Interaction of the Dr1 inhibitory factor with the TATA binding protein is disrupted by adenovirus E1A

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ABSTRACT Past experiments have shown that the adenovirus E1A_{12S} product activates the hsp70 promoter, dependent on the TATA element and dependent on N-terminal E1A sequences. Other experiments have identified a factor termed Dr1 that interacts with and inhibits the transcriptional activity of the TATA-binding protein (TBP). We now find that the E1A_{12S} protein can disrupt the interaction of the Dr1 factor with the TATA-specific TBP factor, allowing the productive interaction of TBP with TFIIA. This E1A-mediated disruption is dependent on N-terminal sequences that are also essential for the TATA-dependent trans-activation of the hsp70 promoter. Moreover, we also find that Dr1 expression in transfected cells can inhibit transcription from the hsp70 promoter and that this can be overcome by coexpression of the wild-type E1A protein, dependent on N-terminal sequences. We conclude that the activation of hsp70 through the TATA element may be mechanistically similar to the activation of the E2 promoter via E2F, in each case involving a release of a transcription factor from an inactive complex.

The adenovirus E1A gene encodes multiple protein products that activate transcription of viral and cellular genes (1). The CR3 domain, which is unique to the so-called E1A_{13S} product, is clearly responsible for the majority of transcriptional activation of viral genes during a lytic infection. Nevertheless, it is also clear that the E1A_{12S} product possesses transcriptional activating function. For example, recent experiments have shown that the E1A_{12S} protein can activate transcription by releasing the E2F transcription factor from inhibitory complexes containing proteins such as the retinoblastoma gene product (2). The ability of the E1A_{12S} protein to activate the E2F factor is dependent on protein sequences within the CR1 and CR2 domains but independent of the N-terminal sequences. The E1A_{12S} product also activates transcription in an E2F-independent manner, an example being activation via the hsp70 promoter (3). In this case, the TATA element is the critical target for activation. Moreover, in contrast to the requirement for the CR1 and CR2 domains for the activation of E2F (4), it is the N terminus of the E1A protein that is necessary for the TATA-dependent activation of transcription (3).

Recent experiments have identified an activity termed Dr1 that inhibits the activity of the TATA-specific binding protein (TBP) transcription factor (5). This inhibition involves a direct physical interaction of Dr1 with TBP and, as a consequence of this interaction, TBP is unable to form interactions with other components of the transcriptional machinery such as TFIIA and/or TFIIB. Given the fact that the ability of E1A to activate transcription through the E2F factor involves the release of E2F from inhibitory complexes with proteins such as Rb, we have explored the possibility that E1A-mediated activation of transcription through the TATA element in-

volves a similar event with respect to the TBP–Dr1 interaction.

MATERIALS AND METHODS

DNA-Binding Assays. DNA-binding assays were performed as described using a probe derived from the adenovirus major late promoter (6). E1A protein was synthesized in a rabbit reticulocyte lysate as described (7).

Transfection Assays. Hsp70-luc was generated by inserting a 1270-bp *HindIII*/*Sal* I DNA fragment from hsp70-CAT between the *Sac* I/*Xho* I sites of a luciferase reporter, GL2-basic (Promega). E1A expression plasmids CMV-E1A_{12S} and CMV-E1A_{d12-36} have been described (3). The Dr1 expression vector CMV-Dr1 was generated by inserting the Dr1 cDNA between the *Kpn* I/*Xba* I sites of a cytomegalovirus expression vector, CMV4 (8). HeLa cells were transfected via lipofectamine according to the manufacturer's specifications (BRL). Cells were plated on 35-mm dishes and received a total of 2.5 μ g of DNA and 2 μ g of lipofectamine per plate. Cells were harvested 48 hr after transfection. Luciferase assays were performed with a kit purchased from Promega.

Plasmids. Plasmids encoding glutathione *S*-transferase (GST) E1A_{12S} fusion proteins constructs were made by PCR using previously described E1A_{12S} wild-type (WT) and mutant cDNAs as template (4). E1A cDNAs were cloned in frame with GST between the *Bam*HI and *Eco*RI sites in pGEX-3X. The ATG start codon of E1A occurs 3 amino acids downstream from the factor Xa cleavage site in the fusion proteins. The fusion protein constructs use the E1A stop codon followed immediately by an *Eco*RI site. The deletion mutants are named for the deleted residues in E1A_{12S} (e.g., d12-36 lacks amino acids 2–36 of the WT E1A_{12S}). Deletion mutant d138-67 contains a 12-nt insertion resulting in the substitution of 4 amino acids (SSRD) for the deleted region. The double point mutant pm928/961 results in the double amino acid substitution, glycine for cysteine at residue 124 and lysine for glutamic acid at residue 135.

Preparation of GST Fusion Proteins. GST fusion proteins were purified from bacterial lysates as described (9).

Protein Interaction Assays. A total of 1 μ l (equal to $\approx$ 4 μ g of eluted protein) of glutathione-Sepharose 4B bound-GST-E1A_{12S} WT or mutant was mixed with 24 μ l of a 50% slurry of glutathione-Sepharose 4B beads (Pharmacia) prewashed in BC 100 buffer (20 mM Tris, pH 7.9/20% glycerol/1 mM EDTA/0.2 mM phenylmethylsulfonyl fluoride/0.1 KCl). This was added to 2.5 μ g of recombinant Dr1, along with 20 μ g of bovine serum albumin and 0.15% Nonidet P-40 (final concentration). The reaction mixtures were gently rocked at 25°C for 1.5 hr. Beads were spun 30 sec at 10,000 rpm and washed twice in 0.5 ml of BC-100 buffer, resuspended in 20 μ l of 1.3 $\times$ SDS/PAGE gel loading buffer, boiled 10 min, and

run on a 15% SDS/PAGE gel. Proteins were transferred to nitrocellulose and probed with anti-Dr1 (a rabbit polyclonal antibody to recombinant Dr1) (5). Detection of bands via avidin/biotin conjugates was accomplished using the Vectastain ABC kit (Vector Laboratories, catalog no. PK-4001) and the peroxidase TMB (3,3',5,5'-tetramethylbenzidine) substrate kit (Vector Laboratories catalog no. SK-4400) as per the manufacturer's instructions.

RESULTS

E1A-Mediated Disruption of the TBP-Dr1 Interaction. Dr1 was identified as a 19-kDa phosphoprotein that inhibits TATA-dependent transcription through an interaction with the TBP (5). Dr1 can remove TFIIA from TBP-TFIIA complexes, suggesting competition between Dr1 and TFIIA for the same recognition site on TBP. As a consequence of this interaction, the assembly of a transcriptionally competent complex of general transcription factors is prevented and transcription is inhibited. Although the interaction of TBP with the TATA element by gel retardation assay is weak and somewhat difficult to detect, it is possible to readily assay the formation of a TBP-TFIIA complex that binds to the TATA element (Fig. 1A). Addition of the adenovirus E1A_{12S} product, produced in a reticulocyte lysate, had no effect on the interaction of TBP and TFIIA with the TATA element. As shown previously (5), the addition of Dr1 to the TBP-TFIIA mixture results in a reduction or loss of the TBP-TFIIA complex and the production of several additional complexes that contain the Dr1 protein in association with TBP (Fig. 1B). The various TBP-Dr1 complexes appear to result from the multimerization of Dr1 (5). Addition of the E1A protein abolished the complexes containing the Dr1 protein, restoring the TBP-TFIIA complex as the major component. Thus, it would appear that the E1A protein can disrupt the TBP-Dr1 interaction but not the TBP-TFIIA complex.

A direct assay for the interaction of TBP and Dr1 reveals the formation of at least two distinct DNA-protein complexes (Fig. 1C). Addition of a Dr1-specific antibody to the binding assay demonstrates the presence of Dr1 in these complexes. Once again, addition of the E1A protein, in the form of a GST-E1A fusion protein, disrupts the TBP-Dr1 complexes (Fig. 1D). The slowest migrating TBP-Dr1 complex was readily disrupted, whereas the faster migrating complex required the addition of larger amounts of E1A. In contrast, addition of a control GST protein only slightly diminished the slower migrating complex, whereas the same quantity of E1A resulted in the total loss of both complexes. It would thus appear that E1A does indeed possess the ability to disrupt the interaction of TBP with the inhibitory Dr1 activity.

The N Terminus of E1A Is Essential for Disruption of TBP-Dr1 and for Binding to Dr1. Previous experiments have shown that sequences at the N terminus of the E1A protein are critical for TATA-dependent activation of transcription, whereas sequences within the CR2 domain are not essential (3). To define the relationship of the E1A-mediated disruption of the TBP-Dr1 complex with the E1A-mediated TATA-dependent transcription activation, a set of E1A mutants previously assayed for transactivation activity (Fig. 2A) was assayed for TBP-Dr1 dissociation. Whereas the WT E1A protein, the E1A_{d173-120} mutant, and the E1A_{pm928/961} mutant could efficiently disrupt the TBP-Dr1 complex, the E1A_{d12-36} mutant was inactive and the E1A_{d138-67} mutant was impaired (Fig. 2A). The E1A_{d138-67} mutant begins to disrupt the TBP-Dr complex when added in increasing quantities, while the E1A_{d12-36} mutant totally lacks TBP-Dr dissociating activity.

These results closely coincide with the TATA-dependent transactivation phenotype of these mutants (3) (summarized in Fig. 2A). The E1A regions required for TBP-Dr1 complex

FIG. 1. The TBP-Dr1 interaction is disrupted by E1A. (A) The interaction of TBP with TFIIA is not altered by E1A. DNA-binding assays used a 3' end-labeled TATA-containing fragment from the adenovirus major late promoter (Ad-MLP), recombinant yeast TBP (yTBP) 20 ng, and TFIIA (0.12 µg) prepared from HeLa cells (6). Reticulocyte lysate programmed with E1A_{12S} (0.5 µl) or an equivalent amount of bromo mosaic virus (BMV) programmed control lysate was added to the binding reaction, incubated 30 min at 25°C, and then loaded on the gel. (B) The interaction of TBP with Dr1 is disrupted by E1A, leaving the TBP-TFIIA complex. Complexes were formed on the Ad-MLP as described in A using yTBP, TFIIA, and Dr1 (25 ng) (HeLa phenyl Superose fraction) (5). Reticulocyte lysate programmed with E1A_{12S} or BMV control was added as in A. (C) Formation of a TBP-Dr1 complex. Formation of a TBP-Dr1 complex, using recombinant human TBP (≈10 ng) and Dr1 (≈25 ng) (HeLa Superdex S200 column fraction), was assayed using the Ad-MLP. The presence of the Dr1 protein in the complexes was demonstrated by the addition of an anti-Dr1 antibody (0.2 µg) (5). An equal amount of anti-large T antibody (T Ab-2, Oncogene Science) was added in the control lane. (D) The interaction of TBP with Dr1 is disrupted by E1A. TBP-Dr1 complexes were formed as in C. Increasing concentrations (1.5–12 µg) of a GST-E1A_{12S} fusion protein or GST control protein (12 µg) were added.

disruption sharply contrast with the regions required for disruption of the E2F complexes (4). For instance, the N-terminal mutant E1A_{d12-36} is as active as the WT E1A protein in disrupting E2F complexes, whereas the E1A_{pm928/961} mutant is inactive (4). The E1A_{d138-67} mutant is impaired for E2F complex dissociation as it is for disruption of the TBP-Dr1 complex, thus demonstrating an overlapping requirement for CR1 sequences working both with the N terminus as well as with CR2. Given the fact that the mutants that are inactive in one assay are active in the other assay provides strong evidence that the failure of the E1A_{d12-36} mutant protein to disrupt the TBP-Dr1 complex is not a consequence of an altogether inactive protein.

The finding that the E1A protein could disrupt the TBP-Dr1 complex suggested the possibility of a direct interaction

FIG. 2. E1A N-terminal sequences are essential for disruption of the TBP-Dr1 complex. (A) Disruption of the TBP-Dr1 complex by E1A mutants. Shown at the top is a schematic depiction of the E1A mutants employed for TBP-Dr1 disruption assays and their activity in activation of TATA-dependent transcription using the hsp70 promoter. The ability of each E1A mutant to trans-activate the hsp70 promoter in comparison to the WT E1A protein is indicated (3). Shown below are assays for the formation of the TBP-Dr1 complex and the ability of GST-E1A_{12S} WT or mutant to disrupt the complex. Assays were as described in the legend to Fig. 1 C and D. GST-E1A_{12S} fusion proteins were added in increasing amount from 1.5 μ g to 7.5 μ g as depicted at bottom. GST control protein was added at the highest quantity (7.5 μ g) to the last lane. (B) Direct interaction of E1A with Dr1, dependent on N-terminal E1A sequence. GST fusion proteins, either WT E1A_{12S} protein or the indicated mutant E1A proteins (dl2-26, dl38-67, dl73-120, pm928/961), each bound to glutathione-Sepharose 4B, were incubated with 2.5 μ g of recombinant Dr1. As a control, the same amount of Dr1 was incubated with GST bound to Sepharose. After washing and elution, the proteins were analyzed in an SDS/acrylamide gel and blotted to nitrocellulose. Dr1 (indicated by arrow) was detected through the use of a specific antibody (see text). An amount of recombinant Dr1 equal to that used for the binding assays (2.5 μ g) was run in an adjacent lane.

of E1A with Dr1. To address this possibility, we assayed for the ability of a WT GST-E1A_{12S} fusion protein and E1A mutants to interact with Dr1. The various E1A proteins were

absorbed onto glutathione-Sepharose 4B beads and then incubated with a preparation of recombinant Dr1 protein. The beads were washed and boiled for 10 min to release associated proteins, and then the released proteins were analyzed in an SDS/acrylamide gel. Following transfer of the proteins to nitrocellulose, the Dr1 protein was detected with a specific antibody. As shown in Fig. 2B, the Dr1 protein did indeed interact with the WT E1A protein, whereas there was no interaction with the control GST protein. Of most importance, the E1A_{dl2-36} mutant, which was defective for disruption of the TBP-Dr1 complex as well as TATA-dependent transcription activation, was also defective for interaction with Dr1. The E1A_{dl38-67} mutant, which is impaired for TBP-Dr1 complex disruption but which at higher concentrations displays some ability to disrupt, can interact with Dr1 under the conditions of this assay. The E1A_{dl73-120} mutant and the E1A_{pm928/961} mutant were capable of interacting with the Dr1 protein just as they were capable of disrupting the TBP-Dr1 complex. This is in contrast to the fact that the E1A_{pm928/961} mutant is unable to bind to the Rb protein. We thus conclude that the ability of E1A to disrupt the TBP-Dr1 complex, which coincides with the activation of TATA-dependent transcription, reflects the ability of E1A to directly interact with Dr1.

Dr1-Mediated Inhibition of hsp70 Promoter Activity Is Reversed by E1A. The experiments utilizing purified TBP and Dr1, demonstrating an ability of E1A to disrupt the TBP-Dr1 interaction, imply that Dr1 would inhibit TATA-dependent transcription activation and that E1A should reverse the inhibition. As a direct test of this expectation, we assayed the ability of a cotransfected Dr1-expressing plasmid to affect expression of a luciferase gene under the control of the hsp70 promoter, a promoter that we have previously shown is activated by E1A in a TATA-dependent manner (3, 10). Although the HeLa cells utilized for these assays do express the human papilloma virus E7 product, which has functional properties related to adenovirus E1A in terms of Rb association and E2F activation, our previous experiments have nevertheless shown that the activity of the hsp70 promoter is not altered by the E7 product (11). As can be seen in Fig. 3, Dr1 expression results in a 10-fold inhibition of hsp70 promoter activity. Cotransfection of a plasmid expressing the adenovirus E1A_{12S} gene product reverses this inhibition in a

FIG. 3. Dr1-mediated inhibition of the hsp70 promoter is reversed by adenovirus E1A. HeLa cells were transfected with a hsp70-luciferase plasmid (1.5 μ g) alone, together with the CMV-Dr1 plasmid (0.5 μ g), or with the Dr1 plasmid and with increasing amounts of either the CMV-E1A_{12S} or the CMV-E1A_{dl2-36} plasmid (0.1, 0.2, and 0.3 μ g). Extracts were prepared 48 hr after transfection and assayed for luciferase activity. The luciferase activity of cells transfected with hsp70-luc reporter plasmid alone was set as 100%.

FIG. 4. Role of E1A in TBP-dependent transcription activation.

dose-dependent manner. Moreover, cotransfection of the E1A_{Δ12-36} mutant fails to reverse the repression. We thus conclude that the ability of E1A to disrupt a TBP–Dr1 complex as seen in *in vitro* assays coincides with the ability of E1A to reverse the Dr1-mediated inhibition of transcription in an *in vivo* context.

DISCUSSION

The E1A_{12S} product has been shown to liberate the E2F transcription factor from various multicomponent complexes, activating the transcriptional potential of E2F (2). The E1A-mediated TATA-dependent activation of the hsp70 promoter appears to be a similar event, coinciding with the release of TBP from a complex with Dr1. Although we do not yet know the basis for the previously observed TATA specificity evident in the E1A activation (3, 10), we suspect it may relate to the presence of distinct TFIID complexes that may recognize TATA elements differentially (12, 13). Our results also demonstrate that the N-terminal domain of E1A, which is essential for TATA-dependent transcription activation, is also required for TBP–Dr1 disruption. Previous experiments have demonstrated the interaction of a 300-kDa polypeptide with the N-terminal E1A sequences (14–16). Although an involvement of p300 in TATA-dependent transcription activation has not been demonstrated in previous assays, it remains possible that p300 could be involved, either through an association of Dr1 or through an interaction with TBP.

At least part of the activity provided by the adenovirus E1A CR3 sequence, unique to the E1A_{13S} product, appears to involve the TATA element (17), and recent evidence shows that the E1A_{13S} product can interact with the TBP transcription factor (18, 19). Based on these observations, we speculate that the net result of the targeting of the TBP–Dr1 complex may depend on the nature of the E1A protein involved. As schematically depicted in Fig. 4, we suggest that through an interaction of the E1A N-terminal sequences with the Dr1 factor, there is a displacement of TBP from the TBP–Dr1 inhibitory complex. This would then leave Dr1 associated with E1A or, alternatively, this interaction may dissociate to leave the free components. If the E1A_{13S} protein targets the complex, the CR3 sequences may then contact the TBP molecule. If this interaction were more stable than the interaction with Dr1, one could then envision a stable complex remaining involving E1A and TBP. In either case, there would be a displacement of an inhibitory activity from TBP, but in the case of the E1A_{13S} protein, an activating region would be left in association with TBP.

Finally, it is also worth mentioning that these findings demonstrate an activity of the E1A protein that correlates with the transforming capacity of E1A, defined by the requirement for the N-terminal domain. If the events of Dr1 inactivation and transformation are indeed related, this might suggest a role for Dr1 in regulating cell proliferation.

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1. Moran, E. & Mathews, M. B. (1987) *Cell* **48**, 177–178.
2. Nevins, J. R. (1992) *Science* **258**, 424–429.
3. Kraus, V. B., Moran, E. & Nevins, J. R. (1992) *Mol. Cell. Biol.* **12**, 4391–4399.
4. Raychaudhuri, P., Bagchi, S., Devoto, S. H., Kraus, V. B., Moran, E. & Nevins, J. R. (1991) *Genes Dev.* **5**, 1200–1211.
5. Inostroza, J. S., Mermelstein, F. H., Ha, I., Lane, W. S. & Reinberg, D. (1992) *Cell* **70**, 477–489.
6. Maldonado, E., Ha, I., Cortes, P., Weis, L. & Reinberg, D. (1990) *Mol. Cell. Biol.* **10**, 6335–6347.
7. Bagchi, S., Raychaudhuri, P. & Nevins, J. R. (1990) *Cell* **62**, 659–669.
8. Andersson, S., Davis, D. L., Dahlback, H., Jornvall, H. & Russell, D. W. (1989) *J. Biol. Chem.* **264**, 8222–8229.
9. Taylor, D. A., Kraus, V. B., Schwarz, J. K., Olson, E. N. & Kraus, W. E. (1993) *Mol. Cell. Biol.* **13**, 4714–4727.
10. Simon, M. C., Fisch, T. M., Benecke, B. J., Nevins, J. R. & Heintz, N. (1988) *Cell* **52**, 723–729.
11. Phelps, W. C., Bagchi, S., Barnes, J. A., Raychaudhuri, P., Kraus, V. B., Munger, K., Howley, P. M. & Nevins, J. R. (1991) *J. Virol.* **65**, 6922–6930.
12. Timmers, H. T. & Sharp, P. A. (1991) *Genes Dev.* **5**, 1946–1956.
13. Brou, C., Chandhoury, S., Davidson, I., Lutz, Y., Wu, J., Egly, J.-M., Tora, L. & Chambon, P. (1993) *EMBO J.* **12**, 489–499.
14. Stein, R. W., Corrigan, M., Yaciuk, P., Whelan, J. & Moran, E. (1990) *J. Virol.* **64**, 4421–4427.
15. Whyte, P., Ruley, H. E. & Harlow, E. (1988) *J. Virol.* **62**, 257–265.
16. Egan, C., Jelsma, T. N., Howe, J. A., Bayley, S. T., Ferguson, B. & Branton, P. E. (1988) *Mol. Cell. Biol.* **8**, 3955–3959.
17. Wu, L., Rosser, D. S. E., Schmidt, M. L. & Berk, A. (1987) *Nature (London)* **326**, 512–515.
18. Horikoshi, N., Maguire, K., Kralli, A., Maldonado, E., Reinberg, D. & Weinmann, R. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5124–5128.
19. Lee, W. S., Kao, C. C., Bryant, G. O., Liu, X. & Berk, A. J. (1991) *Cell* **67**, 365–376.