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# Asp-286 → Asn-286 in Polyomavirus Large T Antigen Relaxes the Specificity of Binding to the Polyomavirus Origin

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**We isolated revertants of a polyomavirus whose origin of DNA replication contains a point mutation in the palindrome to which large T antigen binds. Four independent second-site revertants contain an Asp-286 → Asn-286 substitution in large T antigen. This mutant large T antigen activates replication of DNAs containing the mutant polyomavirus origin as well as replication of DNAs containing the wild-type origin; however, replication of DNAs with enhancer mutations is not activated by this large T antigen. The Asn-286 mutation occurs in a positively charged region of large T antigen near the location of several mutations which inactivate DNA replication. We suggest that this region of large T antigen is responsible for recognition of specific DNA sequences at the origin and that ionic forces are important for this interaction.**

Initiation of polyomavirus DNA replication in vitro requires the interaction of at least three components: the origin of replication (*ori*), the virus-encoded large T antigen, and one or several cellular proteins, including DNA polymerase-primase (14, 42, 53). The core *ori* (nucleotide [nt] 5295 to 64) contains a 34-base-pair palindrome with multiple large-T-antigen-binding sites and an A+T-rich sequence (Fig. 1A). The arrangement of these sites is essential for the formation of a functional initiation complex comprising large T antigen, DNA polymerase-primase, and possibly several other proteins. Additional DNA sequences required for DNA replication in vivo include the enhancer and large-T-antigen-binding site A, on either side of the *ori* palindrome (2, 9, 15, 30, 35, 41, 53, 63, 65). Cellular factors interact with the enhancer (5, 22, 32, 38, 50, 51; reviewed in reference 27) and perhaps promote large T antigen binding to the *ori* (13, 53, 63), while site A may serve as an efficient binding site for large T antigen to recruit DNA polymerase-primase (Tang and Folk, unpublished data).

Large T antigen expresses several distinct biochemical activities, including specific binding to multiple sites (5'-GPuGGC) at the *ori* (7, 8), an ATPase (23), and an ATP-dependent DNA helicase (42, 60). As in the action of simian virus 40 (SV40) large T antigen at the SV40 *ori* (10, 16), of the *Escherichia coli* DnaA, DnaC, and DnaB proteins at the *E. coli oriC* (1), and of bacteriophage lambda proteins O and P at the lambda *ori* (17), polyomavirus large T antigen binds to the core *ori* to form a specialized ATP-dependent nucleoprotein complex. The polyomavirus *ori* probably is unwound through the large-T-antigen DNA helicase reaction. This preinitiation complex probably interacts with DNA polymerase-primase to allow priming at the origin of bidirectional replication (25) and subsequent elongation of nascent DNA strands.

In this report, we describe the isolation of a large-T-antigen mutant with reduced specificity of binding at *ori*. This mutant large T antigen contains an isosteric amino acid change (Asp-286 → Asn-286) within one of two positively charged regions of a potential DNA-binding domain. This mutation suppresses the deleterious effect of different point mutations in several mutant origins. Isolation of this mutant provides strong evidence that binding of large T antigen to *ori* is required for the initiation of viral DNA replication in vivo and focuses on a region of large T antigen which may be important for sequence-specific contacts with DNA.

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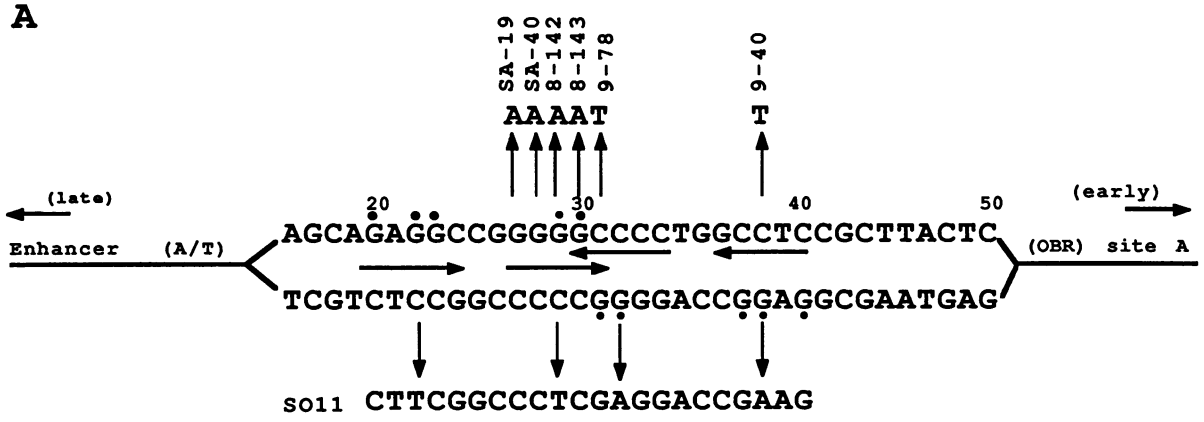
## MATERIALS AND METHODS

**Generation of *ori* mutant SO11 by oligonucleotide-directed mutagenesis.** Mutagenesis was carried out as described by Kunkel et al. (33) with M13mp9DB1 (64) as a template. This clone contains the noncoding regulatory region of the polyomavirus genome between nt 5046 (*BclI*) and nt 200 (*DdeI*). Uracil-containing, single-stranded M13mp9DB1 DNA was annealed with phosphorylated oligonucleotide 5'-GCGGAA GCCAGGAGCTCCCGGCTTCTGCTT (nt 43 to 15; underlined bases indicate sites differing from the polyomavirus core *ori* palindrome) at a ratio of 1 to 6 in 50 mM Tris chloride (pH 7.5)–10 mM $MgCl_2$–5 mM dithiothreitol–1 mM ATP. The mixture was heated at 95°C for 3 min, quickly cooled to 28°C, and incubated at this temperature for an additional 60 min. The solution was adjusted to 0.5 mM deoxynucleoside triphosphate, 10 mM $MgCl_2$, 10 mM Tris-chloride (pH 7.5), 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4), 2 mM dithiothreitol, and 1 mM ATP with 2.5 U of the large fragment of DNA polymerase I and 10 U of T4 DNA ligase. The reaction was incubated at 0°C for 10 min and then at 30°C for 4 h. The DNA products were transformed into *E. coli ung*$^+$ MV1190, and single plaques were purified and screened by DNA sequencing. This mutated *ori* sequence containing the indicated changes was reconstructed into the polyomavirus genome as previously described (63).

**Isolation of large-T-antigen pseudorevertants.** Mutant M13 mp8DB-8-142 (8-142) contains a G → A transition at nt 29 in the large-T-antigen-binding motif of the *ori* palindrome (64). Its *ori* was reconstructed into a full-length polyomavirus genome cloned in pGL101 (63). Plasmids containing polyomavirus with the 8-142 origin were transformed into the *E. coli* mutator strain *mutD5* K1617 and grown in L broth with 10 μg of thymidine per ml (20). DNAs with random mutations were isolated, and DNA fragments from *Hae*II (nt 99) to *Eco*RI (nt 1575) or *Eco*RI (nt 1575) to *Bam*HI (nt 4657) encoding either the N-terminal or C-terminal portions of

A




B

| DNA | wt | SA-19 | SA-40 | 8-142 | 8-143 | 9-78 | 9-40 | SO11 |
|---|---|---|---|---|---|---|---|---|
| replication | ++++ | + | ++ | +/- | +/- | + | +/- | - |
| plaque size | large | N.D. | N.D. | - | N.D. | medium | small | - |

FIG. 1. (A) Features of the polyomavirus *ori* and sequence alterations of *ori* mutants. The sequence of the palindrome at the core origin of polyomavirus strain A3 is numbered as in reference 12. Nucleotides with dots (above or below), when methylated, cause interference of large-T-antigen binding (8). A/T indicates the A+T-rich sequence on the late side of the core origin. OBR indicates origin of bidirectional replication (25). The horizontal arrows indicate the large-T-antigen-binding motif 5'-GPuGGC-3'. (B) Replication and plaque sizes of *ori* mutants. +++, Wild-type replication; ++, 10 to 80% replication; +, 1 to 10% replication; ±, <10% replication; −, no replication. Plaque sizes were determined at day 11 posttransfection. − means no plaques were detected after 15 days. The data for replication of mutant SA-40 are from our previous work (64). N.D., Not determined.

large T antigen, respectively, were fractionated through an 0.8% agarose gel and isolated with a DEAE membrane (NA-45; Schleicher & Schuell, Inc., Keene, N.H.). The unmutated *Eco*RI (nt 1575)-to-*Hae*II (nt 99) or *Bam*HI (nt 4657)-to-*Eco*RI (nt 1575) fragments containing the 8-142 *ori* mutation were also isolated and ligated to the complementary mutated fragments to reconstruct a full-length polyomavirus genome with an 8-142 *ori* and random mutations in the large-T-antigen-coding sequences. These polyomavirus DNAs were transfected into whole mouse embryo (WME) cells with DEAE-dextran, and single plaques were picked, purified, and amplified as viral stocks in WME cells. Viral DNAs were cloned as previously described (63). DNA sequence analysis was performed by dideoxy double-stranded DNA sequencing (68).

**DNA replication assays.** To help investigate the specificity of isolate 8-142N8 large T antigen toward different *ori* mutants, we constructed two helper DNAs, pSO11-wt and pSO11-N8, each containing the noncompetitive mutant SO11 *ori* and expressing wild-type or revertant 8-142N8 large T antigen, respectively. Neither construct replicated in 3T6 cells (data not shown). Use of these DNAs to encode large T antigen in transient replication assays increased the sensitivity with which we can assess the capacity of wild-type or 8-142N8 mutant large T antigen to activate DNA replication of mutant and wild-type *ori* DNAs, for the helper DNA does not compete for *trans*-acting replication factors.

Cultures of $10^6$ 3T6 cells in 60-mm culture dishes were transfected with a mixture of 1 μg of double-stranded M13-polyomavirus (M13-Py) DNAs, 0.2 μg of polyomavirus (SO11)-pMK16 helper DNAs, and 8 μg of salmon sperm DNAs by calcium phosphate coprecipitation (66). At 4 to 5 h after the calcium phosphate coprecipitate was added to the cells, the medium was replaced with 1 ml of 20% (vol/vol) glycerol in Eagle medium for 1 min (48). In some cases, cells were transfected with 0.5 μg of test DNA and 0.1 μg of helper DNA with DEAE-dextran (40). The cells were rinsed and then covered with medium and incubated at 37°C. At 48 h posttransfection, low-molecular-weight DNAs were isolated (26) and digested with pancreatic RNase and proteinase K followed by phenol-chloroform extraction and ethanol precipitation. The purified DNAs were digested with *Dpn*I and *Eco*RI (to linearized *Dpn*I-resistant DNAs). The digested DNAs were fractionated through a 0.8% agarose gel, transferred to nitrocellulose membranes, and hybridized with radiolabeled M13-Py DNA.

**Quantitation of large T antigen.** The accumulation of large T antigen within infected cells was measured by polyacrylamide gel electrophoresis of immunoprecipitates of cell extracts. 3T6 cells ($3 \times 10^6$) were infected with virus at a multiplicity of 5. The viruses used for each infection contained a wild-type *ori* to avoid the complication of different replication capacities. At 44 h postinfection, cells were washed three times with phosphate-buffered saline and then 2.5 ml of methionine-free medium with *trans*-$^{35}$S label (230 μCi/ml; ICN Pharmaceuticals Inc., Irvine, Calif.) was added. After 3 h of incubation at 37°C, the cells were washed and the protein was extracted. Large T antigen was isolated by immunoprecipitation with monoclonal antibody F4 specific for polyomavirus T antigen (47) (kindly provided by Ed Harlow) and analyzed by electrophoresis on a sodium dodecyl sulfate-8% polyacrylamide gel.

## RESULTS

**Identification of functional sequences within polyomavirus *ori* palindrome.** A number of polyomavirus *ori* mutants with base pair transitions have been isolated and grouped by their

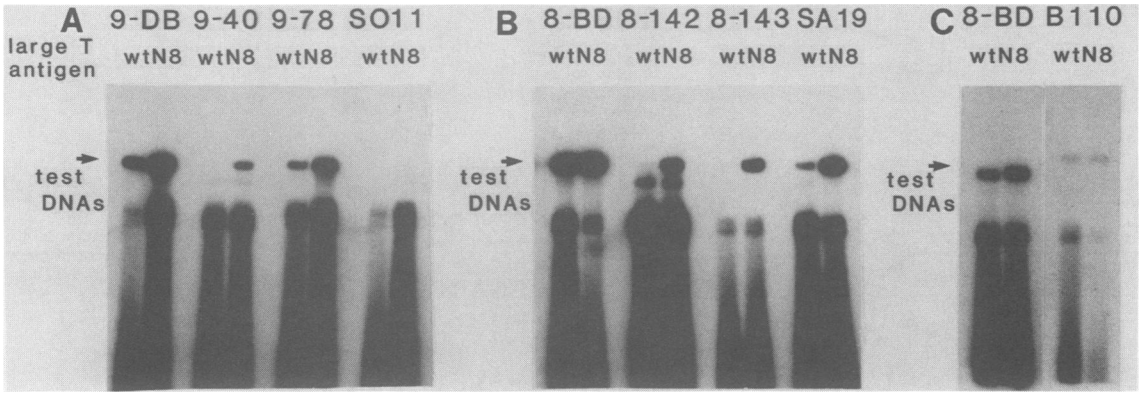



FIG. 2. Complementation of 8-142N8 large T antigen for replication of M13 DNAs containing wild-type or mutated *ori* regions. SO11-wt or SO11-N8 recombinants were used to provide wild-type large T antigen (wt) or N8 large T antigen (N8) so as not to compete with the replication of test DNAs. The indicated test DNAs were cotransfected with helper DNAs into 3T6 cells either with DEAE-dextran (A) and (B) or by calcium phosphate coprecipitation (C). Low-molecular-weight DNAs isolated 48 to 54 h posttransfection were digested with *Dpn*I and with *Eco*RI and then fractionated through 0.8% agarose gels. Digested products were blotted onto nitrocellulose and hybridized to radiolabeled M13mp9-Py DNA.

replication abilities (36, 64). These studies indicate that mutations of the putative large-T-antigen-binding motifs (5′-GPuGGC) of the 34-base-pair palindrome within *ori* severely hamper DNA replication. Analysis of the effects of single base pair substitutions throughout the *ori* palindrome with the noncompetitive helper SO11 (Fig. 1A) to provide large T antigen indicated that base pair transitions at the third or fourth or fifth nucleotide of the GPuGGC large-T-antigen-binding motifs at the core *ori* seemed to have a greater effect on DNA replication (9-40, 8-142, and 8-143, ± for replication) than base pair transitions at the first or second nucleotide (SA-19, + for replication; SA-40, ++ for replication) (Fig. 1B and 2). This agrees with a recent study of SV40 large-T-antigen binding at the SV40 *ori* (11). However, it is worth noting that even though mutant 9-78 contains a mutation whose location relative to the dyad of the *ori* palindrome is the same as that of mutant 8-143, mutant 9-78 replicated better than mutant 8-143 in the transient replication assay (Fig. 3). This difference in replication efficiency might be due to an asymmetry in the function of large T antigen bound to the *ori* palindrome (such as in the requirement for binding of additional proteins) or to sequence differences outside the pentanucleotide large-T-antigen-binding motif.

To ascertain whether the defects of DNA replication of these mutants are due to weakened binding of large T antigen, we performed in vitro DNA-binding assays with S. Triezenberg, A. Cowie, and R. Kamen, using immunoaffinity-purified large T antigen. We observed that wild-type large T antigen had a reduced affinity (30 to 70%) for most mutated *ori* DNAs (data not shown). However, binding of large T antigen to the mutant *ori* DNAs did not quantitatively correlate with the observed extent of inactivation of DNA replication, perhaps because multiple sites for large-T-antigen binding are involved in forming the replication complex at *ori*.

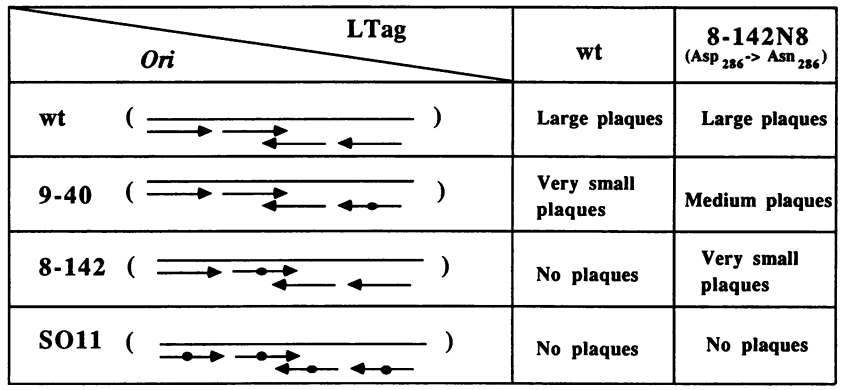
To determine whether binding of large T antigen was responsible for the defective replication of these DNAs and, more generally, to show that binding of large T antigen to the origin was required for initiation of replication of polyomavirus DNA, we attempted to isolate second-site mutations in large T antigen which would suppress the *ori* mutations. The results of such efforts follow.

**Isolation and characterization of large-T-antigen pseudorevertant.** Polyomavirus *ori* mutant 8-142 contains a G → A transition at nt 29 and as result replicates poorly and does not form plaques (Fig. 1). To induce a variety of transitions, transversions, and frameshift mutations in its large-T-antigen-coding sequences, we passed this DNA through an *E. coli* mutator strain (21). Fragments encoding large T antigen were excised from the cloned DNA and rejoined to unmutagenized 8-142 *ori* DNA to reconstitute a complete viral genome, which was transfected into whole mouse embryo (WME) cells. DNAs with random mutants in the first half of large T antigen (amino acid residues 1 to 355) formed a few very small plaques. This virus in several of these plaques were grown up, and their DNAs were cloned for further analysis.

During the preparation of viral stocks of these mutants, we observed that the revertant viruses changed from a small-plaque phenotype to a large-plaque phenotype. Sequence analysis of four independent revertants revealed that they all had A → G reversions at nt 29 restoring the wild-type origin. Although this was disappointing, it was not unexpected, for we had previously observed hypermutability of the polyomavirus origin sequences. Such hypermutability may be a consequence of error-prone priming at *ori* (Tang and Folk, unpublished data) and is being investigated further.

We suspected that these revertants first might have acquired a mutation in large T antigen resulting in a small-plaque phenotype which permitted sufficient viral DNA replication to allow the mutated *ori* to revert to a wild-type *ori*. To verify this hypothesis, polyomavirus recombinants with a portion of the 8-142N8 revertant large T antigen (the *Hae*II [nt 99]-to-*Eco*RI [nt 1575] fragment) and different mutant *ori* regions were constructed and tested for their plaque-forming abilities. We found that the sequences encoding the revertant large T antigen conferred upon DNAs with mutant 8-142 *ori* the capacity to form very small plaques and conferred upon DNAs with mutant 9-40 *ori* the capacity to form medium-size plaques. A recombinant containing the revertant 8-142N8 large T antigen in conjunction with a wild-type *ori* formed plaques the same size as those formed by wild-type virus (Fig. 3A). The plaque phenotype of viruses with the revertant large T antigen was not altered when the temperature of incubation was maintained at

A.

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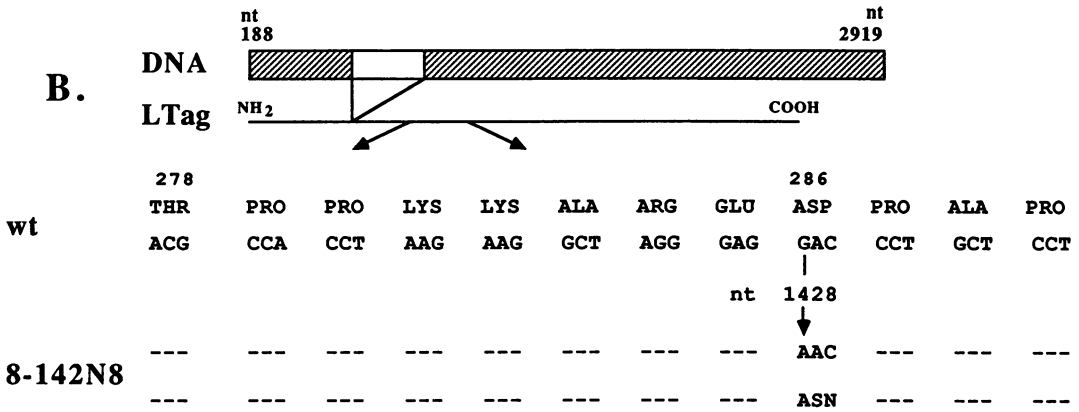


FIG. 3. (A) Viability of *ori* mutants with either wild-type (wt) or 8-142N8 large T antigen. Recombinants were constructed by joining the appropriate *Hae*II-*Eco*RI restriction fragments. The horizontal arrows indicates the large-T-antigen-binding sites at the core *ori*. The dot on the arrows indicates where the large-T-antigen-binding site GPuGGC is changed to GPuAAC. (B) DNA and amino acid sequence of the large T antigen. The upper schematic shows the region of the polyomavirus genome encoding the large T antigen. Dashed boxes signify the exons. The nucleotide sequence and inferred amino acid sequence of wild-type large T antigen is shown below, with the amino acid substitution of 8-142N8 large T antigen.

39.5°C instead of 37°C, indicating that the revertant large T antigen is not thermosensitive.

To localize the site of pseudoreversion in revertant 8-142N8, we generated in vitro recombinants with different portions of the 8-142N8 *Hae*II-*Eco*RI fragment and 8-142 *ori* and measured their resulting plaque sizes. We found that only the *Ava*I (nt 1031)-to-*Eco*RI (nt 1575) fragment could rescue 8-142 *ori* to form small plaques. Sequence analysis of this fragment from four independent revertants of 8-142 revealed the same single base pair substitution: G · C → A · T at nt 1428. This mutation causes an isosteric change from aspartate to asparagine at amino acid 286 of large T antigen but does not change the sequences of middle and small T antigens (Fig. 3B). This mutant large T antigen will be referred to by the virus from which it was isolated, 8-142N8.

**Activation of DNA replication of different *ori* mutants by 8-142N8 large T antigen.** In analyses of the activation of replication of DNAs with a normal *ori* (M13mp8BD, M13mp9DB), we observed no reproducible difference between providing either the wild-type or the 8-142N8 large T antigen (Fig. 2). For DNAs having a single substitution in the large-T-antigen-binding site of the 34-base-pair *ori* palindrome (M13mp9-40, -9-78, -8-142, -8-143, -SA-19), a 5- to 10-fold increase of DNA replication was observed by providing 8-142N8 large T antigen in *trans* as compared with that observed when wild-type large T antigen was provided in *trans*. However, the mutant SO11 origin (which had all four GPuGGC motifs within the *ori* palindrome changed to GPuAGC) was not activated by either large T antigen. Either the 8-142N8 large T antigen could not activate such a severely changed *ori*, or other proteins might be required to bind to this palindrome for DNA replication to be initiated. We tested whether the 8-142N8 large T antigen rescued replication-defective DNAs having mutant enhancers by transfecting pSO11-N8 together with the enhancer mutant B110 (63). We observed no activation of B110 DNA replication by 8-142N8 large T antigen over that provided by the wild-type large T antigen (Fig. 2C).

These results indicate that the 8-142N8 large T antigen restores DNA replication of mutants with base substitutions at the large-T-antigen-binding motif *ori* rather nonspecifically. That the DNA replication of polyomavirus with mutant origins can be activated by such an altered large T antigen indicates that their replication defects are due to weakened or faulty binding of large T antigen.

**Expression of 8-142N8 large T antigen.** The suppression of the replication defects of DNA with mutant origins by the 8-142N8 large T antigen might be due to its excessive accumulation within the nucleus. To determine whether this occurs, we measured the quantity of large T antigen in both wild-type and 8-142N8-infected 3T6 cells by immunoprecip-

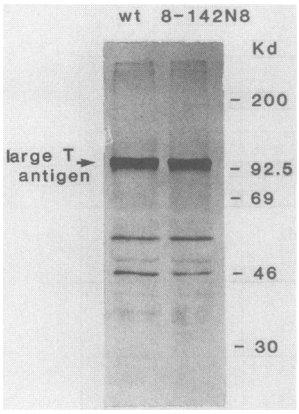


FIG. 4. Comparison of large-T-antigen production of wild-type (wt) and 8-142N8 (N8) viruses. At 44 h postinfection, the infected 3T6 cells were labeled with [$^{35}$S]methionine for 3 h. The large T antigen was quantitated by immunoprecipitation with monoclonal antibody specific to polyomavirus T antigen, followed by electrophoresis on an 8% polyacrylamide gel. Kd, Kilodaltons.

itation. We could detect no difference in the level of large-T-antigen production between the two viruses (Fig. 4). Since pSO11-wt and pSO11-N8 were used as helpers in transient DNA replication assays, the amount of large T antigen produced in cells transfected by these DNAs was compared by immunoprecipitation. Again, no significant difference was observed (data not shown). These results indicate that large-T-antigen accumulation is not affected by the 8-142N8 mutation.

One of several nuclear localization signals contained within the large-T-antigen sequences borders Asp-286 (54). To rule out the possibility that activation of DNA replication by 8-142N8 large T antigen is due to increased nuclear translocation of large T antigen, we examined the localization of large T antigen in the pSO11-wt- and pSO11-N8-transfected cells by immunofluorescence. The nuclei of these transfected cells contained comparable amounts of large T antigen, suggesting that nuclear transport is unaffected by the 8-142N8 mutation (data not shown). Bockus and Schaffhausen have also independently determined that the nuclear localization is unaffected by this mutation (personal communication).

**Noncooperative interaction between wild-type and pseudorevertant large T antigen for polyomavirus DNA replication.** Dean et al. (10) have shown that 8 to 16 SV40 large-T-antigen molecules aggregate at the SV40 *ori* to form a bilobed complex, presumably to form a preinitiation complex for viral DNA replication. It is likely that a similar complex forms at the polyomavirus *ori*. Can wild-type and 8-142N8 large T antigens jointly form an initiation complex for viral DNA replication? To address this question, we cotransfected a constant amount of wild-type (M13mp8BD or M13mp9DB), 9-40, or 8-143 *ori* test DNA with variable amounts of helper DNA capable of expressing wild-type or 8-142N8 large T antigen. The helper DNAs were provided in a range where the amount of test DNA replication is proportional to the quantity of cotransfected helper (0.01 to 2 μg). Each wild-type large-T-antigen monomer should interact with every GPuGGC site in the *ori* palindrome of the test *ori* DNAs (Fig. 1A). We do not know the extent to which the wild-type large T antigen will interact with the mutant GG GGT or GGGAC sites in 8-143 or with the GAAGC site in 9-40 DNA, but the 8-142N8 large T antigen should bind these mutated sites to form a functional complex. Cooperation between the wild-type and the 8-142N8 large T antigens should result in enhanced replication over that observed with either alone. Results typical of several experiments are shown in Fig. 5A and B. It is apparent that both wild-type and 8-142N8 large T antigens act on the wild-type *ori* (lanes 1 and 2 in each panel), but only the N8 large T antigen efficiently activates the 8-143 and 9-40 *ori* (lanes 6 and 7 in each panel). A slight suggestion that both types of large T antigen can interact cooperatively is provided by the increase in replication observed with wild-type (lane 3 in both panels) and 8-143 (lane 8 in panel A) DNAs when both types of large T antigen are present. However, evidence for such cooperative interaction was not observed with 9-40 DNA (lane 8 in panel B). An excess of wild-type large T antigen over 8-142N8 large T antigen proportionally stimulated only

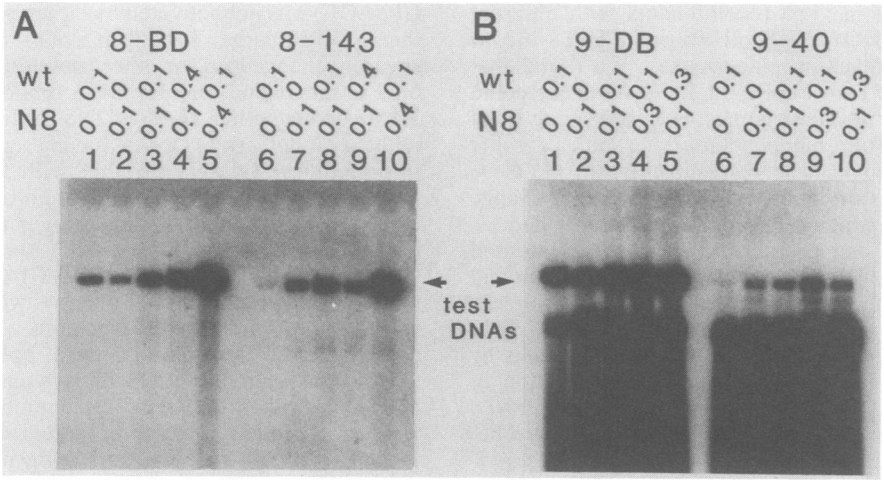


FIG. 5. Cooperativity of wild-type (wt) and pseudorevertant (N8) large T antigen for viral DNA replication. Double-stranded M13 clones with wild-type *ori* (8BD and 9DB) or with mutant *ori* (8-143, 9-40) and variable amounts of helper DNAs (as indicated above lanes in micrograms of DNA) were introduced into 3T6 cells as calcium phosphate coprecipitates (A) or with DEAE-dextran (B). Low-molecular-weight DNAs were isolated at 48 h posttransfection and digested with *Dpn*I and *Eco*RI. Following fractionation through agarose gel, they were blotted and hybridized to radiolabeled M13mp8-Py DNA.

the wild-type *ori*; the 8-143 or 9-40 origins, if anything, were replicated less efficiently (compare lanes 4 and 9 in panel A and lanes 5 and 10 in panel B). This suggests these two large T antigens do not efficiently cooperate in acting at these mutant origins.

## DISCUSSION

The polyomavirus origin of DNA replication is composed of multiple *cis*-acting elements including the enhancer, an A+T-rich sequence, the 34-base-pair palindrome, and the large-T-antigen high-affinity site A (2, 9, 15, 30, 35, 41, 53, 63–65). Replication in vitro depends on these same elements, less the enhancer (54). In vitro footprint analyses have demonstrated that the *ori* palindrome is bound by large T antigen (albeit very weakly) (7, 8, 57). In this report, we extend these analyses by showing that mutations in this palindrome reduce or eliminate DNA replication in vivo and in vitro largely because of altered binding by large T antigen (35, 53, 64).

Nathans and his colleagues (37, 58) previously have demonstrated that the replication of mutant SV40 *ori* DNAs could be suppressed by changes in SV40 large T antigen. We pursued a similar approach to analyze whether polyomavirus large T antigen could suppress inactivating changes in the polyomavirus *ori* and to begin to define domains of the large T antigen responsible for sequence-specific binding. We isolated several *ori* pseudorevertants containing an amino acid substitution (Asp-286 → Asn-286) in large T antigen. This mutant large T antigen suppresses the effects of mutations in the GPuGGC motif of the 34-base-pair palindrome, but does not suppress replication defects caused by enhancer mutations. Suppression of DNA replication by *ori* mutants is not due to overproduction of large T antigen or to its altered cellular localization. The Asp-286 → Asn-286 mutation most likely relaxes the specificity of binding of large T antigen to *ori* and permits a stable preinitiation complex to be formed. By analogy with SV40, this complex is required to unwind *ori* DNA and helps recruit DNA polymerase-primase (43, 59) required to prime and elongate nascent DNA strands.

Similar types of revertants altering DNA-binding specificity have been reported with numerous DNA-binding proteins, such as the phage 434 repressor (69), the *E. coli trp* repressor (S. Bass, Ph.D. thesis, University of Southern California, Los Angeles, 1987), the *E. coli lac* repressor (18, 34), and the *E. coli* catabolite activator protein (19). All those sites involved in altered DNA-binding specificity affect residues contacting specific DNA sequences.

Efforts to define the DNA-binding domain of SV40 large T antigen suggest that the sequences between the nuclear translocation signal (residues 126 to 132) and residue 220 are important for specific DNA binding (29, 49, 61). Other regions such as a putative zinc finger motif (amino acids 302 to 320) may participate in DNA binding (3). A comparison of the putative DNA-binding domain of SV40 and polyomavirus large T antigen is provided in Fig. 6A and B. Specific

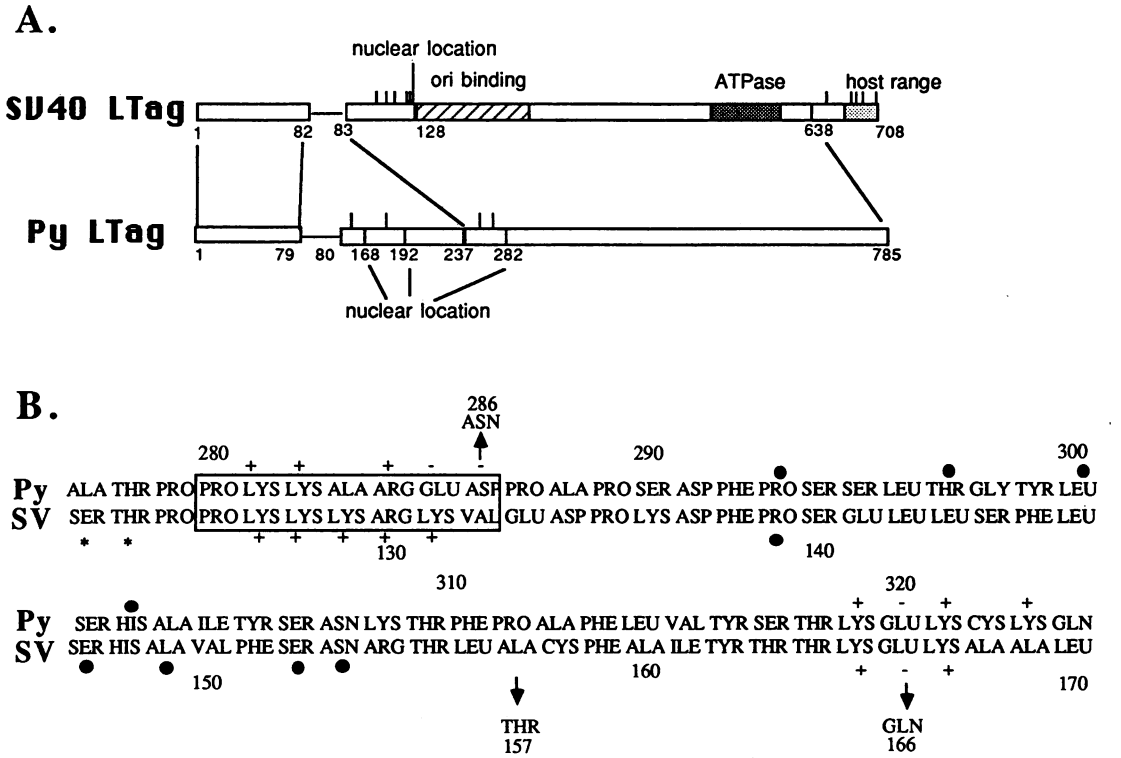


FIG. 6. (A) Comparison of SV40 and polyomavirus large-T-antigen sequences. Regions considered to be related are indicated by connecting lines. | indicates phosphorylation sites (4, 24, 56) (Bockus and Schaffhausen observe no difference between the phosphorylation of 8-142N8 large T antigen and the wild-type large T antigen [personal communication]). (B) Amino acid sequence comparison of the putative *ori* binding domain of SV40 and polyomavirus large T antigen. The nuclear localization signal is boxed (28, 54). Point mutations which abolish DNA binding are indicated by solid circles (6, 49, 52). Point mutations which relax the specificity of DNA binding are indicated by arrows (37, 58). * Indicates phosphorylation sites.

DNA binding is sensitive to amino acid substitutions in this region (Fig. 6B) (6, 49, 52). Large-T-antigen mutant 8-142N8 and the analogous SV40 mutants isolated by Nathans and his colleagues (37, 58) are located within this region, suggesting that this region of large T antigen is part of a DNA-binding domain and is not simply a modulator of DNA-binding activity.

Several types of general DNA-binding structures have been identified in different DNA-binding proteins: (i) the helix-turn-helix structure found in a number of procaryotic proteins, e.g., *E. coli* catabolite gene activator protein and phage lambda *cro* and *c*I repressor (44, 46); (ii) the zinc finger structure found in many eucaryotic transcription activators, e.g., TFIIIA, SP1 (20, 31); (iii) four helices found in the *Eco*RI restriction enzyme which induce a kink in DNA conformation (39, 55); (iv) two helices-finger in *E. coli* polymerase I large fragment (45); (v) two antiparallel beta sheets in HU protein from *Stearothermophilus* species (62). The putative DNA-binding domain of large T antigen bears no obvious homology to any of these structures (that we can detect using current protein structure prediction algorithms) and may comprise a novel structure.

Specific DNA sequence-protein interactions can be mediated by hydrogen bonding (67) and by charge-charge interactions. Interestingly, the putative DNA-binding domain of large T antigen contains two positively charged regions (Fig. 6B) whose net charge is increased in the polyomavirus 8-142N8 large T antigen and one of the SV40 pseudorevertant large T antigens. This suggests that charge-charge interactions are involved in *ori*-large T antigen complex formation. It is worth noting that the DNA-binding domain of restriction endonuclease *Eco*RI contains regions of positively charged amino acids that interact with the phosphate backbone of the DNA and pull the backbones of the two strands away from one another (39, 55). The two positively charged regions of large T antigen might be involved in an analogous activity.

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