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Interaction between T Antigen and TEA Domain of the Factor TEF-1 Derepresses Simian Virus 40 Late Promoter In Vitro: Identification of T-Antigen Domains Important for Transcription Control

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The large tumor antigen (TAg) of simian virus 40 regulates transcription of the viral genes. The early promoter is repressed when TAg binds to the origin and DNA replication begins, whereas the late promoter is activated by TAg through both replication-dependent and -independent mechanisms. Previously it was shown that activation is diminished when a site in the viral enhancer to which the factor TEF-1 binds is disrupted. We show here that the NH_2-terminal region of TAg binds to the TEA domain of TEF-1, a DNA binding domain also found in the *Drosophila scalloped* and the *Saccharomyces cerevisiae TEC1* proteins. The interaction inhibits DNA binding by TEF-1 and activates transcription in vitro from a subset of naturally occurring late start sites. These sites are also activated by mutations in the DNA motifs to which TEF-1 binds. Therefore, TEF-1 appears to function as a repressor of late transcription, and its involvement in the early-to-late shift in viral transcription is discussed. The mutation of Ser-189 in TAg, which reduces transformation efficiency in certain assays, disrupts the interaction with TEF-1. Thus, TEF-1 might also regulate genes involved in growth control.

The large tumor antigen (TAg) of simian virus 40 (SV40) is a multifunctional protein (for a review, see reference 14). Its DNA binding, helicase activities, and ability to interact with the DNA polymerase α-primase complex promote viral DNA synthesis. Its potent cell transformation activity is thought to result from its ability to bind to and sequester the tumor suppressor proteins retinoblastoma (Rb) and p53 (reviewed in references 29 and 39). In addition to DNA polymerase α-primase, Rb, and p53, several other proteins have been shown to have functionally important interactions with TAg. Examples include the transcription factor AP-2, which is inactivated when it binds TAg (38), the X protein of hepatitis B virus, which, like p53, can interfere with the replication function of TAg (46), and the product of the *mdm2* oncogene (5).

The promoter regulating SV40 late gene expression is one of a number of viral and cellular promoters activated by TAg (3, 4, 24, 32, 41, 51). Transcription from the SV40 early promoter is autoregulated when TAg binds to the viral origin and activates replication (21, 28, 30, 40, 43). Mutations that prevent TAg from entering the nucleus or that disrupt the origin result in activation of early transcription similar to that seen from the late promoter (53).

The promoter elements that mediate activation of SV40 transcription by TAg include two sites within the enhancer to which the transcription factor TEF-1 binds (8, 19, 25, 35, 45). One, the Sph motif, can bind two TEF-1 molecules and consists of a repeated 9-bp sequence (in boldface type), AG(**AAG TATGCA**)(**AAGCATGCA**)TC. The other, the GT-IIC motif, is a single binding site with the Sph sequence conserved in only four of nine positions, CT(**GTGGAATGT**)GT (11, 56). In transfection assays, mutations in the Sph motif reduce transactivation by TAg, and multimerized GT-IIC motifs can be activated by TAg and are sensitive to mutations that prevent TEF-1 binding. These results suggested that TAg might enable TEF-1 to participate in formation of late promoter transcription complexes. Recent studies showed that TAg can bind directly to TEF-1 and to the TATA-binding protein (TBP) in the TFIID complex (20, 34).

TEF-1 is one member of a family of proteins that have in common a DNA binding domain referred to as the TEA domain (6). This domain is predicted to contain three α helices. TEF-1 is thought to be the same as MCAT, a protein involved in activation of genes encoding several muscle-specific proteins, including cardiac troponin T (15). Other TEA domain proteins include TEC1 from *Saccharomyces cerevisiae* (27), which binds to the enhancer of the Ty transposon, *scalloped* from *Drosphila melanogaster* (7), implicated to play a role during neural cell differentiation, and AbaA from *Aspergillus nidulans* (37).

In this paper we describe experiments to test in vitro if a direct interaction between TEF-1 and TAg can affect late transcription. We previously showed that in a cell-free transcription system the SV40 late promoter can be activated by a bacterially expressed NH_2-terminal fragment of TAg encompassing amino acids 1 to 272 (10). This region contains the Rb binding site and the DNA binding domain of TAg. Deletion and point mutation analyses of this region have identified a minimal region required for transcription activation in vitro.

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This region contains the DNA binding domain of TAg and is able to bind to the TEA domain of TEF-1. Interestingly, the interaction is prevented by an amino acid substitution in TAg that diminishes its transformation and DNA synthesis-stimulating activities. Furthermore, we find that TAg disrupts the binding of TEF-1 to DNA, suggesting that TEF-1 might be a negative regulator of transcription from selected late start sites. The significance of these data to the question of how TAg promotes an early-to-late switch in viral transcription is discussed.

MATERIALS AND METHODS

In vitro transcription assay and plasmid construction. The transcription reactions were carried out with whole-cell extracts prepared from HeLa S3 cells as previously described (10, 33). The templates used for transcription were pBEL-1, pBEL-1.4, pBEL-1.22, and pBEL-1.26, all of which have been described previously (25). pGEX.TAg.*Pvu*II was constructed by inserting the *Bgl*I-*Pvu*II fragment of SV40 TAg into pGEX1 digested with *Bam*HI and *Sma*I. To produce deletions from the COOH terminus of TAg, pGEX.TAg.*Pvu*II was digested with *Hin*dIII, treated for various times with *Bal*31, and then digested with *Eco*RI. The products of these reactions were treated with Klenow, ligated with T4 DNA ligase, and used to transform *Escherichia coli* HB101. Clones derived from this procedure were screened for production of fusion proteins of the desired sizes and sequenced. One of these COOH-terminal deletions (i.e., TAg1-249) was chosen as a starting point for producing deletions initiating from the NH_4 end. TAg1-249 was used as a template in a PCR with the oligonucleotides 5′-TAG GATCCATGGATAAAGTTTTAAACAG-3′ and 5′-TTTCACCGTCATCACC G-3′. The PCR product was treated with Klenow to repair the ends, digested with *Bam*HI, and cloned into pGEX2T digested with *Bam*HI, and *Sma*I. This intermediate plasmid was cut with *Bam*HI and treated with *Bal*31. After digestion for various periods, the DNA ends were repaired with Klenow and the DNA was digested with *Pst*I. Deletion fragments were purified from low-melting-point agarose and cloned into *Sma*I-*Pst*I-digested pGEX2T. Clones were screened for production of fusion proteins in the desired size range, and deletion endpoints were identified by sequencing. Plasmids expressing TAg101-249 fusion proteins with various substitution mutations were constructed by PCR amplification of the desired regions from templates containing the various mutations (provided by E. Fanning). Products of PCRs were digested with *Bam*HI and EcoRI and inserted into pGEX2T digested with the same restriction enzymes. Plasmids expressing TEF-1 as a GST-fusion protein were constructed by isolating the appropriate fragment from the cDNA of TEF-1 in the vector pXJ40-TEF1A (56) and inserting them into pGEX2T.

Protein A fusions of TEF-1 and TAg fragments were made with the vector pAGEX2T (48). This vector results in the synthesis of a tripartite fusion, resulting in GST-protein A-peptide. The polylinker in this vector is identical to that in pGEX2T, so appropriate fragments were easily transferred to this vector.

Fusion proteins and transcription extracts. All GST-fusion proteins were purified from bacterial cultures and coupled to glutathione agarose (48). When the proteins were to be cleaved with thrombin, the beads were washed in thrombin digestion buffer (150 mM NaCl, 2.5 mM $CaCl_2$) and then incubated as a 50% suspension in the same buffer with thrombin (1 μg/μl; Sigma no. T4648) for 30 to 60 min at 25°C with gentle shaking. The thrombin was inactivated by adding EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] to a concentration of 5 mM, and the beads carrying the GST moiety were removed by centrifugation. The supernatants carrying the various protein A-peptide fusions to be used were analyzed by sodium dodecyl sulfate (SDS)-gel electrophoresis and Coomassie blue staining to verify concentrations and stored at −80°C.

Bead binding assays were carried out in 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; pH 7.9)–100 mM KCl–5 mM $MgCl_2$–1 mM dithiothreitol–1% milk powder. Samples were incubated at 4°C for 1 h with gentle shaking. The beads were recovered by centrifugation and washed three times in the same buffer without milk powder. They were then suspended in sample buffer and loaded onto SDS–10% polyacrylamide gels. The protein A fusions retained on the beads were detected by standard Western blot (immunoblot) analysis with commercial alkaline phosphatase-conjugated antibody. When the protein of interest was TAg that had not been made as a protein A fusion, the blots were first incubated with a primary anti-TAg antibody (pAb419) and then with a secondary alkaline phosphatase-conjugated antibody. Alkaline phosphatase activity was detected with nitroblue tetrazolium and BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium) according to the manufacturer's instructions. In all instances, the blots were blocked for 30 min with 5% milk powder before the addition of antibody.

Mobility shift assays. Mobility shift assays to test the effect of TAg on TEF-1 DNA binding were performed as follows. The two proteins were incubated together in 30 μl of 10 mM Tris-HCl (pH 7.9)–50 mM NaCl–1.0 mM EDTA–2.0 mM $MgCl_2$–1.0 mM dithiothreitol–0.02% Nonidet P-40–1 mg of dI-dC per ml–10% glycerol. After 40 min on ice, approximately 1 ng of ^{32}P-labelled probe was added and the incubation was continued for 10 min. Ficoll was then added to each sample (2 μl of a 15% solution), and the samples were analyzed on 7.5% polyacrylamide gels in 0.5× Tris-borate-EDTA running buffer.

RESULTS

Mutational analysis of transcription activation and DNA binding activities of TAg. Previously, we demonstrated that an NH_2-terminal 272-amino-acid fragment of TAg can autoregulate SV40 early gene expression and activate transcription in vitro from a subset of the natural SV40 late transcription initiation sites (10). To localize the minimal essential region within this TAg fragment, truncated proteins were purified as GST fusions from bacterial cells and tested for late promoter transcription activation, early promoter autoregulation, and DNA binding functions in vitro.

An NH_2-terminal fragment extending to amino acid 249 retained the ability to activate transcription, while fragments shorter than 249 amino acids were inactive (Fig. 1A). In these assays, transcription from late start sites at nucleotide positions 243 and 264 was predominant. This loss of activation observed when the C terminus of TAg1-249 was shortened was coincident with a loss in DNA binding activity (Fig. 1A). The DNA binding domain of TAg has previously been localized to a segment spanning residues 131 to 246 (reviewed in reference 14), and our observation that DNA binding was lost when residues from 239 to 249 were deleted was consistent with that finding. The loss of DNA binding prevented autoregulation of early transcription (Fig. 1A).

Amino-terminal deletions from TAg1-249 localized the minimal region for transcription activation within residues 101 to 249 (Fig. 1B). The removal of amino acids 95 to 101 already showed some reduction in activity. The N-terminal deletions tested extended to position 133, and all deletion mutants retained an ability to bind DNA and autoregulate early transcription. The gel mobility shift assays used to evaluate DNA binding showed that there were differences in the abilities of the different proteins to multimerize on the DNA as more protein was added. Notably, TAg128-249 was less capable of generating higher-order complexes than either TAg101-249 or TAg133-249.

In these experiments transcription activation from only a subset of the natural SV40 late start sites was observed. A major cap site in vivo is at position 325, and we asked whether its low activity in vitro might result because the template used in our runoff assays was truncated on the late side at position 346. It has been reported that sequences from nucleotide positions 345 to 365 bind a factor required for assembly of initiation complexes at the 325 site (2). When GST–TAg1-249 was tested for late activation from a template extending to position 396 on the late side, we saw an increase in the utilization of the start site at position 325 compared with what was noted with the original template (Fig. 1C); nevertheless, position 325 was not the major start site. Since its response to TAg paralleled those of the start sites at 243 and 264, we used the original template for most experiments.

The data from the analysis of TAg deletion mutants suggested that the DNA binding domain played an important role in transcription activation. However, several analyses suggested that DNA binding per se was not required. First, deletion of the TAg binding sites within the origin did not prevent late activation (Fig. 2A). The templates in Fig. 2A, lanes 5 and 6 and lanes 7 and 8, do not produce early transcripts because the promoter fragments are truncated at the *Stu*I or *Sal*I site, respectively. Second, single-amino-acid substitutions that disrupt specific functions of TAg were tested for their abilities to activate late transcription from the template that had the TAg

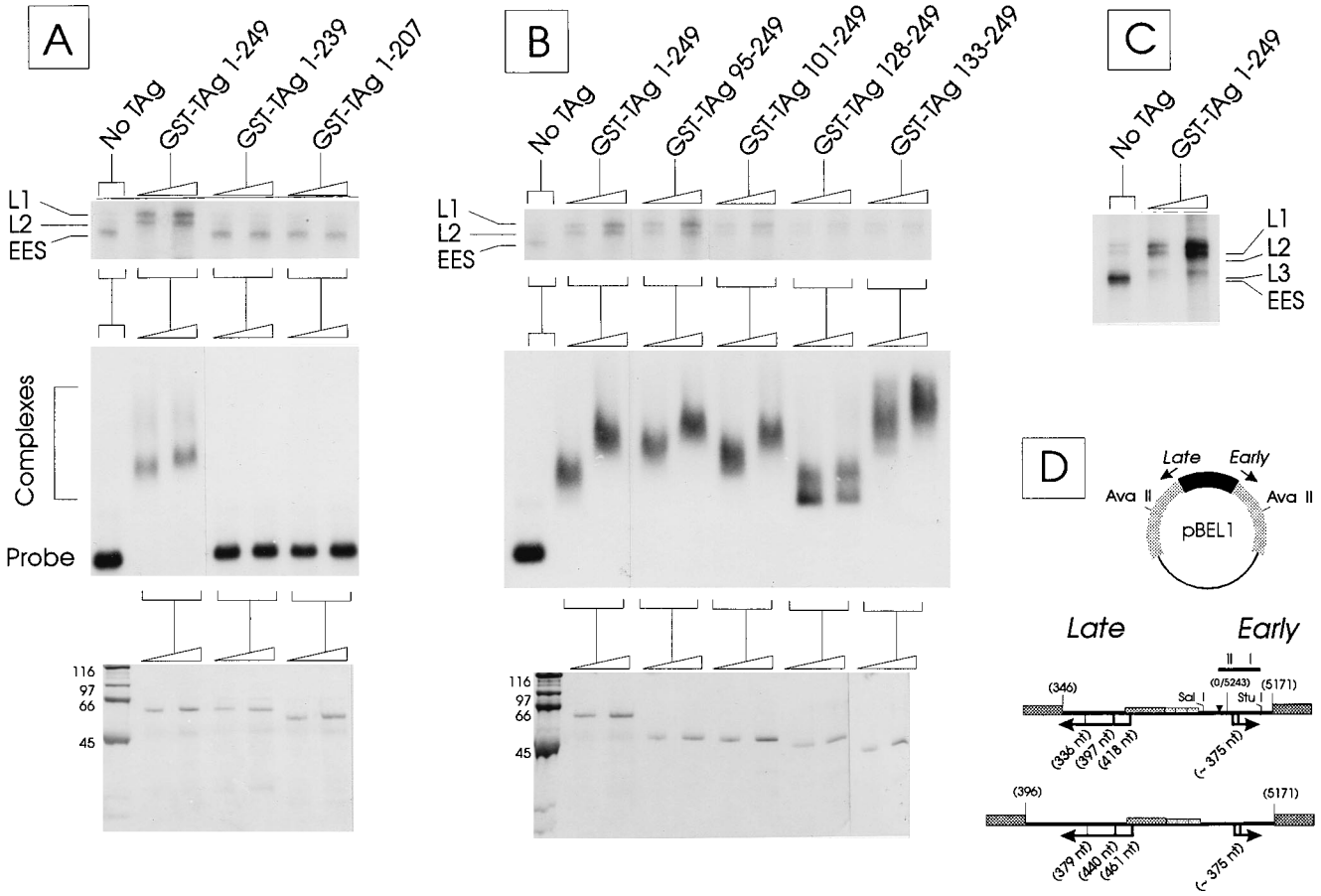

FIG. 1. Autoregulation and transactivation of SV40 gene expression by TAg. GST-TAg fusion proteins with progressive COOH-terminal (A) or NH_2-terminal (B) deletions were added at two concentrations (0.25 and 0.5 μg per reaction mixture) to transcription reaction mixtures with the pBEL1 template linearized with *Ava*II (0.18 μg per assay). EES designates the early transcripts of approximately 375 nucleotides, while L1 and L2 are the late starts from positions 243 and 264, respectively, generating transcript lengths of 418 and 397 nucleotides. Below each transcription assay is a DNA binding assay for the TAg proteins, and below each DNA binding assay is a Coomassie blue-stained gel showing the integrity of the TAg proteins used. The DNA binding assays were carried out as described in Coulombe et al. (10) with a probe that contained the 21-bp region, the TATA box and promoter, and the complete origin of replication up to position 5171. (C) Transcription reactions with the pBEL-1 template carrying additional late promoter sequences to position 396. L3 corresponds to the late start at position 325, and with this template L1, L2, and L3 are 461, 440, and 379 nucleotides, respectively. (D) Vectors used in the runoff assays. The lengths of RNA transcripts from each start site are shown in parentheses below each site. The lower template is the one used in panel C, and it contains additional viral sequences with diaminopimelic acid sites downstream of the late start sites. The upper template was used in most experiments. TAg binding sites I and II are indicated above the upper template. The inverted filled triangle indicates the TATA box. Arrows indicate directions of transcription activation.

binding sites deleted (the template used in lanes 7 and 8 of Fig. 2A). Two of these are shown in Fig. 2B. The proteins TAg101-249(N153T) and TAg101-249(S189N) both carry substitutions that disrupt the DNA binding function relative to that of the wild-type fragment. The replacement of asparagine at position 153 with threonine had no effect on late transcription activation, supporting the suggestion that DNA binding is not required. In contrast, the substitution of serine for asparagine at position 189, a mutation in the DNA binding domain that in some assays reduces the transformation and stimulation of DNA synthesis activities of TAg (12, 23), abolishes late transcription activation by the TAg101-249 fragment. Third, one TAg deletion mutant, TAg1-260, was defective in DNA binding, presumably because of a defect in how it becomes folded. This fragment did not autoregulate early transcription, but retained the transcription activation function (Fig. 2C). Thus, DNA binding is not required for the activation, and with certain TAg molecules it is possible to selectively eliminate DNA binding, late activation, or both.

TAg binds to the TEA domain of TEF-1. The TAg fragments tested for transcription activation in vitro were also tested for their abilities to interact directly with TEF-1. Several approaches were used in these experiments. The first method employed a modified pGEX expression vector, pAGEX (48), to synthesize proteins of interest as fusions with a fragment of protein A. Such fusions are detected with commercial conjugated antibodies. Initially, different regions of the TEF-1 protein were synthesized as protein A fusions and tested for their abilities to be retained on glutathione agarose beads carrying a GST fusion of TAg1-260. A fragment of TEF-1 from amino acids 1 to 167 was found to be selectively retained on these beads (Fig. 3A). A series of GST-TAg beads were then tested for their abilities to bind this protein A–TEF-1 fusion. As shown in Fig. 3B, the TAg133-249 beads retained the ability to bind to this fragment of TEF-1. Deletions from the C terminus that remove part of the DNA binding domain of TAg prevented TEF-1 binding (e.g., GST–TAg1-217). Beads loaded with a GST-TBP fusion protein did not bind TEF-1 in these assays (Fig. 3B) nor did beads that had been loaded with GST fusions of TAg239-367, TAg336-537, and TAg447-708 (data not shown).

The TAg101-249 proteins carrying amino acid substitutions

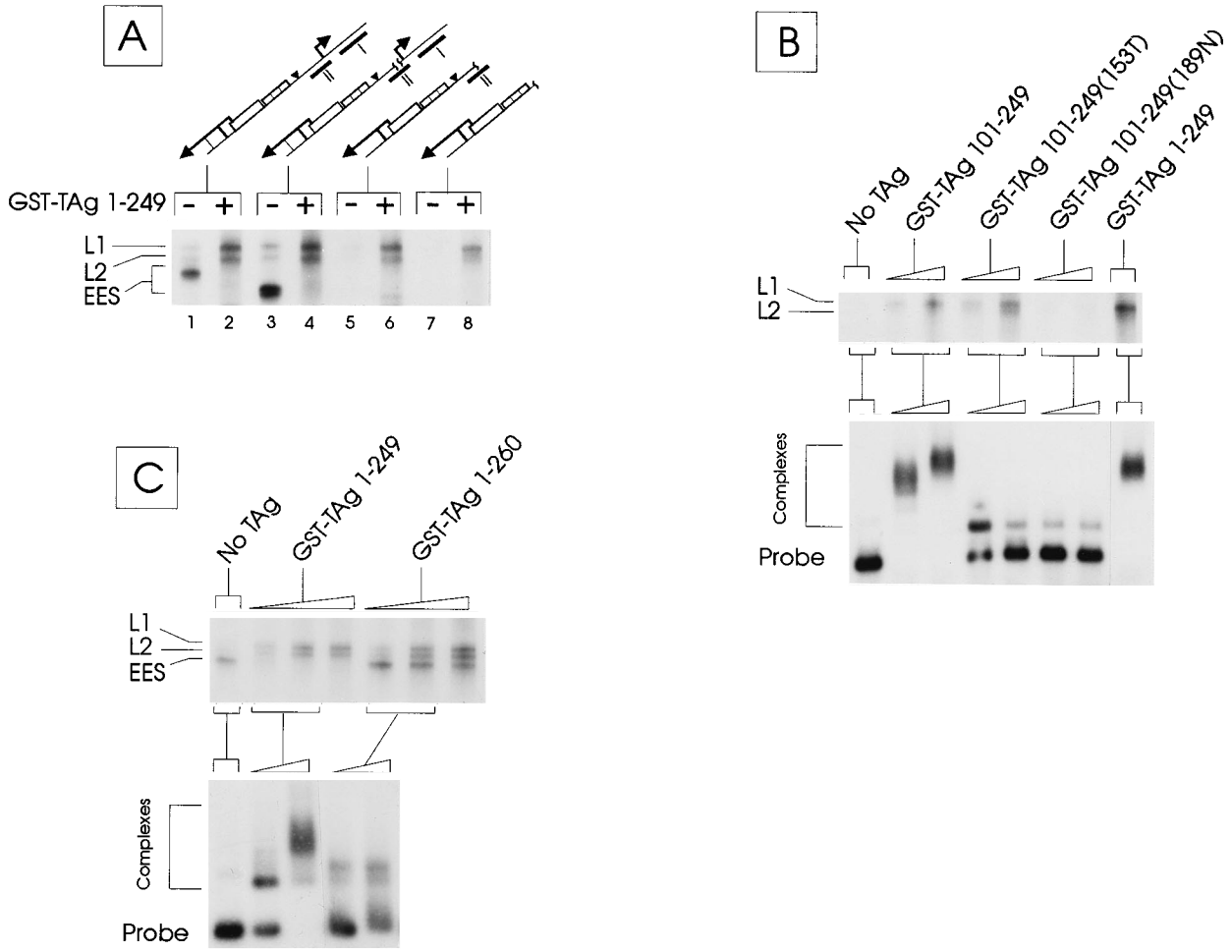

FIG. 2. Activation of late transcription in vitro does not require binding of TAg to DNA. (A) Transcription reactions with various deficiencies in TAg binding sites. Lanes 1 and 2 contain the wild-type pBEL-1 template, lanes 3 and 4 use a template that carries a 6-bp deletion in TAg binding site II (at the *Bgl*I site; reference 18) that makes the origin defective for replication, lanes 5 and 6 use a template truncated at the *Stu*I site, and lanes 7 and 8 use a template truncated at the *Sal*I site. (B) Comparison of different GST-TAg fusion proteins and the DNA binding assays for each of them. (C) Comparison of transcription activation and DNA binding activities of GST–TAg1-249 and GST–TAg1-260. Transcription reactions were carried out as described in the legend to Fig. 1. The lanes in panel A with TAg received 0.5 μg of protein per reaction. The lanes in panel B contained 0.25 and 0.5 μg of each protein tested, except for GST–TAg1-249 for which only 0.5 μg was used. The lanes in panel C contained 0.25, 0.5, and 0.75 μg of TAg fusion protein. EES, early transcripts.

were also tested for their abilities to bind protein A–TEF-1. Beads carrying the TAg with the N153T mutation bound TEF-1, whereas those with the S189N mutant TAg did not (Fig. 3C). A replacement of residue 107 (glutamic acid to lysine), a mutation that disrupts the ability of TAg101-249 to bind the retinoblastoma gene product (12), had no effect on the ability of TAg101-249 to bind TEF-1 (Fig. 3C). In transcription assays this protein activated late start sites as well as the wild type (not shown). Also shown in Fig. 3C is an experiment demonstrating that all of the TAg101-249 fragments bind to TBP with similar efficiencies. The binding of TBP to TAg101-249 has been more thoroughly characterized by Johnston et al. (22a).

These results predicted that if TEF-1 was made as a GST fusion protein and coupled to beads, it should be able to bind TAg. A GST–TEF-1 fusion containing the C-terminal two-thirds of TEF-1 (amino acids 167 to 426) does not bind TAg (Fig. 3D). This was also observed when this region of TEF-1 was synthesized as a protein A fusion and tested for its ability to be retained on TAg beads (data not shown). However, the region of TEF-1 from amino acids 28 to 104, which comprises only the TEA domain of the protein, can bind TAg nearly as efficiently as the full-length TEF-1 molecule.

These experiments demonstrated that the DNA binding regions of the two proteins are important for the interaction. In control experiments (data not shown) to confirm that the interaction was not being mediated by DNA, the binding assays were also carried out successfully in the presence of ethidium bromide (26).

Effect of TEF-1 on late promoter activity in vitro. These data show that a fragment of TAg that is able to activate transcription is also able to interact with TEF-1. It was anticipated, on the basis of the in vivo transfection data showing that TEF-1 sites were involved in mediating transcription activation by TAg, that this interaction would have an effect on transcription in vitro.

The addition of GST–TEF-1 to transcription reaction mixtures resulted in inhibition of transcription activation by TAg (Fig. 4). The inhibition does not require full-length TEF-1 but only the NH_2-terminal 167-amino-acid fragment containing the TEA domain. The C-terminal two-thirds of the protein has little effect. The late transcription start site at position 264 (L1)

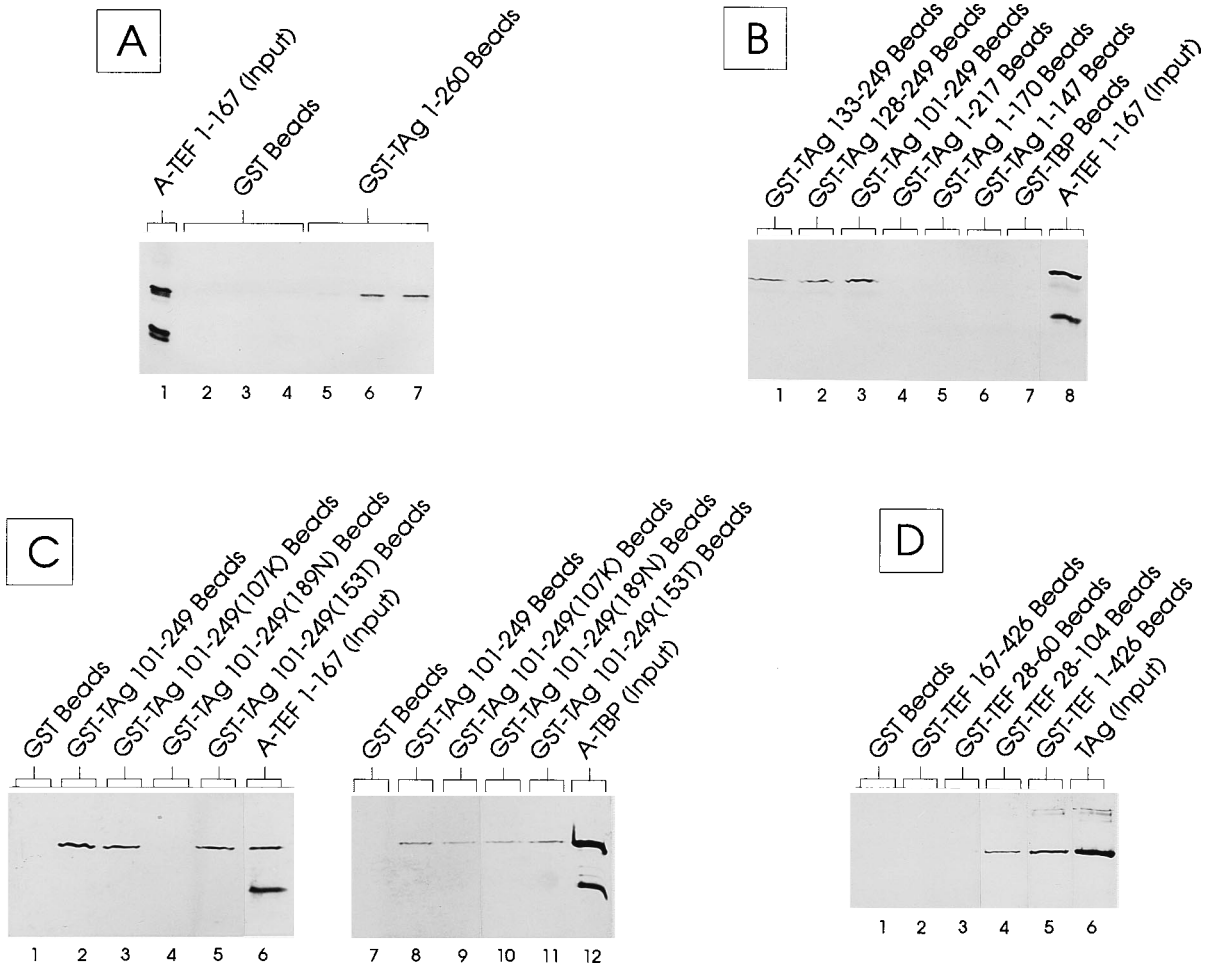

FIG. 3. Evidence for direct interaction between TAg and TEF-1. (A) A protein A fusion carrying the NH_2-terminal region of TEF-1, with the TEA domain, was purified and tested for its ability to bind to beads loaded with either GST protein (lanes 2 to 4) or GST–TAg1-260 (lanes 5 to 7). The material retained was analyzed by Western blot, as described in Materials and Methods. Lane 1 contains an aliquot of the protein A-tagged TEF-1. The lower doublet represents proteolytic fragments. They do not bind TAg. (B) An experiment similar to that shown in panel A tests a range of the TAg deletion mutants that were constructed for their abilities to retain protein A–TEF1-167. Also included is an experiment using beads loaded with GST-TBP. (C) Comparison of TEF-1 (lanes 1 to 6) and protein A-TBP (lanes 7 to 12) binding to TAg molecules carrying amino acid substitutions at the positions indicated. (D) Ability of full-length TAg expressed in baculovirus to bind to various beads loaded with GST fusion proteins of different regions of TEF-1. In all of the bead binding assays, care was taken to ensure that beads to be compared were loaded with similar amounts of GST fusion protein.

is more easily inhibited by TEF-1 than that at position 243 (L2). In Fig. 4, lanes 2 to 4, it is also seen that in the absence of TAg, increasing amounts of TEF-1 inhibit the basal level of late transcription in the extract. The ability of TAg to autoregulate early transcription is unaffected by the increase in TEF-1 concentration.

The amount of late transcription activation increases as more TAg is added, but at all concentrations there is evidence

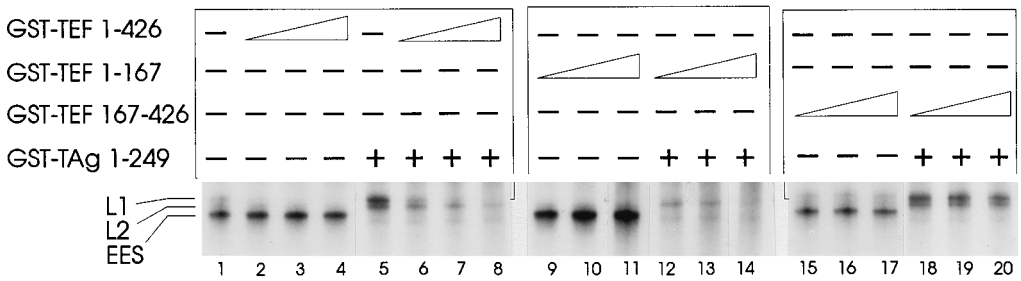

FIG. 4. TEF-1 regulates transcription of SV40 early and late genes. Various GST fusions of TEF-1 (0.1, 0.2, and 0.4 μg in each series of three increasing concentrations) and GST–TAg1-249 (0.35 μg per reaction mixture where indicated by a plus sign) were added as indicated above the figure. EES, early transcripts.

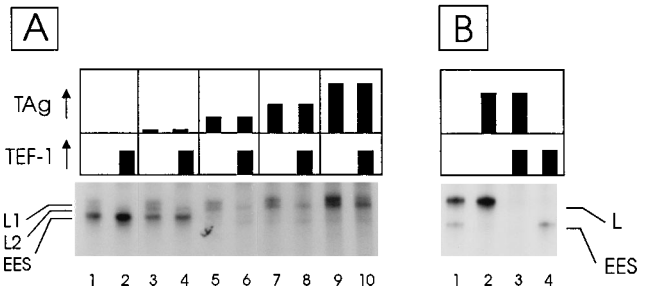

FIG. 5. Transcription activation in the presence of a constant amount of GST–TEF1-426 and increasing amounts of GST–TAg1-249 (represented schematically with bar graphs above the gels). (A) TAg1-249 was added to in vitro transcription assays in the following amounts: 0.03 μg (lanes 3 and 4), 0.13 μg (lanes 5 and 6), 0.25 μg (lanes 7 and 8), and 0.5 μg (lanes 9 and 10). GST–TEF1-426 (lanes 4, 6, 8, and 10) was included at 0.2 μg per reaction mixture. The template was pBEL-1 (0.18 μg per reaction mixture). (B) Late transcription from a template containing multimers of the GT-IIC site in place of the SV40 enhancer sequences up to the *Pvu*II site at position 270 is also repressed by TEF-1 in vitro. This plasmid (0.18 μg) was used as the template in this assay. GST–TAg1-249 (lanes 2 and 3) and GST–TEF1-426 (lanes 3 and 4) were included at 0.35 and 0.2 μg per reaction mixture, respectively. EES, early transcripts.

of inhibition by TEF-1 (Fig. 5A). This, and the experiments reflected in Fig. 4, suggests that the amount of late promoter activity is a function of the relative amounts of TAg and TEF-1. When the SV40 enhancer, which contains binding sites for a number of different cellular proteins, is removed and replaced with a multimerized TEF-1 binding site (consisting of six tandem copies of the Sph region, containing 12 TEF-1 binding sites), the same result is obtained (Fig. 5B). With this template there is a single late start site which, on the basis of its size, originates near the 21-bp-repeat region. Its base-level activity is higher than that of the wild-type template, but it is still activated by TAg and both activated and basal levels are inhibited by TEF-1 (Fig. 5B). Thus, in the absence of other enhancer-binding proteins, the amount of late transcription can be modulated by varying the ratio of TAg to TEF-1, confirming the results with the wild-type template.

TAg blocks the DNA binding activity of the TEA domain. In an effort to understand how the interaction between TAg and TEF-1 was activating late transcription, we examined the effect of TAg on the affinity of TEF-1 for its binding sites. The data shown in Fig. 6 demonstrate that when TAg is present in binding reaction mixtures containing TEF-1 and a TEF-1 binding site probe, there is a reduction in the amount of TEF-1–DNA complex formed. TAg carrying the mutation at position 189 has little effect on the amount of complex, whereas TAg with the mutation at position 153 competes for complexes like the wild-type protein. Figure 6, lanes 2 to 8, utilize a probe with a single GT-IIC motif, and lane 1 has a probe with a mutation that blocks binding of the GST–TEF-1 fusion protein. The two bands in complex A result from some proteolysis of the full-length bacterial protein, but both are absent in Fig. 6, lane 1, and are therefore specific complexes. Figure 6, lanes 9 to 15, utilize the wild-type Sph motifs I and II as probes, and the dimerized binding site permits two TEF-1 molecules to bind cooperatively (56), resulting in larger complexes (B complexes). Figure 6, lane 16, contains a probe with mutations in the Sph motifs and confirms that these complexes are specific.

Lanes 17 to 20 of Fig. 6 show mobility shift assays with native mammalian TEF-1 in HeLa cell extracts. The complexes

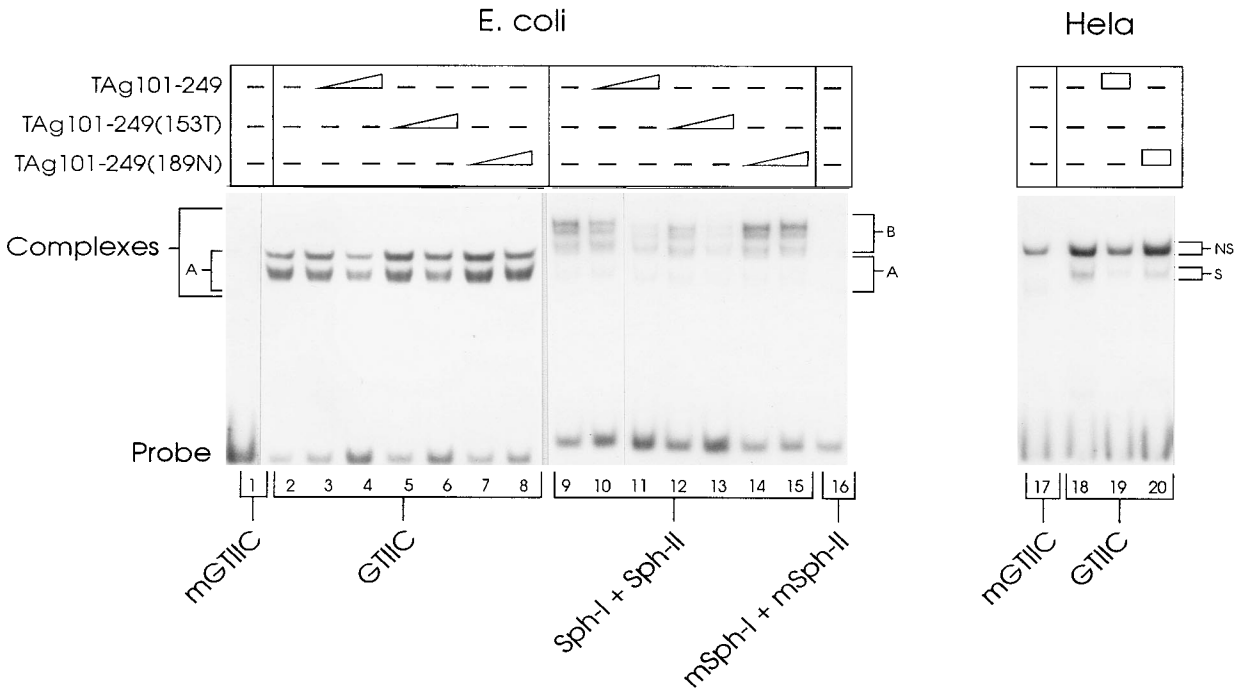

FIG. 6. The interaction between TAg and TEF-1 diminishes the ability of TEF-1 to bind DNA. The binding assays were carried out with bacterial GST–TEF-1 fusion protein (lanes 1 to 16; 0.1 μg of template per reaction mixture) or HeLa whole-cell extract (lanes 17 to 20) as a source of TEF-1. The probes used in each series are indicated below the panels. Various competitors, namely GST-TAg101 proteins with or without point mutations, were added at concentrations of 0.15 and 0.75 μg per reaction mixture. In the lanes with the mammalian extract as a source of TEF-1 protein, only the highest concentration of the TAg competitor (0.75 μg) was used where indicated. The mutations present in the binding sites in the oligonucleotide probes are those previously used by Xiao et al. (56). Similar amounts of each of the probes were used. In lanes 17 to 20, the GT-IIC and mutant GT-IIC (mGTIIC) probes were run just past the bottom of the gel. A, A complexes; B, B complexes; NS, nonspecific complexes; S, specific complexes.

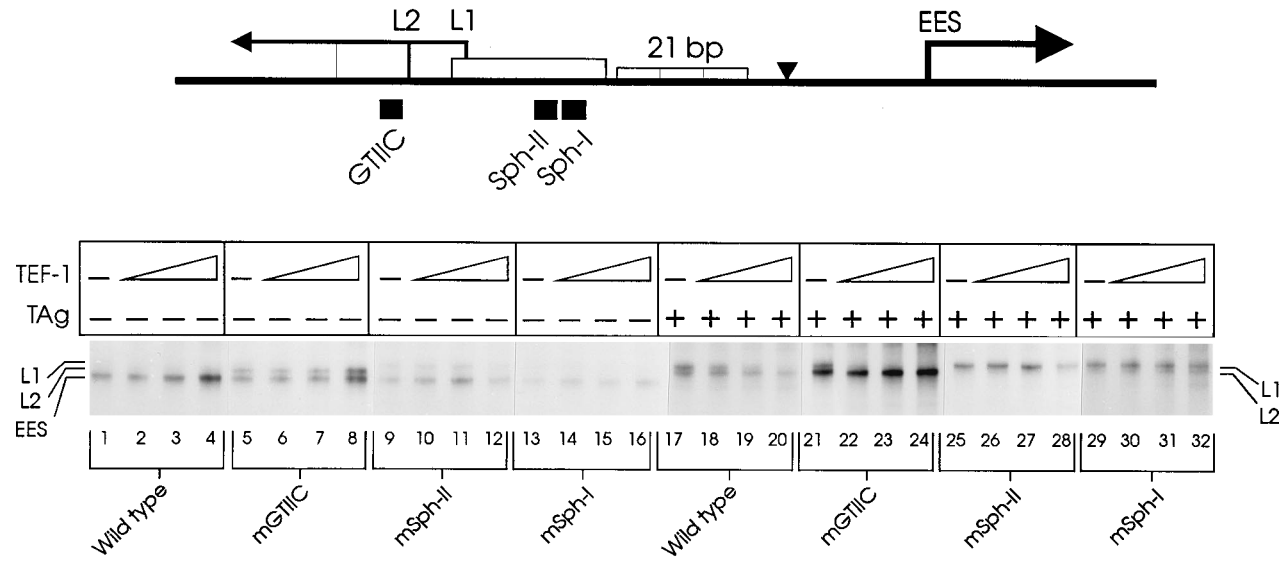

FIG. 7. Effect of mutations in TEF-1 binding sites in the transcription template on activation by TAg. The mutations tested are indicated below the panels. Lanes 17 to 32 contained 0.35 μg of GST–TAg1-249, and where indicated (+), GST–TEF1-426 was added at increasing concentrations of 0.1, 0.2, or 0.4 μg. The templates carrying the various mutations have been described previously (25). Each mutation consists of a triple point mutation in the binding site. EES, early transcripts.

formed are similar to those described by Xiao et al. (56) in that they are nonspecific complexes that migrate more slowly than the specific complex. The wild-type TAg101-249 competes effectively for the formation of this specific complex, while the S189N mutant does not. Thus, the ability of TAg to bind to TEF-1 and block the TEA domain from interacting with DNA is observed with both the natural mammalian protein in crude extracts and purified bacterial fusion proteins.

These data are therefore consistent with the bead binding assays (Fig. 3) showing that TAg interacts with the DNA binding domain of TEF-1 and that this interaction does not require DNA binding activity of TAg per se. We conclude that TAg can mask the DNA binding activity of the TEA domain.

TEF-1 acts as an inhibitor of late transcription. The repression of late transcription, both basal and TAg-activated, by TEF-1 suggested that mutations in TEF-1 binding sites in the promoter might result in increased late promoter activity. Transcription assays testing mutant promoters were run both with and without the addition of the GST fusion protein TAg1-249 (Fig. 7). With all templates, early transcription was autoregulated by TAg (Fig. 7, compare lanes 1 to 16 with lanes 17 to 32). With the wild-type promoter, the addition of TEF-1 (Fig. 7, lanes 17 to 20) resulted in inhibition of TAg-mediated late activation, similar to that seen in Fig. 4. Mutation of the GT-IIC motif resulted in elevated base-level activity from L2 that was not repressed by the addition of TEF-1 (Fig. 7, lanes 5 to 8). Nevertheless, with this mutant both L1 and L2 were activated by TAg, with the result that L2 was now highly active (Fig. 7, compare lanes 5 and 21). L1 was repressed by TEF-1, either in the presence or absence of TAg. Thus, it appears that the repression of late transcription from L2 requires that TEF-1 be able to bind to the GT-IIC motif. The repression is lost if the binding of TEF-1 to that motif is disrupted, and this disruption can occur either by mutating the DNA itself or by the addition of TAg to the extract to block the DNA binding activity of TEF-1.

The Sph motifs appeared to play similar roles, although the results were more complex. Mutation of either the Sph-I or Sph-II motif resulted in slightly reduced levels of early transcription (Fig. 7, lanes 9 to 12 and 13 to 16). It has previously been noted that these motifs can contribute to early transcription in vitro (54). Particularly with mutant Sph-II, increased base-level activity from L1 was noted (Fig. 7, lanes 9 to 12), although longer exposures (data not shown) of the mutant Sph-I template also revealed some basal activity from L1. With either mutant Sph template, the addition of TAg to the transcription reaction mixtures led to an increased level of activity from L1 but had little effect on L2 (Fig. 7, lanes 25 and 29). Unlike with the wild-type promoter, the addition of TEF-1 did not repress activity from L1, except to a small extent at the highest concentration (Fig. 7, lanes 26 to 28 and 30 to 32). Thus, by impairing the ability of TEF-1 to bind to the Sph motifs, there was an increased level of transcription activity from L1, similar to the effect of the GT-IIC mutation on L2 activity. It was noted that with both of the mutant Sph templates, L2 was less active than with the wild type. We wondered whether this suggested that TEF-1 bound to the Sph motifs was required to promote transcription from L2. However, when the GT-IIC motif in the mutant Sph-I template was mutated, L2 was activated by TAg in the same way it was activated on the template that had only the GT-IIC mutation (data not shown).

DISCUSSION

TAg–TEF-1 interaction. The experiments described here demonstrate that a direct interaction between SV40 TAg and TEF-1 interferes with the ability of TEF-1 to bind to sites within the SV40 enhancer, and this results in a relief of repression of viral late transcription. The region of TAg that binds to TEF-1 spans residues 133 to 249 and contains the DNA binding domain of TAg. The S189N mutation disrupted the interaction with TEF-1 and prevented TAg activation of transcription from a subset of naturally occurring late start sites.

The S189N mutation also results in a defective transformation function of TAg (12, 23) and blocks the ability of NH_2-terminal fragments of TAg spanning this region to stimulate DNA synthesis in quiescent cells (12). This correlation between TEF-1 binding and cell growth control properties of TAg suggests that TEF-1 might be involved in the regulation of genes important for growth control. The factor is thought to be

important for transcription of several muscle-specific genes (9, 52), but its functions likely extend beyond those in muscle. It is active during early mouse development when embryonal genes begin to be transcribed (36) and is present in a number of nonmuscle cell lines such as HeLa cells. The *Drosophila* homolog of TEF-1, the product of the *scalloped* gene, plays a role in differentiation of the nervous system (7). There is as yet no evidence that TEF-1, like Rb and p53, which also bind TAg, possesses a tumor suppressor function.

The DNA binding domain (TEA domain) of TEF-1 is conserved among several proteins from diverse species. It does not show homology with any other known types of DNA binding domains. It is not known if other mammalian proteins share this type of DNA binding domain, but considering that the TEA domain alone is sufficient to interact with TAg, it would be likely that other TEA domain proteins could be targeted by large T. The adenovirus E1a protein can target promoters by interacting with a variety of DNA binding domains, including the bZIP domain of ATF-2, the zinc finger domain of Sp1, and the basic helix-loop-helix domain of USF (31). It may be a common feature of viral activators that they interact with transcription factors via the DNA binding domain.

The DNA binding and TEF-1 binding activities of TAg are separable. This was noted with the T-153 mutation and with the TAg1-260 fragment. Both bound DNA poorly but interacted with TEF-1. The fact that the 1 to 260 segment bound DNA less efficiently than the 1 to 249 or 133 to 249 segment presumably results from conformation differences that alter the accessibility of the DNA binding domain. Similarly, the CR3 domain of E1a, which is responsible for interactions with DNA binding domains of transcription factors, is less accessible for these interactions when in the context of the full-length protein (31). The ability of TAg to bind TEF-1 was not inhibited by residues 249 to 260. Thus, overlapping functions within this region of TAg may each have distinct structural requirements.

Transcription activation by TAg. In transfection assays, an NH_2-terminal fragment extending to approximately amino acid 140 retains some ability to activate the SV40 late, Rous sarcoma virus long-terminal-repeat, and adenovirus E2 promoters (49, 58), albeit at generally less than 50% the level of efficiency of the full-length protein. A number of mutations throughout the DNA binding domain have been shown to more seriously disrupt the transactivation function of the protein (58). Many different types of promoters, some of them simple artificial fusions between a TATA box and the binding site for one transcription factor, can be activated by TAg (17, 42). It is not known if TAg targets all of those different promoters in the same way.

TEF-1 was proposed to be important in mediating transcription activation by TAg on the basis of in vivo transfection experiments showing that mutation of the binding sites resulted in loss of activation by TAg and that multimers of a TEF-1 binding site conferred TAg inducibility to a heterologous promoter. Gruda et al. (20) proposed that TAg becomes targeted to the promoter through its interactions with TEF-1 and thereby assists, through interactions with TEF-1 bound to the promoter and with other factors, in the assembly of a transcription complex. Our observations that TEF-1 is a repressor of late transcription and that TAg interferes with its binding to DNA do not support this model. Our mutational analysis of the NH_2-terminal region of TAg shows that the DNA binding domain is critical for transcription activation of selected late start sites, but that DNA binding per se is not. Rather, the domain serves to sequester TEF-1 and relieve late gene repression. Nevertheless, TEF-1 binding by TAg is not sufficient to achieve SV40 late promoter activation. The NH_2-terminal deletion mutants that removed TAg sequences from amino acids 95 to 128 exhibited decreased transcription activation but retained TEF-1 binding and autoregulation functions. Thus, although activation by TAg is facilitated by the removal of TEF-1 from the template, it is likely that interactions between as-yet-unidentified factors and TAg sequences preceding the DNA binding domain are important. Such a conclusion is consistent with the fact that most of the promoters that TAg is reported to activate do not have binding sites for TEF-1. Consistent with the conclusion that titration of TEF-1 is important but not sufficient for late promoter activation, the addition of oligonucleotides with TEF-1 binding sites to transcription reaction mixtures lacking TAg does not lead to late promoter activation (data not shown).

TAg might be involved in several additional interactions that recruit TAg to the promoter for transcription activation. The TBP is reported to bind a TAg5-172 fragment (20), and it also binds our TAg101-249 region. Johnston et al. (22a) found that TBP can bind to an NH_2 138-amino-acid fragment and a fragment spanning residues 133 to 249. The residues responsible for TBP binding within these fragments have not been localized. The region of TAg from amino acids 80 to 120 contains a net negative charge typical of acidic activation domains such as those found in GCN4 and VP16 (10). TBP has been shown to interact with acidic activators (50), and certainly the region from residues 95 to 128, which we have shown to be important for activation, warrants more in-depth investigation as a possible interaction site for TBP. Of the wide range of simple promoters that utilize different transcription factors, TBP is common to all of them. Most promoters responsive to TAg are activated to a similar extent, in the range from severalfold to 10-fold, arguing that there may be a common mechanism underlying them.

TEF-1 is thought to require an additional intermediate factor (TIF) that is present in limiting amounts in HeLa cells and several other cell lines tested. This was inferred from experiments showing that overexpression of TEF-1 or GAL–TEF-1 hybrid proteins leads to squelching, presumably by titrating out the TIF (56). This raises the possibility that TAg could activate such promoters by sequestering soluble TEF-1 and inhibiting squelching. The SV40 late promoter activation in this study cannot be explained by inhibition of squelching, since the TEF1-167 fragment alone is sufficient to repress late transcription (Fig. 4) and this fragment is incapable of squelching or activating transcription (22, 56). Nevertheless, inhibition of squelching could be a mechanism whereby a simple modular promoter having multimerized TEF-1 sites in front of a TATA box (e.g., reference 19) is activated by TAg.

The early-to-late shift in viral transcription. The data presented here are relevant to the question of how transcription shifts from the early to late genes during a lytic infection. There are likely two mechanisms to repress transcription from the early promoter. The first is that the binding of TAg to the viral origin sterically interferes with the assembly of the initiation complex. This was observed in vitro with purified TAg (21, 43) or simply with the DNA binding domain of the protein (this study). The second mechanism involves the DNA replication process itself (28, 30). After the onset of replication, another set of early initiation sites, the late-early start sites, are used (16). They are regulated synchronously with late transcription. In contrast, when autoregulation is achieved in vitro with purified TAg, we do not observe the late-early start sites being activated. Mutations in the early promoter TATA box do result in increased levels of late-early activity in vivo and in vitro (reference 52 and our unpublished observations). The implication is that the initiation of replication may block the

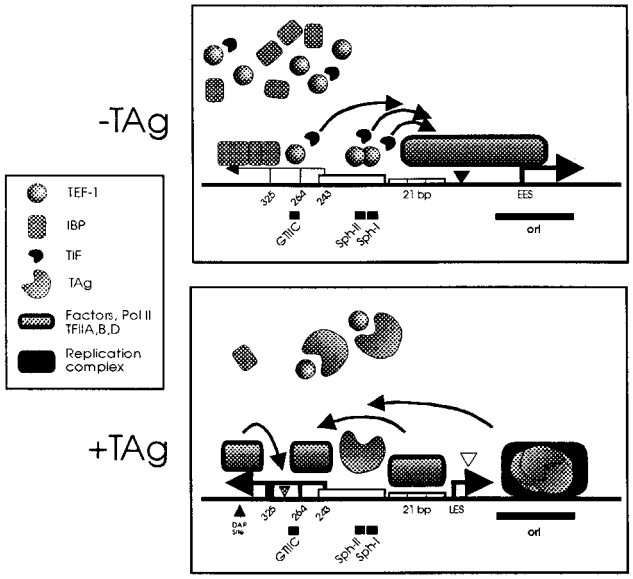

FIG. 8. Diagrammatic representation of some events during the early-to-late switch in SV40 transcription. In the absence of TAg, early transcription is activated by the enhancer region. Although only TEF-1 is shown, many other proteins are known to interact with the enhancer. The transcription factors include Sp1, which binds to the 21-bp region. IBP and TEF-1 repress late transcription from the initiation sites indicated. In the presence of TAg, IBP and TEF-1 are not bound to the template. The diaminopimelic acid (DAP) site is important for initiation from position 325. Arrows indicate directions of transcription activation. EES, early transcripts. LES, late-early transcripts; inverted filled triangle, TBP strongly bound; inverted shaded triangle, TBP weakly bound; inverted open triangle, TBP not bound.

activity of the TATA box, thereby shifting transcription away from the early promoter and to the late-early and late promoters.

Activation of late transcription is maximal in cells that are replicating viral DNA. The replication-dependent contribution has been postulated to result when the increase in template copy number titrates out repressors of late transcription (1, 55). One putative repressor of the late promoter, termed IBP, has multiple binding sites in the vicinity of the −325 start site (55). IBP is thought to be a collection of several members of the steroid hormone receptor superfamily. Our results with TEF-1 indicate that it can also act as a repressor, primarily of initiation from sites at positions 264 and 243. The repression can be achieved with just the NH_2-terminal 167 amino acids of the protein containing the DNA binding (TEA) domain. This fragment does not on its own possess a transcription activation function (22). TEF-1 could, like IBP, be titrated out by increasing template copy number or be sequestered directly through interaction with TAg.

Figure 8 is a diagram which summarizes our interpretation of the data reported here and of data presented in earlier literature on viral late transcription. Prior to the appearance of TAg in the cell, the sites in the enhancer to which TEF-1 binds play important roles in the activation of early transcription (57). The appearance of TAg and initiation of DNA replication suppresses transcription from the early promoter. TAg will be able to interact with TEF-1 and interfere with its binding to the DNA and thereby weaken the ability of the enhancer to promote early transcription. The TAg–TEF-1 interaction could also occur in the cytoplasm and account for the observation that a TAg defective in nuclear localization can nevertheless activate transcription through the same TEF-1 pathway as that of wild-type TAg (25). As the template copy number increases, the titration of IBP most likely results in activation of transcription from the major start site at position 325 (55). The loss of TEF-1 binding could result in enhanced transcription from positions 264 and 243. The promoter elements required for late transcription include the GC-rich motifs in the 21-bp-repeat region that bind the factor Sp1 (13). The enhanced levels of Sp1 in response to TAg in the cell (44) would augment this late activation.

Several groups have published data suggesting that on replicating templates the SV40 enhancer is not required for late transcription (13, 47). On wild-type templates, the virus has a strong enhancer (including TEF-1 sites) to attract RNA polymerase for early transcription and permit TAg synthesis. As replication and autoregulation lead to repression of the major early start sites, this transcription machinery may be available to be directed toward late transcription. On templates with mutant TEF-1 binding sites there could therefore be fewer transcription complexes to start with and therefore fewer complexes available for late transcription after TAg appears. This could underly the observation that when the enhancer is mutated, both early and late transcription decline similarly (25, 35). In addition, mutations in TEF-1 sites will result in there being little transcription activation by TAg, since the TEF-1 protein will already be prevented from interacting with the promoter by virtue of the fact that mutation and additional sequestering by TAg will have little effect. In such a scenario TEF-1 need not be present on templates undergoing late transcription, a suggestion supported by the data showing that the enhancer is not required for late transcription on replicating templates.

There remain many questions, such as the identification of which additional factors are involved in transcription activation by TAg. That other factors exist is evident from the observations that TAg residues from amino acids 95 to 128 are required and that the level of late transcription seen in the presence of TAg exceeds that seen in the absence of TAg when TEF-1 binding sites in the template are mutated (Fig. 7). That is, TEF-1 removal from the template is important but not sufficient. Other factors in addition to TEF-1 and IBP may have to be sequestered by TAg. Finally, the observation that a TAg mutant with reduced transformation potential is also defective in TEF-1 binding raises the interesting possibility that TEF-1 plays a role in regulating genes important for growth control.

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