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Mutation of Large T-Antigen-Binding Site A, but Not Site B or C, Eliminates Stalling by RNA Polymerase II in the Intergenic Region of Polyomavirus DNA

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During transcription of the late strand of polyomavirus DNA, RNA polymerase II stalls and accumulates nearby the binding sites on viral DNA recognized by polyomavirus large T antigen. Stalling by RNA polymerases is eliminated when thermolabile large T antigen is inactivated by using a temperature-sensitive virus mutant (J. Bertin, N.-A. Sunstrom, P. Jain, and N. H. Acheson, Virology 189:715–724, 1992). To determine whether stalling by RNA polymerases is mediated through the interaction of large T antigen with one or more of its binding sites, viable polyomavirus mutants that contain altered large-T-antigen-binding sites were constructed. Point mutations were introduced by site-directed mutagenesis into the multiple, clustered G(A/G)GGC pentanucleotides known to be the target sequence for large T-antigen binding. Mutation of the G(A/G)GGC pentanucleotides in the first two binding sites encountered by RNA polymerases in the intergenic region (sites C and B) had no detectable effect on stalling as measured by transcriptional run-on analysis. However, mutation of the two GAGGC pentanucleotides in binding site A, which lies adjacent to the origin of viral DNA replication, eliminated stalling by RNA polymerases. We conclude that binding of large T antigen to site A blocks elongation by RNA polymerase II. Further characterization of virus containing mutated site A did not reveal any effects on early transcription levels or on virus DNA replication. However, the mutant virus gave rise to small plaques, suggesting impairment in some stage of virus growth. Stalling of RNA polymerases by large T antigen bound to the intergenic region of viral DNA may function to prevent transcription from displacing proteins whose binding is required for the normal growth of polyomavirus.

Termination of transcription by RNA polymerase II is a complex, multistep process that involves the cessation of elongation and the release of the RNA transcript and RNA polymerase from the DNA template. In higher eukaryotes, the 3′ ends of all mRNAs are generated by RNA processing events rather than by transcription termination (70). The results of transcriptional run-on experiments have shown that RNA polymerase II continues to transcribe for distances ranging from several hundred to several thousand nucleotides beyond the site which determines the 3′ end of the mRNA transcript (57). However, termination at the end of some genes transcribed by RNA polymerase II has been shown to be dependent on the presence of upstream 3′-end processing signals (13, 16, 44, 45, 72). Sequence analysis of the various regions of termination has not revealed the existence of a common terminator element, and in only a few cases have specific elements involved in terminating transcription by RNA polymerase II been identified (3, 12, 13, 30, 60, 66).

RNA polymerase II has been observed to stall or terminate transcription within several viral and cellular transcription units in regions on the template DNA which contain binding sites for proteins (3, 9, 13, 17, 18, 47, 58, 62). It has been proposed that DNA-binding proteins may function as stalling or termination signals which impede the progression of RNA polymerases through specific regions of a transcription unit. Interestingly, not all proteins have the ability to impede elongation by RNA polymerase II, suggesting that blockage may be a specific property of only some DNA-binding proteins (17, 18, 41). The mechanism used by DNA-binding proteins to block elongation by RNA polymerase II is currently not understood. However, several DNA-binding proteins have been shown to block elongation in an orientation-dependent manner, suggesting that the blocking protein must be specifically oriented with respect to the transcribing RNA polymerase (3, 18). DNA-binding proteins have been shown to stall or terminate transcription by other RNA polymerases, such as *Escherichia coli* RNA polymerase (24, 52, 59, 61), eukaryotic RNA polymerase I (5, 49), and mitochondrial RNA polymerase (41). Therefore, binding of specific proteins to template DNA appears to be a signal used by both prokaryotes and eukaryotes to block elongation by RNA polymerases during transcription.

The early and late genes of polyomavirus are transcribed by RNA polymerase II in divergent directions from within a 300-nucleotide (nt) intergenic region that contains transcriptional enhancer elements and the viral origin of DNA replication (29). Because polyomavirus DNA is circular, RNA polymerases are able to transcribe around the DNA template and through the intergenic region. Although most RNA polymerases transcribing the late DNA strand terminate upstream of the intergenic region (9, 62), some RNA polymerases do transcribe through this region and traverse the entire circular genome, producing multigenome-length transcripts (1). However, transcription through the intergenic region on some viral DNA templates is blocked, resulting in the accumulation of stalled RNA polymerases (62). Stalling occurs within a 164-nt region which lies between the early transcription start site and the origin of viral DNA replication (9) (Fig. 1A). This region contains four adjacent sites (C,

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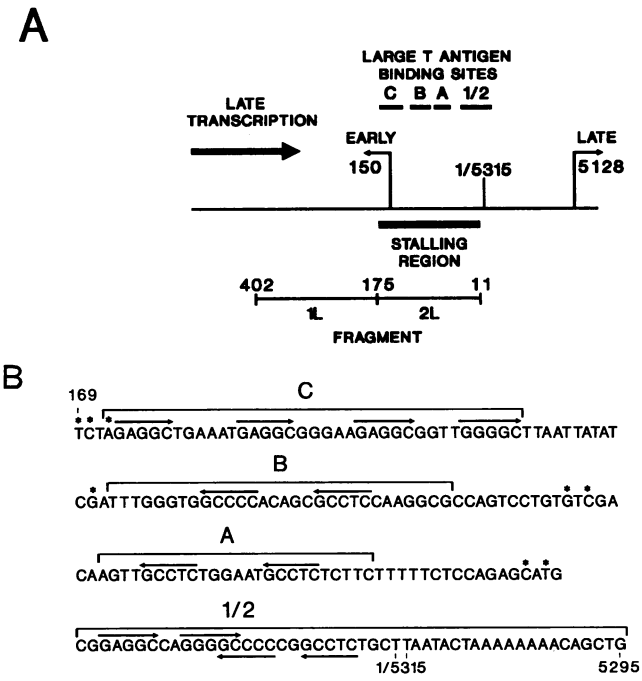

FIG. 1. (A) Locations of stalling-region and large T-antigen-binding sites on polyomavirus DNA. The early and major late transcription start sites, and fragments 1L and 2L used to assay for stalling levels by transcriptional run-on analysis, are shown. (B) Nucleotide sequence of the region containing large T-antigen-binding sites C, B, A, and 1/2 in virus strain AT3-Modori. Differences in nucleotide sequence compared with virus strain AT3 (62) are indicated with asterisks. The regions protected by large T antigen binding in DNase I protection assays (20) and the G(A/G)GGC pentanucleotide sequences (arrows) recognized by large T antigen in each binding site are indicated.

B, A, and 1/2) to which large T antigen binds (20, 28, 33, 54), leading to the initiation of viral DNA replication (55) and probably to the repression of early gene expression (15, 23, 31, 32, 40). Inactivation of polyomavirus large T antigen by use of a temperature-sensitive mutant virus eliminates stalling by RNA polymerases in this region, suggesting that the interaction of large T antigen with these DNA-binding sites is responsible for the block to transcription elongation (9). However, it remained possible that large T antigen interferes with transcription elongation by a mechanism that is not dependent on the binding of large T antigen to the DNA template.

To gain insight into the mechanism used by polyomavirus large T antigen to block elongation by RNA polymerase II, we have introduced mutations into the pentanucleotide G(A/G)GGC sequences that are known to be the target for DNA binding (21, 53). In this study, we show that mutation of both GAGGC pentanucleotides in large T-antigen-binding site A, which is located adjacent to the origin of viral DNA replication, eliminates stalling by RNA polymerases. In contrast, mutation of the first two binding sites (sites C and B) encountered by RNA polymerases transcribing the late DNA strand had no effect on the level of RNA polymerase stalling. Virus containing mutated site A replicated its DNA at rates comparable to those of wild-type virus and did not show any change in early transcription levels. However, mutation of site A caused a significant reduction in the size of plaques formed by the mutant virus. Therefore, stalling of RNA polymerases in the intergenic region may be necessary for the normal growth of polyomavirus.

MATERIALS AND METHODS

Cells and viruses. Growth of mouse 3T6 and primary baby mouse kidney cells and production of virus stocks were carried out as described previously (9). The wild-type polyomavirus strain (AT3-Modori) used in this study contains four additional restriction sites which were introduced, by oligonucleotide-directed mutagenesis, into strain AT3 adjacent to large T-antigen-binding sites C, B, A, and 1/2 (63). The locations of the additional restriction sites are as follows: *Sph*I, nt 32; *Sal*I, nt 72; *Cla*I, nt 118; and *Xba*I, nt 164 (Fig. 1B).

Construction of polyomavirus mutants. Mutant polyomavirus strains were constructed by site-directed mutagenesis (43), using a procedure provided by Bio-Rad, Inc. All mutations were introduced into polyomavirus strain AT3-Modori cloned via an *Eco*RI site into plasmid pGEM-3Zf(−) (Promega Biotec, Inc.). The sequences of the oligonucleotides used for mutagenesis are as follows: oligonucleotide C, 5′-GAT ATA ATT AAT TCC CAA CCT TCT CTT CCC TTC TCA TTT CAT TCT CTA GAC C-3′; oligonucleotide B, 5′-CAC AGG ACT GAA GCC TTG GAG AAG CTG TGG GAA CAC CCA AAT C-3′; oligonucleotide A, 5′-AGA AGA GAT GTA TTC CAG AGT TAA CTT GTC-3′; oligonucleotide A0, 5′-AAA AAG AAG AGA TGA ATT CCA GAG GC-3′; oligonucleotide A1, 5′-CAT TCC AGA GTT AAC TTG TCG A-3′; oligonucleotide A2, 5′-AAA AAG AAG AAA GGC ATT CCA GAG GC-3′; and oligonucleotide A1A2, 5′-AAA AAG AAG AAA GGC ATT CCA GAG TTA ACT TGT CGA-3′. Plasmids containing the desired mutations were screened as indicated: C, additional *Ase*I site; B, missing *Bgl*I site; A, additional *Hpa*I site; A0, additional *Eco*RI site; A1, additional *Hpa*I site; A2, no restriction site altered, mutant identified by sequencing; A1A2, additional *Hpa*I site. The polyomavirus genome containing the desired mutation was released from pGEM by digestion with *Eco*RI and transfected into baby mouse kidney cells (100 ng of DNA per 10^7 cells) to produce mutant polyomavirus stocks. Presence of desired mutations and absence of defective DNA were confirmed by restriction endonuclease digestion and sequencing of DNA extracted from cells infected at high multiplicity (1 to 50 PFU per cell) with each virus stock.

Transcriptional run-on assay. Viral transcription complexes were isolated from infected cells as described previously (62). Transcriptional run-on assays were performed by extending nascent RNA chains for 2 min at 26°C in a 250-μl reaction mix containing 0.35 μM [α-^{32}P]CTP (3,000 Ci/mmol; Amersham). ^{32}P-RNA was hybridized in solution to an excess of single-stranded M13 DNA containing the late or early strand of various fragments of polyomavirus DNA (9, 62). Hybridizations to fragment 2, which contains the large T-antigen-binding sites (Fig. 1A), were done in each case with DNA containing the respective mutations introduced into wild-type virus by site-directed mutagenesis (Fig. 2). The relative transcriptional activity in each region assayed was normalized for the number of C residues in each DNA fragment (Table 1).

DNA-binding assay. The specific DNA-binding activity of large T antigen with DNA fragments containing mutant large T-antigen-binding sites was determined according to a modified immunoprecipitation assay (48) as described previously (9, 64). A 670-nt DNA fragment (nt 32 to 702) containing

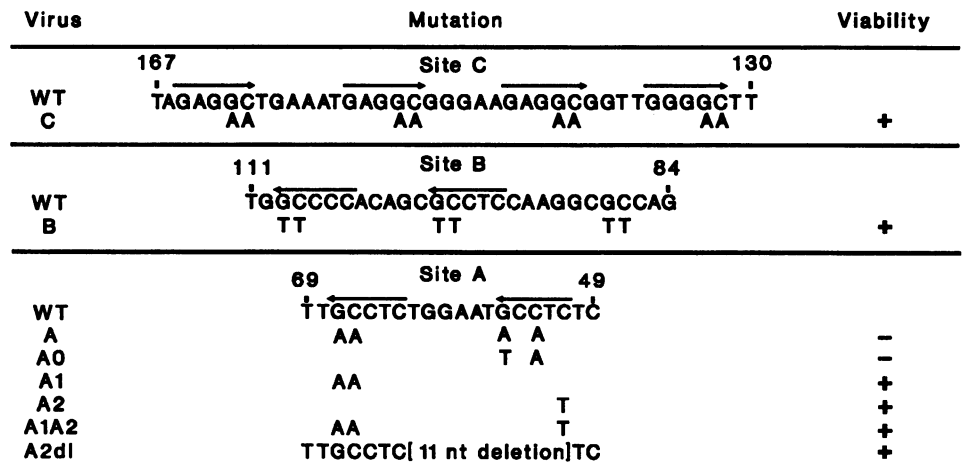

FIG. 2. Mutations introduced into large T-antigen-binding sites.

large T-antigen-binding site A and wild-type or mutant large T-antigen-binding sites B and/or C was gel purified and end-labeled with ^{32}P-labeled nucleoside triphosphates by the Klenow fragment of DNA polymerase I. Duplicate samples of large T antigen-DNA complexes were immunoprecipitated with a polyclonal antibody raised against large T antigen. The amount of DNA bound by large T antigen was quantitated by liquid scintillation counting, and binding levels were corrected for nonspecific binding by using nuclear extracts prepared from uninfected cells.

RESULTS

Construction of polyomavirus mutants. Polyomavirus large T antigen binds specifically to four sites designated C, B, A, and 1/2 in the intergenic region of polyomavirus DNA (20, 28, 33, 54) (Fig. 1A). Each site contains tandem repeats of the pentanucleotide G(A/G)GGC known to be the target sequence for DNA binding by large T antigen (21, 53) (Fig. 1B). The guanine residues at positions 1, 3, and 4 in the pentanucleotide sequence and at position 5 on the opposite DNA strand are critical contact points since methylation of any of these nucleotides interferes with large T-antigen binding (21).

Because our goal was to generate viable mutant viruses, we were restricted to mutating those binding sites not required for the initiation of viral DNA replication. Large T-antigen-binding sites B and C have previously been shown to be nonessential for viability (7). Double-point mutations replacing the final GC with AA were therefore introduced into the four G(A/G)GGC pentanucleotide sequences present within site C and into the two pentanucleotide sequences present within site B, as well as into a degenerate CTGGC present at one end of site B (Fig. 2). Viable viruses containing mutant site B or C or both B and C together (mutant virus BC) were generated.

The polyomavirus origin of bidirectional DNA replication has been mapped to a region located between sites 1/2 and A (37). The minimal origin of viral DNA replication, as defined by deletion mutants, contains binding site 1/2 and part of site A (23, 39, 55). Although site 1/2 is absolutely required for DNA replication, the role of site A remains unclear. Essen-

TABLE 1. Effects of mutations in large T-antigen-binding sites on RNA polymerase stalling

Virus	Fragment	No. of C residues	Correction factor[a]	Avg cpm hybridized[b]	Transcriptional activity[c]	Stalling ratio[d]
Wild type[e]	1	59	0.73	145	106	11
	2	43	1.0	1,214	1,214	
B[f]	1	59	0.68	208	142	13
	2	40	1.0	1,890	1,890	
C[g]	1	59	0.66	162	107	12
	2	39	1.0	1,301	1,301	
BC[h]	1	59	0.61	201	123	11
	2	36	1.0	1,360	1,360	
A1A2[i]	1	59	0.70	160	112	2.2
	2	41	1.0	251	251	

[a] Number of C residues in fragment 2 divided by the number of C residues in the fragment used.
[b] After subtraction of background.
[c] Counts per minute hybridized × correction factor.
[d] Ratio of transcriptional activity in fragment 2 to that in fragment 1.
[e] Input, 25,000 cpm. Background bound in presence of M13 DNA, 51 cpm.
[f] Input, 26,400 cpm. Background, 69 cpm.
[g] Input, 22,800 cpm. Background, 60 cpm.
[h] Input, 35,400 cpm. Background, 81 cpm.
[i] Input, 17,600 cpm. Background, 57 cpm.

tial sequences for DNA replication are present within site A, as certain mutations reduce or abolish replication. However, large T-antigen binding to this region may not be essential (67). Mutation of the GAGGC pentanucleotide farther from site 1/2 (nt 62 to 66) allowed virus growth (mutant A1 virus, GAGGC to GAGTT; Fig. 2). However, mutation of the GAGGC pentanucleotide closer to site 1/2 (nt 51 to 55) to GATGT or GATGA (mutant A and A0 viruses, respectively; Fig. 2) did not allow virus growth. A virus (A2*dl*) recovered after transfection of baby mouse kidney cells with mutant A0 DNA had undergone a deletion of 11 nt (nt 51 to 61) that removed the mutated GATGA pentanucleotide. As a result of this deletion, mutant virus A2*dl* has a single GAGGC pentanucleotide in site A located in precisely the same position relative to site 1/2 as the proximal GAGGC (nt 51 to 55) in wild-type virus.

Previous studies showed that mutation of the GAGGC pentanucleotide proximal to site 1/2 to AAGGC does not affect virus viability (46) or viral DNA replication (67), even though it interferes with large T-antigen binding to site A (21). This mutation was therefore introduced into the pentanucleotide proximal to site 1/2, and a viable virus was generated (mutant A2 virus; Fig. 2). We also combined the A2 mutation with the A1 mutation to generate a mutant virus in which both GAGGC pentanucleotides in site A were mutated (mutant A1A2 virus; Fig. 2).

Transcriptional run-on assay. The level of RNA polymerase stalling in the intergenic region of polyomavirus was measured by performing a transcriptional run-on assay (9, 62). In this assay, viral transcription complexes are isolated from infected cells and RNA polymerases are allowed to extend their nascent RNA chains for a short time (2 min) in vitro in the presence of [^{32}P]CTP. Radioactive RNA is hybridized in solution to single-stranded recombinant DNA. The relative levels of hybridization, corrected for the number of C residues in each DNA fragment used, correspond to the average density of active RNA polymerases within the regions assayed. RNA polymerases that stall in infected cells while traversing the large T-antigen-binding region on polyomavirus DNA accumulate there and can resume elongation in vitro under conditions used for the run-on assay [150 mM $(NH_4)_2SO_4$, 0.1% Sarkosyl]. This results in a higher level of transcription from the stalling region (fragment 2L; Fig. 1A) than from adjacent upstream (fragment 1L) or downstream regions (9).

Mutation of large T-antigen-binding site A but not site B or C eliminates stalling by RNA polymerase II. To determine whether binding of large T antigen to viral DNA is required to block elongation by RNA polymerase II, we examined the level of RNA polymerase stalling on the late strand of viral DNA templates containing altered large T-antigen-binding sites. Results from representative experiments are shown in Table 1 and Fig. 3. When transcription complexes were isolated from cells infected with wild-type virus (AT3-Modori), the density of RNA polymerases within the stalling region (fragment 2L) was 11-fold higher than that in the adjacent upstream region (fragment 1L). The ratios of RNA polymerases in fragment 2L to those in fragment 1L were between 8 and 16 in eight independent experiments using wild-type virus (data not shown). These levels of RNA polymerase stalling in the intergenic region are similar to those previously observed for polyomavirus wild-type strains AT3 and A3 (9). The AT3-Modori polyomavirus wild-type strain used in this study contains several mutations located adjacent to large T-antigen-binding sites C, B, and A (Fig. 1B); therefore, the presence of these mutations does not affect stalling by RNA polymerase.

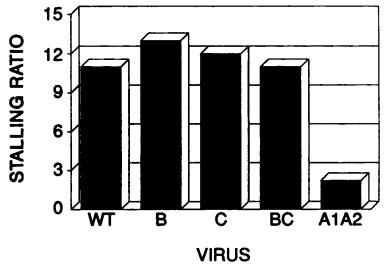

FIG. 3. Mutation of large T-antigen-binding site A, but not sites B and C, affects stalling. Viral transcription complexes were isolated from cells infected for 30 h with wild-type (WT) virus or with mutant B, C, BC, or A1A2 virus. Transcriptional run-on assays were performed as described in Materials and Methods, and the stalling ratio (relative density of RNA polymerases in fragment 2L to that in fragment 1L) for each virus was determined. Results are expressed as the ratio of transcriptional activity in DNA fragment 2L to that in fragment 1L. Data are taken from the experiment shown in Table 1.

We next examined the effects of mutating large T-antigen-binding sites A, B, and C on stalling. In the experiment shown, the ratios of RNA polymerases in fragment 2L to those in fragment 1L were 13 when site B was mutated, 12 when site C was mutated, and 11 when both binding sites were mutated (Table 1 and Fig. 3). Since these ratios fall within the wild-type range, we conclude that the mutations introduced into the G(A/G)GGC motifs present within binding sites B and C have no detectable effect on RNA polymerase stalling. However, mutation of both GAGGCs in binding site A (mutant A1A2 virus) essentially eliminated stalling by RNA polymerases (Table 1 and Fig. 3). In this experiment, the ratio of RNA polymerases in fragment 2L to that in fragment 1L was 2.2, which represents a fivefold reduction in the level of stalled RNA polymerases relative to that observed with wild-type virus. In five independent experiments, the stalling ratio was between 2 and 3 when virus A1A2 was assayed for RNA polymerase stalling levels (data not shown). These ratios fall in the range we observed previously when large T antigen was inactivated by using a temperature-sensitive mutant virus (9). We conclude that stalling by RNA polymerases in the intergenic region of polyomavirus DNA is dependent on the binding of large T antigen to site A but not site B or C.

To rule out the possibility that the reduction in stalling levels observed with mutant A1A2 virus was due to an overall reduction in RNA polymerase levels on the late DNA strand, we compared the level of RNA polymerases in fragment 2L with those in fragments located just downstream of the major late transcription start site (fragments 3L and 4L; Fig. 4A). The density of RNA polymerases decreases along the late DNA strand from a maximum at the transcription start site to the lowest level just prior to the stalling site (9, 62). On transcription complexes isolated from cells infected with wild-type virus, the density of RNA polymerases in fragment 2L was greater than that in fragments located just downstream of the major late transcription start site (Fig. 4B), as was previously observed (9). When binding site A was mutated (mutant A1A2 virus; Fig. 2), the ratio of RNA polymerases in fragment 2L relative to that in fragment 1L decreased from 8 (wild type) to 2 (A1A2) in this experiment, while levels in the other fragments (1L, 3L, and 4L) remained approximately the same (Fig. 4C).

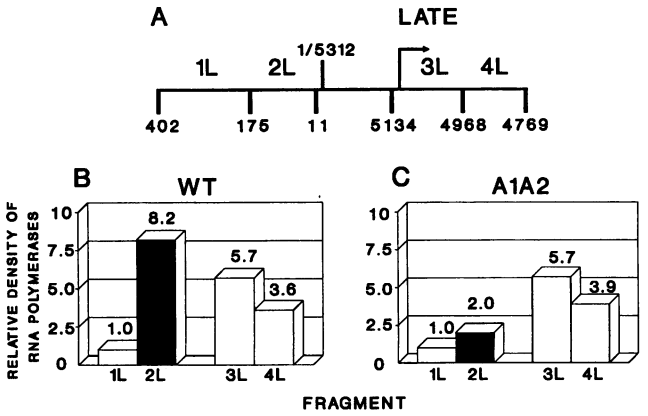

FIG. 4. Comparison of the level of stalled RNA polymerases with the density of polymerases downstream of the late transcription start site. (A) Nucleotide numbers delimiting fragments 1L, 2L, 3L, and 4L are shown. The arrow shows the position of the major late transcription start site (nt 5128). Cells were infected with wild-type virus or with mutant A1A2 virus for 40 h, and viral transcription complexes were isolated. Transcriptional run-on assays were performed, and the relative density of RNA polymerases in each fragment was determined for wild-type (WT) virus (B) and mutant A1A2 virus (C).

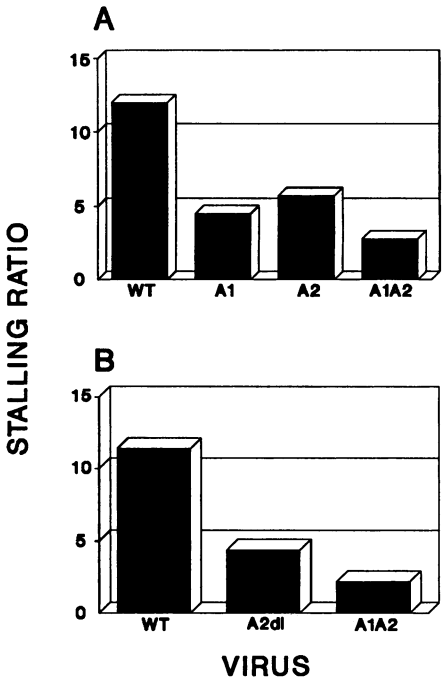

FIG. 5. Both GAGGC pentanucleotides in large T-antigen-binding site A play a role in stalling. Cells were infected with wild-type (WT) or mutant viruses for 30 h, and transcription complexes were isolated. Transcriptional run-on assays were performed, and the stalling ratio (relative density of RNA polymerases in fragment 2L to that in fragment 1L) for each virus was determined and compared with those for wild-type virus and mutant virus A1A2. (A) Results from an experiment with wild-type, A1, A2, and A1A2 viruses; (B) results from a separate experiment with wild-type, A2*dl*, and A1A2 viruses.

This result shows that the A1A2 mutation reduced the level of stalled polymerases in fragment 2L but had no detectable effect on initiation by RNA polymerase at the late promoter, some 200 nt downstream.

We also examined whether both GAGGC pentanucleotides in binding site A were necessary for stalling. The stalling ratios were determined for mutant A1 and A2 viruses (Fig. 2) and compared with those for wild-type virus and mutant A1A2 virus (Fig. 5A). In the experiment shown, mutation of either of the two GAGGCs in binding site A reduced the stalling ratio from 12 (wild-type virus) to 4.5 or 5.7 (mutant A1 and A2 viruses, respectively). Mutation of both GAGGC pentanucleotides in site A (mutant A1A2 virus) further reduced the stalling ratio to 2.8. In two other experiments (data not shown), we also observed intermediate stalling ratios for mutant A1 and A2 viruses relative to wild-type virus and mutant A1A2 virus. In addition, the stalling ratio for mutant A2*dl* virus, which has only one GAGGC pentanucleotide in site A (Fig. 2), was also found to be in this intermediate range (4.4; Fig. 5B). We conclude that stalling by RNA polymerases can occur if only one GAGGC pentanucleotide is present in site A, but that both GAGGC pentanucleotides are required for maximal accumulation of RNA polymerases in the intergenic region.

Mutation of sites B and C interferes with large T-antigen binding in vitro. The introduction of mutations into the G(A/G)GGC pentanucleotides present within binding sites B and C had no effect on stalling by RNA polymerases (Fig. 3). One possible explanation for this result is that the mutations introduced into these binding sites did not interfere with large T-antigen binding. Since a previous study showed that a mutation within a G(A/G)GGC pentanucleotide sequence interferes with large T-antigen binding in vitro (21), we consider this explanation highly unlikely. Nevertheless, we verified that the mutations introduced into binding sites B and C interfere with the binding of large T antigen to these sites in vitro by performing a DNA-binding assay (9, 64). In this assay, a radiolabeled DNA fragment containing large T-antigen-binding sites A, B, and C was mixed with a nuclear extract containing large T antigen. The amount of DNA fragment immunoprecipitated with antibodies against large T antigen is a measure of the affinity of large T antigen to its binding sites. In the experiment shown (Fig. 6),

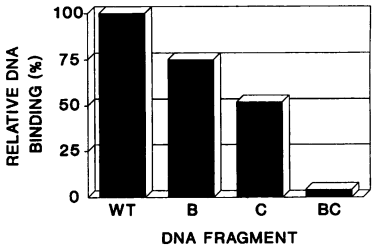

FIG. 6. Immunoprecipitation of DNA fragments containing mutations in large T-antigen-binding sites B and C. The relative amounts of immunoprecipitated radiolabeled DNA fragments containing mutant large T-antigen-binding sites (see Fig. 2 for mutations) are shown. DNA fragments were incubated with nuclear extracts containing large T antigen, and the in vitro DNA-binding activity was determined as described in Materials and Methods. Under these conditions, approximately 10% of an input DNA fragment containing binding sites A, B, and C (wild type [WT]) was immunoprecipitated. In the experiment shown, this amount was standardized to 100% for comparison with other DNA fragments. DNA fragments assayed: WT (binding sites A, B, and C present); B (site B mutant, sites A and C present); C (site C mutant, sites A and B present); BC (sites B and C mutant, site A present).

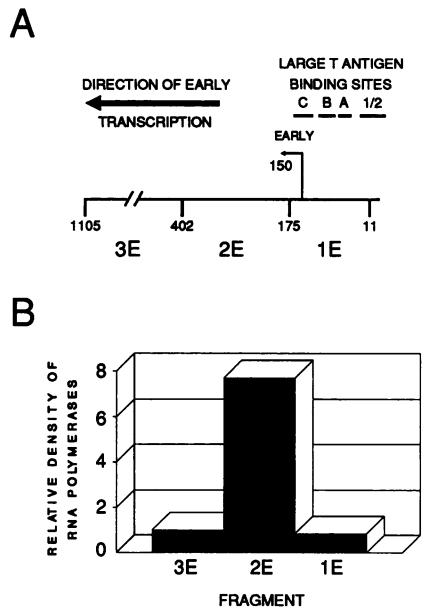

FIG. 7. Densities of RNA polymerases on the early DNA strand. (A) Map of region on polyomavirus strain AT3 (62) analyzed by transcriptional run-on assays. Large T-antigen-binding sites, the early transcription start site, and nucleotide numbers delimiting fragments 1E, 2E, and 3E, which were separately cloned into M13 bacteriophage vectors, are shown. (B) Viral transcription complexes were isolated from cells incubated at 32°C for 3 days after infection with polyomavirus strain AT3. Results are expressed as the ratio of transcriptional activity in each DNA fragment to that in fragment 3E.

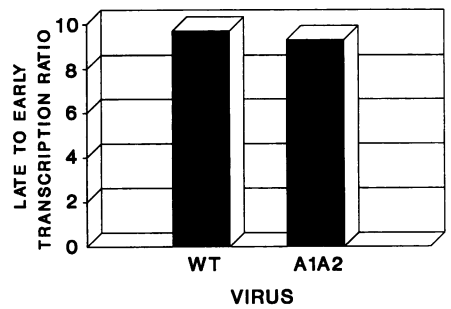

FIG. 8. Late-to-early transcription ratios. Cells were infected with wild-type (WT) or mutant A1A2 virus for 30 h, and viral transcription complexes were isolated. Transcriptional run-on assays were performed (RNA chains extended for 10 min), and the corrected levels of hybridization to the early (nt 1498 to 2617) and late (nt 2991 to 3466) DNA strands were determined. Results are expressed as ratio of RNA polymerases on the late DNA strand relative to the early DNA strand.

mutation of site B or site C reduced the amount of DNA immunoprecipitated by 25 or 50%, respectively. This result is consistent with previous findings showing that site C [containing four G(A/G)GGCs] has a higher affinity for large T antigen than does site B or A [containing two G(A/G)GGCs each in this virus strain] and therefore contributes most strongly to the interaction of large T antigen with this DNA fragment (20, 64). Mutation of both binding sites B and C virtually eliminated binding by large T antigen to this DNA fragment (96% reduction). It should be noted that under the conditions used, binding by large T antigen to site A alone could not be detected (63); this requires higher concentrations of large T antigen than are available in the nuclear extract. We conclude that the mutations introduced into sites B and C strongly reduce or eliminate specific binding by large T antigen to these sites in vitro.

Large T antigen does not appear to block elongation by RNA polymerases transcribing the early DNA strand. We next examined whether large T antigen blocks elongation by RNA polymerases transcribing the early DNA strand of polyomavirus. RNA polymerases have been shown previously by transcriptional run-on analysis to accumulate on the early DNA strand within a 621-nt region which includes the binding sites for polyomavirus large T antigen (62). We decided to map more precisely the distribution of RNA polymerases found within this region on the early DNA strand. Three adjacent fragments from this region of polyomavirus wild-type strain AT3 (62) were cloned into M13 vectors (Fig. 7A), and the density of RNA polymerases associated with each fragment was determined by transcriptional run-on analysis. In the experiment shown (Fig. 7B), and eightfold excess in RNA polymerase density was observed in fragment 2E (nt 175 to 402) relative to adjacent fragments 3E (nt 402 to 1100) and 1E (nt 11 to 175). Therefore, the accumulation of RNA polymerases previously detected on the early DNA strand by Skarnes et al. (62) is located in fragment 2E, a 225-nt region which lies immediately downstream of the early transcription start site (Fig. 7A). The relative density of RNA polymerases in the three DNA fragments examined further suggests that transcription is not blocked in the region which contains the large T-antigen-binding sites (fragment 1E). Furthermore, inactivation of thermolabile large T antigen by using a temperature-sensitive virus had no effect on the relative density of RNA polymerases in fragment 1E or on the accumulation of RNA polymerases in fragment 2E (data not shown). Therefore, large T antigen does not appear to affect elongation by RNA polymerases transcribing the intergenic region on the early DNA strand.

Effect of mutations in binding site A on early transcription and virus growth. The binding of large T antigen to site A has been proposed to play a role in the repression of early gene expression (23) and in the initiation of viral DNA replication (55). We were therefore interested in determining whether the mutations introduced into large T-antigen-binding site A (mutant A1A2 virus; Fig. 2) had any effect on these functions.

To compare the ratio of late to early transcription between wild-type virus and mutant A1A2 virus, the levels of RNA polymerases transcribing the early and late DNA strands for each virus were determined by transcriptional run-on analysis. The densities of RNA polymerases in the regions immediately preceding the poly(A) sites of both the early (nt 1498 to 2617) and late (nt 2991 to 3466) transcription units were measured. Analysis of transcription complexes from wild-type virus revealed a 9- to 10-fold ratio of RNA polymerases traversing the late transcription unit compared with the early transcription unit (Fig. 8, WT). Analysis of mutant A1A2 virus transcription complexes also revealed a 9- to 10-fold higher level of RNA polymerases on the late versus the early transcription unit (Fig. 8, A1A2). Therefore, the mutations introduced into large T-antigen-binding site A had no effect on levels of early transcription.

Several stocks of A1A2 virus generated by transfection of primary baby mouse kidney cells with viral DNA had titers

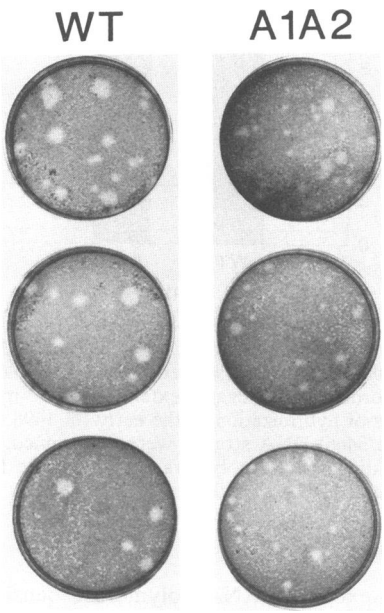

FIG. 9. Comparison of plaque size of wild-type and mutant A1A2 viruses. Plaque assays were performed on primary baby mouse kidney cells infected with wild-type (WT) or mutant A1A2 virus. Plaques were visualized by neutral red staining. Plaques formed by two different wild-type polyomavirus strains are shown; the first two plates are AT3-Modori (63), and third plate is AT3 (62).

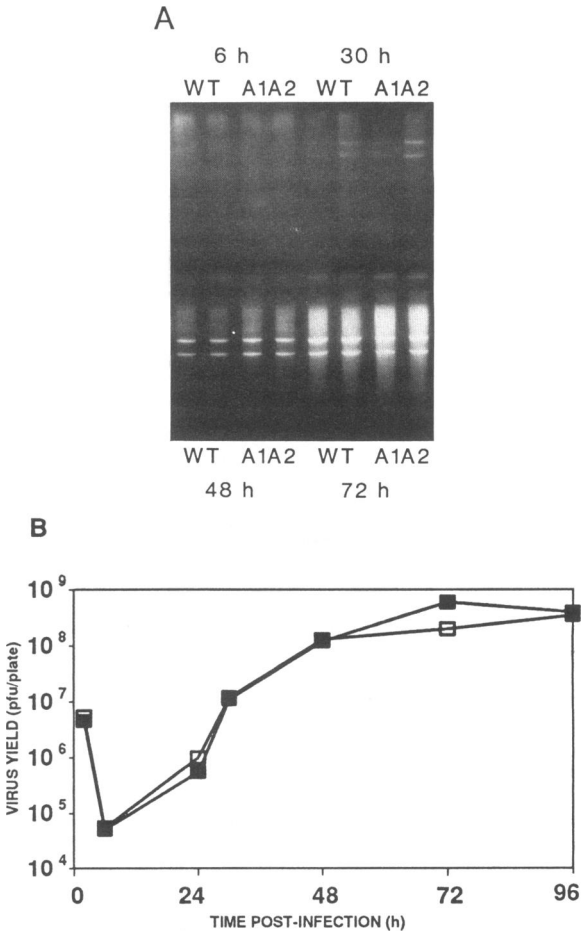

FIG. 10. Growth properties of wild-type and mutant A1A2 viruses. Primary baby mouse kidney cells were infected with either wild-type (WT) or mutant A1A2 virus at a multiplicity of 1 PFU per cell. (A) Amount of viral DNA extracted from infected cells (38) at 6, 30, 48, and 72 h postinfection. Viral DNA was digested with the restriction endonuclease *Hin*dIII, analyzed on a 1% agarose–Tris-borate-EDTA gel, and visualized by staining with ethidium bromide. Duplicate samples from each time point are shown. (B) Growth curves. At the indicated times, the amount of infectious progeny virus was determined by plaque assay. Points represent averages of duplicate or triplicate samples of wild-type-infected cells (filled squares) and A1A2-infected cells (open squares).

(1×10^6 to 8×10^6 PFU/ml) 50- to 100-fold lower than those of equivalent stocks of wild-type virus. A1A2 virus also gave rise to smaller plaques than did wild-type virus (Fig. 9). These observations implied that A1A2 virus grows less well than wild-type virus. We therefore measured viral DNA replication and virus production after infection of baby mouse kidney or 3T6 cells with either wild-type or A1A2 virus at an input multiplicity of 1 PFU per cell. Hirt extraction of infected monolayers revealed no detectable differences between wild-type and A1A2 virus in the accumulation of viral DNA by 24, 30, 48, or 72 h after infection of baby mouse kidney cells (results for 6, 30, 48, and 72 h are shown in Fig. 10A). Similar results were obtained after infection of 3T6 cells (data not shown). Yields of infectious progeny virus measured by plaque assays again revealed no significant differences between wild-type and A1A2 viruses (Fig. 10B). Restriction endonuclease digestion and DNA sequencing (data not shown) showed that the bulk of progeny DNA retained mutations in both GAGGC pentanucleotides in the A1A2 virus in these experiments. We conclude that A1A2 virus replicates its DNA genome and synthesizes infectious progeny virions at rates comparable to those of wild-type virus in both baby mouse kidney and 3T6 cells. The reasons for small plaque size and low titers of virus stocks generated by transfection remain obscure; these observations could be explained by delayed release of A1A2 virus or by a small decrease in the rate of virus DNA replication (see Discussion).

DISCUSSION

We have examined the mechanism by which large T antigen blocks elongation by RNA polymerase II in vivo during transcription of the polyomavirus late DNA strand. The results presented in this study suggest that stalling by RNA polymerases in the intergenic region is mediated through the binding of large T antigen to the viral DNA template at site A. Site A lies adjacent to the origin of viral DNA replication and consists of two GAGGC pentanucleotide sequence elements separated by six nucleotides (Fig. 1B). The interaction of large T antigen with both GAGGC pentanucleotides in site A appears to be essential to form an effective block to transcription elongation, since mutation of either of the GAGGC elements reduces stalling by RNA polymerase, and mutation of both elements eliminates stalling (Fig. 5). Although we have not performed an extensive mutational analysis of large T-antigen-binding site A, the introduction of mutations adjacent to this site was observed to have little or no effect on stalling levels (AT3-Modori sequence; Fig. 1B and Fig. 3). Therefore, the GAGGC pentanucleotides in site A appear to be essential sequence elements required for large T antigen to block elongation by RNA polymerase.

Several other DNA-binding proteins have been shown to block elongation by RNA polymerases in an orientation-dependent manner (3, 18, 25). In this study, an accumulation of RNA polymerases opposite site A on the early DNA strand was not observed (Fig. 7). This result implies that large T antigen may block only elongation by RNA polymerases transcribing through the intergenic region on the late DNA strand. However, a more detailed analysis will be necessary to determine whether large T antigen functions in an orientation-dependent manner.

Large T antigen also binds to three other nearby sites (B, C, and 1/2). Surprisingly, binding to sites B and C, which lie upstream of site A in the direction of late transcription, has no measurable effect on RNA polymerase elongation, since these sites can be inactivated by mutation without reducing stalling, and their presence in the absence of an intact site A is not sufficient to induce stalling. Several explanations for these results can be proposed. First, the GAGGC pentanucleotides in binding site C are oriented in the opposite direction to those in sites A and B (see Fig. 1B). If the orientation of bound large T antigen with respect to elongating RNA polymerases were important, RNA polymerases elongating in the late direction would not be blocked at site C. However, this could not explain the inability of large T antigen bound at site B to block elongation. Second, bound large T antigen may dissociate rapidly from sites C and B in the infected cell yet remain stably bound to site A. In this case, a larger proportion of DNA templates being actively transcribed may have large T antigen bound to site A than to site B or C. Little is known about the occupancy of these sites in vivo. Third, the capacity of DNA-bound large T antigen to block elongating RNA polymerase II may depend on flanking sequences nearby each site, which could influence the tertiary structure of large T antigen or its dissociation rate. These explanations are not mutually exclusive. We have not been able to test directly the dependence of stalling on the integrity of binding site 1/2, as it is required for DNA replication and therefore for virus viability. It is known that large T antigen forms a complex double-hexamer structure around site 1/2 and that this double hexamer enables DNA replication to begin by unwinding the DNA helix and by attracting cellular proteins to this site (10, 71). Clearly, site 1/2 by itself is not sufficient to block RNA polymerase elongation efficiently in the absence of an intact site A. However, it may be that blockage requires site 1/2 in addition to site A.

Large T antigen bound to site A may block elongation on the late DNA strand by physically preventing RNA polymerases from transcribing through the DNA-protein complex. The *lac* repressor protein has been shown to block elongation by *E. coli* RNA polymerase and eukaryotic RNA polymerases I and II (24, 25, 42, 61), suggesting that blockage in these cases occur by simple steric interference. Alternatively, large T antigen may interact with a specific domain of RNA polymerase II which leads to stalling. The murine TTFI protein, which terminates transcription by RNA polymerase I (5), does not terminate transcription by RNA polymerase II or prokaryotic RNA polymerases (42). In addition, the termination and DNA-binding functions of TTFI appear to reside in different protein domains (5). These results imply that a specific protein-protein interaction between the blocking protein and the transcription elongation complex is necessary for termination to occur. It will be of interest to determine whether large T antigen bound to site A is able to block elongation by other eukaryotic and prokaryotic RNA polymerases.

RNA polymerases may respond in a number of ways to a blocking protein. First, RNA polymerases may stall but retain the ability to continue elongation following the dissociation of the blocking protein (52). Alternatively, termination of transcription may occur, resulting in the dissociation of RNA polymerase and its nascent RNA chain from the DNA template (24, 42). The characteristics of the blocking proteins that determine their ability to stall or terminate transcription by RNA polymerases are not yet understood. The accumulation of RNA polymerases in vivo nearby large T-antigen-binding sites and their subsequent reactivation in vitro on viral transcription complexes indicate that large T antigen blocks elongation by a stalling mechanism (9, 62; this study). However, it is possible that stalled RNA polymerases eventually terminate transcription and are released from the DNA template. An accumulation of stalled or paused RNA polymerases has been observed within the termination regions of several genes transcribed by RNA polymerase II (30, 47, 69, 73).

Transcription by RNA polymerases through prokaryotic promoters (2, 34, 35) and eukaryotic promoters (6, 19, 22, 36, 50, 56, 68) has been shown to disrupt the formation of initiation complexes, causing promoter occlusion. RNA polymerase II has recently been observed to stall or terminate transcription in the 5′ flanking regions of several viral and cellular genes (3, 13, 17, 62). Interestingly, specific DNA-binding proteins are responsible, at least in part, for mediating these blocks to transcription elongation (3, 9, 11, 18, 58). It has therefore been proposed that certain DNA-binding proteins may function to prevent RNA polymerases from reading through downstream promoter regions and displacing proteins that are necessary for the initiation of transcription (3, 11, 17, 18). DNA-binding proteins that bind upstream of RNA polymerase I promoters have been shown to prevent such interference from occurring (36, 49). Large T-antigen-binding site A is located some 200 nt upstream of the polyomavirus late promoter, suggesting that large T antigen may function to prevent RNA polymerases transcribing the late DNA strand from disrupting protein binding at the late promoter. However, mutation of large T-antigen-binding site A had little or no effect on late promoter function (Fig. 4). Two other regulatory elements are also located downstream of binding site A: the origin of viral DNA replication and the enhancer region, which augments viral DNA replication and transcription from the early promoter (26, 27, 51, 65). Perhaps large T antigen bound to site A functions to prevent the displacement of proteins assembled at these regulatory elements by readthrough transcription.

The binding of large T antigen to site A, besides blocking elongation by RNA polymerases, has been proposed to augment the initiation of viral DNA replication at adjacent site 1/2 (55) and may play a role in repressing transcription initiation at the early promoter (23). In this study, mutation of both GAGGC pentanucleotides in site A had no apparent effect on the repression of early transcription (Fig. 8). However, several nucleotides within the GAGGC pentanucleotide proximal to site 1/2 were found to be absolutely required for virus growth (mutations A and A0; Fig. 2). In addition, mutant A1A2 virus produced small plaques, suggesting some impairment of virus growth (Fig. 9). Unexpectedly, further characterization of the A1A2 mutant virus did not show any reduction in the levels of viral DNA synthesized (Fig. 10A) or in the production of progeny virions (Fig. 10B). Perhaps the reduction in plaque size that we have observed with the A1A2 mutant virus is due to only a small reduction in viral DNA synthesis that is not detectable by

the assay used in this study. Several polyomavirus mutants having small-plaque phenotypes have been isolated and characterized (7, 8, 46, 65). Some of these mutant viruses show only a two- to threefold reduction in the level of DNA synthesis relative to wild-type virus. Alternatively, the release of infectious progeny virions from infected cells may be affected by mutations in site A. This impairment would account for the small-plaque phenotype (Fig. 9) and our inability to obtain high-titer virus stocks, in four attempts, by transfection of cells with mutant A1A2 virus DNA (data not shown). In both of these situations, repeated cycles of virus infection and release are required, thereby revealing any impairment in virus release. Since all cells were initially infected during the DNA synthesis and growth curve experiments performed in this study, no effect on the level of virus DNA synthesized or on virus yield would be expected, as we have observed (Fig. 10).

CP1, MAZ, and TBF1 are other examples of DNA-binding proteins which have been shown to stall or terminate transcription by RNA polymerase II (3, 11, 17, 18, 58). In addition to blocking elongation, CP1 and MAZ proteins function as activators of transcription initiation (4, 14). The TBF1 protein, which binds to a site within the P2 promoter of c-*myc* (58), may also participate in the initiation of transcription. Displacement of these proteins by elongating RNA polymerases might therefore be expected to interfere with transcription initiation. The binding of large T antigen to site A, besides blocking elongation by RNA polymerases, also appears to be required for normal virus growth, as suggested by the small plaque size of the A1A2 mutant virus (Fig. 9). Perhaps the stalling of RNA polymerases by large T antigen functions primarily to prevent the displacement of large T antigen from site A, thereby retaining an important protein-DNA interaction required for the normal growth of polyomavirus.

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