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Duplication of Noncoding Sequences in Polyomavirus Specifically Augments the Development of Thymic Tumors in Mice

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A 40-base-pair duplication of noncoding sequences in polyomavirus specifically augmented the development of thymic epitheliomas following inoculation of virus into newborn mice. Virus strains carrying only one copy of this sequence induced a full spectrum of tumors except for overt thymic tumors. This 40-base-pair repeat, on the early side of the replication origin, constituted a tissue-specific regulatory determinant for tumor induction.

PTA and A2 are highly tumorigenic strains of polyomavirus, each capable of rapidly inducing a broad spectrum and high frequency of tumors upon inoculation into newborn mice. A remarkable difference exists between these strains, however, in that PTA induces overt thymic epitheliomas and A2 does not (4).

A comparison of the nucleotide sequences of the noncoding regions of the PTA and A2 viral strains is shown schematically in Fig. 1. PTA contains a 33-base-pair (bp) duplication on the late side of the origin in the B enhancer region (5, 10, 11, 14) and a 40-bp duplication on the early side of the origin just upstream of the early promoter (7). A2 contains neither of these duplications (13). In a study of viruses constructed from segments of the high-tumor strain PTA combined with segments of the low-tumor strain RA, it was shown that differences in their enhancer regions do not contribute to differences in the tumor profiles, while differences on the early side of the replication origin do (7). Aside from two single-base-pair changes near the origin, the only difference between PTA and A2 on the early side of the origin is the 40-bp duplication.

In vitro DNA-binding studies have shown that the sequence G(A/G)GGC is bound by polyomavirus large T antigen (2, 3, 12). Three adjacent 11-bp elements containing these sequences are present in both A2 and PTA, located 40 to 70 nucleotides from the replication origin. Other wild-type strains, such as A3 and RA, contain only two of these 11-bp elements (7, 8). Two additional G(A/G)GGC sites are contained in sequences flanking the *Bgl*I site 95 nucleotides from the replication origin in A2; these also were shown to be bound by large T but with lower affinity. PTA has four additional sites due to the duplication (7, 13). Although these sequence elements clearly constitute large T-binding sites in vitro, there has been no report indicating that large T antigen binds to them in vivo. The major early mRNA start site (nucleotide 150) is downstream of the *Bgl*I site(s) (1, 9). Analysis of the sequence in PTA including the duplication shows that even if possible upstream early start sites are utilized (6, 9), no open reading frames with initiating ATG codons are present. We therefore consider the 40-bp repeat a noncoding sequence with potential regulatory function.

To ascertain whether the 40-bp duplication plays a role in the induction of thymic epitheliomas, we removed one copy of the duplicated sequence from PTA and inserted it into A2 at the *Bgl*I site. The A2 and PTA viral genomes were cloned into the vector PiAN7 at the *Eco*RI site; this vector contains no *Bgl*I sites. Cloned PTA DNA was digested with *Bgl*I and ligated under dilute conditions to promote self-closure. This resulted in the clone PTA(−). The 40-bp fragment generated by the *Bgl*I digestion was inserted into the unique *Bgl*I site of the A2 viral DNA to give A2(+). The viral DNAs were excised from the vector, ligated under dilute conditions, and transfected into NIH 3T3 cells. Lysates from these cells were used to grow virus stocks (4, 7).

Four virus strains, A2(+), A2, PTA, and PTA(−), were inoculated subcutaneously into newborn C3H/BiDa mice (less than 18 h old). The mice were monitored twice weekly for tumor formation, and necropsies were performed when the mice were moribund. Tumor tissue as well as apparently normal tissue from sites susceptible to tumor formation were removed and fixed for histological examination (4, 7). The overt and occult tumors induced by each virus are shown in Tables 1 and 2. Overt tumors were detected by gross examination at the time of necropsy; their size ranged from several millimeters to over a centimeter in diameter. Tumors classified as occult are those that were detected only microscopically and were usually less than a millimeter in diameter.

As shown in Table 1, the A2(+) virus induced overt thymic tumors in each of the 26 mice inoculated. Thus, the A2 strain has the necessary coding determinants for efficient induction of thymic epitheliomas but apparently lacks the appropriate regulatory element(s). The insertion of a second copy of the 40-bp sequence in the noncoding region sufficed to promote efficient induction and rapid growth of this tumor type. A2(+) and A2 induced the other major epithelial tumor types at similar frequencies. The tumor profile of the cloned A2 virus used here was similar to that reported earlier for an uncloned A2 virus stock, including the induction of occult thymic tumors (4). In 1 of 15 recipient mice studied here, a single thymic tumor reaching the overt stage was recorded. Although this tumor was identified by gross inspection at the time of necropsy and was therefore recorded as an overt tumor, it was on the borderline of detectability. Histological examination of thymic tissue from mice inoculated with A2 revealed two thymic tumors in the occult stage, indicating that at least in these cases virus had reached the thymus and transformed thymic epithelial cells. Thus, the duplication may act either to increase the likelihood of transformation in this tissue or to promote more rapid growth of tumors following the initial transformation event or both.

Some differences between the A2 and A2(+) strains were

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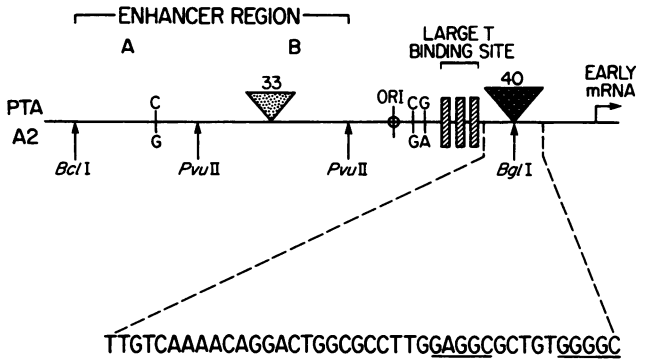

FIG. 1. Schematic diagram of the noncoding regions of PTA and A2, indicating the sequence differences between the two strains (2, 7, 13, 14). Duplications are indicated by shaded triangles and the number of base pairs. The nucleotide sequence of the 40-bp duplication is shown, with the G(A/G)GGC sequences underlined. Single-base-pair differences are indicated with vertical lines. For reference, the enhancer regions A and B (5, 10, 11, 14), the origin of replication, the major large T antigen-binding sites (2, 3), and the early mRNA start site (1, 9) are shown. The locations of the restriction enzyme sites for *BclI* (nucleotide 5021), *PvuII* (nucleotides 5128 and 5262), and *BglI* (nucleotide 87) are indicated (13).

noted with respect to mesenchymal tumors. The higher frequency of most mesenchymal tumors induced by A2 can be accounted for by the fact that the mice receiving this virus were older at necropsy than the mice that received A2(+); overt thymic tumors cause early mortality, while most mesenchymal tumors typically take longer to develop and are less life-threatening (4, 7). The reason for the high frequency of renal tumors induced by A2(+) is unclear. It is, however, unlikely to be related to the 40-bp duplication, since PTA(−), which carries only one copy of this sequence, induced a higher frequency of renal medullary sarcomas than PTA (Table 2).

As a further test of the importance of the 40-bp duplicated element in induction of thymic tumors, we compared the cloned isolate of PTA containing the duplication with the derivative from which one copy of the element was removed, designated PTA(−). The results (Table 2) show that the tumor profile of PTA(−) was very similar to that of the parental PTA virus, except that PTA(−) induced no thymic tumors that reached the overt stage of development. Histological examination, however, revealed that 8 of 16 mice inoculated with PTA(−) had occult thymic tumors. These animals were sacrificed at an average age of 94 days because tumors at other sites had led to a moribund condition. This period of time is more than adequate for development of overt thymic tumors with A2(+) and other isolates of PTA (4, 7). In this study, the PTA isolate required an average of 145 days for the development of overt thymic tumors, and only 39% of the inoculated animals, compared with 80 to 100% in earlier studies, developed this tumor type (4, 7). Nevertheless, results with the PTA virus pair confirm the essential conclusion reached with the A2 pair as to the importance of the 40-bp duplication in development of overt thymic epitheliomas. This conclusion is also consistent with the results of a recent study comparing recombinant viruses constructed from PTA and RA, a low-tumor strain. Among four recombinants that were able to induce epithelial tumors, two carried the duplication and induced overt thymic tumors; the other two lacked the duplication and induced no thymic tumors (7).

PTA and PTA(−) induced other epithelial tumor types at similar frequencies. Thus, as in the case of the A2 strains, the presence of one versus two copies of the 40-bp element in PTA had little or no effect on the ability of the virus to induce other epithelial tumor types. Frequencies of mesenchymal tumors were also similar, except for renal medullary sarcomas.

We conclude that the 40-bp repeat on the early side of the replication origin, when present in either a PTA or an A2 virus strain background, constitutes a major determinant for induction of thymic epitheliomas. Addition of a second copy of the 40-bp element to the highly oncogenic but non-thymic tumor-inducing strain A2, itself containing a single copy,

TABLE 1. Profiles of tumors induced by A2 and A2(+)[a]

	Virus			
	A2		A2(+)	
Dose (PFU)	5×10^7		5×10^7	
No. of mice with tumors/no. of mice inoculated	15/15		26/26	
Mean age (days) at necropsy (range)	103 (40–189)		74 (49–120)	
	No. (%) of mice with tumor[b]			
	Overt	Occult	Overt	Occult
Epithelial tumors				
Thymus	1 (7%)	2 (13%)	26 (100%)	—[c]
Hair follicle	12 (80%)	1 (6%)	22 (85%)	0
Mammary gland	5 (33%)	0	10 (38%)	1 (4%)
Salivary gland	11 (73%)	2 (13%)	16 (62%)	3 (12%)
Mesenchymal tumors				
Subcutaneous connective tissue	2 (13%)	4 (27%)	0	1 (4%)
Vascular endothelium	2 (13%)	0	0	0
Bone	10 (67%)	—	8 (31%)	—
Renal medulla	1 (6%)	6 (40%)	16 (62%)	7 (27%)

[a] A2(+) is the same as A2 except for the insertion of a second copy of the 40-bp sequence at the *BglI* site.
[b] Tumors are scored as the number and percent (in parentheses) of mice inoculated with the designated virus which developed a particular tumor type. When several tumors of a particular tumor type were found in a single animal, the tumor type was scored only once.
[c] —, Not determined.

TABLE 2. Profiles of tumors induced by PTA and PTA(−)[a]

	Virus			
	PTA		PTA(−)	
Dose (PFU)	1×10^7		1×10^7	
No. of mice with tumors/no. of mice inoculated	18/18		16/16	
Mean age (days) at necropsy (range)	146 (68–203)		128 (76–217)	
	No. (%) of mice with tumor[b]			
	Overt	Occult	Overt	Occult
Epithelial tumors				
Thymus	7 (39%)	6 (33%)	0	8 (50%)
Hair follicle	15 (83%)	1 (5%)	15 (94%)	0
Mammary gland	6 (33%)	1 (5%)	6 (38%)	0
Salivary gland	3 (17%)	0	4 (25%)	3 (19%)
Mesenchymal tumors				
Subcutaneous connective tissue	4 (22%)	4 (22%)	3 (25%)	3 (19%)
Vascular endothelium	5 (28%)	0	4 (25%)	0
Bone	6 (33%)	—	8 (50%)	—
Renal medulla	0	5 (28%)	4 (25%)	3 (19%)

[a] PTA(−) is the same as PTA except for removal of one copy of the 40-bp duplication.
[b] See Table 1, footnotes *b* and *c*.

yielded a recombinant virus that induced thymic epitheliomas reaching the overt stage in 100% of inoculated mice. Conversely, removal of one copy of the duplicated element from the thymic tumor-inducing strain PTA resulted in complete absence of overt thymic tumor induction by this virus. Considering the data on occult as well as overt thymic tumors, it appears that viruses with and without the duplication gain access to the target tissue and that the effect of the duplication is expressed not solely in transforming thymic epithelial cells, but also in promoting rapid growth and development of large thymic tumors.

These results suggest the presence of a factor in thymic epithelial cells which, by direct interaction with the duplication or in some other manner, acts positively on viral DNA replication or early gene expression. The duplication could be important for efficient transcription from the early promotor or suppression of late gene expression in thymic epithelial cells, either of which might be expected to increase the tumor-inducing potential of the virus in this tissue. The duplication may also act positively on viral DNA replication in the thymus, increasing the effective gene dosage.

It is clear that the 40-bp duplication must interact with viral as well as cellular factors in the thymus, as indicated by the fact that introduction of a second copy of the 40-bp sequence to an RA virus background does not lead to induction of thymic or any other epithelial tumors (7). Thus, PTA and A2 but not RA have a structural determinant(s) that must be present along with the duplication in order for thymic tumors to develop. Since the duplicated sequence contains two potential large T antigen-binding sites, it is possible that the large T of high-tumor virus strains such as PTA and A2 acts synergistically with a thymus-specific cellular factor(s) in recognizing the duplication. Additional experiments are necessary to identify the putative cellular and viral factors, to define their interactions with the duplicated sequence, and to determine how such interactions might affect replication or transcription of the viral genome, resulting in development of thymic epitheliomas.

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