Simian Virus 40 Small t Antigen *trans* Activates the Adenovirus E2A Promoter by Using Mechanisms Distinct from Those Used by Adenovirus E1A

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As reported previously for simian virus 40 small t antigen, polyomavirus small t antigen stimulates transcription directed by the adenovirus E2A and VA-I promoters during transient transfection assays. To determine whether papovaviral small t antigens might employ biochemical mechanisms during transcription activation that are either similar to or distinct from other viral *trans* activators, I compared the abilities of simian virus 40 small t antigen and adenovirus E1A to regulate the E2A promoter during transient transfection assays. I determined that, whereas activation of the E2A promoter by E1A involves the transcription factors ATF and E1IF, activation by small t antigen involves only E1IF. The effects of cotransfecting maximal concentrations of plasmids encoding small t antigen with E1A suggested that they activate the E2A promoter by different mechanisms. To determine whether small t antigen employs a mechanism different from that encoded in E1A domain II, domain III, or both, I compared the effects of transfecting plasmids expressing small t antigen, the 12S product of E1A, or the 13S product with a mutation in domain II on *trans* activation of the E2A promoter in two cellular backgrounds. On the basis of these comparisons, it appears that small t antigen does not activate transcription by a mechanism similar to either of the activities encoded in E1A. This suggests that papovavirus small t antigens belong to a distinct class of *trans*-acting proteins.

Study of transcription regulation by DNA tumor viruses such as simian virus 40 (SV40) and adenovirus (Ad) has greatly contributed to our understanding of eukaryotic gene regulation. Whereas much work has been focused on transcription regulation by SV40 large T antigen, it has only recently been recognized that the other SV40 early-gene product, small t antigen, can also regulate transcription. Small t antigen *trans* activates the Ad E2A promoter, which is transcribed by RNA polymerase II, and the Ad VA-I promoter, which is transcribed by RNA polymerase III (21). Since the amino acid sequence of papovavirus small t antigens is highly conserved (12, 31), it is possible that *trans* activation is a conserved function among small t proteins, perhaps being important during viral infection *in vivo*.

Several other viral *trans*-acting proteins such as Ad E1A, E4, pseudorabies virus immediate-early protein, and human papillomavirus E7, also stimulate transcription from the E2A or VA-I promoter (14–16, 27, 29). Since none of these *trans* activators, including small t antigen, binds directly to DNA, it is likely that they stimulate transcription by affecting the ability of transcription factors to form active transcription complexes. There is little specific information available regarding how various viral *trans*-acting proteins interact with transcription factors and how many different mechanisms might be involved. It has been recognized, however, that papovaviral small t antigens, E1A, and human papillomavirus E7 all contain a cysteine-rich sequence in domains which are required for *trans* activation (10, 11, 21). In this study, I examined by transient transfection analysis whether the *trans*-activation property of SV40 small t antigen was conserved in at least one other papovaviral small t antigen, polyomavirus (Py) small t antigen. I further compared *trans* activation of the E2A promoter by small t antigen with that by Ad E1A to determine whether it might employ a mechanism similar to or different from either of the activities encoded in E1A.

Transcription regulation is a property which is conserved among at least two papovavirus small t proteins. To determine whether a small t antigen encoded by another papovavirus might also *trans* activate, I tested Py small t antigen when cotransfected with plasmids containing the Ad E2A and VA-I promoters. Cells were cultured in 35-mm-diameter plates and transfected as described elsewhere (22, 23), using 2 µg of chloramphenicol acetyltransferase (CAT) plasmids, 2 µg of plasmids expressing small t antigen or E1A, and calf thymus DNA (Clontech) to a total of 15 µg of DNA. In this and subsequent experiments, cell extracts were used at dilutions which would generate CAT activity within the linear range of enzyme activity (1 to 60%). Transfections were repeated on at least three separate occasions with plasmid DNA purified by double banding on cesium chloride-ethidium gradients from at least two separate preparations. As shown in Fig. 1A, Py small t antigen (encoded by pPy-St, containing the Py enhancer-promoter [34]) can stimulate CAT enzyme activity directed by the E2A promoter approximately 10-fold, which is comparable to the level of *trans* activation observed in response to SV40 small t antigen and Ad E1A.

I also tested the ability of Py small t antigen to *trans* activate the RNA polymerase III-transcribed VA-I promoter as measured by S1 analysis. Following transient transfection, RNA was prepared by using the commercial reagent RNazol (9), and 10 µg of whole-cell RNA were assayed for VA-I RNA by S1 analysis as described elsewhere (6). A 96-nucleotide (nt) probe, 75 nt of which would be protected by VA-I RNA, was used. As shown in Fig. 1B, transient transfection of plasmids expressing either Py or SV40 small t proteins resulted in potent *trans* activation of a VA-I plasmid. In fact, the small t proteins were more effective than Ad E1A in this regard. These data demonstrate that a *trans*-activation function appears to be conserved among small t proteins from at least two papovaviruses.

FIG. 1. (A) *trans* activation of the Ad E2A promoter by Ad E1A, SV40 small t antigen, or Py small t antigen. A CAT plasmid linked to the Ad E2A promoter was transfected into CV-1 cells with only carrier DNA (lane -) or was cotransfected with plasmids expressing Ad E1A (lane E1A) (16), SV40 small t antigen under the control of the Rous sarcomavirus long terminal repeat (lane SV40 t) (6), or Py small t antigen (lane Py t) (34). (B) *trans* activation of the Ad VA-I promoter by Ad E1A, SV40 small t antigen, or Py small t antigen. CV-1 cells were cultured in 60-mm-diameter dishes and transfected with 10 μ g of VA-I plasmid (13) with only carrier DNA (lane -); were cotransfected with 10 μ g of plasmids expressing Ad E1A (lane E1A), SV40 small t antigen (lane SV40 t), or Py small t antigen (lane Py t); or were mock transfected (lane M). Carrier DNA was added to all transfections to maintain a total DNA concentration of 40 μ g. Control lanes include probe incubated with 10 μ g of tRNA and digested with S1 nuclease (lane P + S1) and 3,000 cpm of intact probe (1/10 of that incubated with cell RNA) (lane P). The positions of the undigested probe and that protected by VA-I RNA are indicated by arrows. The sizes of molecular standards are indicated on the right in nucleotides.

It is not clear why papovavirus small t antigens would *trans* activate the Ad E2A and VA-I promoters, since sequences resembling these promoter elements are not contained in papovaviral genomes. It is possible that activation of cellular promoter elements resembling the E2A and VA-I promoters occurs during papovavirus infection. Since both SV40 and Py small t antigens have been found to associate with the cellular enzyme type 2A phosphatase (26, 32), it is reasonable to speculate that regulation of type 2A phosphatase activity could affect the phosphorylated states of proteins that regulate the E2A and VA-I promoters and cellular promoters containing similar control elements.

trans activation by SV40 small t antigen involves binding sites for transcription factor E1IF but not for ATF. I am interested in eventually understanding the biochemical mechanisms by which small t antigen regulates transcription and determining whether other *trans* activators employ mechanisms the same as or different from those of small t antigen. To investigate this small-t-antigen function, it is necessary to determine which promoter elements or transcription factor binding sites are required. Optimal basal and E1A-*trans*-activated expression of the E2A promoter requires a binding site for the transcription factor ATF and duplicated binding sites for the transcription factor E1IF (17, 22, 24, 33). *trans* activation of the E2A promoter by coexpressed SV40 large T and small t antigens also requires these same transcription factor binding sites (22). To determine which of these transcription factor binding sites is required for small-t-antigen-mediated *trans* activation, I employed wild-type or mutant E2A promoter-CAT plasmids which contained base substitutions at either the ATF or both E1IF sites (22), which are illustrated in Fig. 2. As displayed in

FIG. 2. Diagram of the wild-type E2A promoter and mutants. The inducible enhancer region of the Ad E2A promoter is located between nt -85 and -30. The d1ATF mutant contains base substitutions in the ATF site (between nt -80 and -70) but contains wild-type E1IF sites, whereas the d1E1IF mutant contains base substitutions within the E1IF sites (nt -64 to -60 and nt -45 to -36) but contains a wild-type ATF site. These plasmid constructions have been described elsewhere (22).

Table 1, pE2-d1ATF, a plasmid with a mutated ATF site (and, thus, containing the E1IF sites) exhibited reduced basal and *trans*-activated activity but could still be *trans* activated by small t antigen. The fact that the amount of CAT activity of pE2-d1ATF in response to small t antigen was less than that of pE2A-CAT could indicate either that some of the *trans*-activation effects of small t antigen are mediated by ATF or that optimal transcription requires activation of the E1IF factors and also occupation of the ATF binding site. It appears that the latter explanation is the case with regard to *trans* activation by small t antigen, since pE2-d1E1IF, a plasmid with mutations in both E1IF sites (and, thus, containing only an ATF site) could not be *trans* activated by small t antigen. This was not simply due to loss of basal activity upon mutation of the E1IF sites, since, as

TABLE 1. CAT activity in CV-1 cells

Expt	% CAT activity ^a				
	pE2 wt	pE2-d1ATF	pE2-d1E1IF	pE4-CAT	pE2A-CAT
1					
Basal	3.4	1.3	0.35		
+E1A	37.6	7.9	1.1		
+t	42.6	8.7	0.38		
2					
Basal	3.2	2.3	0.3		
+E1A	41.1	14.2	0.94		
+t	64.0	13.3	0.37		
3					
Basal				5.0	3.0
+E1A				72.0	25.0
+t				8.0	53.0
4					
Basal				4.0	ND
+E1A				45.0	
+t				8	

^a From two experiments in which CV-1 cells were transfected with CAT plasmids containing a wild-type (wt) E2A promoter or E2A promoters with mutations in the ATF site or E1IF sites and two experiments in which cells were transfected with CAT plasmids containing a wt E2A or E4 promoter. CAT plasmids were transfected with only carrier DNA (basal) or with plasmids expressing Ad E1A (+E1A) or SV40 small t antigen (+t). Data are expressed as the percentage of total ¹⁴C-chloramphenicol added to the assay which was acetylated. ND, not determined.

previously determined (22), this plasmid was still *trans* activated at least threefold by E1A. This demonstrated that *trans* activation of the E2A by small t antigen requires the EIIF sites. The ATF site of the E2A promoter does not seem to be required for *trans* activation by small t antigen, and the ATF site in the absence of the EIIF sites is not responsive to small t antigen.

To examine whether small t antigen might activate a different promoter containing ATF sites, I transfected a CAT plasmid containing the Ad E4 promoter (kindly provided by Michael Green [18]) with a plasmid expressing small t antigen or E1A. As also shown in Table 1, E1A efficiently *trans* activated the E4 promoter, while small t antigen had no effect. Since E1A, but not small t antigen, can *trans* activate promoters containing ATF sites, we concluded that E1A encodes at least one *trans*-activation function not encoded by small t antigen.

Different mechanisms might be involved in *trans* activation of EIIF sites on the E2A promoter by small t antigen and E1A. The previous results demonstrated that *trans* activation of the Ad E2A promoter by either E1A or small t antigen involves binding sites for the transcription factor EIIF, but they did not determine whether the effects of both *trans* activators on EIIF were the same or different. I reasoned that if small t antigen and E1A act identically, then when E1A-dependent processes are fully saturated, expression of small t antigen should not further activate the E2A promoter. On the other hand, if they act by different mechanisms, then E1A-dependent and small-t-antigen-dependent processes might be additive, even when one of the processes is fully saturated. To determine which was the case, increasing amounts of plasmid expressing either E1A or small t antigen were cotransfected with a constant amount of the E2A-CAT plasmid, keeping the total amount of DNA constant with carrier DNA (Fig. 3A). The concentration of plasmid DNA expressing E1A or small t antigen which produced maximal *trans* activation was determined and was found to be 10 μ g for E1A and 8 μ g for small t antigen. The limitation for *trans* activation by small t antigen or E1A was the E2A-CAT plasmid, not expression of small t antigen or E1A, since further CAT activity could be observed by increasing the concentration of transfected pE2A-CAT (Fig. 3B). When the E2A-CAT plasmid was triple transfected with maximal concentrations of E1A and small-t-antigen plasmids, approximately additive levels of CAT activity were obtained (Fig. 3B). Since small t antigen and E1A, which are individually in excess, together activate a limiting concentration of pE2A-CAT to a greater extent than either alone, this suggests that the effects of small t antigen and E1A are mediated by different mechanisms.

Differences between small t antigen and E1A may be due to two *trans*-activation functions encoded in the E1A protein, both of which are different from that of small t antigen. The E1A expression plasmid which had been used in experiments up to this point expresses two major protein products, a 243-amino-acid product translated from a 12S mRNA species and a 289-amino-acid product translated from a 13S mRNA species. These two mRNA products arise as a result of differential splicing of a primary transcript (5). These two protein products appear to encode two distinct *trans*-activation functions, one of which involves conserved domain II and one of which involves conserved domain III (2). I considered that differences between the effects of small t antigen and E1A could be explained by these two distinct transcriptional regulatory activities encoded in E1A, at least one of which functions differently from small t antigen. I speculated that if either of the E1A functions was small t

FIG. 3. (A) Effect of increasing concentrations of plasmids expressing small t antigen or E1A on CAT activity of 0.5 μ g of pE2A-CAT, maintaining a constant total DNA concentration with carrier DNA. (B) Solid bar, 0.5 μ g of pE2A-CAT transfected with 10 μ g of a plasmid expressing Ad E1A plus 8 μ g of a plasmid expressing SV40 small t antigen (concentrations that were saturating in panel A; note difference in y-axis scale from that in panel A); open bar, 1 μ g of pE2A CAT transfected with carrier DNA or 8 μ g of plasmid expressing small t antigen (hatched bar) or 10 μ g of E1A (dotted bar). In all cases, the total concentration of DNA was kept constant at 20 μ g with carrier DNA.

antigen-like, it would be the domain III-encoded function, because it contains a cysteine-rich sequence similar to one in the unique region of small t antigen that is required for activation of the Ad E3 and E4 promoters (10, 19, 25). In contrast, small t antigen and the 12S product do not have obvious sequence similarity.

To test whether small t antigen contains an activity similar to or distinct from those encoded in either domain II or domain III of E1A, I obtained E1A plasmids which express only 12S or 13S mRNA species of E1A (encoding domains I and II or I, II, and III, respectively) or one which expresses the 13S mRNA species with a mutation in amino acid 928 (kindly provided by Betty Moran). This mutation encodes a Cys-to-Gly substitution in domain II and produces a transformation and *trans*-activation-defective E1A protein (25). I then compared the functions of E1A proteins containing only domains I and II, I and III, or I, II, and III with that of small t antigen. I performed these experiments with two different

lines of CV-1 cells which are grown in this laboratory. These two lines had been previously propagated in two different laboratories and had been successfully used for production of SV40 virus. Unexpectedly, I observed that in one of these cell lines, CV-1H, cotransfection of a small-t-antigen expression plasmid with pE2A-CAT did not stimulate CAT activity. This does not appear to be due to a lack of expression of small t antigen in CV-1H cells, since I observed equivalent amounts of small t antigen to be immunoprecipitated following transient transfection of both cell types and since small t antigen can stimulate accumulation of RNA polymerase III-transcribed VA-I RNA to similar levels in both cell types (20). These two cell lines also differed with regard to the ability of cyclic AMP analogs to stimulate cyclic AMP response element-containing promoters (1). As shown in Fig. 4, *trans* activation by small t antigen occurred in only one line of cells, CV-1N. In contrast, E1A products containing either domain II (12S) or domain III (13S 928) could *trans* activate the E2A promoter in each of the CV-1 cell lines. Because either domain II or domain III of E1A can *trans* activate the E2A promoter in CV-1H cells, whereas small t antigen cannot, perhaps small t antigen *trans* activates the E2A promoter by mechanisms different from those of either of the E1A functions. In additional experiments, I found that the 13S E1A product with a domain II mutation can stimulate pE4-CAT, distinguishing *trans* activation by E1A domain III from that by small t antigen (20), and that small t antigen does not stimulate CAT activity controlled by the hsp 70 promoter (20), distinguishing *trans* activation by E1A domain II (30) from that by small t antigen.

I reasoned that it was possible that small t antigen might *trans* activate the E2A promoter by using a mechanism similar to that of E1A domain II or III but that there might be additional structures contained in the E1A protein but not in small t antigen which were also required for *trans* activation in CV-1H cells. If so, it might be possible to "complement" *trans* activation by small t antigen by coexpression of E1A structures. If small t antigen encoded a domain II-like function, then cotransfection of the small-t-antigen expression plasmid with the plasmid expressing the 13S product with the domain II mutation should result in *trans* activation to levels similar to those achieved in the presence of the wild-type 13S product, whereas if small t antigen encoded a domain III-like function, then cotransfection of the small-t-antigen and the 12S E1A expression plasmids should *trans* activate to levels similar to those achieved by the 13S product. As shown in Fig. 4, cotransfection of the plasmid encoding the 12S product with the plasmid encoding the 13S product with the domain II mutation resulted in the same level of E2A promoter-driven CAT activity as did the wild-type 13S product, demonstrating that domains II and III appear to encode independent activities that will complement each other whether they are expressed in *cis* or in *trans*. However, cotransfection of the plasmid encoding small t antigen did not complement either the 12S E1A product or the 13S product with the domain II mutation. In fact, expression of small t antigen seemed to inhibit *trans* activation in response to these E1A products. This suggests that small t antigen does not encode either an E1A domain II-like function which can complement E1A domain III or a domain III-like function which can complement E1A domain II.

It is not clear why small t antigen did not activate the E2A promoter in CV-1H cells. It is possible that EIIF is complexed with an inhibitor such as the retinoblastoma protein (3, 4, 7, 8) in these cells but not in the (permissive) CV-1N cells. Perhaps small t antigen cannot dissociate the complex

FIG. 4. CAT activity directed by the Ad E2A promoter in CV-1N and CV-1H cells when cotransfected with plasmids expressing SV40 small t antigen or Ad E1A mutants. CV-1H cells were obtained from Ulla Hanson, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Mass., and CV-1N cells were obtained from the late George Khoury's laboratory at the National Institutes of Health, Bethesda, Md. One microgram of CAT plasmid was transfected with 7 μ g of small t antigen or E1A expression plasmids (concentrations which had been determined to produce maximum *trans* activation), keeping the total DNA constant with carrier DNA. Plasmids expressing a 12S E1A cDNA, 13S cDNA, or both with a nt 928 mutation, each under the control of the E1A enhancer (25), were kindly provided by Elizabeth Moran.

in the nonpermissive CV-1H cells. In this regard, small t antigen could resemble the Ad E4 open reading frame 6/7 17-kDa protein, which directly complexes with EIIF and stabilizes its interaction with DNA (14, 15, 28) but requires release of EIIF from association with a cellular inhibitor to do so (2). However, if small t antigen acted by an E4-like mechanism, expression of the 12S E1A product and small t antigen together in CV-1H cells would be expected to stimulate the E2A promoter. Instead, small t antigen inhibited the 12S E1A-dependent activation of the E2A promoter. It is also possible that a cellular factor is required to interact with small t antigen but not with either of the E1A domains in order to activate the E2A promoter. This factor may be expressed in CV-1N cells but not in CV-1H cells, presumably as a result of different culture conditions in the laboratories from which the cell lines were obtained. The difference in the abilities of small t antigen and E1A to *trans* activate the E2A promoter in different cellular backgrounds reported here is reminiscent of our earlier observation that E1A, but not coexpressed SV40 large T and small t antigens, could *trans* activate the E2A promoter in COS-1 cells (23). This could indicate that several different mechanisms are employed by *trans*-acting proteins, leading to regulation of the same transcription factors, and that some of these mechanisms are not operational in all cell backgrounds. In the future, it will be important to determine the effect of small t antigen on factors that mediate its transcriptional effects.

The results presented here demonstrate that the ability to *trans* activate the Ad E2A and VA-I promoters is shared by at least two papovavirus small t antigens, suggesting that this function may be important during infection. *trans* activation of the E2A promoter by SV40 small t antigen involves the E1F but not ATF transcription factor. The mechanism of *trans* activation by SV40 small t antigen is different from those employed by domains II and III of Ad E1A. It is likely that activation of certain host cell genes containing control elements resembling those of the E2A or VA-I promoters is important during infection by several different viruses and that each virus has developed diverse biochemical mechanisms by which their *trans*-acting proteins can produce the same end result. Since small t antigen appears to employ processes in *trans* activation that are different from those employed by Ad E1A, study of the regulation of transcription by small t antigen may reveal novel regulatory mechanisms.

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