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Host Species Specificity of Polyomavirus DNA Replication Is Not Altered by Simian Virus 40 72-Base-Pair Repeats

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The simian virus 40 72-base-pair repeats substituted for the polyomavirus enhancer, allowing replication and transcription in mouse 3T6 but not monkey CV-1 cells. A polyomavirus genome containing the entire simian virus 40 control region replicated at low levels in 3T6 and CV-1 cells; however, transcripts were detected only in 3T6 cells. Our results suggest that the simian virus 40 72-base-pair repeats are unable to alter the host species specificity of the complete polyomavirus genome.

Polyomavirus sequences from position 5021 to 5262 are required in *cis* for early gene expression and DNA replication (2, 4, 9, 14, 18). This genetic element is designated the enhancer because it allows transcription of heterologous genes regardless of position or orientation (3, 12, 22). Similar functions are ascribed to the 72-base-pair (bp) repeated sequences contained within the origin region of simian virus 40 (SV40) DNA (2, 4, 20, 22). It has been proposed that the enhancers of SV40 and polyomavirus may enable these viruses to replicate specifically in primate and mouse cell lines, respectively (1, 2, 10, 22). However, in past experiments (1, 2, 4, 10), the SV40 or polyomavirus enhancer has been placed into a recombinant plasmid carrying a heterologous gene, and the levels of expression have been measured after transfection. Under these conditions, the papovavirus enhancer is in a heterologous genetic environment completely removed from the natural background of the virus genome. Although these observations are significant, these experiments may not permit general conclusions concerning the hierarchy of the efficiency of the enhancer elements in determining virus host species specificity due to the heterologous nature of the recombinants used. Nevertheless, there is compelling evidence of a role for enhancer elements in the tissue (as opposed to species) specificity exhibited by polyomavirus. Polyomavirus is unable to productively infect undifferentiated murine embryonal carcinoma cells (5, 6, 12). Polyomavirus mutants that infect murine embryonal carcinoma cells have sequence modifications within the enhancer (5), suggesting that tissue specificity of polyomavirus is at the level of enhancer function.

We examined the host species specificity (for replication and transcription) of two polyomavirus genomes that contained different versions of the SV40 enhancer. Unlike the results with heterologous genes (2), the SV40 72-bp repeats substituted to allow polyomavirus DNA replication and early gene transcription in murine 3T6 cells, but not in primate cells.

Polyomavirus genomes (Fig. 1) were generously provided as plasmid recombinants by Robert Kamen (4, 18). Viral DNA was separated from bacterial sequences (15) and self ligated before transfection with DEAE-dextran and chloroquine phosphate (13). Approximately 40 to 48 h after transfection, low-molecular-weight DNAs were extracted from the nuclear pellet with sodium dodecyl sulfate and sodium chloride (8). Replicated polyomavirus DNA was

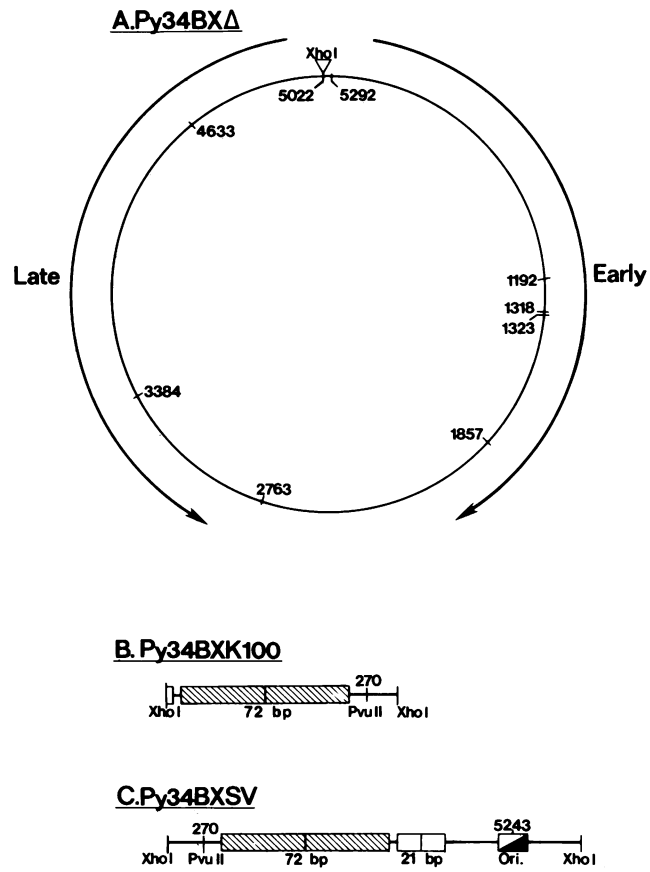

FIG. 1. Structure of polyomavirus genomes containing SV40 enhancer sequences. (A) The circular map of the polyomavirus enhancer deletion (Py34BXΔ) is shown; the positions of the *Mbo*I cleavage sites are given under the A2 numbering system (16). The polyomavirus origin of replication is at position 5292, and the direction of early and late transcription is shown by arrows. The normal polyomavirus enhancer sequences have been deleted as a 241-bp fragment stretching from the *Bcl*I site at position 5021 to the *Pvu*II site at 5262. An *Xho*I linker is inserted in place of the deleted sequences at polyomavirus position 5021 (2, 4, 18). Two derivatives of the SV40 enhancer were inserted into the polyomavirus genome at the unique *Xho*I site. (B) The Py34BXK100 construct contains a 200-bp fragment of SV40 stretching from position 100 to 300 of the SV40 sequence (4), and encodes the 72-bp repeats (72 bp). A *Pvu*II site at position 270 of the SV40 sequence is shown to orient the fragment. (C) The Py34BXSV genome contains a 366-bp fragment of

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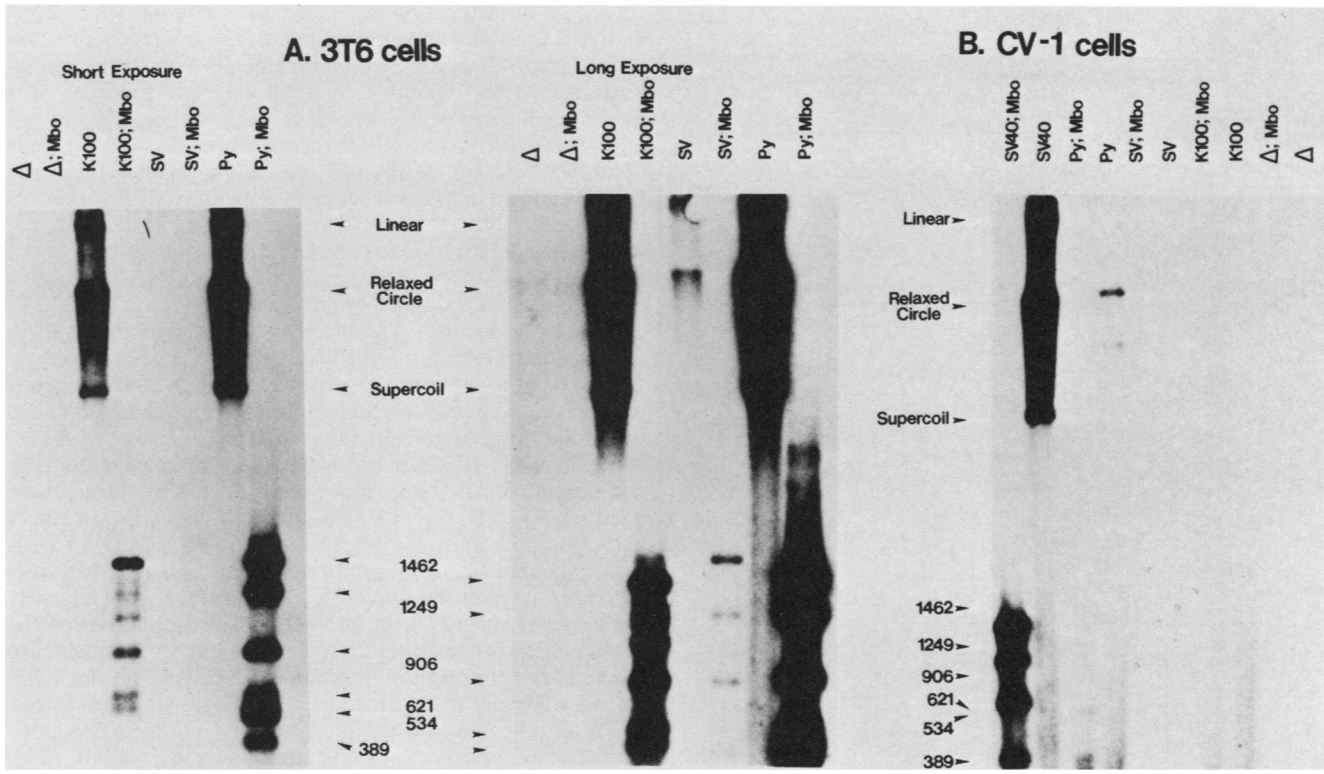

FIG. 2. DNA blot hybridization of nuclear Hirt extracts prepared from 3T6 cells and CV-1 cells transfected with polyomavirus genomes containing SV40 enhancers. (A) Mouse 3T6 cells were transfected with polyomavirus genomes lacking normal enhancer sequences (△); containing the SV40 72-bp repeats (K100); containing the SV40 72-bp repeats, 21-bp repeats, and origin of replication (SV); and with wild-type polyomavirus as a positive control (Py). (B) Monkey CV-1 cells were transfected with the same polyomavirus DNAs, and with SV40 DNA as a positive control (SV40). DNAs were isolated from cell nuclei, and 5-μg samples were analyzed after *Mbo*I digestion (Mbo) and without digestion by electrophoresis in 1% agarose gels. The rationale for this assay is that *Mbo*I does not cleave methylated DNA isolated from *dam*$^+$ bacteria; however, after replication in transfected mammalian cells the DNA becomes sensitive to digestion (2, 4, 18). In this experiment, lanes containing DNA from CV-1 cells (B) transfected with SV40 DNA were cut from the nitrocellulose after transfer and probed with nick-translated, ^{32}P-labeled SV40 DNA cloned into the *Bam*HI site of pBR322. Autoradiography was for 2 days (A, short exposure; B) or 8 days (A, long exposure) in the presence of a DuPont Cronex Lightning Plus intensifying screen. The positions of the linear, relaxed circular, and supercoiled forms of viral DNA are indicated, with the sizes of the polyomavirus A2 *Mbo*I digestion products in bp.

detected after digestion with endonuclease *Mbo*I (New England Biolabs, Inc., Beverly, Mass.), agarose gel electrophoresis, and transfer to nitrocellulose membranes (17).

Deletion of polyomavirus enhancer sequences resulted in a nonviable genome that did not replicate to detectable levels (Fig. 2A and BΔ). In the experiment shown, no polyomavirus DNA was detected in nuclear extracts prepared from cells transfected with the enhancer deletion. This is consistent with observations showing that sequences on the late side of the polyomavirus origin of replication are essential for DNA replication (2, 4–6, 9, 18).

The SV40 72-bp repeats substituted for the normal enhancer to allow polyomavirus DNA replication in 3T6 cells (Fig. 2A, K100). Densitometry scanning of a short autoradiographic exposure indicated that the SV40 72-bp repeats allowed replication to levels ca. 35% of those of wild-type polyomavirus DNA (Fig. 2A, Py). Endonuclease *Mbo*I digested these DNAs to completion, suggesting that replicated DNA was the predominant species in these extracts with little transfection inoculum remaining. We consistently observed that the SV40 72-bp repeats substituted in either orientation for the normal enhancer to allow replication of polyomavirus DNA in 3T6 cells (data not shown). In contrast to a published report (2), we have not observed the SV40 72-bp repeats to allow polyomavirus replication in CV-1 cells (Fig. 2B, K100) or human HeLa cells (data not shown). The lack of replication was not explainable by some difficulty in the transfection protocol or by the physiological state of the CV-1 cells, since SV40 DNA transfected under identical conditions replicated to high levels in these cells (Fig. 2B, SV40). We conclude that although the SV40 72-bp repeats substituted for the polyomavirus enhancer to allow DNA replication in 3T6 cells, the SV40 enhancer cannot change the species restriction and allow replication in primate cells.

Low levels (2% of wild type) of polyomavirus DNA replication were observed in 3T6 cells transfected with the Py34BXSV genome (Fig. 2A, SV). The replication of this DNA in 3T6 cells may be inhibited by elements contained within the SV40 control region, since the SV40 fragment in the opposite orientation allowed replication to levels ob-

SV40 DNA stretching from position 5172 to 300 of the SV40 sequence. This portion of the SV40 genome encodes the SV40 origin of replication (Ori), 21-bp repeats (21 bp), and the 72-bp repeats (72 bp). The orientation of this element is such that the SV40 origin and polyomavirus origin (position 5292) are juxtaposed; in the Py34BXK100 polyomavirus genome, the SV40 72-bp repeats are in the opposite orientation.

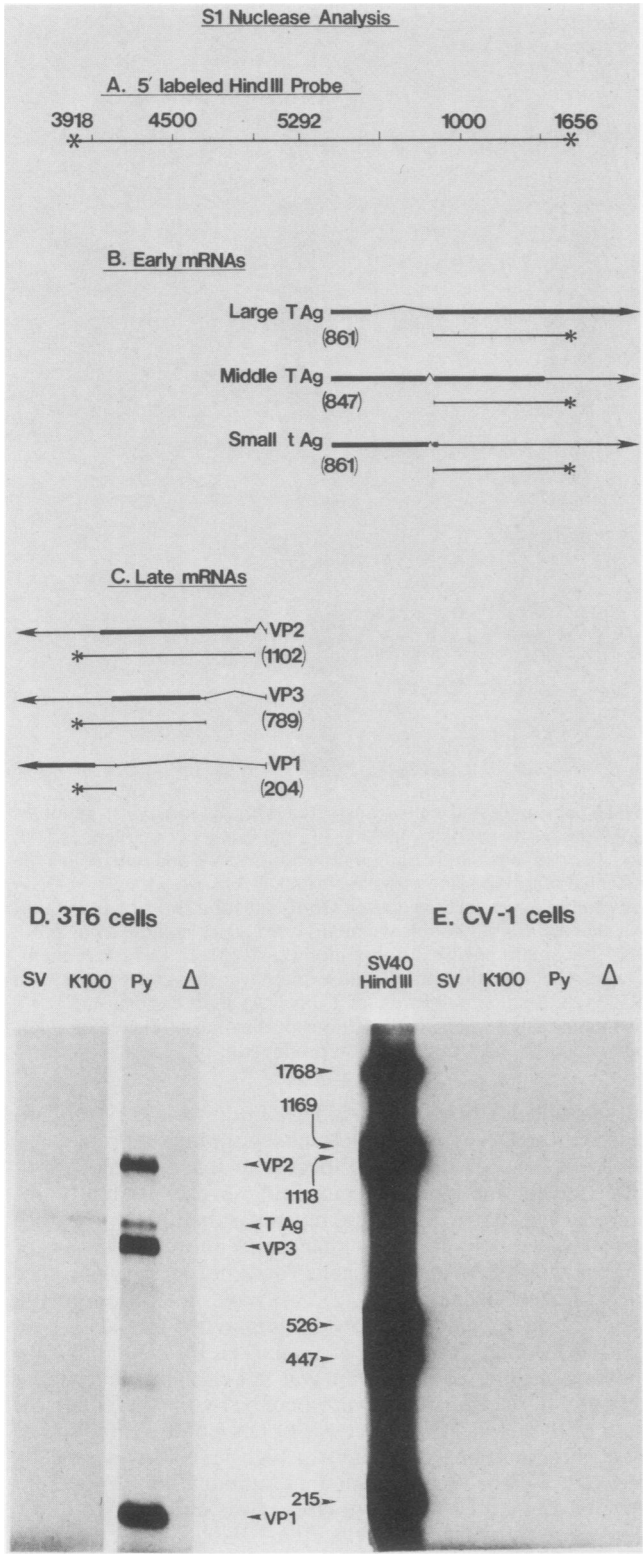

FIG. 3. Detection of polyomavirus RNAs in cytoplasmic extracts prepared from 3T6 cells and CV-1 cells transfected with polyomavirus genomes containing SV40 enhancers. (A to C) Strategy for S1 nuclease analysis of polyomavirus RNAs. (A) The nucleotide coordinates of the 3,023-bp polyomavirus *Hin*dIII-A fragment used as probe to analyze the cytoplasmic RNAs are from the sequence for the A2 strain (16). (B and C) Structures of the spliced regions of polyomavirus mRNAs and the anticipated sizes of the probe fragments protected by the transcripts from (B) the early region and (C) the late region. Heavy lines represent protein coding sequences within the messages; medium lines show noncoding regions. Intervening sequences removed during RNA processing are shown by light carets. The probe fragment protected from S1 nuclease digestion by a given RNA is shown immediately below the respective transcript, and the predicted size of the fragment in nucleotides is in parentheses. (D and E) Detection of polyomavirus specific RNAs in cytoplasmic extracts prepared from transfected 3T6 (D) and CV-1 (E) cells. Cells were transfected with polyomavirus genomes lacking normal enhancer sequences (Δ); containing the SV40 72-bp repeats (K100); containing the SV40 72-bp repeats, 21-bp repeats, and origin of replication (SV); or with wild-type polyomavirus as a positive control (Py). Autoradiography was for 7 days. Sizes (in nucleotides) of the 5' labeled SV40 *Hin*dIII markers are shown. In addition, the positions of the probe fragments protected by polyomavirus transcripts from the early region (T antigens) and late region (VP1, VP2, and VP3) are indicated.

served with the 72-bp repeats (data not shown). Long autoradiographic exposure revealed that low levels of replication occurred in CV-1 cells transfected with the polyomavirus Py34BXSV genome; in addition, this polyomavirus mutant replicated in the presence of SV40 large T antigen after transfection into SV40 transformed COS-1 (7) cells (data not shown). Although replication of polyomavirus DNA occurred in primate cells, levels were very low relative to 3T6 cells. Nevertheless, these results suggested that the host species restriction for replication may lie at the level of the replication origin, since polyomavirus DNA containing both the normal and SV40 origins replicated in CV-1 and 3T6 cells. *Mbo*I-sensitive polyomavirus DNA was detected in CV-1 cells transfected with wild type DNA (Fig. 2B, Py). However, this was probably not replicated DNA, since it was present in low levels and the transfection inoculum used was unmethylated and sensitive to *Mbo*I.

Cytoplasmic RNAs were isolated from transfected cells and polyomavirus transcripts were detected by S1 nuclease analysis (19, 21; Fig. 3A to C). Deletion of the enhancer abolishes polyomavirus transcription in 3T6 or CV-1 cells (Fig. 3D and E, Δ), confirming that the polyomavirus enhancer is required for both DNA replication and early gene expression (2, 4, 6, 14, 18). Either derivative of the SV40 enhancer substituted for the polyomavirus enhancer to allow early transcription in mouse 3T6 cells (Fig. 3D, K100 and SV). Densitometry tracings of the autoradiogram shown in Fig. 3 revealed that the level of early mRNAs produced from the polyomavirus DNA containing the SV40 72-bp repeats was 60% of wild type, whereas the level of early transcripts produced from the polyomavirus PyBXSV genome was 25% of wild type. These results suggested that the transcriptional ability of the mutant genomes was quite good, since (as shown above) relative to wild type, the levels of DNA replication for the mutant genomes were much lower. No late transcripts were obtained from 3T6 cells transfected with the polyomavirus genomes containing SV40 enhancers (Fig. 3D, K100 and SV). It is likely that this can be attributed to deletion of sequences comprising the polyomavirus late promoter (5' cap sites, TATA box, and late leader sequence [16]). CV-1 cells transfected with polyomavirus DNA or genomes containing SV40 enhancers did not express detectable levels of mRNA (Fig. 3E).

Our results show that the SV40 72-bp repeats will substitute for the normal enhancer to restore DNA replication and early gene expression in mouse 3T6 cells. When present as components of recombinant plasmids, the enhancers of

SV40 and polyomavirus will promote expression of heterologous genes after transfection into primate and murine cell lines, respectively (1–3, 9, 10, 12, 18, 20, 22). A popular interpretation is that enhancers may control the host range of the virus which is restricted via genetic mechanisms. Several other published studies (10, 11, 22), however, counter this notion. The enhancer from the long terminal repeat of cloned Moloney murine sarcoma virus will substitute for the 72-bp repeats to yield an SV40 mutant that grows in primate cells, albeit to levels lower than wild-type SV40 (11). In mouse cells, the mutant genome expresses large T antigen at levels equivalent to that of wild-type SV40 (10). Similarly, the polyomavirus enhancer will substitute for the 72-bp repeats to yield an SV40 mutant that grows in monkey cells, does not grow in mouse cells, but produces large tumor antigen to levels equivalent to wild-type SV40 in mouse cells (22). These observations show that the Moloney murine sarcoma virus and polyomavirus enhancers are not able to alter the host range of SV40, because the mutants do not alter the SV40 phenotype in mouse cells. Rather, the only phenotypic change exhibited by the mutant genomes is that they have a reduced ability to replicate in primate cells compared with wild type (11, 22). This is expected since enhancers, like other elements that compose a virus genome, have undergone continual selection by propagation in a given host species. It is likely that the host range of a virus is the consequence of multiple genetic elements which interact in a hierarchical fashion with cellular components. Our results suggest that the enhancer region is not high on this hierarchy of control.

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