

Regulation of polyomavirus transcription by large tumor antigen

(RNA polymerase II/adenovirus late promoter/S1 nuclease mapping)

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ABSTRACT We have analyzed the regulation of viral transcription by the large tumor antigen in cells infected by several viable deletion and insertion mutants of polyomavirus. We find that deletion of the early promoter "TATA box" and associated large tumor antigen binding site has only a small effect on the balance of early and late mRNAs. Furthermore, transcription of a polyomavirus containing a heterologous adenovirus promoter in place of the normal TATA box and cap sites is regulated by the large tumor antigen. We conclude that repression of polyomavirus early transcription cannot occur simply by binding of the large tumor antigen to DNA sequences at the site of transcription initiation and must involve the interaction of the large tumor antigen binding at other sites.

During replication within their host cells, transcription of polyoma and simian virus 40 (SV40) viruses shifts from an emphasis upon mRNAs encoding tumor antigens (T-Ags; at early times) to mRNAs encoding capsid proteins (at late times). The *cis*-acting DNA sequence elements that are known to influence initiation of transcription of polyomavirus early genes include a transcriptional enhancer region, a "TATA box", and the principal *in vivo* mRNA cap site (1–5). Polyomavirus early gene transcription is regulated in *trans* by the large T-Ag (4, 6, 7); however, little is known about how this is mediated. With SV40, numerous studies indicate that the large T-Ag inhibits the initiation of early region transcription by binding to promoter-proximal DNA sequences, including the origin of DNA replication, early region TATA box, and principal mRNA cap site (8–17). Recent evidence suggests that large T-Ag is also capable of directly stimulating SV40 late region transcription (18–21).

In the polyomavirus genome, several sites recognized by large T-Ag have been identified (refs. 4, 22–24; A. Cowie and R. Kamen, personal communication). High-affinity sites include the early TATA box and *in vivo* mRNA cap sites [site I (23) or site C (24)], and sequences bordering the viral origin of DNA replication [site II (23) or site A (24)], located ≈50 base pairs (bp) upstream. Other low-affinity sites span the origin of DNA replication (A. Cowie and R. Kamen, personal communication). We have examined the 5' termini of early region mRNAs and the relative levels of polyomavirus early and late region transcripts in several mutants with deletions and insertions in the large T-Ag binding site I. Our results show that deletion of the TATA box and associated high-affinity large T-Ag binding site I does not dramatically alter regulation of viral transcription by large T-Ag. Furthermore, transcription of a virus containing a heterologous adenovirus promoter replacing the polyomavirus TATA box, cap sites, and T-Ag binding site I is regulated effectively by large T-Ag. We conclude that regulation of polyomavirus transcription cannot occur simply by large T-Ag binding to site I and must involve sequences apart from this element. It is possi-

ble that repression occurs by large T-Ag interfering with RNA polymerase movement to the site of initiation of transcription.

METHODS

Cells and Enzymes. Cell culture and virus assay procedures have been described (25, 26). All enzymes were used in accordance with the directions provided by the commercial suppliers.

Construction of Ad175 and Ad175/ts-25E Insertion Mutants. The adenovirus major late promoter was isolated by *Bst*NI and *Pvu* II double digestion of a pBR313 clone of the *Sma* I fragment of adenovirus 2 (27). The *Bst*NI end was filled in with *Micrococcus luteus* DNA polymerase, *Cla* I linkers were attached, and the fragment was inserted at the single *Nar* I site [nucleotide (nt) 99] of polyomavirus dl75 cloned in the vector pGL101 (28).

The Ad175/ts-25E recombinant was constructed by replacing the *Hind*III-2 fragment of Ad175 with the analogous fragment obtained from polyomavirus mutant ts-25E (29).

Quantitation of Viral Transcripts. For quantifying viral transcripts, RNAs were prepared essentially as described by Nevins (30) with the addition of vanadyl ribonucleoside complexes. The probes for detecting early and late transcripts were 5'-labeled fragments made single-stranded by hybridization to their complements in M13mp8 clones and separated from the partially duplex M13 DNA on strand separation gels. The early probe comprised polyomavirus sequences from nt 1427 to nt 1271 (Fig. 1) joined to M13mp8 sequences between the *Sma* I site and the *Sau*96 I sites. The late probe comprised polyomavirus sequences nt 3763 to nt 3943 joined to M13mp8 sequences between the *Hind*III site and the *Pvu* II sites. In each case, polyomavirus RNA will protect the label at the 5' end of these probes but will not hybridize to the entire length of DNA, so part of the probe will remain susceptible to S1 nuclease. Thus, hybridized probe is distinguishable from undigested input probe DNA.

Quantitation of Pulse-Labeled RNA. Infected 3T6 cells (at 34 hr postinfection) were pulse labeled with [³H]uridine (4 × 10⁻⁴ Ci/ml, 42 Ci/nmol; 1 Ci = 37 GBq) for 2 hr at 37°C. RNA was isolated with slight modifications of the procedure described by Nevins (30). The appropriate strand-specific polyoma-M13mp7 bacteriophage DNA and [³H]uridine-pulse-labeled RNA were combined, ethanol precipitated, and resuspended in 10 μl of 0.4 M NaCl/0.05 M Pipes, pH 6.5/0.002 M EDTA, then sealed in silanized glass capillaries, heated to 106°C for 5 min, and incubated at 68°C for 4 hr. Unhybridized RNA was removed by diluting the hybridization reaction into 0.5 ml containing 0.35 M NaCl, 0.01 M

Abbreviations: SV40, simian virus 40; T-Ag, tumor antigen; WME, whole mouse embryo; nt, nucleotide(s); bp, base pair(s).

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Tris-HCl (pH 7.5), 40 μ g of RNase A per ml, and 32 units of RNase T1 per ml and incubating at 37°C for 30 min. Proteinase K was added to 20 μ g/ml and incubated an additional 30 min at 37°C. The RNase-resistant hybrids were diluted into 10 ml of loading buffer (1.0 M KCl/0.3 M NaCl/0.1 M Tris-HCl, pH 7.5/0.002 M EDTA) and collected on nitrocellulose filters. The filters were washed with 50 ml of loading buffer, dried, and assayed for radioactivity.

Analysis of Viral Minichromosomes. Viral minichromosomes were prepared from the nuclei of 2×10^7 infected whole mouse embryo (WME) cells by a procedure modified from that of Garber *et al.* (31). Following sedimentation through a 17.5–35% glycerol gradient, the fractions containing viral DNA were identified by hybridization to a polyoma-specific DNA probe and the minichromosome peak fractions were pooled, digested with proteinase K (100 μ g/ml, 60°C, 2 hr), phenol extracted, and ethanol precipitated. The viral DNA was linearized by digestion with *Eco*RI, treated with RNase A, fractionated on a 0.8% agarose gel, and identified by Southern blotting (32) and hybridization. The bands of viral DNA were excised and assayed for radioactivity in a scintillation counter.

RESULTS

Properties of the Deletion and Insertion Mutants. The structure and properties of mutant dl75 have been described (4, 33, 34). It lacks the early region TATA box, principal mRNA cap sites, and all of large T-Ag binding site I (refs. 22, 24; Fig. 1). Mutant HB3 lacks sequences deleted in mutant dl75 up to the TATA box, but sequences from there to the initia-

tion codon have been restored (Fig. 1). Its growth properties are indistinguishable from wild-type A3 virus.

We replaced the DNA sequences deleted in mutant dl75 with a 166-bp DNA segment comprising the adenovirus 2 major late transcription promoter. This places the adenovirus TATA box, principal cap site, and 33 bp coding for the first late mRNA leader in a position directly adjacent to the polyomavirus early translation start codon. The resulting virus (Ad175; Fig. 1) is viable, produces normal-sized plaques on WME cells, and encodes 3-fold more early proteins than wild-type virus, as measured by immunoprecipitation from infected cell extracts with antitumor serum (unpublished data).

The early region mRNAs synthesized (at 24 hr postinfection) by mutants dl75 and HB3 are found to initiate at a highly heterogeneous set of positions, many of which lie upstream of large T-Ag binding site II (ref. 4; unpublished data). Mutant Ad175 early region mRNAs initiate 28 nt downstream from the TATA box located in the inserted adenovirus sequences (unpublished data), or at about the same site as occurs with authentic adenovirus late mRNA (37). A detailed analysis of the early region transcription initiation sites utilized by these mutants will be presented elsewhere.

Regulation of Viral RNA Synthesis by Large T-Ag. During the course of a productive viral infection, polyomavirus gene expression follows a temporal program that is regulated at the transcriptional level. Initially, viral gene expression favors the early gene transcription unit but, as the infection progresses, transcriptional emphasis shifts so that eventually late strand transcripts represent about 95% of the total viral-

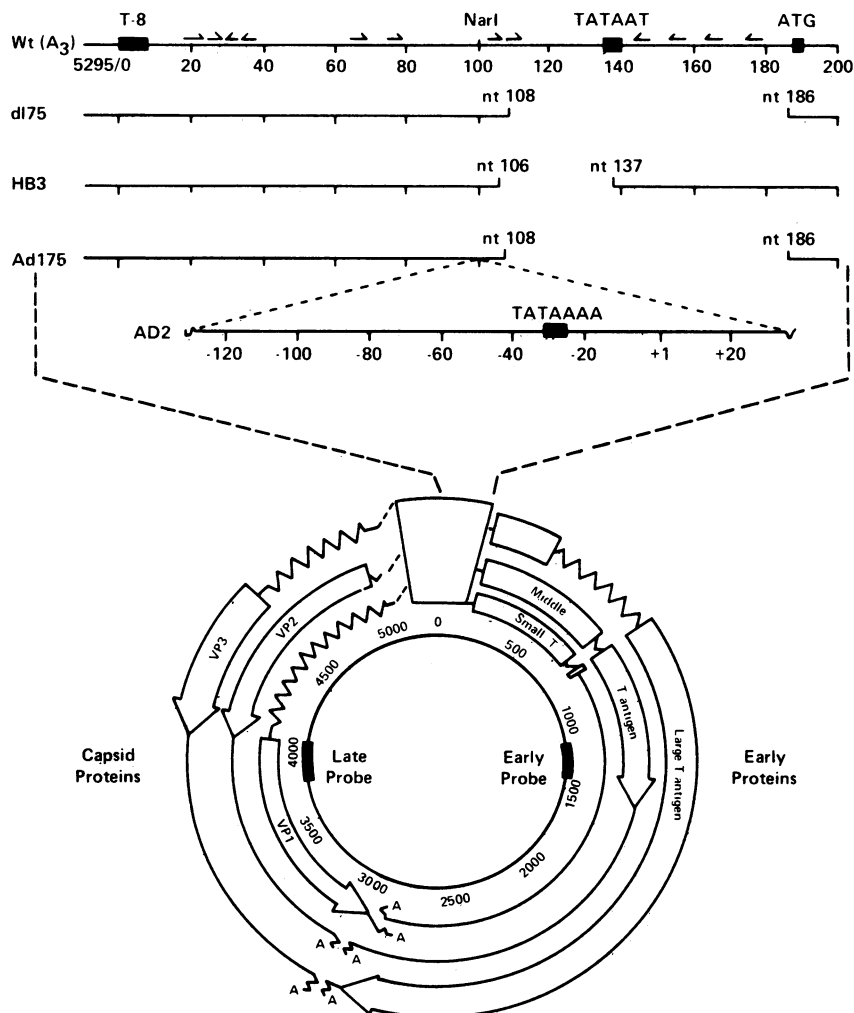


FIG. 1. Schematic of polyomavirus genome and DNA-origin proximal map of viral DNAs. Nucleotides are numbered according to Deininger *et al.* (35). The "core" region of the origin of DNA replication is located between nt 1 and nt 50 (36). The arrows indicate the location and the direction (5' to 3') of G-R-G-G-C (where R = purine) sequences. The adenovirus 2 major late promoter segment is numbered with respect to the principal late mRNA cap site (37).

specific RNA molecules found in the nucleus (38, 39). To define the role of large T-Ag in transcriptional regulation, we examined viral transcription in both the absence and presence of a functional large T-Ag. To do this we simultaneously measured the steady-state levels of early and late region virus-specific transcripts using internal sequence probes (Fig. 1) that "sum" the signals generated by mRNAs that have heterogeneous 5' termini. To minimize RNA-RNA self-annealing by symmetrically transcribed RNA, such as is present late in infection (40-42), single-stranded DNA probes in excess were hybridized under aqueous conditions. We also examined the rate of transcription by pulse labeling polyomavirus-infected mouse 3T6 cells with [³H]uridine and measuring the cytoplasmic RNA that hybridizes to early- or late-strand specific polyoma-M13 clones.

Transcription in the Absence of a Functional Large T-Ag. First, to establish the relative levels of early and late region transcription in the absence of a functional large T-Ag, we examined RNAs from cells infected by a virus encoding a thermolabile large T-Ag (ts-25E; refs. 29, 43). At permissive temperature (24 hr postinfection at 33°C), early region transcripts represent about 55% of total viral transcripts. After the shift to nonpermissive temperature (24 hr at 33°C followed by 24 hr at 39.5°C) the fraction of early region transcripts increases 87% (Fig. 2). It is noteworthy that this is due to a large increase in the amount of accumulated early mRNAs as well as to an apparent decrease in the amount of accumulated late mRNAs. A similar change in balance was observed with [³H]uridine-pulse-labeled RNA. Under these conditions the fraction of early region specific transcription (which is high even at 33°C compared to that of wild-type virus at 37°C) increases from 33% to 86% with the shift to nonpermissive temperature (Table 1). Both types of analysis indicate that when viral transcription occurs under conditions where large T-Ag is functionally inactivated early region transcripts are about 6-fold more abundant than late region transcripts. We consider transcription of the ts-25E mutant at 39.5°C to represent the state of viral transcription in the absence of large T-Ag-mediated regulation.

Effect of Large T-Ag on Late Region Transcription. The apparent reduction in ts-25E late region transcription at 39.5°C (Fig. 2, Table 1) could be due to an absolute reduction in the intranuclear supply of viral minichromosomes. Measurement of the amount of viral minichromosomes from the nuclei of WME cells infected with ts-25E both before and

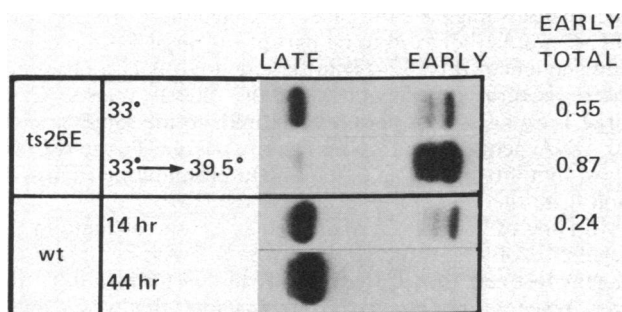


FIG. 2. Early and late region transcripts from ts-25E and A3 viruses. Total cytoplasmic RNA was extracted from A3 virus-infected cells after incubation at 37°C for 14 hr and 44 hr. Single-stranded 5'-end-labeled probe DNAs that were homologous to 192 nt and 156 nt, respectively, of late or early region transcripts were hybridized in excess to 5 µg of 14-hr and 2.5 µg of 44-hr A3 RNA and to 2.5 µg of 33°C or 39.5°C ts-25E RNA. Nonhybridizing probe DNA was removed by nuclease S1 digestion and the products were fractionated by electrophoresis on a 6% denaturing polyacrylamide gel. The fraction of total viral transcription that was early region specific was determined by densitometric scanning of an appropriate autoradiographic exposure.

Table 1. Hybridization of pulse-labeled RNA

| Viral RNA | Complementary DNA | | % early |
|----------------------|-------------------|-------------|---------|
| | M13Py late | M13Py early | |
| ts-25E 33°C | 0.26 | 0.13 | 33 |
| ts-25E 33°C → 39.5°C | 0.02 | 0.12 | 86 |
| Wild-type (A3) | 0.06 | 0.01 | 14 |
| dl75 | 0.17 | 0.04 | 19 |
| Ad175 | 0.35 | 0.10 | 22 |
| HB3 | 0.13 | 0.02 | 13 |

Each hybridization reaction included 4×10^4 to 1.3×10^5 cpm of input total cytoplasmic RNA and all hybridization values have been corrected for 0.02-0.04% background hybridization to M13mp8 DNA. The complementary DNAs, M13Py late and M13Py early, are derived from the vector M13mp7 into which have been inserted ≈1.4 kbp of the polyomavirus late- or early-gene region, respectively. Values given are percent of input cpm that hybridized to the complementary DNA. Wild-type, dl75, HB3, or Ad175 virus-infected mouse 3T6 cells (maintained at 37°C) were pulse labeled with [³H]uridine from 34-36 hr postinfection. The ts-25E 33°C RNA was prepared from ts-25E infected 3T6 cells maintained at 33°C and pulse labeled with [³H]uridine from 21 to 23 hr postinfection. The ts-25E 33°C → 39.5°C RNA was prepared from ts-25 infected 3T6 cells maintained at 33°C for 23 hr and then shifted to 39.5°C and pulse labeled with [³H]uridine from 21 to 23 hr following the temperature shift.

after the shift to nonpermissive temperature revealed that there was little change from the amount present at 24 hr at 33°C to that found 24 hr following the shift to 39.5°C (the ratio of 39.5°C/33°C = 0.97). This contrasts with a 30-fold increase in viral minichromosomes in the nuclei of wild-type virus-infected WME cells after a similar temperature shift. If the fraction of minichromosomes engaged in transcription is unchanged between samples (an assumption that requires verification) the apparent decrease in late region specific transcription (Fig. 2, Table 1) following the thermal inactivation of large T-Ag may be explained by the loss of a stimulatory effect of large T-Ag on late region transcription. Such a stimulatory effect of SV40 large T-Ag upon SV40 late transcription has been reported recently (18-21).

Transcription in the Presence of a Functional Large T-Ag. During the course of a productive polyomavirus infection, large T-Ag is first detected at about 12 hr postinfection and it accumulates until it reaches a maximum at about 24 hr postinfection (44). Our objectives were (i) to observe any change in viral transcription as large T-Ag accumulates in the nucleus and (ii) to establish, under our assay conditions, the maximal regulatory effect of large T-Ag on viral transcription. We measured viral-specific transcripts in total cytoplasmic RNA extracted from wild-type polyomavirus-infected WME cells at 14 hr and at 44 hr postinfection. The results (Fig. 3) reveal that early region transcripts represent about 24% of the total viral transcripts at 14 hr postinfection. At 44 hr postinfection, when the intracellular concentration of large T-Ag is high, the balance of viral transcription greatly favors the late region. We also find early region transcripts to be only 14% of the viral-specific RNA arriving in the cytoplasm during a 2-hr [³H]uridine pulse label (Table 1). Together, these results suggest that late region transcripts outnumber early region transcripts by a ratio of at least 6:1 during the period in virus infection when large T-Ag-mediated repression is reaching a maximum.

The results with the ts-25E mutant and with wild-type virus show that over the course of a productive viral infection, large T-Ag causes at least a 36-fold shift in RNA synthesis from the early to the late transcriptional unit. This is due in part to inhibition of transcription from the early promoter and probably to activation of transcription from the late promoter.

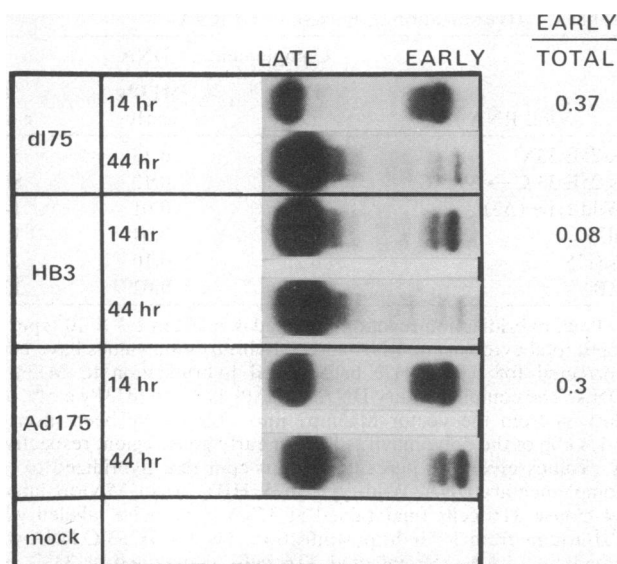


FIG. 3. Early and late region transcripts from deletion and insertion mutants. Total cytoplasmic RNA was prepared from deletion mutants dl75 and HB3 and from the insertion mutant Ad175 at 14 and 44 hr postinfection. Hybridization, nuclease S1 digestion, and quantitation were as described in the legend to Fig. 2.

Effect of Sequence Alterations on Regulation by Large T-Ag. Because the deletions in dl75 and HB3 include all or part of the polyoma large T-Ag binding site I and hence might alter regulation by large T-Ag, we tested their effect on early region transcription *in vivo*. The dl75 deletion causes an approximate 1.5-fold increase in the fraction of early region transcripts (Fig. 3). This effect is most evident at 14 hr postinfection and in the pulse-labeled RNA, but it persists in the RNA extracted at 44 hr postinfection. Mutant HB3 behaves transcriptionally like wild-type polyomavirus, although early in infection the fraction of early transcripts is reduced about 30%. At present we do not understand the significance of this change. However, it is evident from these data that T-Ag binding site I is not the sole, or even a major, contributor to the regulation of transcription that occurs in polyomavirus. The small change in repression that is observed with mutant dl75 may be due directly to the loss of these sequences or to the lowered levels of large T-Ag and viral DNA replication known to occur in dl75-infected cells (34).

Insertion of the adenovirus major late promoter into dl75 functionally replaces the polyomavirus early TATA box and principal early mRNA cap sites with the analogous adenovirus sequence elements. As this virus replicates as well as wild-type polyomavirus, it provides a way to examine regulation of transcription from an efficient heterologous promoter. Early in infection the balance of early transcripts is slightly higher in Ad175 (30%, Fig. 3) than in wild-type polyomavirus (24%, Fig. 2). The [³H]uridine pulse-labeling data for Ad175 also indicate a small increase in the fraction of early region specific transcription (Table 1). However, this level is far below that observed in the unregulated state (ts-25E at the nonpermissive temperature; Fig. 3). To ensure that transcription in Ad175 is subject to T-Ag-mediated regulation, we constructed a virus containing the Ad175 promoter sequences that also encodes a thermolabile large T-Ag (Ad175/ts-25E). Viral transcription by this mutant was measured at both the permissive and the nonpermissive temperatures. The fraction of total viral transcription from the early region at each temperature in this double mutant is similar to that determined for the ts-25E single mutant (Fig. 4). Thus, RNA polymerases utilizing the adenovirus promoter se-

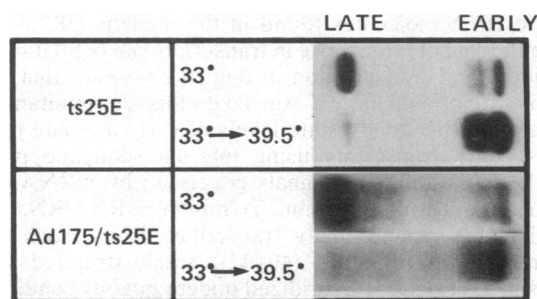


FIG. 4. Transcription of Ad175 in the presence and absence of a functional large T-Ag. The double recombinant Ad175/ts-25E contains the adenovirus major late promoter and encodes a thermolabile large T-Ag. Total cytoplasmic RNA was prepared from Ad175/ts-25E-infected WME cells after 24 hr at 33°C or after an additional 24 hr at 39.5°C. Hybridization and nuclease S1 digestion were as described in the legend to Fig. 2. Analysis of ts-25E transcripts is included for comparison.

quences in the polyomavirus Ad175 construct appear to be subject to regulation by polyomavirus large T-Ag.

DISCUSSION

Large T-Ag is clearly the major determinant of the balance of early and late region transcription in polyomavirus. Our results indicate that a 30- to 40-fold shift in utilization of the polyomavirus early promoter relative to the late promoter occurs as large T-Ag accumulates during productive infection. A significant part of this control is due to repression of transcription from the early promoter. The prokaryotic paradigm for how such repression might be exerted suggests that large T-Ag would sterically hinder the binding of RNA polymerase II or of a putative positive-acting transcription factor (45-47) to the site at which initiation of transcription occurs—i.e., near the TATA box and cap sites. Consistent with this notion is the demonstration that large T-Ag does indeed bind to DNA sequences containing the TATA box and cap sites (4, 22-24). To our surprise, however, we find that deletion of these sequences (in mutant dl75 and HB3) or their replacement by foreign sequences (Ad175) has only a marginal effect upon the control of early gene transcription by large T-Ag. Although, in the latter instance, we have not shown that the large T-Ag is incapable of binding to the adenovirus TATA box and cap sites, there is no reason to suspect that this might be the case. Although there are two G-R-G-G-C motifs (believed to be part of the large T-Ag recognition sequence; refs. 22-24) in the adenovirus late promoter insert, several experiments with the closely related SV40 large T-Ag or D2T protein (which binds to the same or similar DNA sequences as polyomavirus large T-Ag; ref. 48) show that it is incapable of binding to or inhibiting transcription from the adenovirus promoter (10, 13).

Binding of large T-Ag to site I may be one element in the regulation of viral transcription by large T-Ag, but it is insufficient to exert the 30- to 40-fold shift in utilization of the early promoter relative to the late promoter that we calculate by comparing transcription of mutant ts-25E at the nonpermissive temperature to transcription of wild-type virus late in infection. Repression must be exerted by binding at other sites as well. Activation of late gene transcription may also contribute to the transcriptional shift. The mechanism and DNA sequences responsible for such activation remain to be defined; however, they are apparently not at site I.

If site I is not the major sequence element responsible for regulation of transcription by large T-Ag, then what is? Site II, ≈60 bp upstream of the TATA box (Fig. 1), may contribute significantly, as may the low-affinity sites spanning the origin of DNA replication. It is perhaps significant that the

polyomavirus "CAAT box" sequence is directly adjacent to one of the G-R-G-G-C motifs present at binding site II.

If binding of large T-Ag to these upstream sequences causes repression of early transcription, the mechanism must be distinctly different from what we know occurs in the bacterial *lac* and λ systems in which repressors sterically hinder binding of RNA polymerase to the -35 and -10 sequences. In SV40, the early promoter overlaps the origin of DNA replication, and several T-Ag binding sites at or near the origin of DNA replication are responsible for repression (14-17). In this instance, RNA polymerase II access to the site of initiation of transcription may be blocked by large T-Ag. If it is these analogous regions in polyomavirus that contribute to repression by large T-Ag, large T-Ag bound to these upstream sites might instead block the association of a positive-acting transcription factor similar to SP 1 (46, 47) or it might act as a barrier to RNA polymerase molecules as they traverse chromatin toward the transcription initiation site. For both SV40 and polyomavirus, binding of large T-Ag to these same sequences may directly or indirectly help activate late transcription.

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