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Polyomavirus Tumor Induction in Mice: Influences of Viral Coding and Noncoding Sequences on Tumor Profiles

ROBERT FREUND, GAIL MANDEL,† GORDON G. CARMICHAEL,‡ JOHN P. BARNCASTLE, CLYDE J. DAWE, AND THOMAS L. BENJAMIN*

Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

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We determined the DNA sequences of the noncoding regions of two polyomavirus strains that differ profoundly in their abilities to induce tumors in mice. Differences between strains were found, both on the late side of the replication origin in the region containing known enhancer elements and on the early side of the origin, affecting the number and location of large-T-antigen-binding sites. By constructing and analyzing recombinant viruses between these high- and low-tumor strains, we attempted to localize determinants which affect the frequency and histotype of tumors. Seven recombinants were constructed and propagated in vitro, and the tumor profile of each was established by inoculation into newborn C3H mice. Recombinants containing noncoding sequences from the high-tumor strain and coding sequences from the low-tumor strain behaved like the latter, inducing tumors at a low frequency and strictly of mesenchymal origin. Reciprocal recombinants with noncoding sequences of the low-tumor strain linked to structural determinants from the high-tumor strain induced several types of epithelial tumors typical of the high-tumor strain but at reduced frequency, in addition to mesenchymal tumors. A high frequency and full diversity of epithelial tumors required, in addition to structural regions from the high-tumor strain, noncoding sequences on the early side of the origin also present in this strain. A high-tumor profile thus resulted from the combined effects of structural and regulatory determinants in the high-tumor strain, with the former affecting primarily the tissue tropism and the latter affecting the frequency of tumors. No differential effects of the enhancer regions from the late side of the origin in the two virus strains were seen in this study.

Interactions of polyomavirus with its natural host encompass a range of biological processes that remain poorly understood, despite the detailed knowledge we have of this virus at the molecular biological level. Tumors are known to develop from more than a dozen different cell types following inoculation of the virus into newborn mice, and productive infection can occur in as many as 30 different tissues (7). Little is known at the molecular level about the pathogenesis of tumors and lytic lesions in these laboratory infections. Similarly, much remains to be learned about various mechanisms accompanying natural infection in which the virus persists without causing tumors and about the roles that the transforming genes of the virus might play in such infection.

In this report we follow up on recent observations showing that tumor induction in mice by mouse polyomavirus involves viral genetic determinants that are, at least in part, different from those involved in cell transformation in vitro. We found that two molecularly cloned and ostensibly wild-type virus strains were profoundly different in their abilities to induce tumors in mice, despite being equally efficient in the transformation of cells in culture. The PTA strain, referred to here as the high-tumor strain, induced multiple tumors in 90 to 100% of recipient animals. The tumors were of epithelial as well as mesenchymal origin and led to a moribund condition of the host within a period of about 12 weeks. The RA, or low-tumor, strain as a rule induced only single tumors in a small fraction of the animals. These tumors were exclusively of mesenchymal origin and led to a moribund condition after an average survival time of over 40 weeks. Simultaneous inoculation of PTA and RA led to a high tumor profile, suggesting that RA does not act to evoke a dominant systemic resistance, for example, via the host immune system (7). As a step toward understanding the basis for these differences in vivo, we constructed a series of recombinant viruses between PTA and RA and studied them in newborn mice. The results reveal two classes of genetic determinants present in PTA, deriving from noncoding and coding regions, that contribute to a high frequency and broad spectrum of tumors.

* Corresponding author.
† Present address: Division of Molecular Medicine, Tufts New England Medical Center, Boston, MA 02111.
‡ Present address: Department of Microbiology, University of Connecticut Health Center, Farmington, CT 06032.

MATERIALS AND METHODS

Subcloning and sequencing. Molecularly cloned viral DNAs from strains PTA and RA (7) were subcloned into pUC13 by using standard recombinant DNA techniques (18). Both the large and small *Bam*HI-to-*Eco*RI DNA fragments from the viral genomes were gel purified and cloned into the appropriately digested vector. (For a restriction enzyme map of polyomavirus, see Griffin et al. [15].) The subclones containing the small *Bam*HI-to-*Eco*RI viral DNA fragment were further subcloned into the *Bam*HI-to-*Pst*I fragment and *Pst*I-to-*Eco*RI fragment. The *Bam*HI-to-*Pst*I fragment, containing the noncoding region of these viral genomes, was subcloned into M13mp8 and M13mp9 for sequencing. The universal M13 primer, as well as synthesized oligonucleotide primers throughout the noncoding viral DNA region, was used to obtain the nucleotide sequence by the dideoxynucleotide sequencing method (22).

Construction of recombinant viruses. Recombinant viruses were constructed by combining the appropriate gel-purified DNA fragments generated from restriction enzyme digestions of the subcloned DNAs. The structures of the viral recombinants are shown in Tables 1, 2, and 3. To digest the

DNAs with *Bcl*I or *Stu*I or both, the clones were first grown in the *dcm dam Escherichia coli* host MM505. Partial digestions were done by limiting the enzyme concentration and time of digestion. To generate molecular clones of the recombinant viral genomes, the appropriate gel-purified fragments were ligated with *Eco*RI-digested pUC13. The cloned DNAs were screened by restriction enzyme digestion and polyacrylamide gel electrophoresis. The cloned viral DNAs were excised from the vector by digestion with *Eco*RI, ligated under dilute conditions, and transfected into primary baby mouse kidney cells by using the calcium phosphate procedure (7, 14). Virus stocks were grown from these lysates, and titers were determined by plaque assay on NIH-3T3 cells.

Mice, virus inoculations, and pathology. All mice were of the inbred strain C3H/BiDa. Litters of newborns were inoculated at less than 18 h of age by subcutaneous injection of 0.05 ml of crude virus suspension. The dose of virus was 5×10^6 to 2×10^7 PFU per animal. Mice were necropsied either when moribund or at approximately 1 year of age. Histological examination of tissues was performed as previously described (7). Tumors scored as overt were seen by gross examination at the time of necropsy and were confirmed by histological examination, except as noted. Tumors scored as occult were revealed by histological examination only. The latter scoring gave a lower limit to the estimate of tumor frequency because of the small amount of tissue that could be examined histologically.

RESULTS

Multiple differences between PTA and RA in the noncoding regions. PTA and RA viral DNAs were cloned, and virus stocks were prepared on baby mouse kidney cells from the cloned viral DNAs (see Materials and Methods). Purified viral DNAs were compared by restriction enzyme digestion by using several enzymes singly and in combination. Differences were noted in the region within a few hundred base pairs on either side of the origin of replication. No differences in restriction enzyme digestion patterns were noted in fragments deriving either from the early or late coding regions (data not shown).

On the basis of the above results, the entire noncoding regions of the two viral DNAs were sequenced, going from the ATG for VP_2 on the late side through the origin of replication and out to the ATG for the T antigens on the early side. The results are shown in Fig. 1. PTA and RA have nonoverlapping duplications of 33 and 31 base pairs (bp), respectively, on the late side of the origin in the known enhancer region (10, 16, 19, 26). The duplication in RA has been previously reported (21). The duplication in PTA includes sequences containing a core element similar to that found in the simian virus 40 72-bp enhancer (16, 25, 26), as well as a DNase I hypersensitive region (16). This region of polyomavirus DNA (between the *Pvu*II site at 5128 and 5262) has been shown to contain enhancer activity by transfection and transient expression with heterologous constructions (10, 16, 25). Host range variants of polyomavirus capable of limited growth on embryonal carcinoma cells have previously been shown to vary in this region (1), although the changes in these variants are different from those seen in either PTA or RA. On the early side of the origin, PTA has three contiguous 11-bp elements containing large-T-antigen-binding sites (4, 5), while RA has only two. This difference has also been seen between polyomavirus strains A2 and A3, which have three and two large-T-antigen-binding sites, respectively (9, 13, 24). PTA has an additional duplication of 40 bp at the *Bgl*I site that is not found in RA or any other strain described thus far. Four base-pair substitutions, one on the late side and three on the early side, were also found (Fig. 1). An examination of PTA and RA sequences in the origin region indicates small changes in preexisting short open reading frames due to the duplications and insertions; however, in no cases were these marked by a methionine codon at the start.

PR recombinants in which noncoding sequences of PTA were linked to coding sequences of RA. Recombinant virus strains in this study are given a two-letter designation, PR or RP (P for PTA and R for RA), with the first letter indicating the strain of origin of most or all of the noncoding sequences and the second letter indicating the origin of coding sequences. Results obtained with a series of three PR recombinant viruses and schematic diagrams of the recombinants are shown in Table 1. These strains were constructed to determine the contribution of noncoding sequences of the high-tumor strain on a background of structural determinants from the low-tumor strain. The first of these, PR-1, contained the enhancer region of PTA on the late side of the origin, while the noncoding sequences on the early side of the origin along with all early and late coding sequences derived from RA. Only a single tumor, a renal sarcoma, was observed. In PR-2, the amount of noncoding sequences derived from PTA was extended in both directions, incorporating amino-terminal sequences of VP_2 on the late side and additional noncoding sequences on the early side up to but not including the 40-bp duplication at the *Bgl*I site. A third recombinant, PR-3, contained the entire noncoding region of PTA, including the *Bgl*I duplication, with all coding sequences coming from RA. A low-tumor profile was found for both of these recombinants, essentially similar to those of PR-1 and the low-tumor parental strain RA. Neither the enhancer region alone of PTA (PR-1) nor the enhancer region plus the array of additional large-T-antigen-binding sites on the early side of the origin (PR-2 and PR-3) was able to direct a high-tumor profile when linked to coding sequences from the low-tumor strain. These results thus point to the existence of one or more structural determinants in PTA essential for a high-tumor profile and specifically for induction of epithelial tumors.

RP recombinant containing noncoding sequences of the low-tumor strain and coding sequences of the high-tumor strain. The PTA tumor profile as reported earlier (7) and that of a single RP recombinant, RP-1, which had the entire noncoding region of RA and all coding regions of PTA, is given in Table 2. Schematic diagrams of PTA and RP-1 are also shown. Most significant in the profile of RP-1 was the appearance of mammary and salivary gland tumors, two of the major epithelial tumor types in the PTA profile. These results thus bear out the prediction of at least one PTA coding determinant required for induction of epithelial tumors. The RP-1 tumor profile, however, showed less diversity and lower frequencies of epithelial tumors than that of PTA; in particular, only a single hair follicle tumor and no thymic tumors were seen in mice inoculated with RP-1. These animals also lived significantly longer before becoming moribund (203 days) compared with those inoculated with PTA (117 days).

Another significant feature of the RP-1 profile was the high frequency of mesenchymal tumors compared with the profiles of the PR recombinants (Table 1) or with that of the RA parental strain itself (7). This suggests an element(s) in PTA coding sequences which favors development of mesenchy-

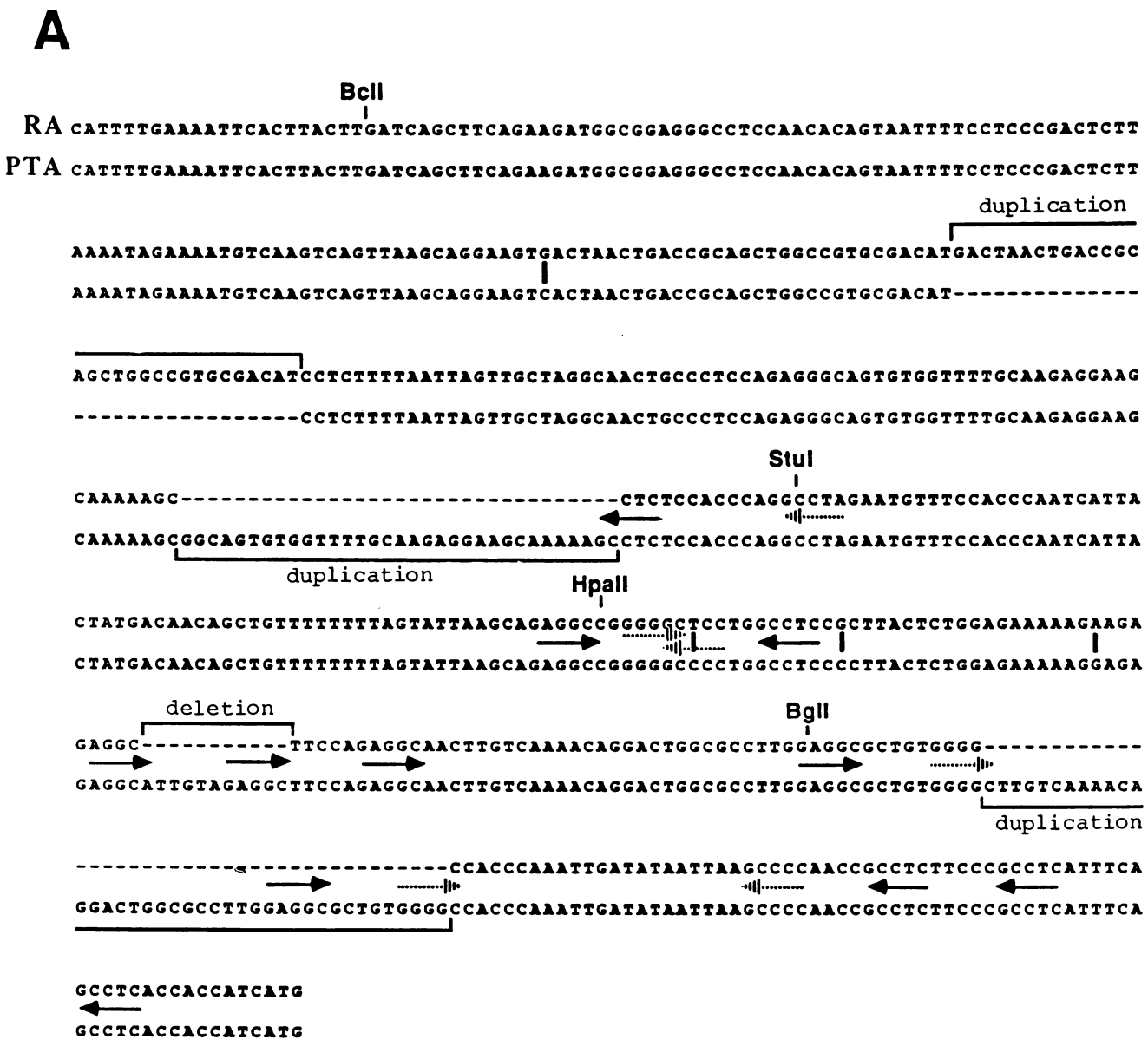
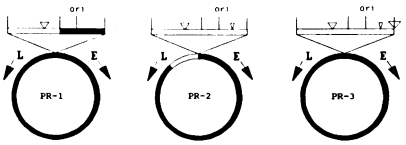

A
BclI
RA CATTTTGAAAATTCACTTACTTGATCAGCTTCAGAAGATGGCGGAGGGCCTCCAACACAGTAATTTTCCTCCCGACTCTT
PTA CATTTTGAAAATTCACTTACTTGATCAGCTTCAGAAGATGGCGGAGGGCCTCCAACACAGTAATTTTCCTCCCGACTCTT
duplication
AAAATAGAAAATGTCAAGTCAGTTAAGCAGGAAGTGACTAACTGACCGCAGCTGGCCGTGCGACATGACTAACTGACCGC
AAAATAGAAAATGTCAAGTCAGTTAAGCAGGAAGTCACTAACTGACCGCAGCTGGCCGTGCGACAT----------
AGCTGGCCGTGCGACATCCTCTTTTAATTAGTTGCTAGGCAACTGCCCTCCAGAGGGCAGTGTGGTTTTGCAAGAGGAAG
----------CCTCTTTTAATTAGTTGCTAGGCAACTGCCCTCCAGAGGGCAGTGTGGTTTTGCAAGAGGAAG
StuI
CAAAAAGC----------CTCTCCACCCAGGCCTAGAATGTTTCCACCCAATCATTA
CAAAAAGCGGCAGTGTGGTTTTGCAAGAGGAAGCAAAAAGCCTCTCCACCCAGGCCTAGAATGTTTCCACCCAATCATTA
duplication
HpaII
CTATGACAACAGCTGTTTTTTTTAGTATTAAGCAGAGGCCGGGGGCCTCCTGGCCTCCGCTTACTCTGGAGAAAAAGAAGA
CTATGACAACAGCTGTTTTTTTTAGTATTAAGCAGAGGCCGGGGGCCCCTGGCCTCCCCTTACTCTGGAGAAAAAGGAGA
deletion
BglI
GAGGC----------TTCCAGAGGCAACTTGTCAAAACAGGACTGGCGCCTTGGAGGCGCTGTGGGG----------
GAGGCATTGTAGAGGCTTCCAGAGGCAACTTGTCAAAACAGGACTGGCGCCTTGGAGGCGCTGTGGGGCTTGTCAAAACA
duplication
----------CCACCCAAATTGATATAATTAAGCCCCAACCGCCTCTTCCCGCCTCATTTCA
GGACTGGCGCCTTGGAGGCGCTGTGGGGCCACCCAAATTGATATAATTAAGCCCCAACCGCCTCTTCCCGCCTCATTTCA
GCCTCACCACCATCATG
GCCTCACCACCATCATG
B
BclI
31
StuI
HpaII
BglI
RA
PTA
G
C
TGA
CCG
33
11
40
Origin
Late
Early

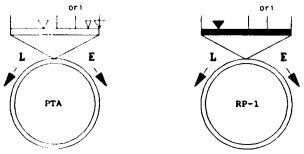
TABLE 1. Schematic diagrams and tumor profiles of PR recombinant viruses[a]



	PR-1	PR-2	PR-3
Fraction of mice with tumors	1/37	3/26	5/34
Mean age at necropsy (days)	292	403	422
Age range at necropsy (days)		393–408	310–550
	No. of mice with gross tumors		
Epithelial tumors			
Hair follicle	0	0	0
Thymus	0	0	0
Mammary gland	0	0	0
Salivary gland	0	0	0
Mesenchymal tumors			
Subcutaneous connective tissue	0	3	4
Vascular endothelium	0	0	1
Renal medulla	1	0	0
Bone	0	1	0

[a] The PR recombinant viruses contain the coding region from RA. The diagrams show the noncoding region expanded. Black segments are from RA, and white segments are from PTA. Vertical lines above the noncoding region are restriction enzyme sites *Bcl*I, *Stu*I, *Hpa*II, and *Bgl*I, from left to right. Triangles indicate insertions or duplications characteristic of the parental strain, as shown in Fig. 1. PR-1 contained the noncoding sequences from *Bcl*I to *Stu*I of PTA and the rest of the viral genome from RA. PR-2 contained all of the sequences from *Bam*HI to *Bgl*I, encompassing the replication origin from PTA. This includes sequences encoding the N-terminal end of VP_2 as well as all the noncoding sequences around the origin from PTA, not including the 40-bp insertion at the *Bgl*I site. PR-3 contained all of the noncoding sequences of PTA from *Bcl*I to *Bgl*I, including the 33-bp duplication in the enhancer, the 11-bp insertion containing the third T-antigen-binding site, and the 40-bp duplication around the *Bgl*I site. All coding sequences were derived from RA. L and E indicate late and early sides of the replication origin, respectively.

TABLE 2. Schematic diagrams and tumor profiles of PTA and RP-1[a]



	PTA	RP-1
Fraction of mice with tumors	37/37	22/22
Mean age at necropsy (days)	117	203
Age range at necropsy (days)	45–225	79–390

	No. of mice with tumors[b]					
	Overt	Occult	Total (%)	Overt	Occult	Total (%)
Epithelial tumors						
Hair follicle	33	1	34 (92)	1	0	1 (5)
Thymus	30	1	31 (84)	0	0	0
Mammary gland	9	1	10 (27)	6	3	9 (41)
Salivary gland	6	9	15 (41)	3	6	9 (41)
Mesenchymal tumors						
Subcutaneous connective tissue	7	1	8 (22)	10	3	13 (59)
Vascular endothelium	0	1	1 (3)	3	0	3 (14)
Renal medulla	4	9	13 (35)	6	1	7 (32)
Bone	10	0	10 (27)	12	0	12 (55)

[a] The recombinant virus RP-1 contained noncoding sequences from RA and coding sequences from PTA. The diagrams show the noncoding regions expanded. Abbreviations and symbols are as described in the footnote to Table 1.

[b] The percentage represents the number of mice with a particular type of tumor divided by the total number of inoculated mice.

mal tumors, particularly renal sarcomas and bone tumors which were not part of the RA tumor profile and occurred only rarely with PR recombinants (Table 1).

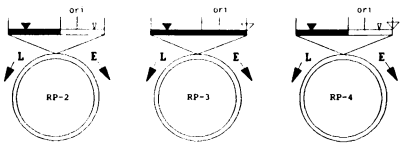
Requirement for noncoding sequences from the early side of the origin of the high-tumor strain. Three additional RP recombinants were constructed to analyze the contributions of noncoding sequences present in the high-tumor strain to the tumor profile and particularly to the frequency and types of epithelial tumors (Table 3). The noncoding regions in these three recombinants all contained the late-side enhancer elements from RA, together with various elements from the early side of PTA, all on a PTA structural background. RP-2 contained origin-proximal PTA sequences, from the *Stu*I site close to the origin out to the *Bgl*I site. RP-3 contained the origin-distal PTA element, consisting of the 40-bp duplication at the *Bgl*I site in an otherwise RA noncoding region. RP-4 contained both segments of PTA sequence from the early side of the origin out to and including the duplication around *Bgl*I.

RP-2 induced a high frequency of both mammary and salivary gland tumors, as did PTA, but only four hair follicle tumors were confirmed histologically, along with a single thymic tumor, which was occult. RP-3 had a profile similar to those of RP-1 and RP-2, with the notable addition of a substantial number of thymic tumors, both overt and occult. When the two early noncoding segments of PTA were combined, as in RP-4, a high-tumor profile was found, essentially similar to the PTA profile with respect to frequency and diversity of tumors as well as average latency. These results establish the importance of early-region noncoding sequences of PTA in increasing both the diversity and frequency of epithelial tumors. The basic similarity between PTA and RP-4 reinforces the conclusion that the enhancer regions of the two parental strains have no differential effect on the tumor profiles.

A more detailed analysis of the data for RP recombinants

FIG. 1. Nucleotide sequences of the noncoding region of PTA and RA. (A) The sequences of strains RA and PTA from the start of translation of the late regions (nucleotide 5000, according to the system of Griffin et al. [15]) through the origin to the start of translation of the early region (nucleotide 175). Single-base-pair differences are indicated by a vertical line. RA contains a duplication of 31 bp (nucleotides 5114 to 5144) on the late side of the origin and an 11-bp deletion (nucleotides 44 to 54) on the early side of the replication origin. The deletion contains the sequence GAGGC which has been shown to bind large T antigen in vitro (4, 5, 20). These T-antigen-binding-site sequences throughout the noncoding region are indicated by dark arrows, and variants of the sequence are indicated by hatched arrows. PTA contains a 33-bp duplication (nucleotides 5183 to 5213) on the late side of the origin. These repeated nucleotides contain the simian virus 40 enhancer core sequence (16, 25, 26) and a DNase I hypersensitive site (16). PTA also contains a 40-bp duplication (nucleotides 67 to 106) on the early side of the replication origin. This region contains two large-T-antigen-binding sites (4, 5). Restriction enzyme sites used in the construction of recombinants are indicated above the sequence. The *Hpa*II site marks the origin of DNA replication. (B) Schematic diagram of the noncoding region of PTA and RA, indicating the differences between the two strains. Duplications and insertions are indicated by triangles and the number of base pairs. Single-base-pair differences are shown with vertical lines. The restriction enzyme sites *Bcl*I (nucleotide 5021), *Stu*I (nucleotide 5211), *Hpa*II (nucleotide 5291), and *Bgl*I (nucleotide 87) shown in panel A are designated for reference.

TABLE 3. Schematic diagrams and tumor profiles of RP recombinant viruses[a]



	RP-2	RP-3	RP-4
Fraction of mice with tumors	16/17	17/17	31/31
Mean age at necropsy (days)	120	112	72
Age range at necropsy	90–157	61–178	55–93

	No. of mice with tumors[b]								
	Overt	Occult	Total (%)	Overt	Occult	Total (%)	Overt	Occult	Total (%)
Epithelial tumors									
Hair follicle	4	0	4 (24)	0	0	0	31	0	31 (100)
Thymus	0	1	1 (6)	6	4	10 (59)	31	0	31 (100)
Mammary gland	5	2	7 (41)	6	0	6 (35)	11	4	15 (48)
Salivary gland	9	5	14 (82)	7	5	12 (71)	3	23	26 (84)
Mesenchymal tumors									
Subcutaneous connective tissue	1	10	11 (65)	5	2	7 (41)	0	2	2 (6)
Vascular endothelium	2	0	2 (12)	1	0	1 (6)	0	0	0
Renal medulla	12	3	15 (88)	14	1	15 (88)	21	5	26 (84)
Bone	9	0	9 (53)	13	0	13 (76)	7	0	7 (23)

[a] Additional RP recombinant viruses containing various portions of the noncoding region on the early side of the origin plus all coding regions from PTA. Abbreviations and symbols are as described in the footnote to Table 1. RP-2 contained noncoding sequences of PTA on the early side of the origin from *Stu*I to the *Bgl*I site; noncoding sequences on the late side of the replication origin from *Bcl*I to *Stu*I were from RA. RP-3 contained the noncoding sequences from *Bcl*I to *Bgl*I from RA, with the addition of the 40-bp duplication from PTA centered at the *Bgl*I site. RP-4 contained the enhancer sequences from *Bcl*I to *Stu*I from RA, with early noncoding sequences from PTA, including the 11-bp insertion and the 40-bp duplication.

[b] The percentage represents the number of mice with a particular type of tumor divided by the total number of inoculated mice.

is shown in Table 4. Here, the total number of gross tumors of a given type in the entire set of animals inoculated with a given virus is tabulated rather than the number of mice with particular tumor histotypes. This analysis better reflects the roles of different viral sequences in the frequencies of induction of specific tumor types. A tumor frequency index, defined as the total number of tumors of a given type or group divided by the number of animals inoculated, is shown to facilitate comparisons among virus strains. Comparisons of RP-1 with RP-2 and RP-3 indicate that each of the early-region noncoding segments of PTA could act independently to increase the frequencies of certain tumors, both epithelial (mammary and salivary gland) and mesenchymal (renal). RP-1, which contained none of the noncoding sequences of PTA, induced fewer total epithelial tumors than RP-2, with the origin-proximal segment of PTA, RP-3, with the origin-distal segment, or RP-4, containing both PTA elements from the early side of the origin. The data in Tables 2, 3, and 4 show that the appearance of thymic tumors correlated strongly with the presence of the duplication in PTA around the *Bgl*I site. Thus, RP-3 and RP-4, which contained the duplication, induced gross thymic tumors, while RP-1 and RP-2, which lacked this element, did not.

Hair follicle tumors are not listed in Table 4 because they could not be accurately enumerated at the gross level except in those animals in which they were few. When present in large numbers, they tended also to be larger in size and confluent or overlapping, making accurate counts virtually impossible. Review of the necropsy records made it evident that for the viruses in this study, there was a wide boundary, quantitatively, between those viruses that induced few hair follicle tumors and the one virus (RP-4) that induced large numbers of this tumor type. When mice infected with RP-1, RP-2, or RP-3 bore hair follicle tumors, there were only 10 or fewer tumors of this type per mouse (Table 5). In contrast, all mice that were infected with RP-4 bore hair follicle tumors and consistently had 50 or more such tumors. Combining the scoring methods in columns 2 and 3 of Table 5, one sees a 5- to 10-fold difference between RP-4 and any of the other RP recombinant viruses, in terms of tumor response per unit of target tissue mass.

TABLE 4. Total number of tumors and tumor frequency indices[a]

	No. of tumors (tumor frequency index) with virus:			
	RP-1	RP-2	RP-3	RP-4
No. of mice inoculated	22	17	17	31
Avg age at necropsy (days)	203	120	112	72
Epithelial tumors				
Thymus	0	0	6 (0.35)	31 (1.00)
Mammary gland	8 (0.36)	13 (0.76)	17 (1.00)	36 (1.16)
Salivary gland	3 (0.14)	16 (0.94)	9 (0.53)	4 (0.13)
Total epithelial	11 (0.50)	29 (1.71)	32 (1.88)	71 (2.29)
Mesenchymal tumors				
Subcutaneous connective tissue	19 (0.86)	2 (0.12)	6 (0.35)	0
Vascular endothelium	3 (0.14)	2 (0.12)	1 (0.06)	0
Renal medulla	9 (0.41)	18 (1.06)	26 (1.53)	33 (1.06)
Bone	18 (0.82)	17 (1.00)	19 (1.12)	10 (0.32)
Total mesenchymal	49 (2.23)	39 (2.29)	52 (3.05)	43 (1.39)
Total tumors	60 (2.73)	68 (4.00)	84 (4.94)	114 (3.68)

[a] Total number of tumors was determined by gross examination. Tumor frequency indices are calculated as the total number of tumors divided by the number of animals inoculated.

The data on the RP recombinants suggest relationships between incidences of particular epithelial and mesenchymal tumors and overall survival time. For example, epithelial tumors of the thymus tended to occur earlier and become life threatening more quickly than other tumors did. Thus, animals injected with RP-4, with the highest frequency of overt thymic tumors, had the shortest overall survival time and, consequently, had relatively few mesenchymal tumors such as fibrosarcomas and bone tumors. Among the animals which did not develop thymic tumors, renal sarcomas tended to cause earliest morbidity. Thus, the average survival was longest for animals inoculated with RP-1, which induced no thymic tumors and the lowest incidence of renal tumors, followed by RP-2, which induced no thymic tumors but a high incidence of renal tumors, and RP-3 and RP-4, which induced both thymic and renal tumors that contributed to the shorter average survival times.

No histological differences between tumors induced by recombinant and parental viruses. In addition to the tabulated histotypes, most of the less common epithelial tumor types known to be inducible by polyomavirus were also

TABLE 5. Hair follicle tumors in RP profiles[a]

Virus	Fraction of mice with hair follicle tumors	Estimate of no. of hair follicle tumors in affected mice
RP-1	3/22	1–10
RP-2	7/17	1–10
RP-3	6/17	1–10
RP-4	31/31	≥50

[a] Number of hair follicle tumors were estimated for each animal at the time of necropsy by gross inspection.

observed. These included tumors of lacrimal glands, thyroid gland, adrenal glands, periurethral glands, bulbourethral glands, prostate gland, and dental epithelium (ameloblastomas). These tumor types were found exclusively in mice that had received RP recombinants. It is noteworthy that ameloblastomas were found only in mice infected with RP-4 (9 of 31 recipients). In view of the small numbers observed, the other less common epithelial tumor types could not be correlated with any particular RP recombinant. No unusual features such as differences in invasiveness or metastasizing ability were noted.

A few unusual neoplasms were discovered at necropsy. These included a microscopic neoplasm of the testis (tentatively classified as a Leydig cell tumor), two hepatocellular neoplasms, and a squamous papilloma of the prepuce. The testicular tumor and the papilloma of the prepuce both occurred in one 109-day-old male that had received RP-3. One of the hepatocellular neoplasms occurred in a 70-day-old female that had received RP-4. Histologically, the neoplasm was not typical of hepatic neoplasms that occur spontaneously and mainly in old males in our C3H/BiDa colony. The other hepatocellular neoplasm occurred in a 158-day-old male that had received RP-3 and was less well differentiated than most spontaneous hepatocellular neoplasms in C3H/BiDa mice. Whether any of the above four neoplasms was induced or accelerated by polyomavirus is unclear.

Lytic infection of kidneys. Evidence of persistent lytic infection of the kidneys was noted in most mice that received recombinants of the RP series but not in those receiving PR virus strains. This evidence consisted of lytic changes mainly in renal tubular epithelium, chiefly in the outer renal cortex. Nuclei exhibited ballooning degeneration and disintegration, and intranuclear inclusion bodies characteristic of polyomavirus lytic infection were often present. Sloughing of tubular epithelium within distal as well as proximal convoluted tubules and occasionally even in collecting tubules was common. Tubular epithelial regeneration was sometimes associated with such lesions. Dense infiltrates of lymphocytes and plasma cells accompanied the lytic tubular lesions, often forming periarterial sleeves or cuffs. Similar tubular and interstitial nephritis has been described in association with infection by a highly virulent strain of polyomavirus that causes high mortality associated with uremia (2). In the present study, two mice that received the RP-3 recombinant died at 30 and 37 days of age and had renal lytic lesions of such severity that the cause of death was attributed to this renal damage.

Renal lytic lesions were scored on a scale of 0 to +4 in an exploratory attempt to correlate their severity with the tumorigenicity of the various recombinant virus strains. The average scores for RP-1, RP-2, RP-3, and RP-4 were, respectively, 1.1, 1.5, 2.2, and 1.6. These scores appear to have no consistent relationship to tumor profiles, but it is interesting that RP-3, the virus that induced renal sarcomas at the highest frequency, also caused the most severe degree of renal lytic lesions as judged by this somewhat subjective method. Renal lytic lesions in mice inoculated with recombinant viruses of the PR series were absent or much less severe than in mice inoculated with the RP series, but a valid comparison is not possible because the PR-infected mice had much higher mean ages at necropsy than the RP-infected animals. By the time of sacrifice, PR mice may have suppressed persistent infections that had earlier been more severe.

Viral lytic lesions were also identified in other organs and tissues, exclusively in mice that had received recombinant viruses of the RP series. Epithelial lytic lesions were found in salivary glands, lacrimal glands, thyroid glands, adrenal medulla, and testicular tubules. Lytic lesions unassociated with inflammatory reactions were seen in smooth muscle of the thoracic aorta and other large arteries. Of particular interest were arterial lesions typical of polyarteritis nodosa, found in 11 of 17 mice that had been infected with the RP-3 recombinant virus, the same recombinant that was associated with the most severe persistent renal lytic lesions and the highest frequency of renal sarcoma.

DISCUSSION

We have begun to investigate the influences of regulatory versus structural determinants of polyomavirus in the frequency and distribution of tumors in mice. The noncoding regions of two cloned virus strains that differ profoundly in their tumor-inducing abilities have been sequenced. Multiple differences (duplications, deletions, and base substitutions) were found, both on the late side of the replication origin in a region containing known enhancer elements and on the early side, affecting the number and position of large-T-antigen-binding sites (Fig. 1). We constructed a series of recombinant virus strains in which all or part of the noncoding regions were exchanged between the parental strains. The tumor profiles of seven such recombinant strains were established by inoculation into newborn mice. Three main conclusions can be drawn from the data thus far. (i) Coding sequences of the high-tumor strain were essential for the appearance of a high-tumor profile and particularly for the development of epithelial tumors. (ii) Noncoding sequences of the high-tumor strain acted to increase tumor frequencies and diversity, but only in conjunction with structural determinants from the same strain and not with those of the low-tumor strain. (iii) Two segments of noncoding sequences from the early side of the origin in the high tumor strain acted in conjunction with homologous coding seqences to induce a high-tumor profile; no effects of the late-side enhancer regions were seen.

Contributions of coding sequences. Although DNA restriction analysis failed to reveal any differences in fragments deriving from the coding regions of the two parental viruses, it is clear from the biological data that such differences must exist. The presence of one or more determinants in the coding regions of PTA essential for induction of epithelial tumors is evident from the fact that the four RP recombinant viruses, all with PTA coding regions, induced at least a low level of epithelial tumors (Table 2 and 3), whereas none of the three PR recombinants with RA coding regions induced any such tumors (Table 1). Coding determinants in PTA may similarly dispose toward a high frequency of certain types of mesenchymal tumors, particularly renal sarcomas and bone tumors.

Comparison of the tumor profiles of PR-3 and RP-1 clearly establishes the importance of structural determinants in tumor tropism. These two strains are reciprocal recombinants, involving exchanges of the entire noncoding regions between PTA and RA. RP-1, with PTA coding and RA noncoding sequences, induced mammary and salivary gland tumors in addition to at least four types of mesenchymal tumors, all of which were found in the PTA tumor profile. In sharp contrast, PR-3 induced only a few mesenchymal tumors. Thus, a recombinant virus containing all of the noncoding sequences of the high-tumor strain linked to coding sequences of the low-tumor strain failed to induce a high-tumor profile.

The coding determinant(s) in PTA that affected tumor tropism did not by itself dispose toward a complete PTA tumor profile. This is evident from the fact that RP-1 did not show the full diversity and high frequency of epithelial tumors typical of PTA. In addition, the RP-1 tumor profile showed an average time to necropsy significantly longer than that for PTA.

Contributions of noncoding sequences on the early side of the origin. Noncoding sequences on the early side of the origin in PTA, acting in conjunction with PTA coding regions, disposed toward a higher frequency and diversity of epithelial tumors than was found when those sequences derived from the corresponding noncoding region of RA. This is apparent from comparisons among the four RP recombinants (Tables 2 to 5). RP-1, deriving all its noncoding sequences from RA, induced the lowest frequency of epithelial tumors. Addition of either the origin-proximal element from PTA, which includes the 11-bp insertion and three single-base-pair differences (RP-2), or the origin-distal element, consisting of the 40-bp duplication at the *BglI* site (RP-3), resulted in higher frequencies of epithelial tumors. The combination of these two sequence elements (RP-4) induced a full PTA-like profile with respect to diversity and frequencies of tumors as well as average survival time.

A particularly strong correlation existed between overt thymic tumors and the presence of the 40-bp duplication at the *BglI* site in PTA. Rare occult thymic tumors were found by histological examination with RP-2 (Table 2) as well as with the A-2 strain reported earlier (7), both of which lacked the *BglI* duplication. This element, which may affect both the frequency of induction and the rate of growth of thymic tumors, is under further investigation.

Lack of effects of the PTA and RA enhancer regions on the tumor profile. While the presence of an enhancer region on the late side of the origin is presumed to be essential for virus growth in vivo and induction of tumors, the particular array of enhancer sequences present in PTA or RA appears not to affect the tumor profile in any clearly discernible way. Two comparisons in the data establish this point convincingly. The first is between the high-tumor strains PTA and RP-4. With structural regions and early noncoding sequences coming from PTA in both of these viruses, a high-tumor profile is seen regardless of whether the enhancer region derives from PTA or RA (RP-4). In particular, the results with RP-4 show that the RA enhancer region did not act negatively in suppressing the appearance of epithelial tumors. The second comparison is between the low-tumor strains RA and PR-1, both with RA coding regions. Low-tumor profiles with no epithelial tumors were seen, regardless of whether the enhancer is from RA or PTA (PR-1). The PTA enhancer region thus does not appear to constitute a positive *cis*-acting element recognized by either viral or cellular factors in any manner different from the RA enhancer.

Possible mechanisms. While current data are not sufficient to reveal precise mechanisms, they do suggest likely interpretations as to the kinds of elements that exist and some general features concerning how they might exert their effects. It is clear that an important determinant(s) of tissue tropisms is contained within viral coding sequences. Whether this determinant(s) involves T antigens or viral structural proteins is not presently known. These possibilities imply different mechanisms, involving interactions of viral proteins with either intracellular or cell surface components, and with specificities of the interactions determined by both the virus strain and the type of tissue. Analysis of additional virus recombinants aimed at dissecting early and late coding regions should help to identify and clarify the nature of these determinants.

Noncoding sequences presumably play a regulatory role. Segments of the early noncoding region of PTA influenced overall tumor frequencies as well as frequencies of individual tumors within the profile. Interactions of both viral and cellular *trans*-acting factors with these sequences are implied in the data. Large-T-antigen protein-origin interactions are well known; specific pentanucleotide sequences have been established as high-affinity large-T-antigen-binding sites by in vitro methods (4, 5, 20; Fig. 1), and genetic and physiological experiments carried out in cell culture systems have shown these interactions to be important in initiation of viral DNA replication (6, 12), repression of early transcription (3, 6, 11, 19), and stimulation of late transcription (17). No direct evidence of in vivo binding to these sequences has been reported, however. The data on recombinant viruses suggest that these interactions may be virus strain-specific to some extent. The early-region noncoding sequences of PTA acted to boost tumor frequencies only when present along with the early coding region of PTA and had no effect when linked to the RA structural background. Thus, one possibility is that large-T-antigen and origin sequences in these two strains have coevolved and differentiated along strain-specific lines, with the consequence that the heterologous combinations function poorly. The coevolution and tight linkage of interacting *cis-trans* elements is familiar and well documented in both bacterial and animal virus systems, including simian virus 40 (23).

Interactions of a cellular factor(s) with noncoding viral sequences can also be inferred from the data. For example, the 40-bp duplication at the *BglI* site in PTA comprised an element necessary for induction of thymic epitheliomas (RP-3), although it was not required for high frequencies of other epithelial tumors (RP-2). Thymic epithelium must therefore express a factor(s) capable of recognizing this duplication. These viral sequences did not act autonomously with respect to the presumptive thymic cell factors, but needed a *trans*-acting viral factor(s) as well. This is indicated by the lack of effect of this duplication when present in an RA structural background, i.e., PR-3 failed to induce thymic tumors. This duplication adds two potential large-T-antigen-binding sites (Fig. 1) and could therefore affect binding in a strain-specific manner as suggested above. Similar mechanisms involving recognition of noncoding viral sequences by factors in mammary gland, salivary gland, kidney, and bone may be involved in tumor formation at these sites, although the quantitative effects were less dramatic than they were for the thymus.

The interpretations given above focus on direct interactions between the virus and the target cell for tumor induction and ignore the role of virus replication in the animal and possible variations in effective virus dose reaching the target site. We have begun to examine relationships between replication and tumor induction by determining directly how well and at what sites different virus strains replicate in vivo. The data thus far indicate that replication to high levels in the kidney, the major site of virus amplification, while important, does not necessarily lead to high levels of tumor induction (T. Dubensky, R. Freund, J. Barncastle, C. Dawe, and T. Benjamin, unpublished observations). A separate approach to this question involves the use of preinfected transplants, in which fragments of target tissue are directly exposed to virus in vitro and then transplanted back to the host (8). Current experiments with salivary gland tissue show that following exposure to PTA or RA at equivalent

high doses, only the PTA-infected tissue fragments give rise to tumors (J. Barncastle, R. Freund, C. Dawe, and T. Benjamin, manuscript in preparation). Thus, the results of two lines of ongoing experiments point to the importance of viral genotype in cell interactions at target sites for tumor formation. Additional data from these as well as other approaches will be needed to fully assess the roles of different viral genetic elements in infections of the natural host.

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