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ORIGINAL ARTICLE

The adenoviral E1A protein displaces corepressors and relieves gene repression by unliganded thyroid hormone receptors *in vivo*

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The human adenovirus type 5 early region 1A (E1A) is one of two oncogenes present in the adenovirus genome and functions by interfering with the activities of cellular regulatory proteins. The E1A gene is alternatively spliced to yield five products. Earlier studies have revealed that E1A can regulate the function of thyroid hormone (T3) receptors (TRs). However, analysis in yeast compared with transfection studies in mammalian cell cultures yields surprisingly different effects. Here, we have examined the effect of E1A on TR function by using the frog oocyte *in vivo* system, where the effects of E1A can be studied in the context of chromatin. We demonstrate that different isoforms of E1A have distinct effects on TR function. The two longest forms inhibit both the repression by unliganded TR and activation by T3-bound TR. We further show that E1A binds to unliganded TR to displace the endogenous corepressor nuclear receptor corepressor, thus relieving the repression by unliganded TR. On the other hand, in the presence of T3, E1A inhibits gene activation by T3-bound TR indirectly, through a mechanism that requires its binding domain for the general coactivator p300. Taken together, our results thus indicate that E1A affects TR function through distinct mechanisms that are dependent upon the presence or absence of T3.

***Keywords*:** adenoviral E1A, thyroid hormone receptor, corepressor, coactivator, chromatin
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Introduction

Thyroid hormone receptors (TRs) are believed to mediate the vast majority of diverse biological effects of thyroid hormone (T3). TRs belong to the superfamily of nuclear hormone receptors [1, 2]. One of the unique features of TRs is that they can constitutively, regardless of ligand availability, bind to T3 response elements (TREs) in the promoter region of T3-response genes. In other words, TRs can adopt either the unliganded or the liganded conformation, and both unliganded and liganded TRs are recruited to TREs [3-6]. Various *in vitro* studies have demonstrated that unliganded TRs repress T3-response genes and liganded TRs activate the same genes. The dual effects of TRs are accomplished by recruiting mutually exclusive sets of coregulators to the target promoters [3, 4, 7-20]. Corepressor complexes composed of N-CoR (nuclear receptor corepressor) or SMRT (silencing mediator of retinoid receptors and TRs) with HDAC3 (histone deacetylase 3), TBL1 (transducin beta-like protein 1)/TBLR1 (TBL1-related protein 1), and GPS2 (G-protein pathway suppressor 2) associate with unliganded TR and deacetylate histones [13-16, 21-31]. The presence of T3 induces a conformational change

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in TR, promoting the release of corepressor complexes and recruitment of coactivator complexes such as those composed of SRCs (steroid receptor coactivators) with p300 and pCAF (p300-associated factor), which increase histone acetylation [10-13, 17-19, 32-38].

T3 is critical for adult organ function and development in vertebrates [2, 12, 39, 40]. The effects of T3 are predominantly mediated by TRs [2, 12, 41], and alterations in the function of cellular proteins such as TR by viral proteins are important mechanisms in disease development and progression. The human adenovirus type 5 early region 1A (E1A) was originally identified as one of two oncogenes that are present in the adenovirus genome and functions by interfering with the activities of cellular regulatory proteins [42-44]. The E1A gene is alternatively spliced to yield five mRNA products (Figure 1). These spliced variants encode proteins ranging in size from 289 residues to 55 residues, among which E1A12S and E1A13S are the major products. E1A proteins, which do not directly bind DNA, associate with various key cellular proteins to regulate gene transcription and cell growth [42-50]. Using the human choriocarcinoma cell line, JEG3 cells, Wahlstrom *et al*. [51] demonstrated that the largest form of E1A, E1A13S, interacts with TR and activates TR-dependent gene transcription both in the absence and in the presence of T3. On the other hand, a recent study using a yeast system revealed that E1A13S protein interacts with TR and activates TR-dependent gene transcription only in the absence of T3 [20, 52]. The presence of T3 reduces the interaction of E1A with TR, but E1A is able to down-regulate TR-dependent gene transcription in the presence of T3 [20, 52]. It was proposed that this apparent discrepancy was due to the difference in the cellular context of co-regulatory proteins between yeast and mammalian cells. In contrast to mammalian cells, yeast is devoid of the p160 and p300/CBP co-activator proteins, as well as the N-CoR and SMRT co-repressor proteins [52].

In this study, we used the reconstituted frog oocyte system to examine the effects of E1A proteins in TR-dependent gene transcription. The frog oocyte system is an excellent model to explore the mechanism of TR-dependent gene regulation. First, minimal expression of endogenous TRs in frog oocytes allows us to define the basal transcription level of a TRE-controlled gene. Second, since frog oocytes contain abundant amounts of endogenous co-regulators that are required for TR-dependent gene regulation, the expression of TR is sufficient to simulate the physiological situation of TR action. Third, since DNA injected into the oocyte nucleus is remodeled into a minichromosomal structure, we can explore TR-dependent gene regulation in the context of chromatin [4, 53]. The results presented here demonstrate that E1A binds to unliganded TR to displace endogenous corepressor N-CoR, thus relieving the repression by unliganded TR. In the presence of T3, E1A does not interact with TR but inhibits gene activation by T3-bound TR indirectly, most likely through its binding to the general coactivator p300. These results thus support the argument that cellular context and ligand can influence the effects of E1A on gene regulation by TR.

Results

Differential effects of different E1A splice forms on TR function in vivo

To study the effects of E1A on gene regulation by TR in the context of chromatin *in vivo*, we analyzed the effect of full-length E1A13S on the transcription of a TR-target gene in the reconstituted *Xenopus laevis* oocyte

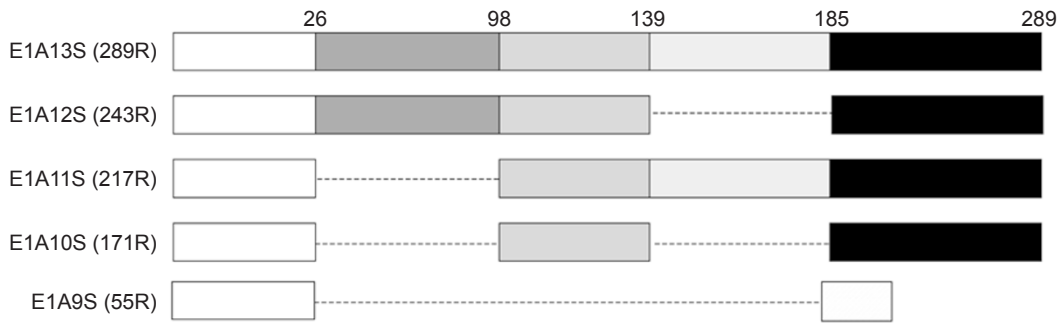

Figure 1 Schematic representation of E1A variants used in this study. The E1A gene is alternatively spliced to yield five mRNA products ranging in size from 13S to 9S. These encode proteins ranging in size from 289 residues (R) to 55 R. The positions for the ends of the regions encoded by alternatively spliced exons are indicated on the top. Note that splicing preserves the reading frame, except where indicated by a hatched box in E1A9S. E1A9S was not used in this study because it lacks most of the highly conserved functional domains.

system [4]. As a reporter for T3-dependent transcriptional activity, a plasmid containing the T3-dependent promoter of *X. laevis* TRβA gene driving the expression of firefly luciferase (TRE-Luc) was microinjected into the oocyte nucleus together with an internal control plasmid driving the expression of *Renilla* luciferase. Since the oocyte has little endogenous TR/RXR, *in vitro* transcribed mRNAs encoding FLAG-tagged *Xenopus* TRα and untagged RXR with or without the mRNA for myc-E1A13S were coinjected into the cytoplasm. After overnight incubation in the presence or absence of T3, the oocytes were lysed and assayed for luciferase activities. The ratio of firefly luciferase activity to *Renilla* luciferase was determined as a measure for transcription level from the reporter gene. In the absence of T3, over-expression of TR and RXR reduced the reporter gene transcription and E1A13S overexpression reversed this repression in a dose-dependent manner (Figure 2, lanes 2-4). When T3 was added to the oocyte culture medium, the repression by unliganded TR/RXR was relieved and the promoter was further activated (Figure 2, lane 5). Interestingly, unlike the promoter activation effect in the absence of T3, overexpression of E1A13S inhibited transcription in a dose-dependent manner in the presence of TR/RXR and T3 (Figure 2, compare lanes 6-7 with lane 5).

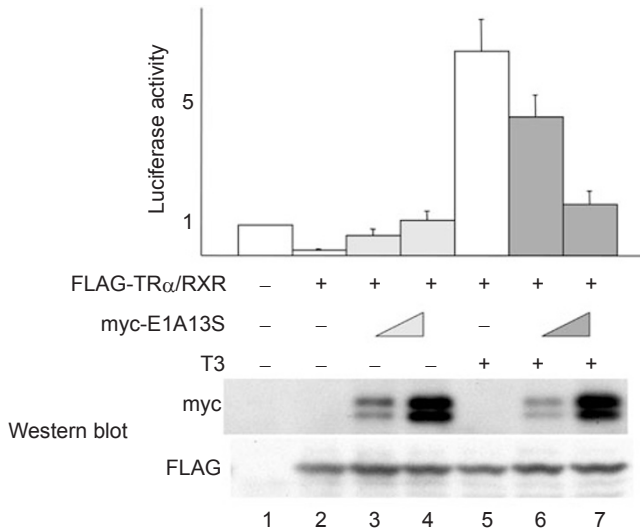

Figure 2 E1A13S protein relieves unliganded TR-induced gene repression and inhibits liganded TR-induced gene activation in the reconstituted frog oocyte system. The mRNAs for FLAG-TRα/RXR (5.75 ng/oocyte each) with or without increasing amounts of myc-tagged E1A13S mRNA (0.92 or 4.6 ng/oocyte) were injected into the cytoplasm of the frog oocytes. The firefly luciferase reporter vector (TRE-Luc) together with the control Renilla luciferase plasmid (tk-Luc) was then injected into the nucleus. After overnight incubation with or without 100 nM of T3, the oocytes were lysed and assayed for luciferase activities (top panel). As a measure of the reporter gene transcription level, the ratio of firefly luciferase activity to Renilla luciferase activity was determined and was normalized, with the basal level in the absence of T3 and TR as 1. The result from each group was expressed as a percentage of the basal transcription level that was obtained from the oocytes without TRα/RXR mRNA injection. This experiment was repeated three times. The same oocyte samples used in luciferase assay were subjected to western blotting with anti-myc and anti-FLAG antibodies to detect the E1A and TR expression, respectively, and representative results are shown in the lower panels, confirming the protein expression of E1A13S and FLAG-TRα.

We next investigated whether different splice forms of E1A had a similar effect on TR function. We analyzed four of the five splice forms. E1A9S was not analyzed as it lacked all of the common functional domains except the N-terminal 26 amino acids (aa), thus making it difficult to interpret the outcome of the experiment. We microinjected mRNA for myc-tagged E1A13S, E1A12S, E1A11S, or E1A10S together with mRNAs for TR/RXR into the cytoplasm of *Xenopus* oocytes, followed by the injection of the reporter DNA as above. Luciferase assays showed that E1A12S behaved similarly as E1A13S, i.e., inhibited both transcription repression by unliganded TR (Figure 3, lanes 5 and 6) and activation by liganded TR (Figure 3, lanes 15 and 16). On the other hand, E1A11S and E1A10S had no effect on the repression by unliganded TR (Figure 3, lanes 7-10). Interestingly, at high concentration, they enhanced the gene activation by T3-bound TR (Figure 3, lanes 17-20), in contrast to the inhibition observed with E1A13S and E1A12S (Figure 3, lanes 13-16). These contrasting effects were clearly due to isoform-specific functions since similar levels of the different isoforms were expressed after mRNA injection (Figure 3, bottom panels).

E1A competes with N-CoR for binding to TR in the absence of T3

Earlier studies have shown that E1A is capable of binding to unliganded TR and that full-length N-CoR could competitively inhibit the binding and functional effects of E1A [20, 52]. To investigate whether E1A affected TR function by competing for binding to TR, we carried out co-immunoprecipitation assay to analyze the binding of endogenous cofactors to TR in the presence or absence of E1A. Owing to the similar effects of E1A12S and E1A13S and the smaller size of E1A12S, we chose E1A12S for the remainder of the studies. We microinjected mRNAs for myc-E1A12S, FLAG-TR, and RXR into *Xenopus* oocytes. After overnight incubation in the presence or absence of T3, oocyte lysates were prepared and subjected to immunoprecipitation with anti-FLAG

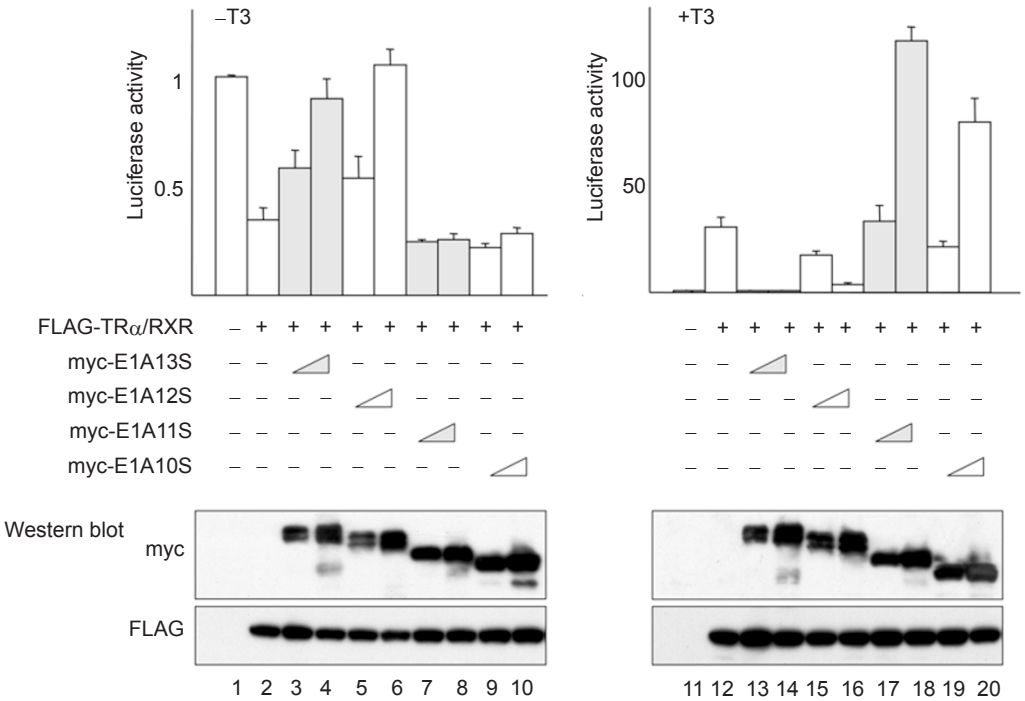

Figure 3 Differential effects of E1A variants on TR-regulated gene transcription. The mRNAs for FLAG-TRα/RXR (5.75 ng/oocyte) with or without increasing amounts of myc-tagged E1A13S, 12S, 11S, or 10S mRNA (0.92 or 4.6 ng/oocyte) were injected into the cytoplasm of the frog oocytes as indicated. After overnight incubation with or without 100 nM of T3, the oocytes were lysed for luciferase assays. The ratio of firefly luciferase activity to Renilla luciferase activity was determined as a measure of the reporter gene transcription level, with the basal level in the absence of TR set to 1 (top panels). The same oocyte samples were subjected to western blotting with anti-myc and anti-FLAG antibodies to confirm the protein expression (lower panels). This experiment was repeated twice with similar results. As observed with E1A13S, E1A12S derepressed unliganded TR-induced gene repression and inhibited liganded TR-induced gene activation. In contrast, the shorter forms of E1A, E1A11S and E1A10S, enhanced liganded TR-induced gene activation. E1A11S and E1A10S had little effect on unliganded TR-induced gene repression. Note that the + and –T3 samples were plotted on different scales to highlight the effects of E1A.

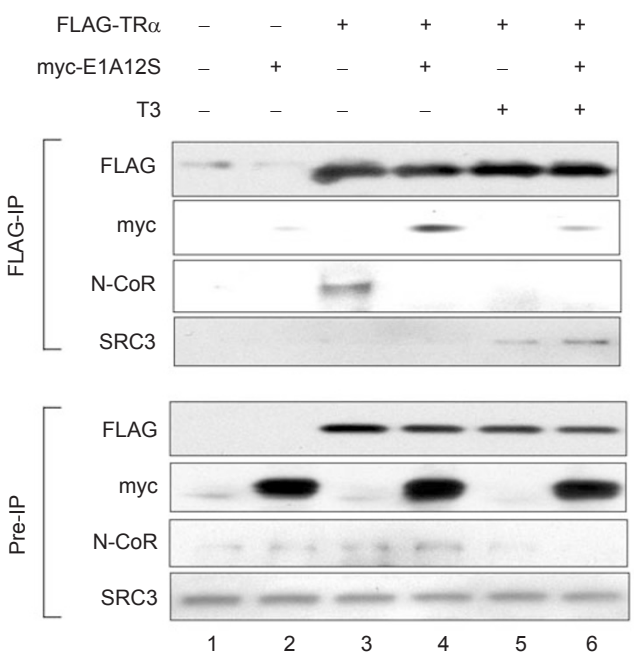

Figure 4 E1A12S competes against corepressor binding to unliganded TR but not coactivator binding to T3-bound TR. The mRNAs for FLAG-TRα/RXR (23 ng/oocyte each) with or without myc-E1A12S mRNA (4.6 ng/oocyte) were injected into the cytoplasm of oocytes as indicated. After overnight incubation with or without 100 nM of T3, the oocytes were lysed and subjected to IP with anti-FLAG antibody against TRα. Pre-IP lysates and IP samples were immunoblotted with anti-FLAG, anti-myc, anti-N-CoR, and anti-SRC3 antibodies. Myc-E1A12S was co-immunoprecipitated with FLAG-TR in the sample without T3 treatment (lane 4). The amount of co-immunoprecipitated myc-E1A12S was markedly reduced in the T3-treated sample (lane 6). Overexpression of myc-E1A12S dissociated the endogenous corepressor N-CoR from FLAG-TR, resembling T3 treatment (lanes 3-5). Unlike T3 treatment, however, the coactivator SRC3 binding to FLAG-TR was not affected by myc-E1A12S in the presence or absence of T3 (lanes 3-6). Note that the N-CoR signal in the pre-IP samples was expected to be the same in all lanes as it was from endogenous N-CoR in the oocyte. However, it appeared to be stronger in the center lanes but weaker in the flanking ones, especially lane 6. This was likely due to difficulty in transferring the large protein, resulting in some variation, with the center lanes being transferred better than the flanking ones. However, this does not affect the conclusion about the competition by myc-E1A12S against endogenous N-CoR for binding to TR, as shown by lanes 3 and 4 in the center.

antibody against the TR. The immunoprecipitates were analyzed by western blot with different antibodies. As expected, similar amounts of FLAG-TR were immunoprecipitated in all samples with mRNA injection (Figure 4, lanes 3-6). Western blot with the antibody against the myc-tag indicated that E1A was co-immunoprecipitated with TR in the absence of T3 (Figure 4, lane 3), while in the presence of T3 the co-immunoprecipitated E1A was significantly reduced (Figure 4, lane 6). Similarly, the corepressor N-CoR was also co-immunoprecipitated with TR in the absence of T3 (Figure 4, lane 3). As expected, N-CoR dissociated from TR in the presence of T3 (Figure 4, lane 5). In the presence of E1A, N-CoR binding to TR was abolished even in the absence of T3 (Figure 4, lane 4 vs 3), suggesting that E1A competed against N-CoR for binding to unliganded TR. On the other hand, the co-activator SRC3 was, expectedly, not bound to unliganded TR (Figure 4, lane 3), but bound to TR in the presence of T3 (Figure 4, lane 5). This ligand-dependent binding of SRC3 to TR was not significantly affected by E1A over-

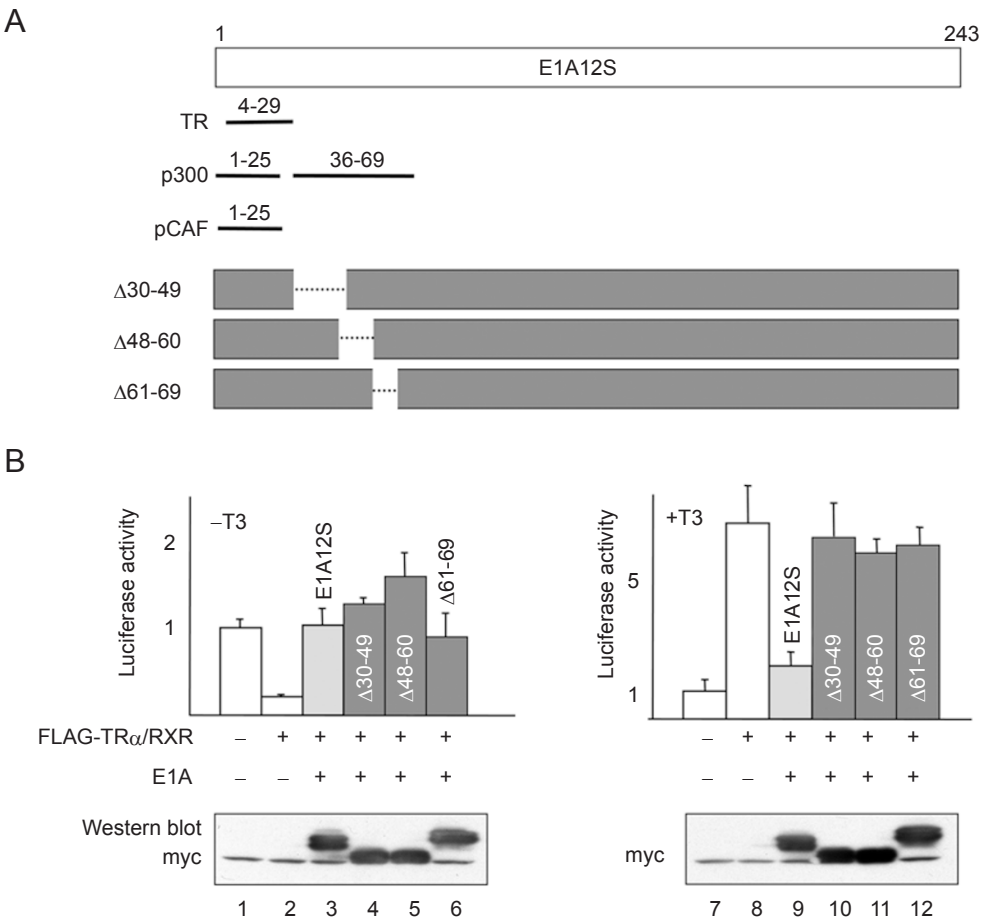

Figure 5 Effects of mutant E1A12S on TR-regulated reporter gene transcription. **(A)** Schematic diagrams of E1A12S and its mutants. The regions in wild-type E1A12S protein that are required for interaction with TR, p300, and pCAF are shown. Three deletion mutant constructs, Δ30-49, Δ48-60, and Δ61-69 were generated in the context of the E1A12S cDNA by PCR-based mutagenesis. All of these mutants retained the TR-binding site (aa 4-29 [52]) and the ability to bind pCAF (aa 1-25 [55, 56]). Mutants Δ30-49, Δ48-60, and Δ61-69 are unable to bind p300 [54]. **(B)** The mRNAs for FLAG-TRα/RXR (5.75 ng/oocyte each) with or without the mRNA for myc-tagged E1A12S, Δ30-49, Δ48-60, or Δ61-69 (4.6 ng/oocyte), were injected into the cytoplasm of the frog oocytes as indicated. The reporter DNA was injected next. After overnight incubation with (lanes 7-12) or without (lanes 1-6) 100 nM of T3, the oocytes were lysed for luciferase assays. The ratio of firefly luciferase activity to Renilla luciferase activity was determined as a measure of the reporter gene transcription level, with the basal level in the absence of TR set to 1 (top panels). The same oocyte samples were subjected to western blotting with anti-myc antibody to show similar levels of the expression of different E1A mutants (bottom panels). As shown in the left panel, all of these mutants de-repressed unliganded TR-induced gene repression just like E1A12S. In contrast to E1A12S, which inhibited liganded TR-induced gene activation, all these mutants had minimal effect on liganded TR-induced gene activation. This experiment was repeated twice, with similar results.

expression (Figure 4, lane 6), consistent with the reduced binding of E1A to TR in the presence of T3.

The p300-binding domain of E1A is required for its ability to inhibit TR function in the presence of T3

The N-terminus of E1A binds to the general transcription coactivators p300 and pCAF, as well as to TR, via a short-peptide CoR-NR interaction motif at aa 20-28 (LDQLIEEVL) (Figure 5A) [20]. The binding of p300 requires E1A aa 1-25 and aa 36-69 [54], and pCAF binding requires aa 1-25 [55, 56]. Thus, to investigate how E1A inhibits gene activation by liganded TR, we analyzed the effects of three E1A mutants that retained the ability to bind to TR and pCAF but not p300 (Figure 5A). As shown in Figure 5B, the wild-type E1A12S again inhibited both the repression by unliganded TR (lane 3) and activation by T3-bound TR (lane 9). All three mutant E1A12S inhibited the repression by unliganded TR (Figure 5B, lanes 4-6), just like the wild-type E1A12S. On the other hand, all these mutants failed to affect the activation by TR in the presence of T3 (Figure 5B, lanes 10-12). Since the mutants bind pCAF but not p300 [55, 56], our results suggest that E1A inhibits gene activation by T3-bound TR by binding p300 but not pCAF.

Discussion

The human adenoviral gene E1A encodes five proteins due to alternative splicing. The E1A proteins have been shown to affect diverse cellular processes, leading to disease development. Earlier studies have shown that the longest form of E1A, E1A13S, is capable of affecting transcriptional regulation by TR. Interestingly, different results were obtained in the yeast model system compared with the mammalian cell culture transfection studies, suggesting that cellular context and/or chromatin structure may influence how E1A affects TR function. By using the reconstituted frog oocyte model system, where the effect of E1A can be studied in the context of chromatin *in vivo*, we have shown for the first time that different E1A isoforms have distinct effects on gene regulation by TR. More importantly, we show that E1A long forms inhibit gene regulation by TR in both the presence and absence of T3, but with distinct mechanisms.

In the reconstituted frog oocyte system, the reporter gene is assembled into chromatin and this allows one to study both gene repression by unliganded TR and activation by liganded TR in the chromatin context [4, 53]. Overexpression of E1A13S inhibited the repression by unliganded TR, as well as the activation by T3-bound TR. While these findings appear to differ from the yeast studies [20, 52] and the transient transfection studies in mammalian cell cultures, a careful analysis suggests that the results are consistent in several key aspects. First, in the absence of T3, E1A enhanced transcription of TR-regulated genes in all three systems. In the transient transfection assays [51], although the authors focused the discussion on the effects in the presence of T3, there was clear upregulation of the reporter gene expression by E1A in the absence of T3. This is consistent with the binding of E1A to unliganded TR to release corepressors, as we have shown in this study. In yeast, there is no repression by unliganded TR due to the lack of equivalent N-CoR type of corepressors that TRs utilize in vertebrate cells and thus, a different mechanism would likely exist there. Second, our immunoprecipitation data demonstrated for the first time that TR binds to E1A *in vivo* in the absence of T3. This is consistent with the two-hybrid studies in yeast and with the *in vitro* GST-fusion protein pull-down assays in earlier studies [20, 52]. Third, our immunoprecipitation data showed that TR binding to E1A *in vivo* was reduced in the presence of T3. While this contrasts with the *in vitro* GST-fusion protein pull-down assays that showed that TR bound to E1A largely independently of T3, it is consistent with the yeast two-hybrid assays that showed a T3-induced dissociation of E1A from TR *in vivo* [20, 52]. Taken together, these data suggest that the different findings on the binding between TR and E1A were likely due to either inappropriate conformation of the proteins in the *in vitro* assays or, more likely, the involvement of other proteins that rendered the T3-dependent dissociation of E1A from TR *in vivo*.

One of the major differences among the three studies conducted in different model systems to date is that we observed an inhibition of T3-induced activation by E1A. In mammalian cells E1A further activated the reporter, while in yeast the constitutive activation induced by E1A was downregulated in the presence of T3 and overcome whenever SRC1 or GRIP1 coactivators were present [20, 52]. This discrepancy is likely due to the difference in cellular context and/or the chromatin structure of the reporter. As indicated above, E1A had much weaker or little interaction with TR *in vivo* when T3 was present. Thus, it is unclear how E1A influences TR function when T3 is present. Our mutational analysis provided one possible mechanism. Any deletions affecting the p300 interacting domains of E1A abolished the inhibition of E1A on transcriptional activation by liganded TR, but not its inhibition on the repression by unliganded TR. This suggests that in the presence of T3, E1A affected TR function through its association with p300, a protein that is absent in the cellular context of yeast. Liganded TR is known to bind coactivators such as SRCs [10-13, 17-19, 35, 36, 38]. SRCs in turn form large complexes contain-

ing p300/CBP [32-34, 37]. Thus, it is possible that E1A interferes with liganded TR *in vivo* by disrupting the SRC-p300 type of coactivator complexes important for gene activation either through squelching or at the target promoter. As yeast lacks the SRC type of coactivators, E1A, therefore, has little effect on the promoter activity in the presence of T3. On the other hand, the different levels and/or compositions of coactivators present in the mammalian JEG cells used in the transient transfection study compared with those in the frog oocyte might underlie the observed enhancement by E1A on gene activation by liganded TR in JEG cells. Alternatively, the SRC-p300 coactivator complexes may play a more critical role in the frog oocyte system, where the promoter DNA is packaged into chromatin. The interaction of E1A with p300 may interfere with the histone acetyltransferase activity important for gene activation on this chromatinized template. In the transient transfection studies in JEG cells, the reporter plasmid likely has a less compact chromatin structure. In this case, the histone acetyltransferase activity of SCR-p300 complexes may thus not be critical. This coupled with different cofactor compositions and levels is likely responsible for the observed activation by E1A, which functions as an activator of viral transcription [42-44]. These cellular context-dependent effects of E1A on TR function clearly deserve further studies in the future as this will not only help our understanding of how TR functions but also have implications for the pathogenic effects of E1A.

E1A has five different spliced forms. With the exception of the smallest form, all differ from each other by the inclusion of different number of alternatively spliced domains. Interestingly, the two longest forms, E1A13S and E1SA12S, both contain the N-terminal TR- and p300-binding domains and can inhibit the repression and activation by TR in the absence and presence of T3, respectively. In contrast, the two shorter forms, E1A11S and E1A10S, lack the intact N-terminal domains for binding to TR and p300, and failed to inhibit either the repression by unliganded TR or the activation by T3-bound TR. Instead, at high levels of overexpression, these short forms actually enhanced gene activation by TR in the presence of T3. These interesting findings may help to explain some of the differences observed in the earlier studies in yeast and JEG cells. First, although *in vitro* GST-fusion protein pull-down assays suggest that E1A has multiple regions that bind to TR [20, 51, 52], a yeast two-hybrid assay showed that the N-terminal 29 aa were essential for the interaction *in vivo* [20], and further studies identified a leucine-rich CoR-NR box consensus motif in the N-terminal aa 20-28 of E1A, similar to those in the receptor-interacting domains of N-CoR and SMRT [52]. Our transcription assay *in vivo* also showed a requirement of this region for interaction with TR *in vivo*. Specifically, the isoforms produced as a result of alternative splicing lack aa 26-98. This removes several key residues required for interaction with TR, and these E1A isoforms lose the ability to inhibit repression by unliganded TR. Thus, while other regions of E1A may interact with TR *in vitro*, the interaction is likely too weak *in vivo* and/or not able to disrupt the interaction between endogenous corepressors and unliganded TR in order to affect repression. Second, the smaller isoforms, E1A11S and E1A10S, also lack the functional domain for binding to p300 and pCAF. Consistent with our studies with the E1A mutants, these forms failed to inhibit activation by T3-bound TR. Furthermore, the ability of these short forms to enhance activation by TR in the presence of T3 also supports the model proposed above. Specifically, SRC-p300 complexes play an essential role in T3-dependent gene activation in the frog oocyte system. In the absence of inhibition caused by E1A interaction with p300, the activation effects of other regions of the multifunctional E1A proteins now become detectable. In JEG cells, SRC-p300 complexes may be less critical for gene activation by liganded TR, or function through a mechanism that cannot be disrupted by E1A binding to p300. E1A thus does not inhibit gene activation by T3-TR through its N-terminal p300-binding domain, but can enhance the transcription through its C-terminal domains. In this regard, it is worth noting that yeast lacks an N-CoR suppressor of TR in the absence of ligand. N-CoR, when coexpressed in yeast, can function as a repressor. Interestingly, however, spliced variants of N-CoR devoid of its repressor domains can act as an activator via its intact receptor-interacting domains (CoR-NR box motifs) [52, 57]. Since the cellular context of yeast also lacks SRC-p300 complexes needed for E1A to inhibit gene activation by T3-TR, E1A functions as a TR coactivator in this model system [20, 52, 57]. Clearly, further studies are needed to clarify all the details of the mechanism.

In conclusion, our present studies suggest that E1A can affect the function of both unliganded TR and T3-bound TR, but through distinct mechanisms, inhibiting repression through direct competition against corepressors for binding to TR and affecting activation indirectly through its interaction with coactivators such as p300. Both E1A and TR are known oncogenes. Unliganded TR mimics the viral oncogene v-erbA, the viral homolog of TR that cannot bind to T3, in promoting cell proliferation and inhibiting cell differentiation, while liganded TR does the opposite in cell cultures [58-61]. Thus, it will be of interest in the future to investigate how wild-type and different mutant E1As may interact with TR in regulating

cell growth and differentiation in different cell types with different cofactor compositions. Such studies will likely provide novel insights on how viruses utilize various cellular mechanisms to transform host cells.

Materials and Methods

Cloning and constructs

JMB312, JMB1024, JMB1338, JMB1390, JMB2218, JMB2221, and JMB2246 vectors, which contain cDNAs for E1A13S, E1A12S, E1A11S, E1A10S, E1A12SΔ30-49, E1A12SΔ48-60, and E1A12SΔ61-69, respectively, were described previously or constructed by PCR-based mutagenesis [20]. The sequences of these E1A variants and E1A12S mutants were tagged with a myc sequence at their 5′-end and subcloned into the T7Ts vector, which contains the 5′- and 3′-untranslated regions of the *X. laevis* β-globin gene flanking the multiple cloning sites [62].

Luciferase assays using frog oocytes

The plasmids pSP64-FLAG-TRα and pSP64-RXRα [4] were used to synthesize the corresponding mRNAs with a SP6 *in vitro* transcription kit (mMESSAGE mMACHINE; Ambion); T7Ts-myc-E1A13S, 12S, 11S, 10S, E1A12SΔ30-49, E1A12SΔ48-60, and E1A12SΔ61-69 were used to synthesize the corresponding mRNAs with a T7 *in vitro* transcription kit (mMESSAGE mMACHINE; Ambion). The mRNAs for FLAG-TRα/RXR (5.75 ng/oocyte each) and/or mRNAs for myc-E1As (5.75 or 23 ng/oocyte) were injected into the cytoplasm of 12 *X. laevis* stage VI oocytes. The reporter plasmid DNA (0.33 ng/oocyte), which contained the T3-dependent *Xenopus* TRβ promoter driving the expression of the firefly luciferase, was injected into the oocyte nucleus, together with a control construct that contains the herpes simplex virus tk promoter driving the expression of *Renilla* luciferase (0.03 ng/oocyte). Following overnight incubation at 18 °C in the absence or presence of 100 nM T3, oocytes were prepared for luciferase assay by the Dual-Luciferase Reporter Assay system (Promega), according to the manufacturer's recommendations. To verify the protein translation from the injected mRNAs, the same oocyte lysates were subjected to western blotting using anti-FLAG antibody (Sigma) for detection of FLAG-TRα and anti-myc antibody (Invitrogen) for detection of myc-E1A proteins.

Co-immunoprecipitation using frog oocytes

The above-prepared mRNAs for FLAG-TRα/RXR (23 ng/oocyte each) and/or mRNA for myc-E1A12S (23 ng/oocyte) were injected into the cytoplasm of 20 *X. laevis* stage VI oocytes. After overnight incubation at 18 °C with or without 100 nM of T3, the oocytes were lysed in IP buffer (20 mM HEPES, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM glycerophosphate, 50 mM NaCl, 0.1% NP-40, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Roche Applied Science)). After centrifugation at 14 000 rpm for 10 min at 4 °C, parts of the supernatant were kept as input samples and the rest were used for immunoprecipitation with Ezview Red ANTI-FLAG M2 Affinity Gel (Sigma). Each lysate was incubated with the gel for 4 h and washed three times in the same IP buffer. The immunoprecipitates were boiled in sodium dodecyl sulfate (SDS) loading buffer, separated on an SDS-polyacrylamide gel, and immunoblotted with anti-myc, anti-N-CoR [63], or anti-SRC3 antibody [36].

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