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Interaction between two transcriptional control sequences required for tumor-antigen-mediated simian virus 40 late gene expression

(*trans*-activation/gene regulation/simian virus 40 enhancer)

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ABSTRACT **Transcriptional control signals required for tumor (T)-antigen *trans*-activation of the simian virus 40 (SV40) late promoter include T-antigen binding sites I and II and the SV40 72-base-pair (bp) repeats. We have used *in vivo* competition studies to examine how these signals function in relationship to one another. *In vivo* competition with recombinant plasmids containing the entire SV40 late regulatory region and promoter sequences [map position (mp) 5171–272] results in quantitative removal of limiting *trans*-acting factor(s) required for late gene expression in COS-1 cells. Deletion of either the T-antigen binding sites (mp 5171–5243) or the 72-bp tandem repeat (mp 128–272) from the competitor plasmid results in markedly less efficient binding of the *trans*-acting factor, as judged by the loss of competition. Cotransfection of two separate plasmids, one containing the T-antigen binding sites I and II and the other containing the 72-bp repeats, fails to compete for the *trans*-acting factors. Insertion of increasing lengths of DNA sequences between the T-antigen binding sites and the enhancer sequences also dramatically reduces the efficiency of competition. These results suggest that efficient binding of *trans*-acting factors requires the presence, in *cis*, of at least two SV40 regulatory domains. Our studies further suggest that the distance separating these two transcriptional signals is important.**

The expression of the simian virus 40 (SV40) late genes is restricted to permissive monkey kidney cells, the normal lytic host for the virus. Efficient transcription from the SV40 late promoter is normally observed only after expression of the early gene product, tumor (T) antigen, and subsequent DNA replication (1–5). In nonpermissive cells, virus infection or DNA transfection leads to a significant level of early gene expression; transcription from the late promoter is weak. These studies indicate a tight regulation of SV40 late transcriptional control signals in eukaryotic cells. This observation is of particular interest because the SV40 late promoter lacks certain transcriptional control sequences located upstream of most other eukaryotic genes (6, 7).

Recently, we (8, 9) and others (10, 11) have found that SV40 late gene expression is activated by the SV40 early gene product, T antigen, in the absence of DNA replication. RNA analysis demonstrates that activation occurs at the transcriptional level (9, 11). Studies in our laboratory using deletion and point mutants have defined two important domains for the SV40 T-antigen-induced late gene expression. One of these includes T-antigen binding sites I and II; the other is located in the SV40 72-base-pair (bp) repeat. Based on these experiments, it was not possible to distinguish whether the transcriptional control sequences represented promoter elements and/or binding sites for *trans*-acting factors. To address this point and to determine how the two upstream control elements interact to effect T-antigen-dependent *trans*-activation, we have used template competition analysis. In the presence of increasing levels of competitor DNA fragments, which are capable of binding limiting *trans*-acting factors, a decrease in expression from a fixed amount of template was observed. Thus, this methodology allows the definition of DNA domains that bind regulatory proteins present at limiting levels in the eukaryotic cell.

Our present studies suggest that efficient binding of the limiting *trans*-acting factor(s) present in COS-1 cells depends on the presence, in *cis*, of at least two SV40 regulatory domains. Moreover, this binding apparently requires a physical interaction between protein(s) and the DNA sequences, because the length of DNA present between the two transcriptional control domains is critical for efficient competition.

MATERIALS AND METHODS

***In Vivo* Competition for *Trans*-Acting Factor(s) Present in COS-1 Cells.** COS-1 cells (12) (10-cm plate) were transfected with the SV40 template and competitor DNAs by the calcium phosphate precipitation method (13). For the competition assays, standard transfection mixtures contained 0.1 μg of supercoiled SV40 template DNA (which yields a 60% maximal response in a 10-cm plate) plus a competitor DNA molecule containing regions of the SV40 T-antigen binding sites and late promoter region in the pBR322 plasmid derivative. All competition plasmids lacked the intact coding region for the major late gene product, VP-1. Competitor DNA was added in parallel assays at a ratio of 1:1 (0.1 μg), 5:1 (0.5 μg), or 10:1 (1.0 μg) relative to template DNA, as indicated in the figure legends. The total DNA present in the transfection mixture was adjusted to 30 μg with salmon sperm DNA. Transfected cultures were maintained in Dulbecco's minimal essential medium with fetal calf serum (10%) and the DNA replication inhibitor cytosine arabinoside (25 μg/ml). Using the sensitive *Dpn* I assay, we do not detect any SV40 replication. At 40 hr after transfection, whole-cell protein extracts were prepared and analyzed by immunoblot analysis using anti-SV40 VP-1 antisera (8). After autoradiography, bands were excised from the blot, and ^{125}I cpm were determined in a γ counter.

RESULTS

T-Antigen-Mediated Late Gene Expression Is Not Influenced by Insertion of TATA Sequence Upstream of the Major Late Cap Site. A prominent feature of the SV40 late gene is its lack of a well-defined Goldberg–Hogness (G-H) or TATA box upstream of the major late start site (mp 325) (14, 15). We have analyzed the effect of base substitutions located 21–31 nucleotides proximal to the SV40 major late cap site. Two of

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Abbreviations: SV40, simian virus 40; bp, base pair(s); T antigen, tumor antigen; mp, map position.

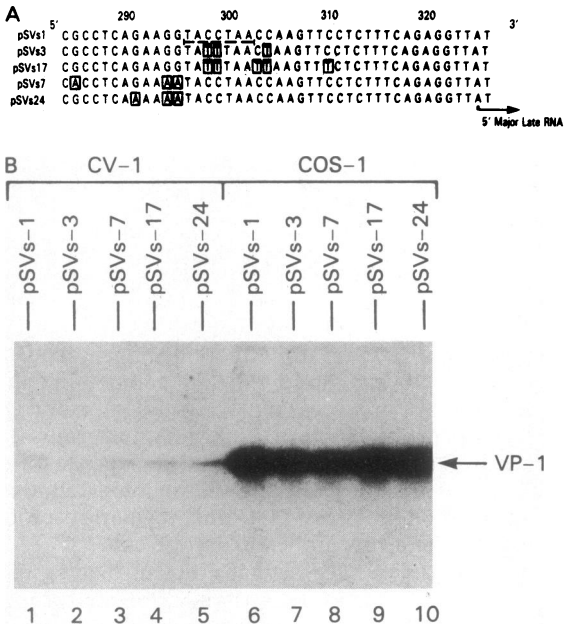

FIG. 1. SV40 late gene expression in CV-1 and COS-1 cells after transfection with wild-type and base-substitution mutants. Parallel cultures of CV-1 and COS-1 cells were transfected with 1 μg of wild-type (pSVs-1) and base-substitution mutants (pSVs-3, -7, -17, and -24) by the calcium phosphate precipitation method. Prior to transfection, SV40 DNA was released from plasmid by restriction endonuclease cleavage with *Bam*HI. Cells were maintained in Dulbecco's minimal essential medium with fetal calf serum (10%) and cytosine arabinoside (25 μg/ml). (*A*) pSVs-3, -7, -17, and -24 are SV40 mutants obtained by sodium bisulfite mutagenesis at the single SV40 *Kpn* I site as described (16). Nucleotide changes are indicated by the presence of a box. (*B*) At 40 hr after transfection, whole-cell protein extracts were prepared and analyzed by immunoblot analysis using anti-SV40 VP-1 antisera (8). The position of migration of VP-1 is indicated by the arrow.

the mutants, pSVs-3 and pSVs-17, have base substitutions that create a TATTTA sequence starting 29 nucleotides upstream of the major late cap site (Fig. 1*A*). This sequence is identical to the functional SV40 early G-H box (17, 18) and increases specific transcription from the major late initiation site ≈10-fold *in vitro* (16). Two other mutants, pSVs-7 and pSVs-24, contain base-substitution mutations immediately prior to the putative SV40 late control region. *In vitro*, these mutations decreased major late initiation by a factor of 2–3.

The efficiency of late gene expression, as determined by immunoblot analysis of the major late protein VP-1, with wild-type SV40 (pSVs-1) and the four mutant templates (pSVs-3, -7, -17, and -24) was compared after transfection of either CV-1 or COS-1 cells. We have previously demonstrated by S1 nuclease and RNA blot analysis that *trans*-activation of the transcription unit occurs at the RNA level and that the immunoblot assay accurately reflects the transcriptional efficiency (9). DNA replication was blocked in these experiments by the addition of cytosine arabinoside (25 μg/ml). Consistent with previous results, the level of late gene expression from a wild-type SV40 DNA template in CV-1 cells, in the absence of template amplification, is extremely low (Fig. 1*B*, lane 1). The insertion of a G-H sequence does not appear to overcome the block in late gene expression (Fig. 1*B*, lanes 2 and 4). Transfection of COS-1 cells resulted in efficient expression of the SV40 late transcription unit for both wild-type (pSVs-1; lane 6) and the substitution mutants (lanes 7–10). SV40 late gene expression from wild-type and mutant templates was approximately equal, suggesting that the insertion of a TATA sequence upstream of the SV40 major late cap site neither overcomes the block to late gene expression in CV-1 cells nor allows more efficient *trans*-activation of the late transcription unit by SV40 large T antigen.

***In Vivo* Competition for *Trans*-Acting Factors Is Influenced by the Presence of the SV40 72-bp Repeat.** In previous studies, we have demonstrated that base-substitution mutants in the SV40 72-bp repeat decrease T-antigen-mediated SV40 late gene expression (9). The role of the SV40 72-bp repeats in binding of positive *trans*-acting factors was examined by *in vivo* competition analysis. This technique examines the ability of molecularly cloned DNA fragments to reduce late gene expression from a cotransfected SV40 template, presumably because of competition for limiting *trans*-acting factors that bind to the competitor DNA fragment. Two plasmids that contain SV40 late regulatory sequences map position (mp) 5171–272 (Fig. 2, competition fragment A) or

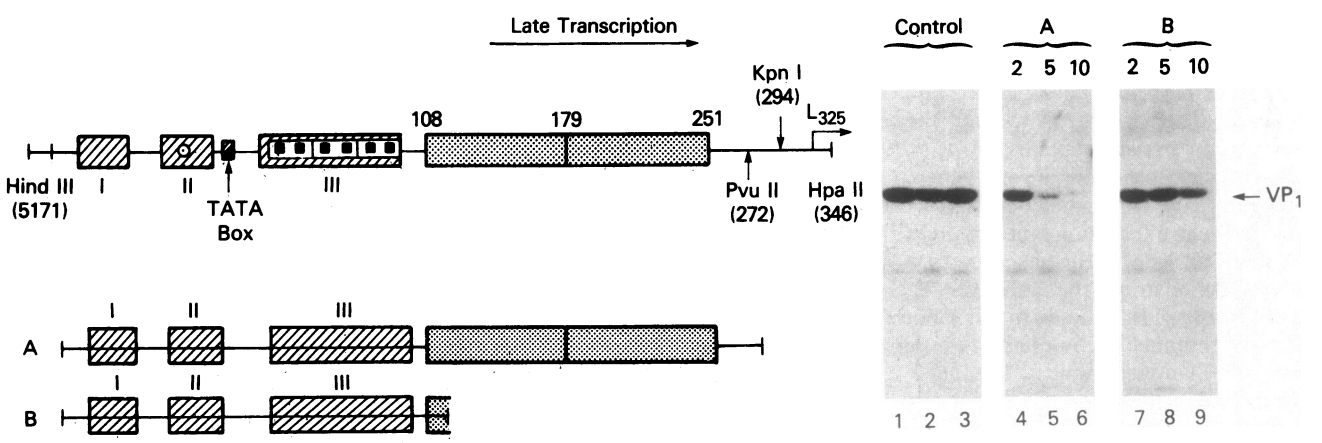

FIG. 2. Effect of the SV40 72-bp repeats on *in vivo* competition for *trans*-acting factor in COS-1 cells. COS-1 cells were transfected with template SV40 DNA and competition plasmids by the calcium phosphate precipitation method. Transfection mixtures contained 0.1 μg of supercoiled SV40 template DNA plus a competitor DNA molecule containing SV40 sequences mp 5171–272 (enhancer +) (competition fragment A) or mp 5171–128 (enhancer −) (competition fragment B) cloned into pBR322. The competitor DNA plasmid was added at a ratio of 1:1 (0.1 μg), 5:1 (0.5 μg), or 10:1 (1.0 μg) relative to the template DNA. The total DNA in each transfection mixture was adjusted to 30 μg with salmon sperm DNA. Transfected cultures were maintained in Dulbecco's minimal essential medium with fetal calf serum (10%) and cytosine arabinoside (25 μg/ml). At 40 hr after transfection, whole-cell protein extracts were prepared and analyzed by immunoblot analysis using anti-SV40 VP-1 antisera (8). Lanes: 1–3, 0.1 μg of SV40 DNA template; 4–6, 0.1 μg of SV40 template and 0.1 μg, 0.5 μg, or 1.0 μg of cloned competition fragment A, respectively; 7–9, 0.1 μg of SV40 DNA template and 0.1 μg, 0.5 μg, or 1.0 μg of cloned competition fragment B, respectively. The position of migration of VP-1 is indicated by the arrow.

mp 5171–128 (Fig. 2, competition fragment B) were compared. Transfection mixtures (10-cm plate) contained 0.1 μg of supercoiled SV40 template DNA plus a competitor DNA plasmid at a ratio of 1:1, 5:1, or 10:1 relative to template DNA (see *Materials and Methods*). Competition experiments in which the competitor DNA fragment contained three T-antigen binding sites (19–21) and the entire late promoter region (Fig. 2, competition fragment A), including the 72-bp tandem repeat, resulted in quantitative competition. The level of late gene expression was proportional to the ratio of template to competitor DNA (Fig. 2, lanes 4–6). For example, at a ratio of 10:1 (competitor/template DNA), SV40 late gene expression was reduced to ≈10% of the level observed with no competitor DNA (Fig. 2, compare lanes 1–3 and lane 6). In contrast, when the SV40 72-bp enhancer element was deleted from the competitor fragment (Fig. 2, competition fragment B), a significant decrease (by a factor of 5) in the competition efficiency was observed (Fig. 2, lanes 7–9). These results indicate that the 72-bp enhancer element is important for efficient binding of the *trans*-acting factor(s) present in COS-1 cells that are involved in late gene expression.

In the absence of the 72-bp repeats, competition for *trans*-acting factors was observed at the higher competitor/template ratios (10:1) (Fig. 2, lane 9). This result suggests that other regulatory sequences (e.g., 21-bp repeats) may either functionally substitute for the enhancer element in its absence or may bind transcriptional factors present at higher concentrations (see *Discussion*).

T-Antigen Binding Sites or the 72-bp Tandem Repeats Alone Are Not Efficient Competitor Molecules. In experiments similar to those described above, we have previously demonstrated that deletion of the T-antigen binding sites I and II (mp 5171–5243) from the control region competitor fragment also dramatically decreases its ability to function as a competitor (9). It was surprising, therefore, that a DNA fragment containing all three T-antigen binding sites was an inefficient competitor molecule (Fig. 2, competition fragment B). Similarly, competition experiments performed with fragments containing only the SV40 72-bp repeats failed to reduce late SV40 gene expression (9). These results suggested that efficient competition of a limiting *trans*-acting factor might involve cooperativity between the two DNA sequences. It was not clear whether the cooperativity required a *cis* or a *trans* interaction. We performed a series of *in vivo* competition experiments with cloned DNA fragments containing the individual SV40 late transcriptional regulatory domains on separate plasmids. The level of SV40 late gene expression observed after transfection of 0.1 μg of SV40 template DNA is represented in Fig. 3*B*, lane 1. As a positive control, competition experiments in which the cloned DNA fragment (cotransfected at a ratio of 1:1, 5:1, and 10:1) contained the T-antigen binding sites and the 72-bp repeats resulted in quantitative competition of SV40 late gene expression (Fig. 3*B*, lanes 2–4). In contrast, competition with a plasmid containing SV40 regulatory sequences from mp 41–294 (Fig. 3, competition fragment 2) resulted in almost no detectable decrease in late gene expression, even at competition ratios of 10:1 (Fig. 3*B*, lanes 5–7). This result confirms that in the absence of T-antigen binding sites I and II the SV40 72-bp repeats (or 21-bp repeats) are not capable of efficiently binding the limiting transcription factors required for T-antigen-mediated late gene expression. Negative results were also obtained when various concentrations of a cloned DNA fragment containing T-antigen binding sites I and II were analyzed as a competitor (Fig. 3, competition fragment 3; lanes 8–10). SV40 template DNA (0.1 μg) was also cotransfected with both competition plasmids at a competitor/template ratio of 1:1, 5:1, and 10:1 for each clone (Fig. 3, competition fragments 2 and 3). The fact that little or no

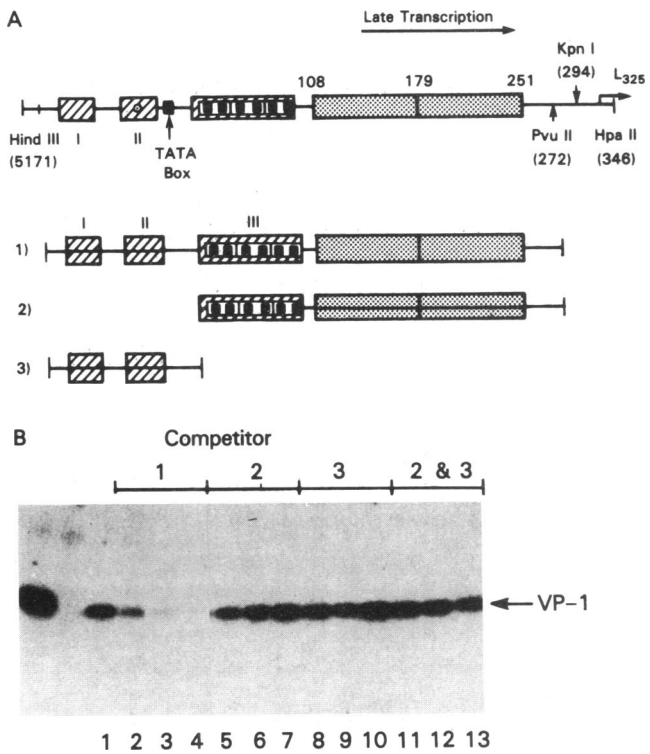

FIG. 3. *In vivo* competition for COS-1 *trans*-acting factor using individual transcriptional control sequences. COS-1 cells were transfected with template SV40 DNA and competition plasmids as described in Fig. 2. Transfection mixtures contained 0.1 μg of supercoiled SV40 template DNA plus a competitor DNA molecule containing cloned SV40 sequences mp 5171–272 (competition fragment 1), mp 41–294 (competition fragment 2), and mp 5171–37 (competition fragment 3). The competitor plasmid was added at a ratio of 1:1 (0.1 μg), 5:1 (0.5 μg), or 10:1 (1.0 μg) relative to the template DNA. Transfected cultures were maintained and assayed as described in Fig. 2. Lanes: 1, 0.1 μg of SV40 template DNA; 2–4, 0.1 μg of SV40 template DNA and 0.1, 0.5, or 1.0 μg of competition fragment 1, respectively; 5–7, 0.1 μg of SV40 template DNA and 0.1 μg, 0.5 μg, or 1.0 μg of competition fragment 2; 8–10, 0.1 μg of SV40 template DNA and 0.1 μg, 0.5 μg, or 1.0 μg of competition fragment 3, respectively; 11–13, 0.1 μg of template SV40 DNA and 0.1 μg, 0.5 μg, or 1.0 μg each of competition fragments 2 and 3, respectively. The position of migration of VP-1 is indicated by the arrow.

competition for VP-1 expression is observed when the two domains are supplied on separate molecules (Fig. 3, lanes 11–13) suggests that any cooperativity between the two transcriptional domains requires a *cis* interaction.

Binding of *Trans*-Acting Factors Is Dependent on Spacing Between T-Antigen Binding Sites I and II and the 72-bp Enhancer Element. The results presented thus far demonstrate that efficient competition for *trans*-acting factors occurs when the regions representing the SV40 T-antigen binding sites and the 72-bp repeats are linked on the same competitor molecule (Fig. 3, compare lanes 2–4 and 11–13). These findings suggest that efficient binding of the *trans*-acting factors requires simultaneous interaction between one or more protein(s) and the two transcriptional domains.

To test this hypothesis, we have analyzed the effect of increasing the distance between the two transcriptional domains on competition efficiency. A series of mutants were recently generated by Innis and Scott (22) by the insertion of DNA sequences at the SV40 *Nco* I site (mp 37) (Fig. 4). Using standard recombinant DNA techniques, we transferred a *Bgl* I/*Sph* I DNA fragment (mp 0–128) from each mutant to a plasmid containing SV40 sequences from the *Hin*dIII site (mp 5171) to the *Pvu* II site (mp 272), thus reconstituting the spacer mutants in plasmids containing the entire control

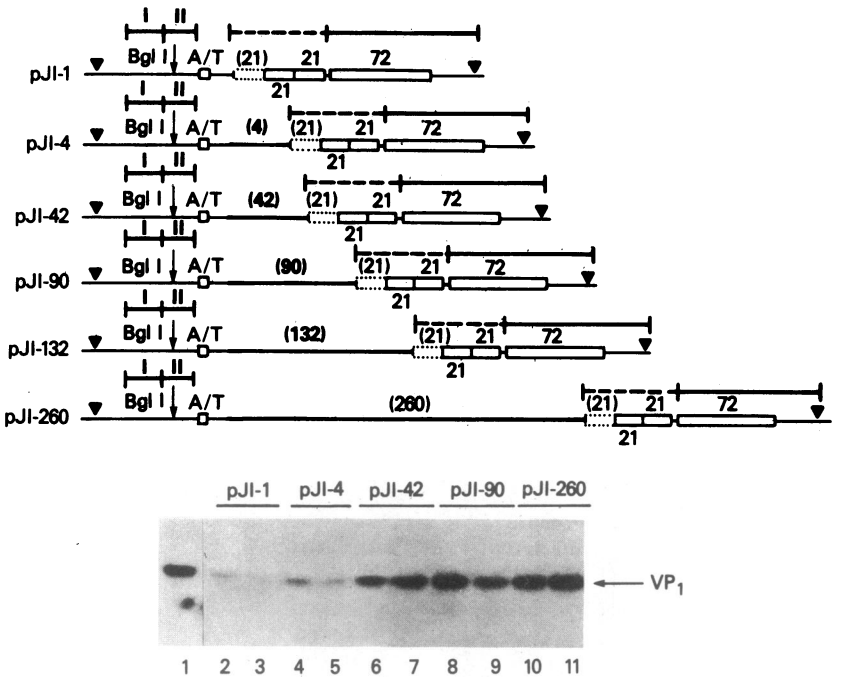

FIG. 4. *In vivo* competition for COS-1 *trans*-acting factor with plasmids containing insertions between T-antigen binding sites I and II and the 72-bp repeats. COS-1 cells were transfected with template SV40 DNA (0.1 μg) and competition plasmids (1.0 μg). Transfected cultures were maintained and late gene expression was assayed as described in Fig. 2. Lanes: 1, 0.1 μg of SV40 DNA template; 2 and 3, 0.1 μg of SV40 template DNA and 1.0 μg of pJI-1 competition plasmid; 4 and 5, 0.1 μg of SV40 template DNA and 1.0 μg of pJI-4 competition plasmid; 6 and 7, 0.1 μg of SV40 template DNA and 1.0 μg of pJI-42 competition plasmid; 8 and 9, 0.1 μg of SV40 template DNA and 1.0 μg of pJI-90 competition plasmid; 10 and 11, 0.1 μg of SV40 template DNA and 1.0 μg of competition plasmid pJI-260.

region (Fig. 4). Competition with plasmid pJI-1, which contained no insertion of DNA sequences between the two transcriptional domains, resulted in a quantitative decrease in SV40 late gene expression (Fig. 4, lanes 2 and 3). Insertion of 4 bp of DNA at the *Nco* I site had a minimal effect on the competition efficiency, reducing it ≈20% (Fig. 4, lanes 4 and 5). In contrast, pJI-42 (lanes 6 and 7), pJI-90 (lanes 8 and 9), or pJI-260 (lanes 10 and 11) were ineffective competitors, producing little or no decrease in the level of late gene expression. These results suggest that efficient binding of the *trans*-acting transcription factors requires a precise physical relationship between both sets of DNA sequences and the putative transcriptional factors with which they interact.

T Antigen Is Not the Limiting *Trans*-Acting Transcriptional Factor. These experiments do not reveal the identity of the limiting *trans*-acting factor in COS-1 cells. An obvious candidate was SV40 T antigen. To investigate this possibility, we attempted to overcome the competition by the cotransfection of a plasmid, RSV-T, which codes for SV40 large T antigen. RSV-T induces SV40 T-antigen expression under the control of the RSV-LTR, a strong transcriptional enhancer/promoter, which is not susceptible to T-antigen autoregulation. Transfection of this plasmid (5 μg) into CV-1 cells results in levels of T-antigen expression comparable to those observed in COS-1 cells (unpublished data). Thus, if T antigen were the limiting transcriptional factor, cotransfection with RSV-T should reverse or offset the competition curve. A typical competition curve, using a plasmid containing the entire regulatory domain (Fig. 3*A*, competition fragment 1), is shown in Fig. 5*A*. A parallel competition assay, in which 5 μg of the RSV-T plasmid was cotransfected with the template and competition fragment, is presented in Fig. 5*B*. Addition of RSV-T antigen failed to decrease the efficiency of the competition. These results suggest that T antigen alone is not likely to be the limiting *trans*-acting transcriptional factor required for activation of SV40 late gene expression in COS-1 cells. We suspect, therefore, that a limiting cellular transcriptional factor binds cooperatively to the SV40 72-bp repeat region, perhaps requiring the presence of SV40 large T antigen.

DISCUSSION

This study extends our findings on the regulation of SV40 late transcription. First, we demonstrate that base substitution mutants creating a TATA box ≈25 nucleotides upstream of the major late RNA initiation site do not modify the timing or level of SV40 late gene expression in CV-1 cells or COS-1

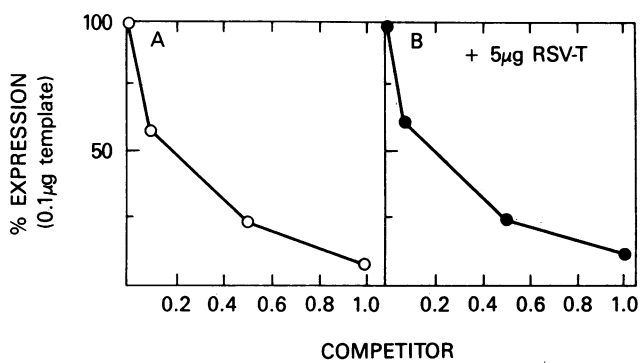

FIG. 5. Effect of exogenous T antigen on *in vivo* competition for COS-1 *trans*-acting factor. COS-1 cells were transfected with template SV40 DNA, competition plasmid, and RSV-T plasmid as described. Each transfection mixture contained 0.1 μg of supercoiled SV40 template DNA and a competitor DNA molecule containing SV40 DNA sequences mp 5171–272 cloned into plasmid pBR322. (*A*) The competitor plasmid was added at a ratio of 1:1 (0.1 μg), 5:1 (0.5 μg), or 10:1 (1.0 μg) relative to the template DNA. (*B*) The competitor plasmid was added as described in *A*. In addition, each transfection mixture contained 5 μg of an RSV-T plasmid. Transfected cultures were maintained and SV40 late gene expression was assayed as described in Fig. 2. After autoradiography, bands were excised from the blot and cpm of ^{125}I were determined in a γ counter. The level of expression obtained with 0.1 μg of SV40 template DNA was arbitrarily set at 100%.

cells. As seen with wild-type SV40, the base-substitution mutants are expressed efficiently only in COS-1 cells (i.e., in the presence of T antigen) or after DNA replication. These results demonstrate two important points. First, the absence or low level of SV40 late gene expression observed before DNA replication in a CV-1 cell is not due to the absence of the polymerase II promoter element, the Goldberg–Hogness sequence. Mutants pSVs-3 and pSVs-17 contain substitutions that create the sequence TATTTA, identical to the SV40 early Goldberg–Hogness box (17, 18). Second, unlike sequences further upstream, the presence/absence of a Goldberg–Hogness sequence does not dramatically affect the level of T-antigen-mediated late gene expression *in vivo*.

Using template-deletion and base-substitution mutants, we have previously shown that at least two upstream control regions are required for efficient *trans*-activation of the SV40 late promoter by T antigen (9). The first sequence consists of T-antigen binding sites I and II; the second sequence is located in the SV40 72-bp tandem repeats. Deletion of either transcriptional control sequence from the template decreases late gene expression by an order of magnitude. The *in vivo* competition studies presented here support these data and contribute additional information toward an understanding of the potential interaction of the upstream sequences with putative regulatory molecules. The ability to compete for transcriptional factors suggests the direct interaction of the factors with the transcriptional control sequences. Efficient binding of the limiting transcriptional factor requires the presence of the two transcriptional control sequences in *cis* and at a critical distance from one another. These results suggest that binding of the limiting transcriptional factor(s) requires a cooperative interaction between the protein(s) and DNA sequences located within these domains.

Although T antigen is required for SV40 late gene activation, it does not appear to be the limiting *trans*-acting factor. This conclusion is based on the inability to reverse or offset the competition of VP-1 synthesis by the addition of a plasmid expressing T antigen. We suspect that the limiting transcription factor binds cooperatively to the SV40 72-bp repeat control region, perhaps requiring the presence of T antigen.

The mechanism by which the upstream transcriptional control sequences activate SV40 late transcription is still unclear. An obvious possibility is the direct interaction of T antigen with T-antigen binding sites I and II. While this explanation is consistent with our previous studies, which showed a correlation between the ability of mutant SV40 T antigens to bind the origin and the efficiency of late gene activation (8), a direct role for T antigen in the induction of VP-1 expression has yet to be rigorously demonstrated. The second region of the SV40 template, which is required for *trans*-activation of the late transcription unit, is the 72-bp tandem repeats. This regulatory element serves as a transcriptional enhancer for SV40 early gene expression (23–27). We have previously demonstrated that base-substitution mutants that quantitatively affect enhancer function in the early orientation similarly affect the level of T-antigen-mediated late gene expression (9, 28). Thus, it seems likely that DNA sequences that control early gene enhancement also are important for late gene activation.

Specific *in vivo* competition for the SV40 early enhancer function apparently involves only the 72-bp tandem repeats (29, 30). This result suggests that the enhancer binding factors required for early gene expression are able to bind independently to the 72-bp element. Our *in vivo* competition studies demonstrate that the putative DNA binding proteins responsible for late gene expression do not efficiently associate with DNA fragments containing either the 21- and 72-bp repeats or the 72-bp repeat element alone. These results raise the possibility that different proteins may interact with the SV40 72-bp repeat region in the induction of early gene expression and T-antigen-mediated late gene expression. Further experiments will be required to demonstrate this point conclusively.

We have shown that a cloned DNA fragment containing the T-antigen binding sites and the 21-bp repeat transcriptional control element (31–35) (mp 5171–128) is able to compete for the limiting *trans*-acting factor in COS-1 cells, albeit at a low efficiency. These results suggest that, in the absence of the 72-bp repeats, the 21-bp repeats bind transcriptional factors required for T-antigen-mediated late gene activation. Dynan and Tjian have recently demonstrated that a cellular eukaryotic transcription factor, SP1, binds specifically to the SV40 21-bp repeats (36–38). It is possible, therefore, that SP1 may potentiate T-antigen-mediated SV40 late gene expression in the absence of the 72-bp repeats.

Our future studies will need to be directed toward the development of *in vitro* transcription systems in which the *trans*-activation of the late promoter can be studied. Such systems will be required to characterize, at the molecular level, the interaction of T antigen and other transcriptional regulatory proteins with the template DNA.

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