JOURNAL OF VIROLOGY, Feb. 1983, p. 693–699
0022-538X/83/020693-07$02.00/0

Vol. 45, No. 2

Loss of Polyoma Virus Infectivity as a Result of a Single Amino Acid Change in a Region of Polyoma Virus Large T-Antigen Which Has Extensive Amino Acid Homology with Simian Virus 40 Large T-Antigen

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Received 10 August 1982/Accepted 25 October 1982

The polyoma virus (Py) transformed cell line 7axB, selected by in vivo passage of an in vitro transformed cell, contains an integrated tandem array of 2.4 genomes and produces the large, middle, and small Py T-antigen species, with molecular weights of 100,000, 55,000, and 22,000, respectively (Hayday et al., J. Virol. 44:67–77, 1982; Lania et al., Cold Spring Harbor Symp. Quant. Biol. 44:597–603, 1980). The integrated viral and adjacent host DNA sequences have been molecularly cloned as three *Eco*RI fragments (Hayday et al.). One of these fragments (7B-M), derived from within the tandem viral sequences, is equivalent to an *Eco*RI viral linear molecule. Fragment 7B-M has been found to be transformation competent but incapable of producing infectious virus after DNA transfection (Hayday et al.). By constructing chimerae between 7B-M and Py DNA and by direct DNA sequencing, the mutation responsible for the loss of infectivity has been located to a single base change (adenine to guanine) at nucleotide 2503. This results in a conversion of an aspartic acid to a glycine in the C-terminal region of the Py large T-antigen but does not appear to affect the binding of the Py large T-antigen to Py DNA at the putative DNA replication and autoregulation binding sites. The mutation is located within a 21-amino acid homology region shared by the simian virus 40 large T-antigen (Friedmann et al., Cell 17:715–724, 1979). These results suggest that the mutation in the 7axB large T-antigen may be involved in the active site of the protein for DNA replication.

Rat cells transformed in vitro by polyoma virus (Py) can contain multiple inserts of integrated viral sequences as well as free viral genomes (1, 2, 8, 11, 12). Passage of such transformed cell lines either in vitro or in vivo usually simplifies the integration pattern and causes the loss of free viral genomes (5, 13). In cell lines transformed by wild-type virus, the loss of free viral genomes is usually accompanied by the loss of a full-size viral replication protein, the 100,000-molecular-weight (100K) large T-antigen (T-Ag). This can occur as a result of interruptions in the integrated viral sequences that code for the 100K large T-Ag either by addition and deletion or by recombination to host cell DNA (1, 5, 10–13, 19; H. E. Ruley, unpublished results). In previous work (13), we inoculated rats with in vitro transformed cell lines containing multiple viral inserts and free viral genomes. The resulting tumors contained simplified integration patterns and no free viral genomes. Although most of the tumors did not contain a 100K large T-Ag, one tumor line, 7axB, did synthesize a full-size 100K large T-Ag species (13). Analysis of the 7axB tumor cells revealed that they contain a single insert of 2.4 genomes of viral sequences in a tandem head-to-tail array (10). The viral and adjacent host sequences in 7axB DNA were cloned into λ phage as three *Eco*RI fragments. The 5.3-kilobase clone 7B-M, containing a complete viral genome, was found to be transformation competent but incapable of producing new infectious virus after DNA transfection (10). In this communication, we report that we have located the mutation in the cloned 7B-M viral DNA to a single base change in viral sequences coding for the C-terminal region of the 100K large T-Ag and have analyzed some of the properties of the mutant large T-Ag. The mutation is in a region of Py DNA that shares extensive amino acid and nucleotide homology with a region of simian virus 40 (SV40) DNA coding for the C-terminal region of SV40 large T-Ag.

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MATERIALS AND METHODS

Chimera construction and assay for plaque formation. Reciprocal chimerae were formed between restriction fragments of 7B-M viral DNA and the Py wild-type strain CR and were tested for their plaque-forming ability on mouse embryo cells as described by Miller and Fried (17). Restriction fragments from either complete (*Eco*RI, *Bam*HI) or partial (*Hha*I, *Hin*cII, *Xba*I) digestion were fractionated by gel electrophoresis in low-melting-temperature agarose (Seaplaque) gels. The appropriate fragments were isolated from excised gel fragments after heating to 65°C and ligated together with T4 DNA ligase (Biolabs) to form hybrid complete viral DNA molecules.

The chimerae were tested for their ability to form plaques on mouse embryo cells by the DEAE dextran technique as previously described (17). Wild-type CR DNA lacks a *Hha*I site at 26 map units (m.u.) (16) which is present in 7B-M DNA. In addition, the CR strain is of the A3 type, containing a shorter *Hpa*II-5 fragment than that of 7B-M DNA (A2 type) (6). These restriction enzyme markers allowed verification of chimera formation.

DNA sequence analysis. Restriction endonuclease cleavage sites were 5′ end labeled after treatment with calf intestine alkaline phosphatase and T4 polynucleotide kinase (23). After secondary enzyme cleavage, labeled fragments were purified from polyacrylamide gels and were sequenced by the chemical degradation method of Maxam and Gilbert (14), except for the guanine plus adenine reaction, which was stopped with 200 μl of Hydrazine Stop buffer and was ethanol precipitated. The sequence was confirmed by sequencing both strands.

Partial proteolysis peptide analysis. Essentially, the method of Cleveland et al. (3) was employed. A 90-mm dish of 3T6 cells infected with A2 wild-type virus (100 PFU per cell) and a confluent 90-mm dish of 7axB cells were washed twice with Dulbecco modified Eagle medium without phosphate. Each dish was then incubated in 1.5 ml of medium without phosphate, but containing 1% horse serum and 1 mCi of ^{32}P (Amersham), for a period of 2 to 4 h at 37°C. The soluble proteins were extracted, and the T-Ags were immunoprecipitated and fractionated on 12.5% polyacrylamide gels as previously described (11, 13). The ^{32}P-labeled large T-Ags were identified by autoradiography, and the bands were excised from dried gels and allowed to swell in 100 to 150 μl of gel elution buffer containing 10 mM sodium bicarbonate, 0.1% sodium dodecyl sulfate, 0.1% β-mercaptoethanol, and 20% sucrose. The swollen gel slice was transferred along with the buffer to a microhomogenizer and was carefully ground to give a smooth suspension. Each suspension was then dispersed (25 to 30 μl) into small plastic Sarsdedt tubes, and 5 μl of serial dilutions of *Staphylococcus aureus* V8 protease (ranging from 0 to 10 mg/ml) was added to each sample. After incubation for 1 h at 37°C, 10 μl of buffer containing 2% sodium dodecyl sulfate and 10 mM dithiothreitol were added. The resultant phosphopeptides were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The dried gels were exposed to X-ray film for 2 to 4 days.

Immunoassay for DNA-binding studies. An immunoassay described by McKay (15) was adapted for studying the DNA-binding properties of Py large T-Ag. Py large T-Ag obtained from crude cell lysates of 3T6 cells infected with wild-type (A2) virus or from Py-transformed rat cells (7axB) was reacted with 5′ end-labeled (23) restriction endonuclease fragments of Py DNA or fragments labeled from 3′ ends by replacement synthesis with T4 DNA polymerase (a kind gift of Alison Cowie; Cowie et al., manuscript submitted for publication). The DNA protein complexes were bound by an anti-T serum and were immunoprecipitated with Formalin-fixed *S. aureus*.

In a typical reaction mix, 100 to 200 μg of crude lysate from either Py-infected 3T6 cells or 7axB cells was added to 5 ng of ^{32}P end-labeled DNA fragments in 1 ml of binding buffer and was incubated at 22°C for 60 min. The binding buffer contained 20 mM sodium phosphate (pH 7.0), 2 mM dithiothreitol, 0.01% (wt/vol) bovine serum albumin, 0.1 mM EDTA, 0.05% Nonidet P-40, 3% dimethyl sulphoxide, and 0.15 M NaCl.

Nonspecific binding was reduced considerably by incubating the DNA protein complexes with 100 μl of a 10% *S. aureus* suspension before immunoprecipitation. For immunoprecipitation, the DNA protein complexes were incubated with 25 to 30 μl of anti-T serum or normal rat serum for 1 h at 22°C. The immune complexes (DNA–T-Ag–antibody) were collected by adsorption to protein A-bearing *S. aureus*. The immunoadsorbent was washed twice with wash buffer containing 20 mM Tris-hydrochloride (pH 8.0), 0.15 M NaCl, 2 mM dithiothreitol, 1 mM EDTA, 0.01% (wt/vol) bovine serum albumin, 10 μg of calf thymus DNA (sheared) per ml, and 0.5% Nonidet P-40. This reduced the binding of DNA to *S. aureus* in the absence of protein. The immune complexes were eluted in Tris-EDTA containing 7% sodium dodecyl sulfate. The bound DNA fragments were extracted twice with Tris-EDTA-saturated phenol and once with chloroform and were ethanol precipitated. The precipitate was applied to 6 to 8% polyacrylamide gels (0.8 mm), and the bound DNA fragment was identified by autoradiography. Dried gels were exposed to X-ray film for 2 to 6 days.

RESULTS

Location of the 7B-M mutation. Figure 1 shows the map of the Py insert in 7axB cellular DNA and the three *Eco*RI fragments (7B-L, 7B-M, 7B-R) previously cloned and characterized (10). The 7B-M clone contains a full-length *Eco*RI linear viral molecule. When the 7B-M insert was cleaved from the procaryotic vector with *Eco*RI, circularized, and transfected into cells, it was found to be transformation competent on rat cells but incapable of inducing plaques on mouse cells (10). To locate the plaque formation lesion of 7B-M, a series of chimerae (17) were formed between 7B-M DNA and wild-type Py (strain CR) DNA. Strain CR (16) has restriction site markers that allow the origin of progeny plaques to be identified (see above). Chimerae formed between various restriction fragments of 7B-M DNA and wild-type Py DNA were tested for their infectivity. The results of the various constructions and their plaque-forming ability are

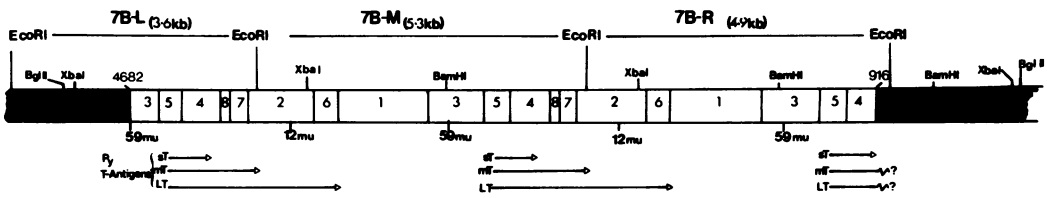

FIG. 1. Map of the tandem array of the integrated viral sequences in the 7axB cell line. The map was determined by Southern blot hybridization, molecular cloning, and DNA sequencing (10, 13). Flanking DNA sequences (black) and viral sequences (white) are shown relative to the *Hpa*II physical map (9). The nucleotide numbers (21) indicate the first discontinuity in the viral DNA sequences. The coding regions for the Py small T-Ag (sT), middle T-Ag, (mT) and large T-Ag (LT) are indicated. The size of each cloned *Eco*RI fragment from 7axB (7B-L, 7B-M, 7B-R) is also shown. Fragments 7B-L and 7B-M contain both viral and flanking host DNA sequences, whereas 7B-M contains just viral sequences (*Eco*RI linear Py DNA).

presented in Table 1. In this way, the 7B-M lesion was located between the *Hha*I site at 14 m.u. and the *Xba*I site at 18 m.u. The chimera containing the 7B-M sequence from the *Eco*RI site at 0 or 100 m.u. to the *Hha*I site at 14 m.u. is infectious, whereas the chimera containing the 7B-M sequence from the *Eco*RI site to the *Xba*I site at 18 m.u. does not form plaques.

DNA sequence analyses were subsequently performed on both 7B-M DNA strands between the *Hha*I site at 14 m.u. and the *Xba*I site at 18 m.u. Two changes from the published sequence of Friedmann et al. (7) were detected at nucleotides 2503 (A to G) and 2504 (C to T) (Fig. 2). The change at 2504 does not alter the amino acid (aspartic acid) encoded by the Py DNA triplet, and a C instead of a T is found at this position in the sequence determined by Soeda et al. (21). The A to G transition at nucleotide 2503 in fragment 7B-M destroys the *Xba*I cleavage site (TCTAGA) at 17 m.u. and results in a substitution of a glycine for aspartic acid in the large T-Ag expressed in 7axB cells (Fig. 2). A similar analysis of sequences of the 7B-R clone (Fig. 1) in this region showed that the same base changes were present as in 7B-M, resulting in the loss of the *Xba*I cleavage site at 17 m.u.

Properties of the 7B-M large T-Ag. The effect of the base change at nucleotide 2403 on both the in vitro DNA binding and the in vivo phosphorylation of the 7axB large T-Ag was examined. It is thought that in vivo replication of Py DNA is initiated by the specific binding of Py large T-Ag to regulatory sequences on the Py genome. In vitro Py large T-Ag binds to at least two sites of Py DNA near the origin of DNA replication (A. Cowie et al., manuscript submitted). One site, located close to the origin, is thought to be involved in DNA replication, whereas the other site, around the start of the Py early mRNAs, is thought to be involved in the autoregulation of early viral RNA synthesis. Both T-Ag binding sites lie in the Py 604-base pair (bp) *Hin*fI fragment which extends from Py nucleotide 5073 to Py nucleotide 385 (21). With a modification of the assay described by McKay (15), no consis-

TABLE 1. Chimerae formed between DNAs of wild-type Py and fragment 7B-M[a]

Enzyme digestion	Chimera				Plaque-forming ability (PFU/μg)
	Py fragment		7B-M fragment		
	Size (%)	Position (m.u.)	Size (%)	Position (m.u.)	
*Eco*RI	100	0–100			1.3×10^5
*Eco*RI			100	0–100	<10
*Eco*I-*Bam*	41	59–100	59	0–59	<10
*Eco*RI-*Bam*	59	0–59	41	59–100	5×10^4
*Eco*RI-*Hin*cII (partial)	74	26–100	26	0–26	<10
*Eco*RI-*Hin*cII (partial)	26	0–26	74	26–100	6×10^3
*Eco*RI-*Xba*I (partial)	82	18–100	18	0–18	<10
*Eco*RI-*Xba*I (partial)	18	0–18	82	18–100	8×10^3
*Eco*RI-*Hha*I (partial)	14	0–14	86	14–100	<10
*Eco*RI-*Hha*I (partial)	86	14–100	14	0–14	6×10^3

[a] Chimerae (17) were formed by ligation of isolated restriction endonuclease fragments of 7B-M and Py DNA as indicated. The chimera DNAs were tested for their plaque-forming ability on mouse embryo cells after DNA transfection.

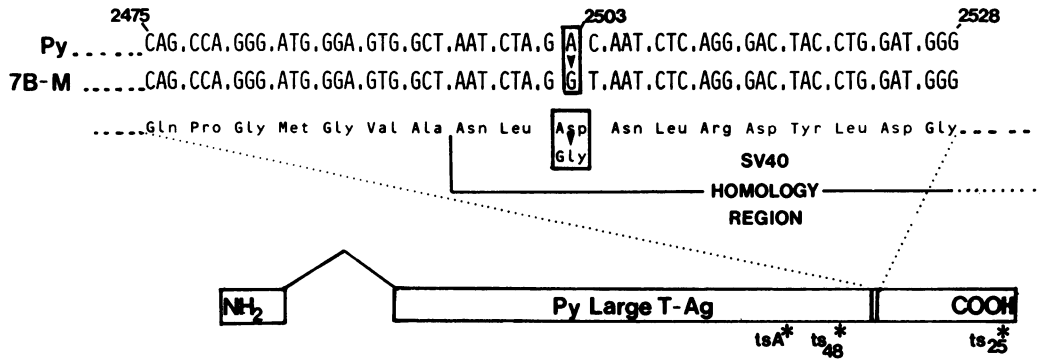

FIG. 2. DNA sequence of the region responsible for the loss of infectivity of 7B-M DNA located in the SV40 homology region. The DNA sequence of the region of fragment 7B-M containing the mutation is compared with that derived by Friedmann et al. for Py DNA (7). Two differences at nucleotides 2503 and 2504 are noted. The nucleotide change at 2504 does not affect the coding capacity of the DNA triplet, whereas the change at 2503 results in the conversion of an Asp to a Gly in the amino acid sequence and the loss of an *Xba*I site. Between nucleotides 2596 and 2613 there is a large region of homology between Py and SV40. This region has 34 of 39 amino acids (87%) and 82 of 117 nucleotides (70%) conserved between the two viruses. The mutation lies in a stretch of 21 conserved amino acids (of which eleven are shown) within this SV40 homology region. At the bottom is indicated the position of the 7B-M mutation in the C-terminal region of the Py large T-Ag in relation to the position of sequence changes found in the three *ts*A mutants *ts*A, *ts*48, and *ts*25 (22).

tent differences in different experiments could be detected between the binding of the 7axB and Py large T-Ags to the 604-bp *Hin*fI fragment at a number of different salt concentrations (Fig. 3). Cleavage of the 1,132-bp Py *Bam*HI-*Sst*I fragment (nucleotides 4362 to 569) with *Bgl*I results in a 750-bp fragment (nucleotides 4632 to 87) thought to contain the DNA replication large T-Ag binding site and a 482-bp fragment (nucleotides 87 to 569) thought to contain the autoregulation large T-Ag binding site. No significant differences could be detected between the binding of the 7axB and Py large T-Ag to these two fragments (Fig. 4). Also, no significant differences could be detected between the binding of the 7axB and Py large T-Ag to either a 87-bp (replication binding site) or a 312-bp (autoregulation binding site) *Hpa*II-*Bgl*I fragment extending from nucleotides 1 to 87 and 88 to 399, respectively (data not shown).

The Py large T-Ag is a phosphoprotein (20). To see whether the pattern of phosphorylation of the mutant 7axB large T-Ag was different from that of Py large T-Ag, the partial proteolytic cleavage method of Cleveland et al. (3) was utilized after different degrees of proteolysis. No significant differences between the cleavage patterns of the two ^{32}P-labeled proteins were detected (Fig. 5).

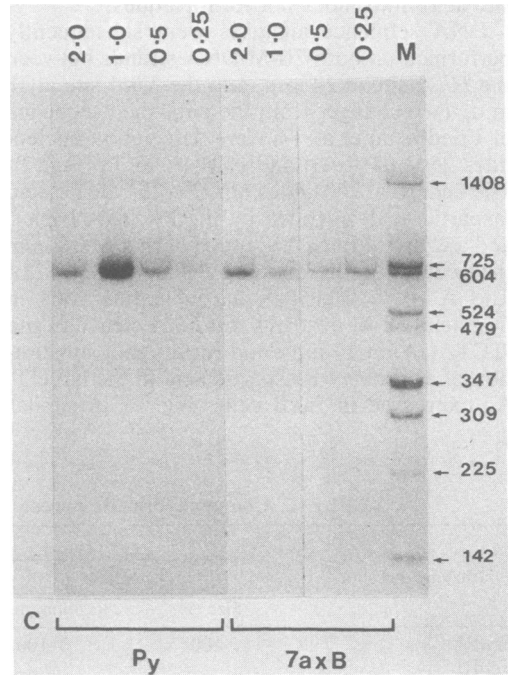

FIG. 3. DNA binding of Py and 7axB large T-Ags to *Hin*fI cleaved Py DNA. Extracts from unlabeled Py-infected mouse 3T6 cells (Py) and 7axB cells (7axB) were reacted with ^{32}P 5′ end-labeled *Hin*fI cleaved Py DNA, immunoprecipitated with Py anti-T serum, and fractionated on a 6% polyacrylamide gel. The numbers at the top of the figure indicate the molarities of NaCl used in the immunosorbent wash. Track C represents the infected 3T6 extract reacted with normal serum; track M contains the end-labeled *Hin*fI-digested Py fragments.

DISCUSSION

Growth in vitro or passage in vivo of wild-type Py-transformed cells can result in the loss of a functional large T-Ag (4, 12). This has been observed to be due to both alteration (addition

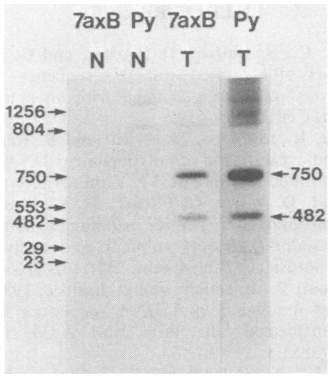

FIG. 4. DNA binding of Py and 7axB large T-Ags to Py DNA digested with *Bam*HI, *Sac*I, and *Bgl*I. Extracts from unlabeled Py-infected 3T6 cells (Py) and 7axB cells (7axB) were reacted with pAT153 plasmid DNA containing a *Bam*HI Py linear DNA after ^{32}P replacement labeling at the 3' ends of the fragments derived by digestion with *Bam*HI, *Sac*I, and *Bgl*I. After immunoprecipitation with either normal serum (N) or anti-T serum (T), the products were fractionated on a 1.6% agarose gel. The sizes of the end-labeled fragments of the pAT153-Py plasmid DNA are indicated.

or deletion) to integrated sequences unique to the large T-Ag and to fusion of the large T-Ag-unique sequences to host DNA (1, 5, 10–13, 19; H. E. Ruley et al., unpublished results). As a result, a full-size (100K) large T-Ag is not produced from such integrated viral sequences. The 7axB cells were derived from a tumor induced by inoculating cells of the in vitro transformed line 7ax (12). These 7ax cells contain free viral genomes and a fully functional large T-Ag (the viral DNA replication protein) (13). The 7axB cells contain an apparently inactive large T-Ag, as a result of which no free viral DNA is present in 7axB cells, even after fusion to permissive mouse cells (L. Lania, personal communication). By analysis of cloned integrated viral sequences, a change of a single base-pair at nucleotide 2503 has been identified as responsible for the inactive 7axB large T-Ag and for the consequent nonviability of fragment 7B-M DNA.

The mutation is situated at nucleotide 2503 and results in the change of a single amino acid. The mutation lies in a region of the genome which is unique to large T-Ag and where a

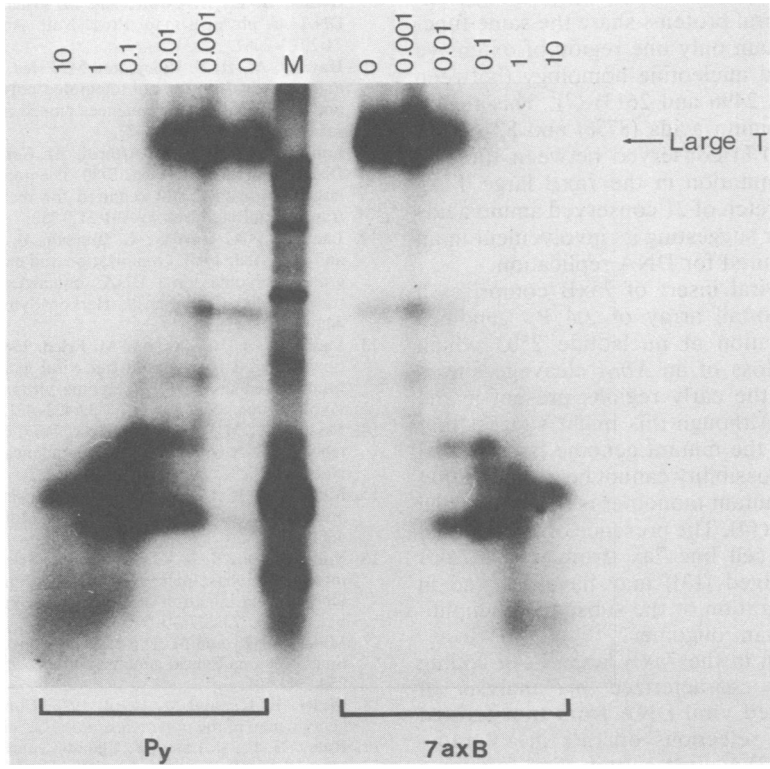

FIG. 5. Partial proteolysis comparison of Py and 7axB large T-Ags. The ^{32}P-labeled large T-Ags from Py-infected 3T6 cells and 7axB cells were isolated from a 10% polyacrylamide gel after immunoprecipitation. The isolated polypeptides were digested for 1 h at 37°C with increasing amounts of *S. aureus* V8 protease, and the products were separated on a 15% polyacrylamide gel. The different protease concentrations used are indicated on the top in milligrams per milliliter. Track M contained markers of known molecular weight.

number of Py *ts*A mutations which code for thermolabile Py large T-Ags have been mapped (18). It is thought that as a prerequisite for viral DNA replication the Py large T-Ag binds to the viral DNA, and in vitro Py large T-Ag has been observed to bind to sequences around the Py origin. The exact relationship between the binding of Py large T-Ag to Py DNA in vitro and events in vivo is unclear at present. Wilson et al. (24) have shown that a number of SV40 temperature-sensitive A mutants produce large T-Ags which are thermolabile in their in vitro binding to DNA sequences at the SV40 origin of DNA replication. Thus, the in vitro binding properties of the SV40 mutant large T-Ags appear to be correlated with their inability to replicate in vivo at the nonpermissive temperature. By contrast, the 7axB mutation does not appear to affect the specific binding of the 7axB large T-Ag to sequences around the Py origin of viral DNA replication (Fig. 3 and 4), nor does it affect the phosphorylation pattern of the protein (Fig. 5). These results suggest that the mutation might affect an active site involved in large T-Ag-promoted DNA replication. The virus-coded proteins required for viral DNA replication of Py and the related SV40 are the large T-Ags. Although these viral proteins share the same function, they contain only one region of extensive amino acid and nucleotide homology (between Py nucleotides 2496 and 2613) (7). This region has 34 of 39 amino acids (87%) and 82 of 177 nucleotides (70%) conserved between the two viruses. The mutation in the 7axB large T-Ag lies within a stretch of 21 conserved amino acids (Fig. 2), further suggesting its involvement in an active site required for DNA replication.

The single viral insert of 7axB comprises a tandem head-to-tail array of 2.4 Py genomes (10). The mutation at nucleotide 2503 which results in the loss of an *Xba*I cleavage site is found in both the early regions present in the viral tandem. Although this might suggest that an oligomer of the mutant genome is the initial integrant, the possibility cannot be excluded that an integrated mutant monomer is at a later stage amplified in situ (4). The presence of a functional large T-Ag in cell line 7ax (from which 7axB cells were derived [13]) may have assisted in either the integration or the subsequent amplification of a mutant oligomer.

The mutation in the 7axB large T-Ag coding sequence was characterized by analysis of cloned integrated viral DNA from transformed cells. Because selections operate in Py-transformed cells (13) against certain viral functions (e.g., replication) that are intact in infecting molecule populations, it is likely that further nonviable papovavirus mutants will be isolated in this way.

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