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Adenovirus E1A Functions as a Cofactor for Retinoic Acid Receptor β (RARβ) through Direct Interaction with RARβ

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Transcription regulation by DNA-bound activators is thought to be mediated by a direct interaction between these proteins and TATA-binding protein (TBP), TFIIB, or TBP-associated factors, although occasionally cofactors or adapters are required. For ligand-induced activation by the retinoic acid receptor-retinoid X receptor (RAR-RXR) heterodimer, the RARβ2 promoter is dependent on the presence of E1A or E1A-like activity, since this promoter is activated by retinoic acid only in cells expressing such proteins. The mechanism underlying this E1A requirement is largely unknown. We now show that direct interaction between RAR and E1A is a requirement for retinoic acid-induced RARβ2 activation. The activity of the hormone-dependent activation function 2 (AF-2) of RARβ is upregulated by E1A, and an interaction between this region and E1A was observed, but not with AF-1 or AF-2 of RXRα. This interaction is dependent on conserved region III (CRIII), the 13S mRNA-specific region of E1A. Deletion analysis within this region indicated that the complete CRIII is needed for activation. The putative zinc finger region is crucial, probably as a consequence of interaction with TBP. Furthermore, the region surrounding amino acid 178, partially overlapping with the TBP binding region, is involved in both binding to and activation by AF-2. We propose that E1A functions as a cofactor by interacting with both TBP and RAR, thereby stabilizing the preinitiation complex.

Transcription of RNA polymerase II promoters requires the assembly of the preinitiation complex, consisting of basal transcription factors. This process begins with the binding of TFIID, consisting of the TATA-binding protein (TBP) and TBP-associated factors, to the TATA box, followed by ordered binding of the other transcription factors (TFIIA, -B, -E, -F, and -H) and RNA polymerase, forming the initiation complex (29). Transcription factors bound to promoter or enhancer sequences modulate the activity of RNA polymerase II promoters. These transcriptional activators are thought to function by directly or indirectly interacting with a component of the basal transcription machinery, thereby stabilizing the preinitiation complex (14, 47). Several activators have been shown to interact with TBP (33, 49), TFIIB (3, 37), or TBP-associated factors (27, 30). However, there is evidence that some activators require additional proteins, termed adapters, cofactors, or bridging proteins (29, 64). The presence of such factors was first hypothesized from experiments in which overexpression of a given activator could inhibit its own activity as well as the activity of activators belonging to the same class of activators, with all of the activators thus functioning by the same mechanism (28, 55, 56). This repression, referred to as squelching, is thought to be caused by the titration of a limiting component required for activation by promoter-bound activators (28).

The adenovirus E1A protein has been shown to activate a variety of both viral and cellular promoters (24), while this protein is unable to bind DNA (23). This has led to the suggestion that E1A could activate transcription through an interaction with various transcription factors and could thus behave as a cofactor-like protein. Comparison of E1A proteins of various adenovirus strains revealed the presence of three conserved regions which fulfill specific functions (48, 55). From the E1A gene, two alternatively spliced mRNAs are transcribed, a 12S and a 13S form encoding proteins of 243 and 289 amino acids (aa), respectively (6). The two proteins differ by the presence of conserved region III (CRIII) in the longer form, which is the most important region for transcriptional activation (24) and which contains a zinc-binding region (15). A direct interaction between E1A and TBP (34, 43) or TFIID (7), which was found to be dependent on the presence of the 13S mRNA-specific region, has been reported. Furthermore, it was shown that this region is responsible for activation by promoter-bound transcription factors (50).

The activity of retinoic acid (RA) is mediated by two types of receptors, the RA receptors (RARs) and the retinoid X receptors (RXRs). Both receptors belong to the steroid-thyroid hormone receptor superfamily and can activate transcription in the presence of their ligands, all-*trans* RA or 9-*cis* RA, the latter being specific for RXR whereas RARs can be activated by both isomers. RAR and RXR, like other members of this family of transcription factors, have similar domain structures: an autonomous activation domain (activation function 1 [AF-1]) in the N-terminal part of the receptor (AB region), a highly conserved DNA-binding domain (C region), and a hormone-binding domain (E region) containing a region involved in dimerization as well as an autonomous AF (AF-2) (22, 44).

RA has profound effects on cell growth and differentiation, and furthermore, an important role for RA in embryonal development has been suggested (20, 21, 31). For the study of the mechanism of retinoids in differentiation, embryonal carcinoma (EC) cell lines have been very helpful, as they can differentiate into a variety of cell types upon administration of RA (54), causing extensive changes in gene expression (31). One of the first events observed upon RA-induced differenti-

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ation of EC cells is the induction of RARβ2. Cloning of the promoter (17) and subsequent analysis of other RA-responsive promoters revealed that an element consisting of two repeats of the sequence (A/G)G(G/T)TCA separated by 2 or 5 bp is required for RA-induced activation of such promoters (reference 44 and references therein). Binding to such an RA response element (RARE) is possible only by RAR-RXR heterodimers, of which RAR has been shown to bind the more 3′ repeat, while RXR binds preferentially to the more 5′ repeat (40, 52, 62).

We have observed that the RARβ2 promoter is highly active in P19 EC cells in the presence of RA, but not in their differentiated derivative, END2 cells (39). In EC cells, E1A-like activity has been identified by the ability of specific adenovirus mutants which are unable to produce functional E1A protein to grow in these cells, while in differentiated EC cells this was not observed (35, 42). Furthermore, several promoters which require E1A are active in EC cells but not in their differentiated counterparts (24). This E1A-like activity is also present in mouse oocytes and preimplantation embryos but is lost during subsequent embryonal development (70). Introduction of E1A proteins in EC cells can result in differentiation (57, 69), whereas RA-differentiated cell lines can be reverted to a less differentiated form (16, 74), suggesting that regulation of E1A-like activity expression is important for EC cell differentiation. This led us to hypothesize that E1A or E1A-like activity may be involved in RARβ promoter activation. It was observed previously that many cell lines do not express the RARβ2 isoform, in either the presence or the absence of RA (72). Transfection experiments with some of these cell lines have revealed that the RARβ2 promoter cannot be activated by RA, while upon cotransfection of the gene product of the 13S mRNA of E1A (E1A 13S), RA-dependent activation could be observed (39). We identified two promoter regions as being involved in this E1A-dependent upregulation: a cyclic AMP response element (CRE)-like element (from positions −99 to −92) and the region from −63 to +156. The first element has been demonstrated to bind members of the CREB/ATF family (38). Some members of this family have been shown to interact with E1A, causing transcriptional activation, possibly by bringing the upstream activator close to the basal transcription machinery (12, 51). The second region contains the transcription start site, the TATA box, and the RARE, of which the last has been shown to be the most important regulatory element for this promoter (17). In this study, we have focused on the mechanism underlying the E1A-dependent upregulation of the RARE-containing promoter element and investigated whether the activity of the RAR-RXR heterodimer is upregulated by E1A. We show here that the activity of AF-2 of RARβ is upregulated by E1A 13S but not by the shorter, 12S form. Transfection experiments using large amounts of AF-2-containing activators indicate that cotransfection of E1A 13S could prevent squelching caused by this overexpression, suggesting that E1A functions as a bridging protein or cofactor. For this upregulation by E1A, a direct interaction between the hormone-binding domain of RARβ and the C-terminal part of CRIII of E1A is required, since all mutants that lost the ability to bind were also impaired in E1A-dependent activation. We suggest that E1A forms a bridge between AF-2 of RARβ and TBP through interactions of these proteins with E1A.

MATERIALS AND METHODS

Plasmids. GST-RARβ (aa 138 to 448) was produced by cloning the blunt *Sal*I-*Xba*I fragment of pSG5 RARβ in the *Sma*I site of pGEX 2T. GST-RXRα (aa 1 to 466) was made by cloning the *Eco*RI fragment of pSG5 mouse RXRα (mRXRα) in pGEX 2T. GST-E1A, GST-TBP, and GST-TFIIB were kindly provided by M. Timmers. The E1A 12S and E1A 13S expression plasmids containing genomic sequences of serotype 5 E1A genes have been described before (38). The Rous sarcoma virus-driven E1A expression constructs E1A, 12S, 13S, 5/3, NC, CX, 3/2, and C3/MX were provided by N. C. Jones (67). The GST-E1A mutants were constructed by amplifying parts of the E1A gene from these plasmids by PCR using a forward primer (P1) containing a *Bam*HI site linked to the first codon of E1A in frame with pGEX 2T and a reverse primer (P2) ending after the stop codon, extended with an *Eco*RI site. The *Bam*HI-*Eco*RI-digested PCR products were subsequently cloned in the corresponding sites of pGEX 2T. An *Eco*RV site inserted at aa 139 and 140 (aa 139-140) and a *Sca*I site at aa 177-178 (inserted without changing the codons) were introduced into E1A 13S by site-directed mutagenesis. Internal deletions were made by using these introduced sites together with the *Sma*I (aa 150-151) or the end-filled *Dra*II (aa 164-165) site. N1 was produced by digesting GST-E1A with *Bam*HI and *Eco*RV (aa 139-140) and ligated back after Klenow treatment. The *Sma*I-*Eco*RI and *Sca*I-*Eco*RI fragments were cloned in *Sma*I-*Eco*RI-digested pGEX 3X to generate N2 and N3, respectively. C1 and C4 were created by cloning the *Bam*HI-*Xba*I (made blunt ended with Klenow fragment) fragment and *Bam*HI-*Sca*I fragment, respectively, in *Bam*HI-*Sma*I-digested pGEX 2T. For C3, a PCR fragment was generated by using P1 and P3 (primer ending at codon 185 extended with an *Eco*RI site), and after digestion with *Bam*HI and *Eco*RI it was cloned in the corresponding sites of pGEX 2T. Digestion of this clone with *Bam*HI and *Dra*III, treatment with Klenow fragment, and ligation resulted in mutant 132-185. C2 was made by cloning the *Bam*HI-*Dde*I (blunt-ended)-digested fragment in *Sma*I-*Bam*HI-digested pGEX 2T. Mutants C157S (with a C-to-S change at aa 157), C174S, C179S, S185R, and V187L were made by PCR using P1 or P2 and primers that alter the codons concerned. These PCR products were digested with *Acc*I-*Sma*I or *Acc*I-*Xba*I and cloned in the corresponding sites of pGEX-E1A. Deletion constructs Δ178-179, Δ179-185, and Δ185-187 were made by using the mutants described above in which we introduced the restriction sites *Sca*I (aa 177-178), *Nae*I (aa 179-180), *Stu*I (aa 184-185), and *Afl*II (aa 186 to 188). Mutant Δ185-187 was made by cloning the *Eco*RI-*Afl*II (blunt-ended) fragment of mutant V187A in *Stu*I-*Eco*RI-digested mutant S185R. Expression constructs were made by cloning the corresponding *Sty*I-*Xba*I fragments from the GST-E1A mutants in the E1A-containing expression vector digested with the same enzymes.

Gal-Luc, the reporter containing five GAL binding sites in front of the E1b TATA box, and the human RARβ (hRARβ) promoter construct −1470+156 Luc were made by cloning the promoter sequences from the reporters GAL-CAT (48) and −1470+156 CAT (38) in the promoterless luciferase reporter construct pLUC.

Cell culture and transient transfection. Cells were cultured as described previously (25), and transient transfections were performed by calcium phosphate precipitation as described previously (25) by using 5 to 8 μg of the indicated reporter with 1 μg of the indicated activator and 1 μg of E1A 12S or 13S when indicated. As an internal control for transfection efficiency, 1 μg of SV2-LacZ (not affected by RA treatment or E1A cotransfection) was added. After 16 h, the precipitate was removed and the cells were cultured for another 24 h in the presence of 1.0 μM RA. Chloramphenicol acetyltransferase (CAT) activity was determined and normalized for transfection efficiency by using the β-galactosidase assay (25). Results are the means (± standard errors of the means [SEM]) of at least five independent or three duplicate experiments.

Luciferase assays were performed as described by Brasier et al. (8), with minor modifications. Extracts for this assay were made by lysing the cells on plates in 400 μl of Triton-lysis buffer consisting of 1% Triton X-100, 25 mM glycylglycin (pH 7.8), 15 mM $MgSO_4$, 4 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 1 mM dithiothreitol. Lysates were transferred to Eppendorf tubes and pelleted by centrifugation (10 min, 12,000 × *g* at 4°C) and directly used to assay luciferase activity. A 75-μl volume of cell extract was added to a 265-μl reaction mixture (3.7 mM ATP, 34 mM glycylglycin [pH 7.8], and 20 mM $MgSO_4$). Reactions were started upon addition of 100 μl of 0.2 mM luciferin, light emission was integrated over 10 s in a Lumac/3M Biocounter, and the results were normalized for transfection efficiency by measuring β-galactosidase activity.

For the β-galactosidase assay, 5 μl of CAT extract or 50 μl of luciferase extract, 145 or 100 μl of 100 mM phosphate buffer (pH 7.8), and 90 μl of LacZ buffer (30 mM $NaPO_4$ [pH 7.0], 3 mM KCl, 0.3 mM $MgSO_4$, 15 mM β-mercaptoethanol, and 20 μg of *ortho*-nitrophenyl-β-D-galactopyranoside) were combined in a microtiter plate well. After 5 to 60 min, β-galactosidase activity was determined by measuring the A_{417} in an enzyme-linked immunosorbent assay plate reader.

GST pull-down assay. Linearized plasmids (pSG5-RARβ and pSG5-RXRα, provided by P. Chambon) were transcribed by using T7 RNA polymerase and translated in vitro by using rabbit reticulocyte lysate in the presence of [^{35}S]methionine as recommended by the manufacturer (Gibco-Life). Glutathione *S*-transferase (GST) fusion proteins were purified as described by Hateboer et al. (33). Binding was performed in 450 μl of binding buffer (250 mM NaCl, 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-KOH [pH 7.5], 0.5 mM EDTA, 0.1% [vol/vol] Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 100 μg of bovine serum albumin per ml) using 1 to 5 μl of in vitro-translated protein together with 1 to 2 μg of GST fusion protein bound to glutathione-Sepharose for 2 h at 4°C. After four washings in

binding buffer (4°C, 1 ml), bound proteins were eluted by being boiled for 5 min in sample buffer and separated on sodium dodecyl sulfate (SDS)–10 to 12.5% (wt/vol) polyacrylamide gels. To compare the binding strength, 1/10 of the total input of in vitro-translated protein was run next to the GST-purified proteins. Quantification of GST-bound complexes was performed with a PhosphorImager (Molecular Dynamics).

Western blotting (immunoblotting). Whole-cell extract from transiently transfected COS cells was prepared by three sequential freeze-thaw cycles (−80 and 4°C) in 50 to 100 μl of lysis buffer (20 mM Tris [pH 7.5], 20% [vol/vol] glycerol, and 400 mM KCl) with 0.2 mM phenylmethylsulfonyl fluoride and protease inhibitors (aprotinin, leupeptin, pepstatin, and chymostatin; final concentration, 1.0 μg of each per ml). Equivalent amounts of extract were loaded, separated by SDS–8 to 12.5% (wt/vol) polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose by using a semidry blot apparatus. The membranes were blocked in 4% (wt/vol) nonfat dry milk in PBST (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, and 1% [vol/vol] Tween 20) for 1 h. The blots were incubated with a monoclonal antibody against either E1A or a hemagglutinin (HA) tag in PBST containing 2% (wt/vol) nonfat dry milk for 2 h. After seven washes in PBST, the blots were incubated with peroxidase-conjugated second antibodies in PBST containing 2% (wt/vol) nonfat dry milk. After seven washes with PBST, the blots were developed with an ECL kit (Amersham).

Immunoprecipitations. A monoclonal antibody to the HA tag (12CA5) was chemically cross-linked to protein A-Sepharose by using dimethylpimelidate. The antibody was bound to protein A-Sepharose beads, washed three times with 0.1 M borate buffer (pH 8.0) and three times with 0.2 M triethanolamine (pH 8.5), and then cross-linked for 1 h at room temperature with 40 mM dimethylpimelidate in triethanolamine (pH 8.5). Subsequently, three washes with 40 mM triethanolamine (pH 8.5) and three washes with 0.1 M borate buffer (pH 8.0) were performed. Immunoprecipitations were performed at 4°C, using 50 μl of chemically cross-linked E1A–protein A-Sepharose and 25 to 100 μg of whole-cell extract of COS cells transfected with E1A 13S, HA-TBP, or HA-RARβ in 750 μl of binding buffer (see "GST pull-down assay" above). After 2 h, the beads were washed four times with the binding buffer. Antigen-antibody complexes were eluted by incubation at 100°C in sample buffer, subsequently loaded on SDS-PAGE gels, transferred to nitrocellulose, and immunoprobed as described above.

RESULTS

AF-2 of RARβ is upregulated by E1A. We and others have previously shown that both RAR and RXR contain two autonomous cell- and promoter-specific AFs (25, 59, 60). To test whether any AF of the RAR-RXR heterodimer is involved in E1A-induced RARβ promoter activation, AFs of both RARβ and RXRα were coupled to the DNA-binding domain of the *Saccharomyces cerevisiae* transcription factor GAL4 (Fig. 1A) and transfected into COS cells together with GAL-CAT, a GAL-responsive reporter (48). As shown in Fig. 1B, the activity of RAR AF-2 is significantly upregulated (sevenfold) by E1A 13S and not by the 12S form, whereas RXR AF-2 is not affected by cotransfection of E1A. Similar results were obtained with a Rous sarcoma virus-driven E1A expression construct (data not shown). No induction by E1A was observed in the absence of RA or activator (data not shown). The ligand-independent AFs (AF-1) of both RARβ and RXRα were both slightly (twofold) upregulated by E1A 13S in the presence or absence of RA (Fig. 1B and data not shown). Cotransfection with increasing amounts of E1A 12S or 13S in the presence of GAL-RAR AF-2 resulted in a slight decrease by the 12S form and a concentration-dependent increase in activity by E1A 13S (Fig. 1C).

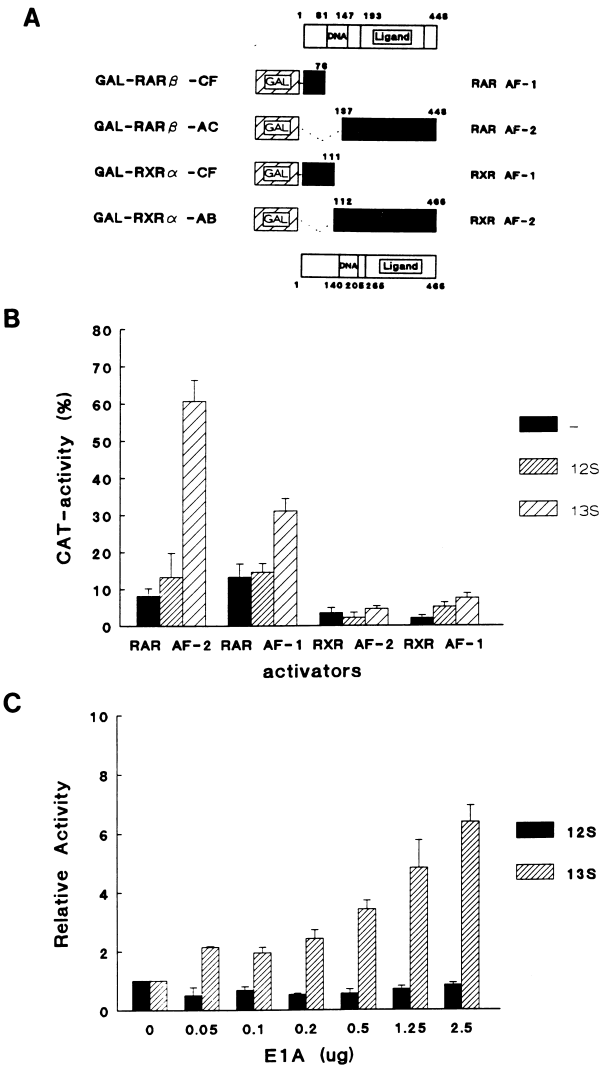

FIG. 1. E1A-dependent transcriptional activation of the AFs of RARβ and RXRα. (A) Schematic representation of the constructs containing RARβ or RXRα AFs coupled to the DNA-binding domain of GAL4 (aa 1 to 147). Numbers correspond to the amino acid residue positions. (B) CAT activity (percent conversion) of COS cells transfected with the GAL-DBD-fused AFs of RARβ and RXRα (1.0 μg) in the absence (solid bars) or presence of the E1A constructs 12S and 13S (1 μg) transfected together with a GAL-responsive reporter (48). Results are the means ± SEM of five independent experiments. (C) Activation of GAL-RAR AF-2 by increasing concentrations of E1A 12S and 13S performed as described for panel B; data are calculated relative to the activity of this activator in the absence of E1A.

Cotransfection of E1A can prevent squelching by overexpressed AF-2. Previous experiments suggested that E1A 13S or E1A-like activity functions as a cofactor in RA-dependent activation by RARβ (5, 39). If E1A can function as a cofactor for RARβ, addition of large amounts of RARβ AF-2 titrates this cofactor out, while addition of E1A should then release squelching. In order to test this hypothesis, we transfected increasing amounts of hRARβ2 in P19 EC cells (containing E1A-like activity), using the hRARβ2 promoter as a reporter. We observed a concentration-dependent decrease in RARβ promoter activity (Fig. 2A) which was prevented by addition of E1A. Only at the highest concentration of hRARβ2 was a decrease in activity, in the presence of E1A, observed, possibly because at this RARβ concentration RXR, the dimerization partner for RAR, is limiting. In order to confirm that this squelching and the E1A-dependent relief are caused by AF-2 of RARβ, we transfected increasing amounts of RARβ AF-2 fused to GAL-DBD, and we observed a repression of RARβ AF-2 activity upon overexpression of AF-2 (Fig. 2B). On the other hand, in the presence of E1A, an increase in activity was observed. On the basis of these observations, we propose that AF-2 can squelch E1A-like activity away from the preinitiation complex, which can be reversed by the addition of E1A 13S functioning as a cofactor for RARβ AF-2. To further confirm that E1A-like activity can be squelched by overexpression of AF-2, we performed similar experiments with cells lacking such

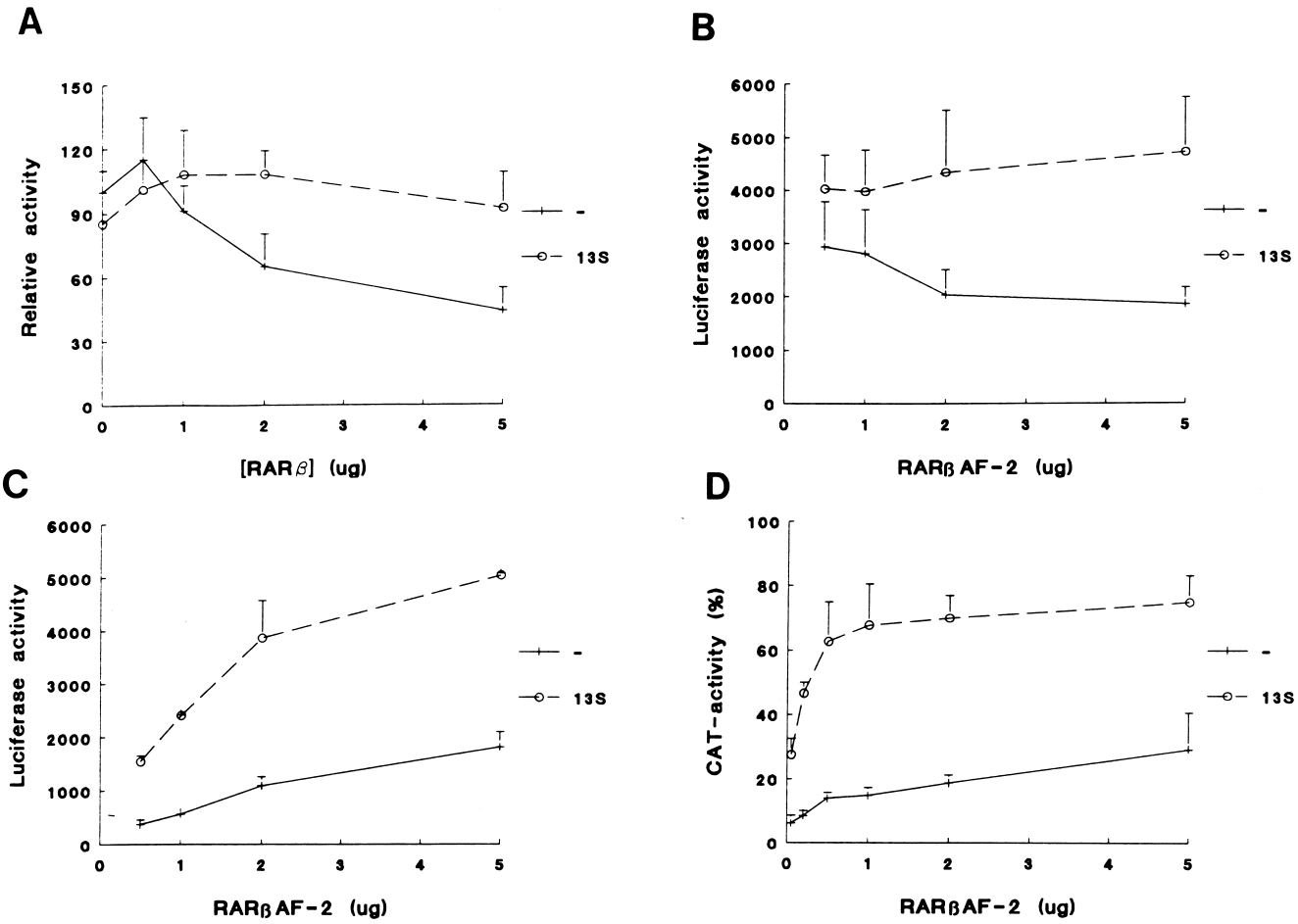

FIG. 2. E1A 13S can prevent squelching caused by overexpression of RARβ AF-2. (A) Increasing amounts of hRARβ were transfected in P19 EC cells with the hRARβ promoter-luciferase construct (−1470/+156 Luc) as a reporter in the presence or absence of E1A 13S (1 μg) (the amount of expression vector was kept constant by adding pSG5 DNA up to 5 μg) as indicated. Results are presented as the mean relative activity of four independent experiments; error bars represent the SEM. The activity of this promoter in the absence of cotransfected E1A or pSG5 RARβ was set at 100%. (B) Transfection in P19 EC cells with increasing amounts of GAL-fused RARβ AF-2 as an activator, pSG424 as a vehicle, and GAL-Luc as a reporter. Results are expressed as the mean luciferase activities (± SEM) of five independent experiments in light units. (C) Transfection as for panel B but performed with P19 EC cells treated with 10 μM RA 30 h before transfection mixture was added. Data are presented as the means ± SEM of duplicate experiments. (D) Transfection experiment performed as for panel B but with COS cells with GAL-CAT as a reporter. Data are presented as percent CAT conversion (means of five experiments ± SEM).

activity: RA-differentiated P19 EC cells (Fig. 2C), the P19 EC cell derivative END2 (data not shown), or COS cells (Fig. 2D), of which the former two have lost their E1A-like activity during differentiation (35, 42) whereas the last lacks such activity completely. In these three cell lines, contrary to the results for undifferentiated EC cells, squelching by overexpression of AF-2 did not take place, but instead a concentration-dependent increase in activity was observed (Fig. 2C and D and data not shown). However, cotransfection of E1A resulted in an enhancement at all concentrations of activator used, but the fold induction by E1A was most pronounced at low concentrations of activator and differed among the various cell lines tested. Possibly, in the presence of large amounts of RARβ AF-2, more E1A is needed for full activation, while other components required for activation might become limiting and thus could prevent a further increase in activation. Together, these transfection experiments indicate that E1A 13S is directly involved in this enhancement, making an indirect effect by release of other factors, as reported for RB/E2F (61) or DrI/TBP (10), less likely.

Direct interaction between RAR and E1A. Our transfection experiments indicate that E1A functions as a cofactor in RA-dependent RARβ activation. To test whether direct interaction between RARβ and E1A 13S could be involved in RA-induced transactivation, we used an in vitro binding assay utilizing in vitro-translated RARβ or RXRα and GST-E1A fusion proteins. As shown in Fig. 3A, comparable amounts of RARβ protein were bound to GST-E1A and GST-RXRα, a dimerization partner for RARs (see reference 44). In contrast, no interaction between RARβ and TBP, TFIIB, or GST was observed. In agreement with the transfection data (Fig. 1B), RXRα, however, did not interact with E1A, nor with TBP, TFIIB, or GST (Fig. 3A). As expected, RXRα readily interacted with GST-RARβ but not with GST-RXRα. The interaction between both RARβ and GST-E1A and RARβ and GST-RXRα was stable in binding buffer containing 500 mM NaCl but was severely diminished in 750 mM NaCl and completely lost in 1 M NaCl (data not shown). Furthermore, these interactions were stable in the presence of ethidium bromide (25 μg/ml of binding buffer), indicating that binding to DNA, possibly present in the reticulocyte lysate, is not required for this interaction. Also, the interaction between in vitro-trans-

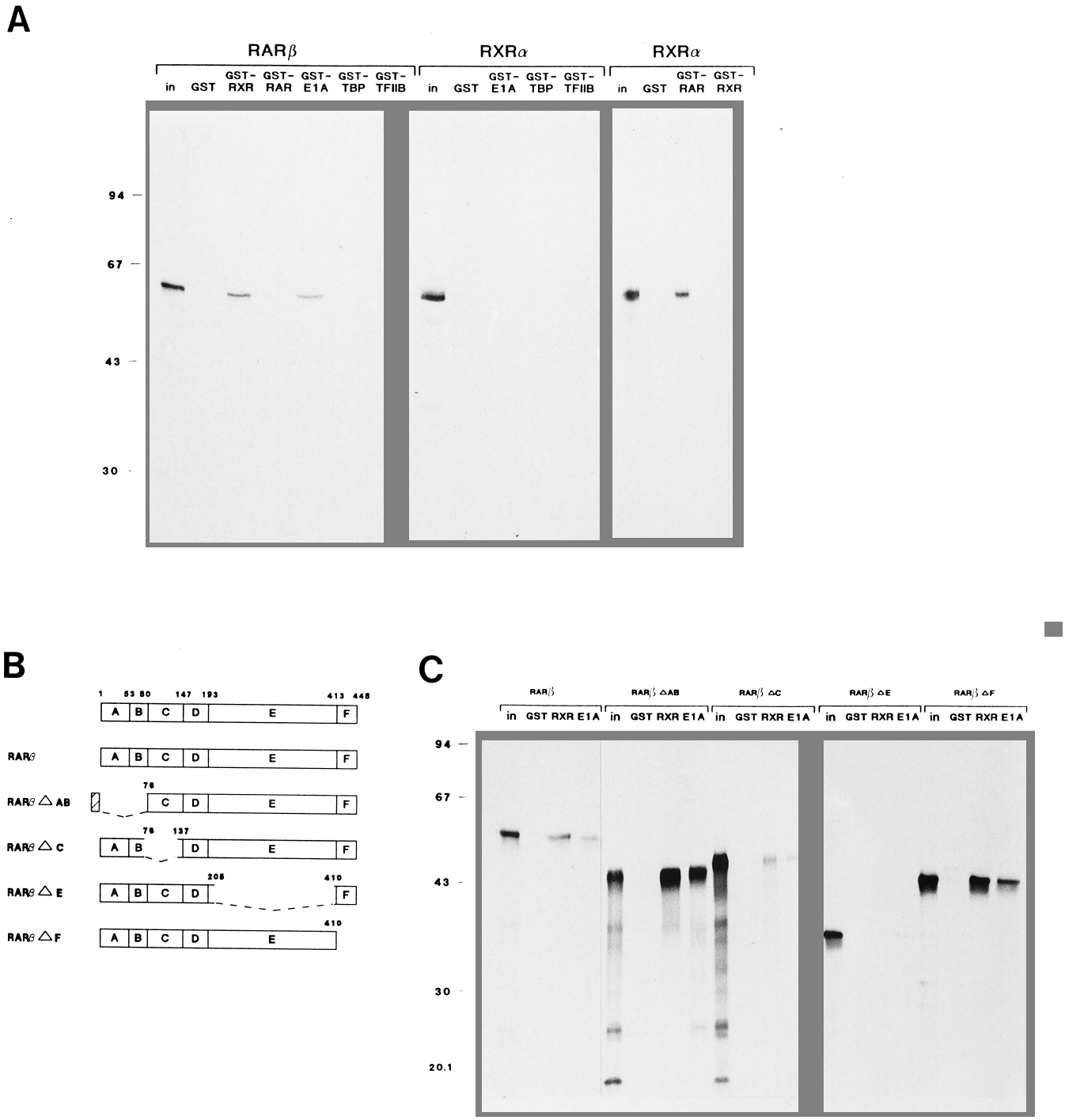

FIG. 3. E1A interacts directly with RARβ mainly through the hormone-binding domain. (A) Interactions between in vitro-translated RARβ or RXRα and the indicated GST fusion proteins. Lanes in, 1/10 of the total input of in vitro-translated protein. Numbers on the left refer to positions of molecular mass markers (in kilodaltons). (B) Schematic representation of the various RARβ deletion constructs; the hatched region of RARβ ΔAB represents an HA tag. Numbers are amino acid positions. (C) Interactions between RARβ deletion constructs and GST, GST-RXRα, or GST-E1A (13S).

lated E1A 13S and GST-RARβ was readily observed, whereas no binding was found with GST-RXR or GST alone (data not shown). We were also able to coimmunoprecipitate E1A 13S in an immunoprecipitation reaction with an antibody against RARβ in the presence of extracts of COS cells transfected with both RARβ and E1A 13S but not when either RARβ or E1A 13S alone was present (data not shown).

Subsequently, various deletion constructs of RARβ (Fig. 3B) translated in vitro were used to map the region interacting with GST-E1A. As shown in Fig. 3C, the F region as well as the AB region (containing AF-1) could be omitted without affecting the interaction with both E1A and RXRα. Unexpectedly, deletion of the DNA-binding domain (C region) reduced binding of RARβ to both RXRα and E1A, while the interaction of

RARα and RXRβ has been shown in similar assays to rely on the hormone-binding domain (53). Possibly, the folding of the ligand-binding domain in vitro in the absence of the DNA-binding domain is altered, resulting in a weaker interaction with both RXRα and E1A. As expected, deletion of the hormone-binding domain resulted in a complete loss of binding between RARβ and RXRα. Similarly, the interaction between RARβ and E1A was almost completely lost, in agreement with the transfection data (Fig. 1B), indicating that direct interaction between RARβ AF-2 and E1A is a requirement for the E1A-dependent activation of AF-2.

Activation and binding of AF-2 by E1A is dependent on CRIII. To identify the region(s) of E1A required for activation, we transfected various deletion constructs of E1A (67) (schematically presented in Fig. 4A) into COS cells together with GAL-RAR AF-2 and a GAL-responsive reporter. Figure 4B shows that deletion of all regions except CRIII permits activation by these E1A constructs. These transfection experiments show that CRI and CRII, which are known to bind p300, RB, and p107 (61), are not required for the upregulation of AF-2. Binding of RARβ to GST-E1A deletion constructs with intact CRIII is comparable with that for full-length E1A or slightly reduced and, in agreement with the activation capacity, completely lost when the 13S mRNA-specific region (CRIII) is deleted (Fig. 4C). Subsequently, constructs with N- and C-terminal deletions as well as internal deletions affecting CRIII were tested for their ability to bind RARβ. The region between aa 151 and 191 was sufficient for optimal binding (N1, C1, and C2), demonstrating that the 13S mRNA-specific region is important for RARβ binding (Fig. 4C).

The behavior of mutants N1 and N2 indicates that possibly the N-terminal part of the protein (aa 1 to 150) stabilizes the interaction between RARβ and E1A, since the binding of these N-terminal deletion constructs is impaired, although alternatively deletion of this region could disrupt the structure of the remaining protein. Deletion of the complete putative zinc finger region resulted in a complete loss of binding (12S, N3, and m3). The region C terminal from the putative zinc finger region (aa 175 to 188), which has been shown to be required for E1A–ATF-2 interaction (51), also contributed to binding of RARβ to E1A, since deletion constructs N3, C4, m3, and 132-185, containing only parts of this region, bound only weakly to RARβ. Finally, the lack of effect of the internal deletion mutant (m1) in binding to RARβ indicates that the N-terminal part of CRIII is not required for binding to RARβ (compare m1 with m2 and m3). Differences in binding of RARβ to these GST-E1A mutants were not caused by variations in the amount of protein added in the GST pull-down assays, as is shown by Coomassie blue staining of such an assay (Fig. 4C).

These findings strongly suggest that the C-terminal part of CRIII is involved in RARβ-E1A interaction. Transfection of these mutants with the RARβ promoter or with GAL-RAR AF-2 on a GAL-responsive reporter in COS cells revealed that all deletions within the 13S mRNA-specific region are deleterious for E1A-dependent upregulation of RARβ AF-2 activity. C3/MX, lacking only 3 aa of CRIII, is impaired in activation, while binding to RARβ was only marginally reduced. Transfection of these E1A mutants with the RARβ2 promoter shows that the same regions are required for upregulation of this promoter by E1A (Table 1).

The zinc finger region of E1A 13S is required for transcriptional activation. In general, we observed a good correlation between the capacity of E1A to interact with RARβ and the ability to enhance transcription by this receptor (Fig. 4; Table 1). However, a few exceptions were observed: the (internal) deletion mutants C3/MX, m1, and m2 could bind RARβ, but their RA-induced AF-2 activation was lost or diminished. This can be explained by the requirement of both the activator and TBP to bind to E1A for transcriptional activation. The importance for an interaction between E1A and TBP for RA-dependent upregulation in activity of the RARs is supported by mutational analyses of TBP which show that the core 1 region, close to the basic region of TBP, is required for activation by E1A-like activity (36). Recently, detailed mutational analyses revealed that the region between aa 147 and 177, containing the putative zinc finger structure, is important for TBP-E1A interaction (26). This implies that m1 and m2, although still capable of binding to RARβ, are unable to activate transcription, because they can no longer bind to TBP.

To test this hypothesis more directly, we made mutants that were shown to affect TBP binding (C157S and C174S) and point mutants and deletion constructs that do not influence TBP binding but were shown to be required for transactivation, presumably by binding upstream activators (73). Transfection of these mutants revealed that most of them were no longer capable of enhancing the activity of AF-2 of RARβ when fused to GAL4-DBD; only C179A and Δ179-185 retained the ability to transactivate (Fig. 5A). To confirm that the observed differences were not caused by different levels of accumulation of the expression constructs, we performed Western blotting of extracts from COS cells transfected with these mutants and immunoprobed with a monoclonal antibody against the C-terminal part of E1A. All of the mutants were expressed to comparable levels, as shown in Fig. 5A. The expression of the Δ179-185 mutant was variable between experiments, explaining the larger variation in transactivation. Analyses of the RARβ binding characteristics of these mutants (Fig. 5B) revealed that all point mutants bound to RARβ. Deletion construct Δ179-185 was partially impaired and Δ178-179 had almost completely lost the ability to bind RARβ, whereas mutant Δ185-187 retained full binding capacity. These differences in binding of RARβ to the GST-E1A mutants were not the consequence of different levels of accumulation of these mutants, as shown by Coomassie blue staining of a representative GST pull-down assay (Fig. 5B). Taken together, binding and transactivation data confirmed the idea that interaction with both TBP and RARβ is a requirement for transcriptional activation mediated by E1A. Finally, a third type of mutant was observed: mutants altered within the C-terminal part of CRIII are unable to enhance the activity of RARβ AF-2, although binding to RARβ (Fig. 5B) and TBP (26) was not diminished (S185R, V187L, and Δ185-187).

DISCUSSION

On the basis of transfection experiments and binding studies, we propose that the E1A-dependent upregulation of the RARβ2 promoter in cells that are otherwise unable to activate the RARβ2 promoter in response to RA is caused by an interaction between RARβ AF-2 and TBP mediated by a direct interaction of these proteins with E1A 13S. On the basis of these data, we propose that E1A-like activity is functioning like E1A, as a cofactor for RARs by simultaneously interacting with RAR and TBP, thereby enhancing the activity of the hormone-dependent AF (AF-2).

E1A is functioning as a cofactor for RARβ. The presence of intermediary factors, cofactors, or adapters in transcriptional activation was proposed initially after the observation that overexpression of activators leads to squelching (64). These cofactors are thought to contact both a component of the basal transcription machinery and the promoter-bound activator,

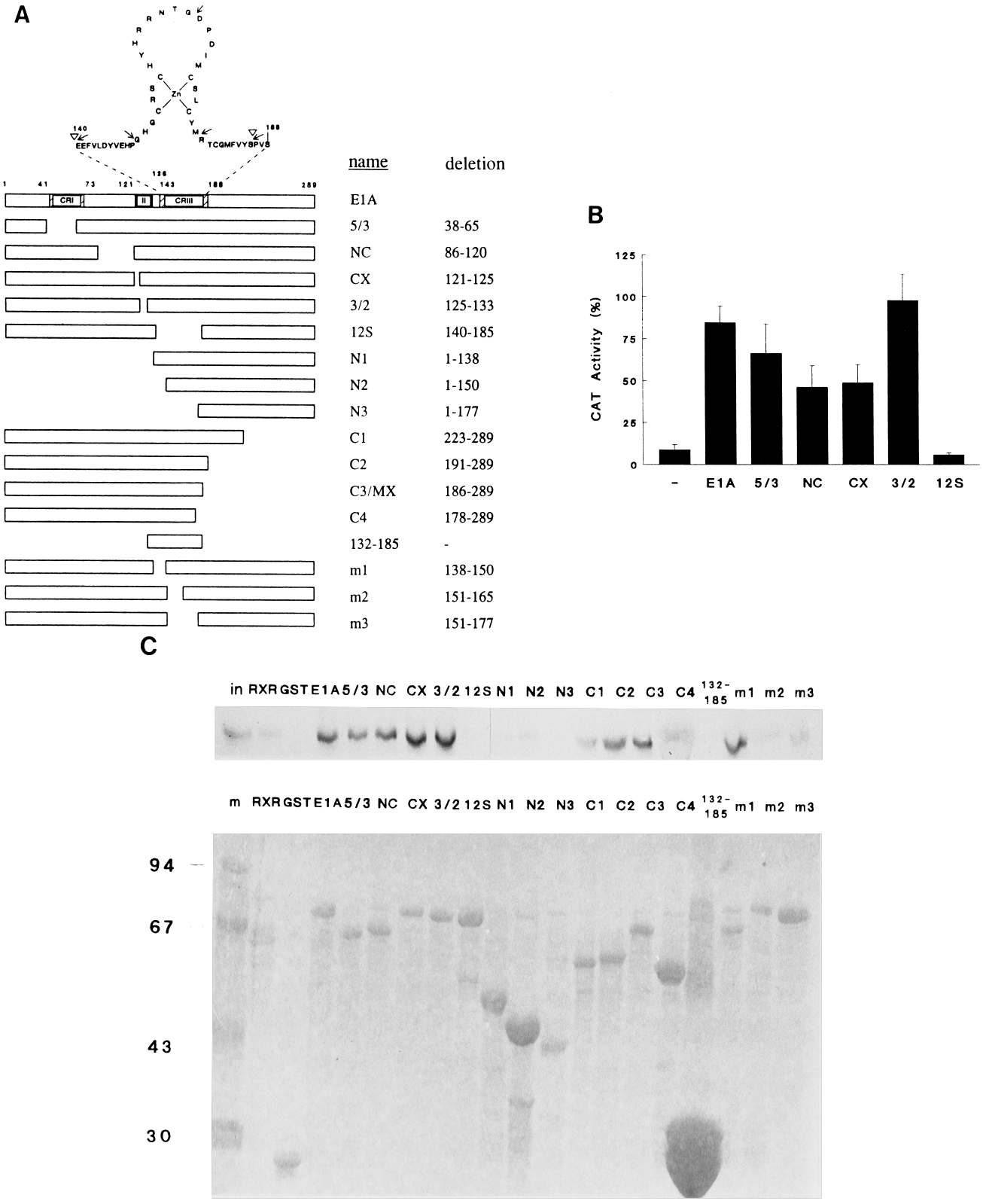

FIG. 4. CRIII is required for E1A-RARβ interaction and activation. (A) Schematic representation of the various E1A deletion constructs used in this study. CRI, CRII, and CRIII are the conserved regions in E1A. The putative zinc finger region within CRIII is depicted; the 13S mRNA-specific region (triangles) and the positions used for the various deletion constructs (arrows) are indicated. (B) COS cells were transfected with different E1A deletion constructs (1 μg) (67), using GAL-CAT as a reporter (8 μg) and GAL-RAR AF-2 (1 μg) as an activator. Results are presented as mean CAT activities (± SEM). (C) Results of a representative experiment of a GST pull-down assay showing interaction between RARβ and various GST-E1A deletion constructs and Coomassie staining of an SDS-PAGE gel from a GST pull-down assay, indicating that all proteins are expressed and migrate according to their expected molecular masses. Numbers on the left refer to the positions of the molecular mass markers (in kilodaltons).

TABLE 1. Binding to RARβ and activation of RA-responsive reporters by E1A mutants

Construct	Deletion (aa)	% Binding[a]	Transactivation[b]	
			GAL-CAT	hRARβ-CAT
None[c]		5	13.8	20.6
Wild-type E1A		100	100.0	100.0
5/3	38–65	65	78.7	52.8
NC	86–120	85	54.5	100.9
CX	121–125	40	58.3	92.0
3/2	125–133	70	113.0	102.4
12S	140–185	5	6.9	17.5
N1	1–138	35	ND	ND
N2	1–150	25	ND	ND
N3	1–177	5	ND	ND
C1	223–289	60	ND	ND
C2	191–289	120	ND	ND
C3/MX	186–289	85	47.4	32.9
C4	178–289	30	ND	ND
132-185	1–131 186–289	10	ND	ND
m1	138–150	85	14.3	36.3
m2	151–165	25	13.3	33.4
m3	151–177	10	13.2	33.4

[a] Quantification of four independent binding experiments performed as described for Fig. 4, using a PhosphorImager (Molecular Dynamics), is shown; the binding of RARβ to GST-E1A was set at 100%, and the binding strength of each mutant was calculated relative to that of this GST fusion protein.

[b] Results of transfection experiments with COS cells with the indicated RAR E1A mutants (1 μg), together with 8.0 μg of GAL-CAT and the activator GAL AF-2 (1 μg) or the hRARβ promoter construct −180/+156 CAT (8 μg) (38) transfected together with 1 μg of RARα and 1 μg of RXRα. Results are the means of at least five independent experiments with SEM of <20%. In all cases, the activity of wild-type E1A was set at 100% and the activity of the mutants was calculated relative to wild-type activity. ND, not determined.

[c] Binding of RARα to GST alone and promoters tested in the absence of cotransfected E1A.

thereby stabilizing the preinitiation complex. Finally, this model predicts that addition of extra cofactors could prevent squelching caused by overexpression of the activator.

Previous experiments by us and others (5, 39) have shown that E1A can enhance transcription of the RAR-RXR complex bound to a RARE. Here, we present evidence that viral activator E1A enhances RA-dependent activation of the RARβ gene, functioning as a cofactor for RARs. This is supported by the following observations. (i) The RARβ2 promoter is inactive in cell lines lacking E1A or E1A-like activity, whereas it is highly upregulated by RA in cells having these proteins, such as P19 EC, F9 EC, HepG2, and 293 cells. Introduction of E1A 13S into cell lines lacking E1A or E1A-like activity enables RARs to further activate this promoter (5, 39). (ii) Transfection experiments using this promoter have shown that hormone-dependent AF-2 of RARs is most important for activation of this promoter (75). We show here that the activity of the ligand-dependent AF (AF-2) of RARβ (but not of RXRα) is upregulated by E1A, while the ligand-independent AFs (AF-1) of these receptors are not affected (Fig. 1). This further strengthens the importance of E1A in the RA-dependent upregulation of the RARβ2 promoter, since the AF that is upregulated by E1A is also the critical AF for RARβ2 promoter activation. (iii) Squelching by overexpression of AF-2 of RARβ could be prevented by cotransfection of E1A 13S (Fig. 2). (iv) The activity of RARβ AF-2 is cell specific (25), and a good correlation between the presence of E1A-like activity or E1A and the activity of this activator in the presence of RA was observed (25). Cotransfection of E1A 13S in cells not expressing E1A or E1A-like activity caused an upregulation in activity, whereas in cells already expressing these proteins, no or only marginal upregulation in activity was observed (24a), indicating that there is a correlation between the strength of this activator and the presence of E1A or E1A-like activity. (v) Using in vitro binding assays, we have shown that RARβ but not RXRα can interact with E1A and that this interaction is dependent on the presence of the 13S mRNA-specific region (CRIII) and the ligand-binding domain of RARβ, containing AF-2, the region shown to be required for the E1A-dependent upregulation in activity (Fig. 3). (vi) Deletion and point mutations within the 13S mRNA-specific region show that this activation is dependent on the TBP binding region, on a distinct region required for RARβ binding, and finally on a region not involved in binding of RARβ or TBP. This indicates that interactions with both RARβ and TBP and a third (hypothetical) protein are all important for E1A-dependent upregulation of AF-2 activity (Fig. 4 and 5; Table 1). All together, these data indicate that E1A fulfills all characteristics for being a cofactor for RARs.

The requirement for cofactors has been shown to be important for members of the steroid-thyroid hormone receptor superfamily both in vivo (56) and in vitro (9, 63), but so far, no cofactor for a member of this family has been cloned. Recently, however, two groups have purified proteins that interact with AF-2 of the estrogen receptor and which may fulfill a cofactor-like function (11, 32). Furthermore, transcriptional enhancement by the SWI genes as well as their human homologs has been reported to function in glucocorticoid receptor-dependent transcriptional activation, probably at the level of chromatin disruption (13, 58, 76).

Mechanisms underlying cofactor action of E1A. In recent literature, there is compelling evidence that certain activators are brought into proximity of the basal transcription machinery by cofactors interacting with both the activator and a target in the basal transcription machinery. The nuclear protein CREB-binding protein (CBP) simultaneously contacts phosphorylated CREB and TFIIB, causing transactivation by the AF of CBP (2, 41). Transactivation by BmFTZ-F1 is mediated by the presence of two proteins, MBF1 and MBF2, which form a dimer that is necessary for complex formation between TBP and BmFTZ-F1 (46). A similar model has been proposed for E1A acting as a cofactor in Oct4-dependent transcription. In gel mobility shift assays, a direct interaction between Oct4 and E1A was observed; in this case, the 13S mRNA-specific region was required for both activation and binding to Oct4, suggesting that E1A functions as a bridging factor (68). For E1A and the CCAAT-binding factor (CBF), an interaction between the CRIII region and CBF was required to mediate transcriptional activation (1).

The interaction of E1A with TBP has been studied in detail. This interaction can take place with both recombinant TBP (34, 43) and holo-TFIID (7) and is dependent on the zinc-binding region of E1A (CRIII) (26). Furthermore, the residues C terminal from this region are also important for transcriptional activation (51, 73). On the basis of transactivation, *trans*-repression, and binding experiments with E1A mutants (51, 73), it was suggested that two discrete functions are present in this region: a binding site for the promoter-bound activator and a different binding site for a limiting factor. For RARβ, the results are similar; both mutants that were reported to prevent TBP binding were unable to activate transcription (C157S and C174S), although they can still bind to RARβ. Also, the mutant unable to bind RARβ (Δ178-179) is impaired in transactivation. Furthermore, binding experiments indicate that the N-terminal part of E1A (aa 1 to 150) contributes to binding of E1A to RARβ. This region has been shown to

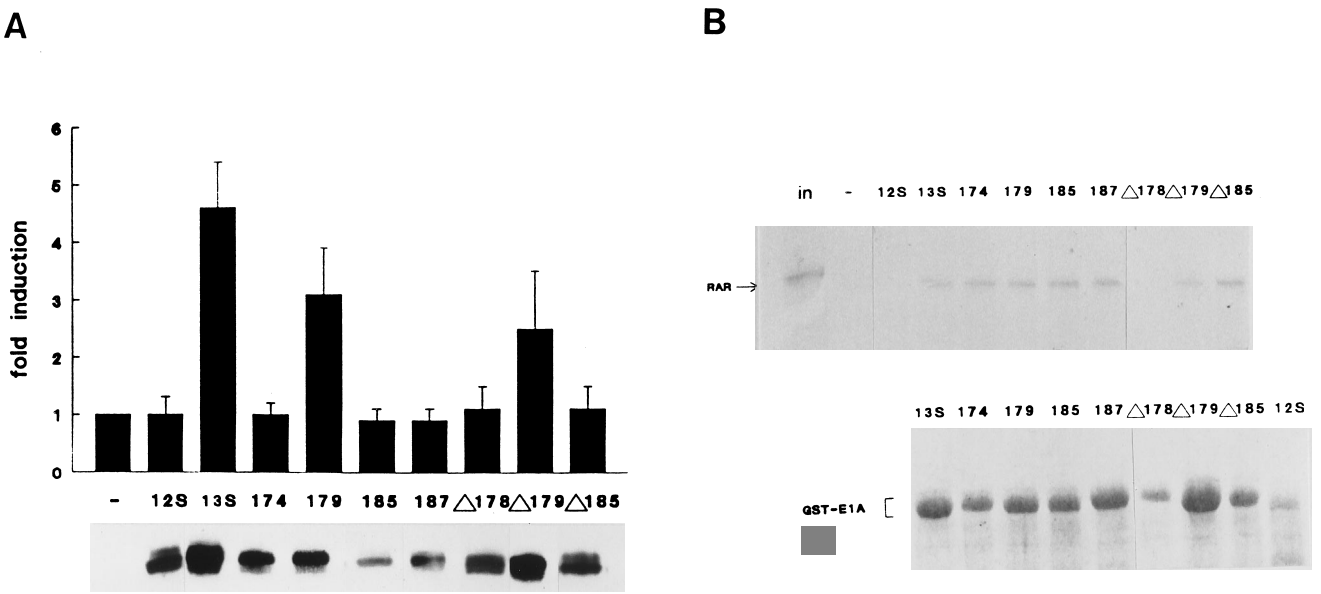

FIG. 5. The C-terminal part of CRIII is required for both interaction with RARβ and E1A-dependent activation of RARβ. (A) Transcriptional activation by the indicated mutant E1A constructs (1.0 μg) with GAL-RAR AF-2 (1.0 μg) as an expression construct and GAL-Luc as a reporter transfected in COS cells. Data are presented as relative luciferase activities compared with activities in the absence of cotransfected E1A. Below the graph is a Western blot using whole-cell extract from COS cells transfected with the various E1A mutants, immunoprobed with a monoclonal antibody against the C-terminal part of E1A (M73), showing that all mutants are expressed. In addition to a control (-), the following mutants were used: C174S (174), C179A (179), S185R (185), V187L (187), Δ178-179 (Δ178), Δ179-185 (Δ179), and Δ185-187 (Δ185). (B) In vitro binding of RARβ to the various GST-E1A mutants. Coomassie staining of an SDS-PAGE gel from a representative GST pull-down assay shows that comparable amounts of GST fusion constructs were used.

interact with various proteins, including RB, p107, and p300 (61), indicating that possibly multiple interactions between RARβ and E1A are required for transcriptional activation by E1A. A third type of E1A mutants not directly involved in RARβ or TBP binding (C3/MX, S185R, V187L, and Δ185-187) created proteins unable to function as a cofactor for RARβ. We suggest that these mutants are unable to bind a third partner within this complex which is required for further formation of the preinitiation complex. This protein could be an additional cofactor, a TBP-associated factor, or a basal transcription factor. It is unclear whether binding of all proteins simultaneously or sequentially is required for the functioning of E1A as a cofactor for RARβ.

Taking these findings together, we suggest that three distinct parts of the 13S mRNA-specific region, each of which binds a different protein, are required for transcriptional activation: the TBP binding region (aa 147 to 174); the RARβ binding region around aa 178, possibly requiring the zinc finger structure, since mutants with part of the zinc finger deleted are defective or impaired in binding to RARβ (although point mutants C157S and C174S, proposed to have the zinc finger structure destroyed, are still capable of binding to RARβ); and, finally, a region that possibly interacts with an additional protein, binding in a region partially overlapping with the RARβ binding region and extending to aa 188. Mutational analysis of ATF2 has shown that deletion of aa 179 to 193 destroys both binding to and activation by E1A (51). On the basis of transactivation and *trans*-repression experiments using E1A mutants, it was concluded that the extreme C-terminal part of the 13S mRNA-specific region (aa 183 to 188) is involved in ATF binding and that the region N terminal from this region, partially overlapping with the TBP binding region, was proposed to bind an unknown limiting factor (26, 51). Detailed mutational analyses using more point mutants will be required to prove that RARβ and ATF2 bind to different regions of E1A and to establish whether different putative limiting factors are bound to the C-terminal part of CRIII of E1A when it acts as a cofactor for RARβ or ATF.

Recently, several groups have identified an autonomous transactivation function within the hormone-binding domain of members of the steroid-thyroid hormone receptor superfamily (4, 19, 45, 66, 71) which is proposed to form an α-helix with hydrophobic residues on one side and negatively charged glutamic acid on the other side of the helix. Since the region interacting with E1A colocalizes with the region required for transactivation, we propose that the activation domain is involved in this interaction. The region of E1A 13S important for RARβ binding contains several hydrophobic residues and one positively charged arginine, suggesting that a stable interaction could be formed by hydrogen bonds or ionic interactions between these proteins. Another possible explanation is that the interaction is not mediated directly by AF-2 of RARβ but, rather, is the consequence of the E1A-RARβ interaction bringing the region required for transcriptional activation in proximity with a target molecule of the basal transcription machinery, which cannot take place or would be much more difficult in the absence of E1A.

Functional importance of E1A. The presence of an E1A-like activity in cells in which the RARβ promoter can be activated by RA (P19 EC, F9, and HepG2) and the inability of the RARβ promoter to be activated in cells that have lost their E1A-like activity (differentiated EC cells) make it likely that this E1A-like protein(s) functions by a mechanism similar to that of its viral counterpart. This was further confirmed by experiments in which squelching, caused by overexpression of AF-2 in P19 EC cells, was prevented by E1A 13S, indicating that E1A can functionally replace E1A-like activity in its ability to activate through AF-2 (Fig. 2). These results suggest that E1A-like activity is also acting as a cofactor mediating activation by interactions with both RAR and TBP. The importance

for an interaction of E1A-like activity with TBP for RA-dependent upregulation of the RARβ promoter became apparent from mutational analyses of TBP showing that a region in core 1 is required for activation (36), although it remains to be seen whether E1A or E1A-like activity indeed contacts the same region (43). If it indeed functions like E1A, this could open the way for cloning E1A-like activity by virtue of its ability to bind to both RARβ and TBP. The identification of E1A-like activity and the subsequent study of the role in RARβ promoter activation and embryonal development can ultimately establish the role of this protein as a cofactor. The availability of the cofactor required for RA-dependent transactivation by RARs could also make it possible to investigate whether the restricted pattern of expression of RARβ during embryonal development (18, 65) is caused by the absence of these cofactors for RARs.

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