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Polyomavirus DNA Replication in the Pancreas and in a Transformed Pancreas Cell Line Has Distinct Enhancer Requirements

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The regulatory DNA (enhancer) of polyomavirus (Py) is a major determinant of tissue-specific DNA replication during acute infection of newborn mice. Previously, we reported that the combination of one of the two Py enhancers (A enhancer) and the repeated Moloney murine leukemia virus (Mo-MuLV) enhancer gave a chimeric Py genome (Py-MuLV) that replicates predominantly in the acinar cells of the pancreas, a tissue not permissive for wild-type PyA2 replication (R. Rochford, B. A. Campbell, and L. P. Villareal, Proc. Nat. Acad. Sci. USA 84:449–453, 1987). In this report, we further examine the combined enhancer requirements for acinar cell-specific Py replication. We also compare enhancer requirements for Py replication in the acinar cells of the pancreas with those of a transformed acinar cell line (266-6 cells). The deletion of sequences within the A enhancer of Py-MuLV (nucleotides 5098 to 5132) results in a virus with 10-fold-reduced levels of pancreas-specific replication. The deletion, however, of one of the 72-bp repeated Mo-MuLV enhancer sequences from Py-MuLV results in a complete loss of pancreas-specific DNA replication. Thus, the Py A enhancer is required for efficient replication of Py in the pancreas without otherwise altering organ specificity, but both of the repeated copies of the Mo-MuLV enhancer are essential for pancreas-specific Py replication. In contrast to the enhancer requirements for in vivo pancreas replication, in transformed acinar cells (266-6), PyA2 wild-type replicated efficiently and the Py-MuLV recombinant replicated inefficiently. These data suggest that the cell-specific control of DNA replication is different between normal pancreas cells and their transformed cell line counterparts and that this difference is apparent in the enhancer requirement of cell-specific Py DNA replication.

Murine polyomavirus (Py) has been used as a model system to study the genetic requirements of regulatory DNA (enhancers) for cell-specific transcription and DNA replication. Py has two enhancers, A and B, found on the late side of the origin of replication (7, 15, 27). Numerous consensus sequences and binding sites for cellular *trans*-acting nuclear factors have been identified within the Py enhancer (for a review, see reference 10). Alterations in enhancer DNA sequences can affect cell-specific viral replication in culture, and they can also affect organ-specific replication in mice during the acute phase of infection (17, 18). This cell type control is generally *cis*-restricted for Py DNA replication and is not due solely to control of early gene transcription (2, 3, 17, 18).

We have previously generated an enhancer variant of Py by replacing the Py B enhancer (nucleotides 5132 to 5269) with the Moloney murine leukemia virus (Mo-MuLV) 72/73-bp repeat enhancer (in either orientation) (17). This variant has a strikingly altered organ specificity and replicates mainly in the acinar cells of the pancreas of mice, cells that are not permissive for either wild-type Py replication or Mo-MuLV replication (9, 17). In addition, this Py-MuLV recombinant did not replicate in the usual target organs for Py replication (kidneys) or Mo-MuLV replication (lymphatic tissues). The orientation independence implies that the combination of the Py A enhancer and the Mo-MuLV enhancer may have been important for high-level acinar cell-specific replication and was not a fortuitous generation of a junction sequence, as proposed by others (14).

Deletion of a single consensus sequence or factor binding site within the enhancer generally has little or no effect on the replication of Py in 3T6 cells; replication in specific mouse organs, however, is often significantly altered and in some cases expanded to include high-level replication in otherwise nonpermissive tissues (18). In this situation, the in vivo results were not predicted by the in vitro replication results. Although primary and transformed kidney fibroblasts are often used to grow Py in culture, these kidney-derived fibroblasts bear no resemblance in cell type to the normal tubule epithelial cell targets for Py replication in vivo (5, 16a). Comparisons of cell-specific Py replication in normal tissues with replication in their transformed-cell counterparts have not been made. Given the major differences in the control of cell replication which accompanies transformation, this comparison would seem worthwhile. Others have recently established a transformed acinarlike cell line which expresses many acinar-specific genes, albeit at lower levels than normal acinar cells do (16). Because infection in vivo with Py-MuLV is acinar cell type specific, we can now examine the enhancer-dependent cell type control of Py DNA replication in normal tissue and compare it with its precise transformed-cell counterpart.

In this report, we provide further evidence that it is the modular combination of the Py A enhancer and the Mo-MuLV enhancer that determines in vivo viral tissue-specific replication in the pancreas. In addition, we compare Py replication in acinar cells in mice with Py replication in an acinar-derived transformed cell line. We show that although

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Py must combine with the Mo-MuLV enhancer for high-level viral replication in the acinar cells in the mouse, wild-type virus without the Mo-MuLV enhancer can replicate at high levels in acinar cells in culture, indicating that control of replication in a tumor cell line is altered relative to its differentiated, but nontumor, in vivo counterpart.

Recombinant Py viruses with additional enhancer alterations were made to further examine the combinatorial requirements for pancreas-specific replication in vivo. The necessity of the Py A enhancer and also the requirement of two copies of the Mo-MuLV 72/73-bp repeat were determined by deleting the appropriate sequences as described in the legend to Fig. 1. In all experiments, BALB/c mice less than 24 h old were inoculated intraperitoneally with approximately 10^8 PFU of the appropriate virus. Mice were sacrificed at 6 days postinfection, and DNA from organs was extracted as previously described (6). Two to four litters were infected with each mutant virus. Ten micrograms of total organ DNA was digested with *Eco*RI restriction enzyme, fractionated by electrophoresis on a 1% agarose gel, and analyzed by DNA blot hybridization with a ^{32}P-labeled, nick-translated Py probe as previously described (6). Quantitative determinations of viral genome copies per cell were made and are shown in Table 1. These values were obtained by densitometer scanning of the properly exposed autoradiograms and comparison with the reconstructed-copy-per-cell control lanes. A representative autoradiogram from a single mouse is shown in Fig. 2.

To determine whether both copies of the Mo-MuLV enhancer are required, a recombinant virus that deleted one copy of the Mo-MuLV enhancer repeats [Py-MuLV(*Pvu*II)] was made. Replication of this virus in the pancreas was reduced 40-fold in mice infected with Py-MuLV(*Pvu*II) relative to the level of replication in mice infected with Py-MuLV, which contains both copies of the Mo-MuLV enhancer (Table 1). This level of pancreas-specific replication is equivalent to that seen with a deletion of the B enhancer alone (PyA/dlB) (Table 1), which contains no Mo-MuLV enhancer (17). Thus, it appears that both copies of the Mo-MuLV 72/73-bp repeat are needed for viral tropism to the pancreas.

A region within the Mo-MuLV enhancer reportedly has a degree of homology (16 of 20 nucleotides) to a putative 20-bp consensus sequence found in the regulatory regions of acinar-specific genes (11, 20). The biological significance of this sequence is unknown, especially since Mo-MuLV does not replicate in the pancreas. As this sequence is believed to be required for acinar-specific transcription, it is possible that it may contribute to the pancreotropism we observe with Py-MuLV. Digestion of the Mo-MuLV enhancer with *Pvu*II, however, disrupts this acinar consensus sequence. As it was possible that decreased replication in the pancreas of Py-MuLV(*Pvu*II) was due to inactivation of this sequence rather than to a reduction of the Mo-MuLV enhancer to a single copy, we made another enhancer recombinant virus [Py-MuLV(*Hpa*II)] that contains a single copy of the Mo-MuLV enhancer by using an *Hpa*II restriction site within the direct repeats to generate a single copy of the enhancer. Such a recombinant contains the putative consensus sequence in the single copy of the Moloney enhancer repeats (Fig. 1). After infection of newborn mice with Py-MuLV(*Hpa*II), patterns of replication similar to those of Py-MuLV(*Pvu*II) were observed at 6 days postinfection, suggesting that it is the reduction to a single copy of the Mo-MuLV enhancer that results in lowered viral replication in the pancreas and not inactivation of this acinar consensus site (Fig. 2). In both

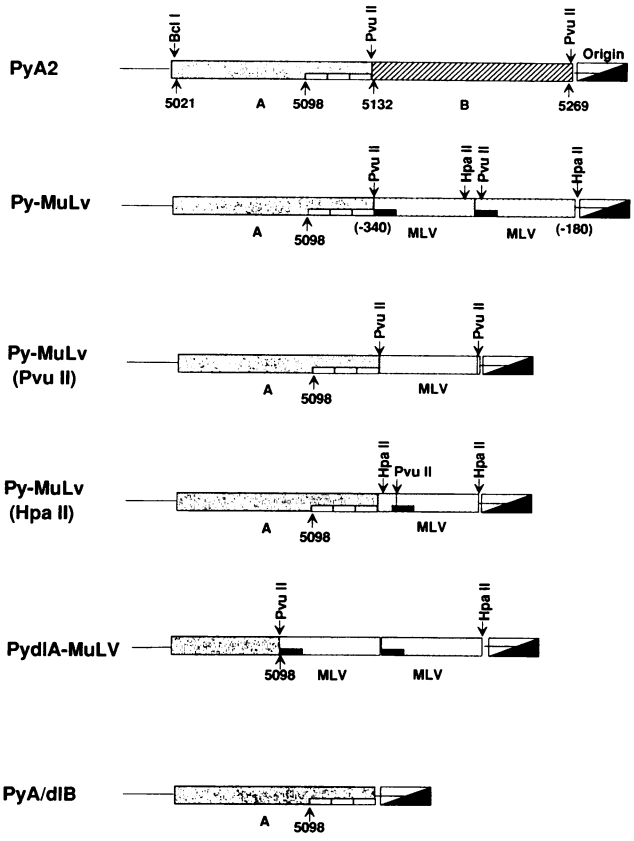

FIG. 1. Structures of enhancer regions of PyA2 (wild-type) and recombinant viruses. Sequence numbers for Py are from GenBank version 64. The locations of the A and B enhancer elements are indicated by shaded (A enhancer) or hatched (B enhancer) boxes. Mo-MuLV sequence numbers are shown in parentheses and correspond to the sequence numbers used for pMo-CAT (4), which was a generous gift of R. Hanecak and H. Fan; the Mo-MuLV enhancer is indicated by stippled boxes. Construction of the Py-MuLV enhancer recombinant was previously described (17). The Mo-MuLV 72/73-bp enhancer was purified from pMo-CAT and cloned into a unique *Xho*I restriction site of pPydl5098-5269 to generate the viral recombinant PydlA-MuLV. pPydl5098-5269 contains the Py genome cloned into the *Eco*RI site of pIBI-31 (International Biotechnologies, Inc.), but the enhancer is deleted (nucleotides 5098 to 5269) and replaced with a unique *Xho*I restriction site. To generate a single copy of the Mo-MuLV enhancer repeats, the Mo-MuLV enhancer cloned into pMo-CAT was excised and digested with *Pvu*II or *Hpa*II restriction enzymes. The corresponding fragment was purified and cloned into pPyA/dlB, which contains the entire Py genome with a deleted B enhancer (nucleotides 5132 to 5269) (17). The enhancer region of the recombinant virus strains was sequenced by the dideoxy method (19) with a primer complementary to Py nucleotides 5041 to 5061. The small white boxes within the A enhancer indicate the functionally important factor binding sites (13, 22), and the small black box within the Mo-MuLV enhancer indicates the acinar consensus sequence.

Py-MuLV(*Pvu*II) and Py-MuLV(*Hpa*II), partial digestion of pancreas DNA with *Eco*RI resulted in three bands in the autoradiogram corresponding to the relaxed, linear, and supercoiled forms of the genome. These are indicated in Fig. 2.

Unlike the original Py-MuLV recombinant, in which no replication in the kidneys was observed, these single Mo-MuLV enhancer recombinants gave lowered but clear levels

TABLE 1. Recombinant Py DNA replication

Virus	Copies/cell[a]			
	3T6	266-6	Mouse pancreas	Mouse kidney
PyA2	10,000	10,000	<5	1,000
Py-MuLV	5,000	200	2,000	<5
Py-MuLV(*Pvu*II)	10,000	5,000	50	300
PydlA-MuLV	5,000	200	200	10
PyA/dlB	5,000	<5	50	50

[a] The copies per cell shown are averages from at least two separate experiments (varied by ±200, except for PyA/dlB in 266-6 cells, PyA2 in mouse pancreas cells, and Py-MuLV in mouse kidney cells) and were obtained by densitometer scanning of the autoradiograms.

of replication in kidneys. This kidney replication was about 30% of wild-type Py levels (Table 1 [18]). It is possible that the two Mo-MuLV enhancer repeats repress Py replication in the kidneys and other tissues and that their deletion alleviates this repression. The apparent alleviation of repressed replication in specific tissues due to enhancer deletions has been previously observed by us (18). It should be noted, however, that Py-MuLV is the only recombinant virus we have tested to date that replicates predominantly in a single organ (the pancreas) and is restricted in most other organs. Although we expected replication in lymphatic tissues due to the Mo-MuLV enhancer, it was not observed, nor was it observed in any of the recombinant Py-MuLV viruses (Fig. 2). Lymphatic replication can, however, be seen with other unrelated Py enhancer variants (12), which establishes that lymphatic tissue can be a permissive tissue and further implies that the recombinant enhancer was responsible for the pancreotropism.

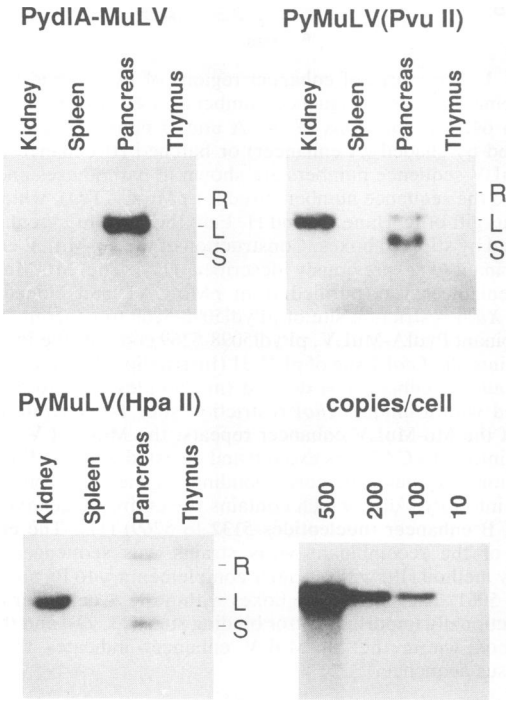

FIG. 2. In vivo replication of enhancer recombinant strains. Mice were sacrificed at 6 days postinfection, and DNA was extracted from the organs indicated. Ten micrograms of organ DNA was digested with *Eco*RI and analyzed by DNA blot hybridization with a Py-specific ^{32}P-labeled probe. Organ-specific replication of the enhancer recombinants PydlA-MuLV, Py-MuLV(*Pvu*II), and Py-MuLV(*Hpa*II) is shown. The genetic structures of their enhancer regions are shown in Fig. 1. Copy-per-cell control lanes are also shown. R, L, and S, Relaxed, linear, and supercoiled forms of the Py genome, respectively. Autoradiograms were exposed for 24 h.

To examine the contribution of the Py A enhancer of Py-MuLV to pancreas-specific replication, we deleted the Py enhancer from nucleotides 5098 to 5269 and inserted the Mo-MuLV enhancer (repeat) in its place to generate the recombinant virus PydlA-MuLV (Fig. 1). This results in a Py virus with only full (repeated) Mo-MuLV enhancer and deletes all of the known factor binding sites within the Py A and B enhancers with established functional effects (10). Newborn mice were injected intraperitoneally with PydlA-MuLV, and levels of viral DNA at 6 days postinfection were measured by Southern blot analysis. A representative autoradiogram is shown in Fig. 2. Although this virus replicated in the pancreas, the levels were reduced 10-fold relative to the levels of Py-MuLV, which contains the Py A enhancer (Fig. 2) (Table 1). In addition, a very low level of replication was observed in the kidneys but not in the spleen or thymus (Fig. 2). Thus, viral replication was observed in the pancreas without the A enhancer present, but high-level replication appears to also require the A enhancer. These data suggest that Mo-MuLV enhancer alone may confer a pancreas specificity but that the adjacent Py A enhancer synergistically stimulates this replication.

To determine whether the acinar cell-specific, enhancer-dependent Py DNA replication we observed in vivo was also seen in transformed acinar cells, we obtained a mouse acinar cell line (266-6) derived from pancreatic neoplasms of mice transgenic for an elastase I promoter-simian virus 40 (SV40) large T antigen (T-Ag) fusion gene (16). Levels of viral DNA replication in 266-6 cells transfected with wild-type PyA2 and Py-MuLV, PydlA-MuLV, PyA/dlB, and Py-MuLV (*Pvu*II) recombinant DNAs were measured by the DEAE-dextran chloroquine transfection method as previously described (1). Nuclear DNA was isolated after 48 h, and the level of DNA replication was determined by restriction digestion with *Dpn*I (which cuts the unreplicated, methylated DNA from *Escherichia coli*) and single-cutting *Eco*RI (which cuts the newly replicated DNA), followed by DNA blot hybridization and densitometer analysis of the resulting autoradiogram as described previously (1). The transfection experiments were repeated at least twice with different DNA preparations. The results from these experiments are shown in Table 1. A representative DNA replication analysis is shown in Fig. 3.

Both wild-type PyA2 and Py-MuLV(*Pvu*II) replicated efficiently in transformed acinar cells. In contrast, PyA2 did not replicate at all in the acinar cells in vivo and Py-MuLV(*Pvu*II) replicated inefficiently in the acinar cells in vivo. In addition, both Py-MuLV and PydlA-MuLV, which were pancreotropic in vivo, replicated poorly in transformed 266-6 cells. Replication of PyA/dlB in 266-6 cells was barely detectable. It is clear that the enhancer requirements for Py DNA replication in the pancreas differ significantly from those for Py DNA replication in transformed acinar cells. There appears to be almost an inverse relationship between pancreas-specific, enhancer-driven Py DNA replication in vivo and that in transformed acinar cells. The Py-MuLV (*Pvu*II) enhancer clearly stimulated replication in 266-6 cells. Furthermore, the combination of the Py A enhancer and a single copy of the Mo-MuLV enhancer did not repress Py replication in these cells. This suggests that the simple

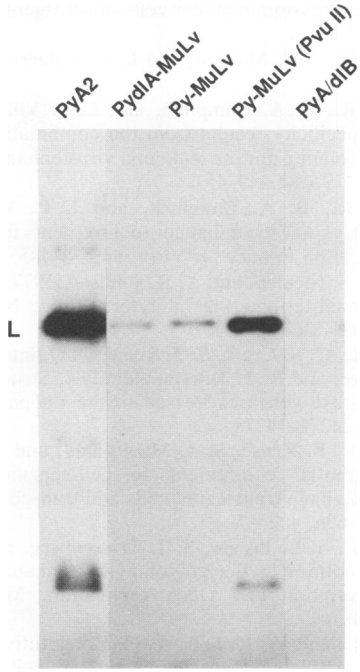

FIG. 3. Replication of enhancer recombinant strains in acinar 266-6 cells. Before transfection, bacterially cloned Py genomes were excised from plasmid sequences by digestion with *Eco*RI, purified by electrophoresis in SeaPlaque (FMC Corp., Marine Colloids Div.) agarose gels, and ligated into circular forms with T4 DNA ligase. Viral DNA (2 μg) was transfected by the DEAE-chloroquine method as described previously (1). Nuclear DNA was extracted 48 h after transfection by the method of Hirt (8), and 10 μg was digested with both *Dpn*I and *Eco*RI. 266-6 cells were transfected with the Py strain indicated and analyzed on a 1% agarose gel. L, *Eco*RI-linearized replicated band. Autoradiograms were exposed for 24 h.

presence of a repressive factor which binds to the Moloney enhancer would not account for the different genetic requirements for Py replication in transformed versus normal pancreas cells.

It is possible and perhaps likely that the expression of the SV40 T-Ag in the 266-6 cells is a major factor in the alteration of the cell specificity of Py replication that we observed. Whether SV40 T-Ag is acting directly or indirectly as a consequence of the transformed 266-6 cell state on Py DNA replication has not been established. Although SV40 T-Ag does not support Py DNA replication, its expression appears to allow Py DNA to replicate in normally nonpermissive monkey cells (23), but this effect appears to be indirect, and Py T-Ag expression is still required to drive Py DNA replication. It seems possible that the transcriptional regulation of Py T-Ag production may be involved in the cell-specific replication of Py DNA by limiting T-Ag-directed DNA synthesis. Because we have previously shown, by using mixed virus infections in vivo (PyA2 and Py-MuLV), that enhancer-dependent pancreas specificity is restricted in *cis* for DNA replication and is not restricted by limiting the production of Py T-Ag, it is unlikely that transcription control of Py T-Ag production is the primary determinant of our current observations (17).

Although normal and transformed (266-6) acinar cells both express acinar-specific genes (i.e., elastase I and other mRNA), 266-6 cells express much-reduced (about 5%) quantities of these genes (16). Thus, there are clear quantitative differences in gene expression between transformed and normal pancreas cells. Studies of enhancer-binding proteins made from pancreatic acinar cells and from 266-6 cells, however, have shown no differences in the activities of factors which bind to a chymotrypsin enhancer (14). Thus, these results do not explain the significant quantitative differences in acinar-specific gene expression. Swift et al. (21) have proposed that there is a simpler genetic requirement for high-level elastase transcription in acinar cells of mice than in 266-6 cells. They suggest that the absence of a *trans*-acting factor in the 266-6 cells, rather than a reduction in the amount of one or more *trans*-acting factors, contributes to the observed difference in genetic requirements for elastase-specific expression between acinar cells in mice and transformed acinar cells in culture. This suggestion seems inconsistent with the low level of pancreas-specific replication that we observed with the Py-MuLV(*Hpa*II) recombinant compared with the high level of replication with Py-MuLV (two copies of MuLV repeat), as both genomes contain the putative acinar consensus sequence. There appear, therefore, to be significant qualitative and not just quantitative differences in the enhancer-dependent control of Py DNA replication between normal and transformed acinar cells. It is possible that the control of transcription by *cis*-acting DNA is distinct from the control of DNA replication in acinar cells even though both processes are enhancer specific and are regulated by similar genetic elements. The uncoupling of enhancer-dependent transcription from DNA replication has been previously observed by us; thus, transcription and DNA replication can be differentially regulated (3). The possibility that acinar cell-specific control of DNA replication may be involved in gene expression was not considered.

The fact that cell-specific Py DNA replication was markedly different between these two cell states could indicate that transformed cells have generally aberrant DNA replication control. In acinar cells in vivo, the rate of [^{3}H]thymidine uptake indicates a very low rate of DNA synthesis, with an estimated cell doubling time of 7 years (25, 26), yet these cells can support high-level, enhancer-dependent Py DNA replication in newborn animals but not with wild-type viral enhancers. The transformed acinar cells, however, are very active for cell division and efficiently amplify wild-type Py DNA but not the pancreotropic Py recombinants. Thus, these transformed acinar cells appear to have aberrant control of enhancer-dependent Py DNA replication. It is possible that an aberrant control of DNA replication generally exists in transformed cells. Such a view is suggested by a marked increase in the ability of various transformed cells, but not of their normal untransformed precursors, to amplify various cellular, drug-resistant DNAs (24, 28).

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