replication occasionally seen by in situ hybridization. If so, then such a state might be reactivation and might not formally be considered a persistent infection. Accordingly, we propose that there may be three different types of viral DNA persistence and that the enhancer variants may differentially affect these three states: (i) a low-level maintenance of episomal DNA, which is often seen but may not necessarily correlate with viral production; (ii) a reactivation from this maintenance state, seen as clusters of positive cells and intermediate levels of viral DNA; and (iii) a newly (here) described state in which a high (almost acute) level of Py DNA is maintained directly after the acute phase for at least 30 days and which is correlated with significant cytopathology and runtiness.

High-level persistent infections. The histological and in situ hybridization analyses of the runty animals established that they generally had significant kidney damage and that high levels of viral DNA were present in a substantial number of tubule epithelial cells and some other cell types. Much of the cellular damage appeared to be due to the inflammatory reaction, as the levels of viral DNA replication were lower than those in an acute infection, in which there was relatively little inflammation or necrosis. The physiological reason for runtiness has not been further examined. It seems likely that the degree of chronic kidney damage is large enough to restrict animal growth. However, other explanations (e.g., hormonal effects) are also possible. How such high-level persistence is achieved, however, is puzzling and raises a dilemma; how can a high-level persistent infection be stably maintained in the presence of a vigorous and damaging cellular immune response? It is possible that high-level persistence is actually an extended acute phase of replication. If so, then why were the acute infection and the resulting high-level persistent infection not cleared? The reactive inflammation seen in runty mice was not apparent in nonrunty, acutely infected mice or in putative centers of reactivation during persistence. In addition, the runty mice replicated Py DNA to high levels even as adults. In normal or athymic adult mouse kidneys, acute Py replication is not observed (1, 8, 27). We have proposed that because newborn kidney cells are actively differentiating, they may generally be more permissive than quiescent adult kidney cells as sites for Py replication (27). If this proposal is correct, then situations which increase cellular differentiation may make the adult kidney more permissive. It seems likely that the inflammation in runty animals kills infected and possibly uninfected tubule cells. Such killing may in turn lead to increased rates of tubule epithelial cell replacement and differentiation, similar to what is seen following ischemic kidney damage (19). This elevated rate of differentiation may in turn be more permissive, as proposed, and result in increased rates of Py replication. Thus, a vigorous cellular immune reaction is associated with an increased permissive cellular environment for Py replication. What then might be the difference between the runty mice and their nonrunty littermates? Although runtiness appeared to be virus induced, the virus, the mouse strain, the time of infection, and the amount and route of inoculation were all the same. Why was there such a different outcome in individual mice? We have no results which bear on this question, but perhaps the individual newborn immune status, such as the induction of tolerance, was involved.

B enhancer requirements and persistent infections. The B enhancer is not specifically needed for efficient in vitro or acute in vivo infection. Viruses with deletions of the B enhancer along with A enhancer duplications or substitutions replicate well during an acute infection of the kidneys and other sites, so one might question the natural function of the B enhancer (31). Our results now imply some B enhancer participation in either reactivation or maintenance of a persistent infection, as mutations within the B enhancer had little effect on acute kidney infections but often had a significant effect on persistent kidney infections. Several enhancer variants (*dl*AdE1A, *dl*SV40, and *dl*GRE) persisted only at very low levels. Thus, the loss of these core sequences appears to negatively affect either the reactivation or the high-level maintenance of persistent infections. Previously, elements within the A and B enhancer sequences were shown not to be required for replication in 3T6 cells and were called redundant, as either the A or the B enhancer alone would suffice for replication (28). Alterations of these sequence elements did, however, alter non-kidney organ specificity in acute infections (31). The two variants (PyF101 and PyF441) selected for growth in undifferentiated embryonal carcinoma cell lines and especially the *dl*cfos mutant

did, in contrast, often cause a high-level persistent infection correlated with runtiness of animals. These variants have in common alterations to B enhancer sequences. It thus appears that Py enhancer requirements for acute and persistent infections are distinct and that the B enhancer is important for the wild-type pattern of persistent infections. This suggestion leads to the question of why there is a difference between the regulatory DNA requirements of acute versus persistent DNA replication in the same cell type. Such a question is not specific to Py and has been examined with the bovine papillomavirus and Epstein-Barr virus systems. It has been reported that the episomal DNA maintenance of bovine papillomavirus (23, 24) and Epstein-Barr virus (18, 26, 34) has requirements for *cis*-acting DNA different from those of high-level DNA replication seen in differentiating keratinocytes (7) and lymphocytes (18), respectively. We recently proposed the existence of two types of DNA replication, cell cycle-regulated and unregulated runaway DNA replication, which could represent persistent and high-level DNA replication, respectively (35). Although we currently have no direct evidence that low-level persistent Py DNA replication is cell cycle regulated (as is bovine papillomavirus and Epstein-Barr virus replication), simian virus 40 origin-directed DNA replication can be cell cycle regulated (6), and arguments in support of this view have been presented for Py (35). These considerations may be relevant to recent observations with JC virus and BK virus. These viruses isolated from persistently infected kidneys have a nonduplicated archetypal enhancer sequence (15, 17, 25). The propagation of these kidney isolates in cultures rapidly leads to the selection of a different enhancer sequence with a deletion of some and a duplication of other control elements. It is possible that BK virus and JC virus and under different enhancer selection conditions in persistent infections than in acute infections, similar to what we observed with Py in vivo, and that the subsequent propagation of these isolates in cultures rapidly selects against the archetypical enhancer present in persistently infected tissue and selects for an enhancer more active for acute infection and high-level DNA replication.

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