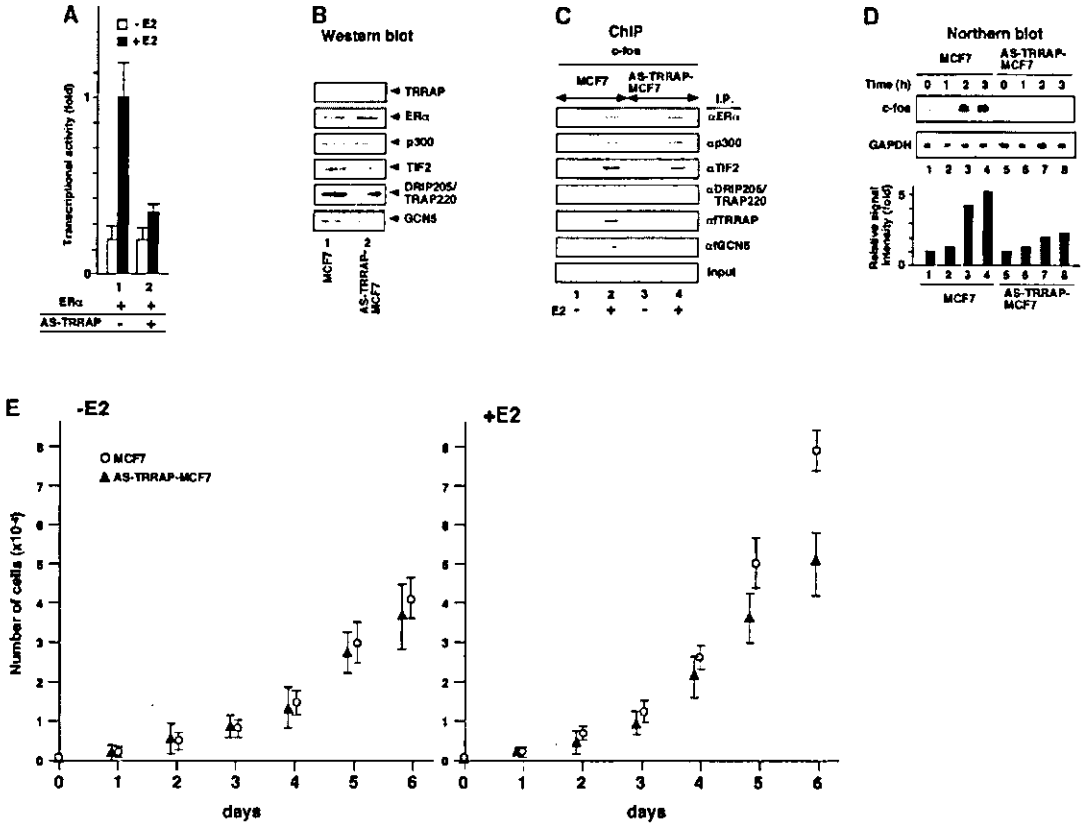

Figure 5. A TFTC-Type TRRAP/GCN5 Complex Is Involved In E_2-Dependent Growth of MCF-7 Cells

(A) Reduced ligand-dependent transactivation function of ERα by expression of TRRAP antisense RNA. COS-1 cells were transfected with ERα (0.5 μg), pGL-ERE-AdML (1.0 μg), and pML-CMV (10 ng), along with pcDNA-AS-TRRAP (1.0 μg) in the presence or absence of E_2 (10^{-8} M), and the cell extracts used in a luciferase assay. pcDNA-AS-TRRAP was transfected into MCF-7 cells and stable transformants selected by G418 (AS-TRRAP MCF7).

(B) Decreased TRRAP protein level in AS-TRRAP-MCF7 cells. Unchanged expression levels of ERα and certain other coactivators were observed by Western blot analysis, whereas TRRAP expresssion level was decreased in the AS-TRRAP-MCF7 cells.

(C) Lack of the TFTC-like complex recruitment to the *c-fos* promoter in the AS-TRRAP-MCF7 cells. No recruitment of the TFTC-like complex components (TRRAP and GCN5) was seen by ChIP assay in MCF7 cells expressing antisense TRRAP RNA. In contrast, under the same conditions, normal recruitment of the p300/TIF2 and the DRIP/TRAP complexes was observed to the *c-fos* promoter by ChIP assay.

(D) Reduced expression of an estrogen target gene in AS-TRRAP-MCF7 cells. The *c-fos* gene expression was examined by Northern blotting with GAPDH expression as an internal control. Densitometric analysis of the *c-fos* gene is shown in the lower panel.

(E) Involvement of TRRAP/GCN5 complexes in E_2-dependent growth of MCF-7 cells. Control MCF-7 cells and AS-TRRAP-MCF7 cells were incubated for the indicated times in the absence (left panel) and presence (right panel) of E_2 (10^{-8} M) and examined by counting cell numbers. Data shown as means ±SD of triplicate cultures of one representative clone used for analyses shown in panels (A)–(D) among five tested clones.

here we demonstrate that the TFTC-type GCN5/TRRAP-containing HAT complexes can function both in vitro and in vivo as coactivator complexes for NRs, and thus, we have defined a third class of coactivator complex important for NR function. The three identified LXXLL motifs in TRRAP protein are responsible for the direct and ligand-dependent interactions with the LBDs of NRs. This finding is in agreement with the suggested accessible location of TRRAP in the TFTC complex as assumed from its structural analysis (Brand et al., 1999a). The ChIP assay suggested that the GCN5 HAT-containing multiprotein complex acts, after recruitment to liganded ERα, about the same time as the DRIP/TRAP complex, and following the p160/CBP complex dissociation from liganded ERα. These data raise two alternative possibilities for the function of these TFTC-type complexes. In the first case, the TFTC-type complex would be functionally identical to that of the DRIP/TRAP complex in potentiating NR function. Note however that differences exist between the ligand-dependent recruitment of the TFTC-type complexes and that of the DRIP/TRAP complex when comparing proteins interacting with liganded LBDs of either ERα (Figure 1B) or VDR (data not shown). Furthermore, the TFTC-type complexes acetylate histone H3 in a nucleosomal context, while the DRIP/TRAP complex does not. Thus, we would prefer a second model in which both of the complexes may be simultaneously (or very quickly each after the other) recruited to ERα, and probably to many other receptors, to allow the formation of an efficient, large transcription initiation complex on chromatin templates, though the molecular mechanism underlying the switch-

ing of the complexes remains elusive. Thus, our findings, together with recent data showing that a TFTC-type TRRAP/GCN5 complex acts as a cofactor complex for c-Myc-mediated transformation (McMahon et al., 1998; Park et al., 2001) and that yeast Tra1 serves as a common target for acidic activators (Brown et al., 2001), raise the interesting possibility that the TFTC-type complex-dependent recruitment of acetyltransferase activity is critical for regulation of gene expression in general.

The lowered expression of endogenous TRRAP by its antisense RNA caused reductions in the E2-dependent cell growth of breast cancer cells (Figure 4F), indicating possible roles of TFTC-type complexes in the estrogen-dependent cell growth of breast cancer cells with enhanced function of c-Myc in cell proliferation. It is of particular interest to clarify the molecular mechanisms of the estrogen-dependent cell growth and the development of acquired E2-antagonist resistance in breast cancer. By identifying components of TFTC-type complexes in breast cancer, it will allow us a better understanding of the tumor development.

Experimental Procedures

Plasmid Construction

TRRAP cDNA (McMahon et al., 1998) was cloned into the pcDNA3 vector (Invitrogen) in an antisense orientation to generate pcDNA-AS-TRRAP. Three consensus estrogen response elements (3x ERE) or eight GAL4 binding elements (8 × 17 M) were inserted into a luciferase reporter plasmid bearing an AdML promoter to give pGL-ERE-AdML and pGL-17M-AdML, respectively. To produce chimeric GST-fusion proteins, cDNA fragments corresponding to LBDs of the NRs, the GAL4-DNA binding domain (1-147 aa) or VP16 transactivation domain were inserted into pGEX 4T-1 vector (Pharmacia Biotech).

Cell Culture

Human MCF-7 breast cancer cells and transformants were routinely maintained in phenol red-free DMEM (Life Technologies, CA) supplemented with 10% FBS (Hyclone, UT). To establish stable transformants, parent MCF-7 cells were transfected with either pcDNA-FLAG-GCN5 or pcDNA-AS-TRRAP by calcium phosphate precipitation (Yanagisawa et al., 1999) and cultured for 2 weeks in the presence of 500 μg/ml G418 for transformant selection (Watanabe et al., 2001). Reduced levels of TRRAP protein were confirmed by Western blotting and the growth rate of cells estimated in the presence or absence of E_2 (10^{-8} M). FLAG-GCN5 transformants were replated into fresh dishes and further cultured for 2 weeks in 500 μg/ml G418. Individual colonies were selected and expanded for further analysis.

Purification and Separation of ERα-Associated Complexes

HeLa nuclear extracts were loaded onto a P11 phosphocellulose column. After extensive washing with washing buffer (20 mM Tris-HCl, pH 7.9, 150 mM KCl, 0.2 mM EDTA, 0.05% NP40, 10% glycerol, 0.5 mM PMSF, and 1 mM DTT), bound proteins were eluted by elution buffer (20 mM Tris-HCl, pH 7.9, 1 M KCl, 0.2 mM EDTA, 0.05% NP40, 10% glycerol, 0.5 mM PMSF, and 1 mM DTT). Immobilized GST-ERα LBD fusion proteins were preincubated for 1 hr at 4°C in GST binding buffer (20 mM Tris-HCl, pH 7.9, 180 mM KCl, 0.2 mM EDTA, 0.05% NP40, 0.5 mM PMSF, and 1 mM DTT) containing BSA (1 mg/ml) and E_2 (10^{-8} M). Bead-immobilized proteins were then incubated at 4°C for 6–10 hr with P11 column-eluted fractions in the presence of 10^{-8} M E_2. After washing with GST wash buffer (GST binding buffer with 0.1% NP-40) three times, the beads were further washed with a GST wash buffer containing 0.2% *N*-lauroyl sarkosine (Sarkosyl, Sigma, St. Louis, MO). Complexes bound to the E_2-bound-liganded ERα were eluted with 15 mM reduced glutathione in elution buffer (50 mM Tris-HCl, pH 8.3, 150 mM KCl, 0.5 mM EDTA, 0.5 mM PMSF, 5 mM NaF, 0.08% NP-40, 0.5 mg/ml BSA, and 10% glycerol). For purification of the FLAG-GCN5 complex from the MCF-7 stable transformant, elutants from GST-ERα column were further loaded onto 2.5–5 ml of M2 anti-FLAG agarose (Kodak, FL). After washing with binding buffer, the bound proteins were eluted from agarose by incubation for 60 min with 2.5–5 ml of the FLAG peptide (Kodak) in the binding buffer (0.2 mg/ml). For fractionation on glycerol gradient, elutants were layered onto the top of a 4.5 ml linear 100%–40% glycerol gradient in GST binding buffer and centrifuged for 16 hr at 4°C at 40,000 rpm in a SW40 rotor (Beckman, CA). Protein standards used were ovalbumin (44 kDa), β-globulin (158 kDa), and thyroglobulin (667 kDa) (Watanabe et al., 2001).

HAT Assay

The purified fractions were incubated with or without 10 μg calf thymus histones (Type IIA, Sigma) and ^{3}H-labeled acetyl CoA (4.7 Ci/mmol, Amersham, UK) for 30 min at 30°C, spotted onto Whatman P-81 filters, and washed extensively with sodium carbonate buffer (pH 9.1). Radioactivity remaining on the filter was then quantitated by liquid scintillation counting.

GST Pull-Down Assay

GST-fusion proteins were expressed in *Esherichia coli* and bound to glutathione-sepharose 4B beads (Pharmacia Biotech, UK). The in vitro-translated proteins were then incubated with beads in NET-N buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, and 0.5% NP40) with 1 mM PMSF. Bound proteins were separated by 7.5% SDS-PAGE, lightly stained with Coomasie brilliant blue to verify equal amounts of fusion protein, and then visualized by autoradiography (Endoh et al., 1999).

Immunoprecipitation

After washing MCF7 cells twice with ice-cold phosphate-buffered saline, the collected cells were resuspended in 1 ml ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP40), incubated on ice for 30 min, then centrifuged again for 5 min at 500 × g. Sedimented nuclear fractions were resuspended in TNE buffer (10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM EDTA, and 1% NP40) and incubated for 30 min on ice. After centrifugation, the supernatants were used as MCF7 whole-cell extracts for immunoprecipitation using anti-ERα antibody (anti-ER Ab-1; NEO MARKERS) following Western blotting using anti-ERα antibody, anti-TRRAP antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-GCN5 antibody raised against the N-terminal region (Santa Cruz Biotechnology) (Yanagisawa et al., 1999).

In Vitro Transcription

Transcription assay was performed according to previously published methods (Kundu et al., 2000). Briefly, 200 ng DNA was incubated with or without purified activators in 10 μl reaction volumes containing 0.02 mM PMSF and 0.2 mM DTT for 15 min at room temperature; after which, 6 μl rat liver nuclear extract (Conaway et al., 1987) (9 mg/ml protein) was added to each reaction. After incubation for 15 min at room temperature, 9 μl reaction mix containing 5 μl of 5× buffer (50 mM HEPES-KOH, pH 7.6, 15% glycerol, 128 mM KCl, and 30 mM $MgCl_2$, rNTPs), RNase inhibitor, α-^{32}P CTP, and RNase T_1 was added, and incubated for 45 min at 30°C. Reactions were stopped by addition of stop buffer (20 mM Tris-HCl, pH 7.5, 0.25 M NaCl, 1% SDS, 5 mM EDTA, and 200 μg/ml tRNA) and treated with proteinase K for 15 min at 37°C. ^{32}P-labeled RNA was extracted by phenol-chloroform, precipitated by ethanol, analyzed on 6% acrylamide 8.3 M urea gels, and visualized by autoradiography.

Transfection and Luciferase Assay

Cells at 40%–50% confluence were transfected with the indicated plasmids using Lipofectamine regent (GIBCO-BRL, CA) in 12-well petri dishes. Total amounts of DNA were adjusted by supplementing with empty vector up to 1.0 μg. Luciferase activity was determined using the Luciferase Assay System (Promega, WI). As a reference plasmid to normalize transfection efficiency, 25 ng of pRL-CMV plasmid (Promega) was cotransfected in all experiments (Takeyama et al., 1999).

ChIP Assay

FLAG-GCN5 transformant cells were cultured for the indicated periods in the presence of E_2 (10^{-8} M) and soluble chromatin prepared using the Acetyl-Histone H4 Immunoprecipitation assay kit (Upstate Biotechnology, CA). Soluble chromatin was immunoprecipitated with antibodies against the indicated proteins. Specific primer pairs were designed to amplify Cathepsin D (5′-TCCAGACATCCTCTCTG GAA-3′ and 5′-GGAAGCGGAGGGTCCATTC-3′) (Shang et al., 2000) and c-*fos* (5′-GAAGAGTGGAGAAGGG-3′ and 5′-GAAGCTGTGCT TACGG-3′) from extracted DNA. Optimal PCR conditions to allow semiquantitative measurement were 20 cycles of 30 s at 96°C, 15 s at 58°C, and 1 min at 72°C. PCR products were visualized on 2% agarose/TAE gels.

Northern Blot Analysis

Total cellular RNA was isolated from the indicated cells using ISOGEN reagent (Wako Co., Japan), and 20 μg RNA used for Northern blot analysis with cDNA of c-*fos* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as probes (Takeyama et al., 1997).

Antisense Oligonucleoside Phosphorothioate (AS PS-ODN) Preparation

The sequence of AS-TRRAP PS-ODN was 5′-CTGTGTTGCAA CAAACGCCAT-3′. The control PS-ODN was 5′-CTGAAATGCCG CAAACTTCAT-3′. MCF-7 cells were cultured for 48 hr with PS-ODNs before addition of E_2 (10^{-8} M). After a 24 hr culture with or without E_2, DNA synthesis of these cells was measured using 5-Bromo-2′ deoxy-uridine (BrdU) incorporation method.

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TATA-binding Protein-free TAF-containing Complex (TFTC) and p300 Are Both Required for Efficient Transcriptional Activation*

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Initiation of transcription of protein-encoding genes by RNA polymerase II was thought to require transcription factor TFIID, a complex comprising the TATA-binding protein (TBP) and TBP-associated factors (TAFs). In the presence of TBP-free TAF complex (TFTC), initiation of polymerase II transcription can occur in the absence of TFIID. TFTC contains several subunits that have been shown to play the role of transcriptional co-activators, including the GCN5 histone acetyltransferase (HAT), which acetylates histone H3 in a nucleosomal context. Here we analyze the coactivator function of TFTC. We show direct physical interactions between TFTC and the two distinct activation regions (H1 and H2) of the VP16 activation domain, whereas the HAT-containing coactivators, p300/CBP (CREB-binding protein), interact only with the H2 subdomain of VP16. Accordingly, cell transfection experiments demonstrate the requirement of both p300 and TFTC for maximal transcriptional activation by GAL-VP16. In agreement with this finding, we show that *in vitro* on a chromatinized template human TFTC mediates the transcriptional activity of the VP16 activation domain in concert with p300 and in an acetyl-CoA-dependent manner. Thus, our results suggest that these two HAT-containing co-activators, p300 and TFTC, have complementary rather than redundant roles during the transcriptional activation process.

Transcription initiation of protein-encoding genes by RNA polymerase II was thought to require transcription factor TFIID, which comprises the TATA-binding protein (TBP)[1] and a series of TBP-associated factors (TAFs) (1–3). However, we have previously shown that initiation of polymerase II transcription can occur in a TFIID-independent manner in the presence of a novel human (h) multiprotein complex, termed TFTC for TBP-free TAF complex (4). TFTC is able to direct preinitiation complex assembly from different TATA box-containing and TATA-less promoters *in vitro* on naked DNA templates. TFTC contains no TBP but is composed of several TAFs and other proteins that have been shown to mediate transcriptional activation or are important in correct initiation site selection (4, 5). The three-dimensional structure of TFTC resembles a macromolecular clamp that contains five globular domains organized around a solvent-accessible groove of a size suitable to bind DNA (6).

A large number of recent studies have provided a direct molecular link between histone acetylation and transcriptional activation (reviewed in Refs. 7 and 8). In these reports, it has been shown that several previously identified co-activators of transcription possess intrinsic HAT activity. Among these co-activators are yeast Gcn5 (9), human GCN5 (10), PCAF (11), TATA box-binding protein-associated factor 250 (TAF1; formerly $TAF_{II}250$) (12), p300/CBP (13), ACTR (14), and steroid receptor co-activator 1 (SRC-1) (15). Many of these chromatin-modifying activities have been found within large multiprotein complexes that also contain several components with homology or identity to known transcriptional regulators. In *Saccharomyces cerevisiae* the co-activator protein Gcn5 is part of two large multisubunit complexes, the 1.8–2-MDa SAGA complex and the 0.8-MDa ADA (alteration/deficiency in activation) complex (16). Yeast SAGA, similar to the TFTC-type complexes, comprises products of at least four distinct classes of genes: (i) the Ada proteins (yAda1, yAda2, yAda3, yGcn5 (yAda4), and yAda5 (ySpt20); (ii) the TBP-related set of Spt proteins (ySpt3, ySpt7, ySpt8, and ySpt 20); (iii) a subset of TAFs, including scTAF5, scTAF6 scTAF9, scTAF10, and scTAF12; and (iv) the product of the essential gene *Tra1*, which has been shown to be a component of SAGA (8) (for new TAF names see Ref. 3). The yeast SAGA complex has been shown to mediate activation by the acidic activators yGcn4 and VP16 and to potentiate transcription activation in an acetyl coenzyme A (acetyl-CoA)-dependent manner on chromatin templates *in vitro*, whereas the ADA complex failed to do so (17–20).

Mammalian homologues of yGCN5 include PCAF and GCN5

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[1] The abbreviations used are: TBP, TATA-binding protein; TAF, TBP-associated factor; TF, transcription factor; TFTC, TBP-free TAF complex; HAT, histone acetyltransferase; SAGA, Spt-Ada-GcN5-acetyltransferase complex; ADA, (alteration/deficiency in activation; h, human; TRRAP, transformation/transcription domain-associated protein; CBP, CREB-binding protein; AD, activation domain; AS, antisense; HIV, human immunodeficiency virus; GST, glutathione *S*-transferase; PCAF, p300/CBP-associated protein.

(11, 21). In human (h) cells a number of GCN5- and PCAF-containing multiprotein complexes have been characterized: such as TFTC (5), the PCAF and GCN5 complexes (22), and the SPT3-TAF$_{II}$31-GCN5 acetyltransferase complex (STAGA) (23, 24), that all contain either GCN5(L) or PCAF as catalytic HAT subunit, as well as the human ADA proteins hSPTs, hTAFs, and hTRRAP. TRRAP was originally isolated as a Myc-associated transcriptional co-activator (25). The SAGA, TFTC, PCAF/GCN5, and STAGA HAT complexes preferentially acetylate histone H3 in both a free and a nucleosomal context (5, 22, 24, 26). Although the human TFTC, PCAF/GCN5, and STAGA complexes share several subunits, they are not identical (5, 24, 27), suggesting the existence of overlapping but also different functions between these complexes. Moreover, a TFTC-type HAT complex was shown to be required as a co-factor for nuclear receptor function both *in vitro* and *in vivo* (28).

CBP and p300 are distinct but functionally related co-activator proteins with intrinsic HAT activities, involved in both proliferative and differentiating pathways (Ref. 29 and references therein). CBP/p300 efficiently acetylate the N-terminal tails of the four histones, however with a preference for histones H3 and H4 as compared with H2A and H2B (13, 30). In addition to modifying histones, CBP/p300 proteins have been shown to acetylate non-histone proteins including transcriptional activators, general transcription factors, and chromatin-associated proteins (31).

The fact that that TFTC (i) mediates transcriptional initiation and activation on naked DNA templates, (ii) contains the hGCN5 HAT as well as several human homologues of yeast SAGA subunits that have been shown to be important for transcriptional activation and correct initiation site selection in different genetic screens, (iii) preferentially acetylates histone H3 on chromatin templates (4, 5), and (iv) is required as a co-activator for nuclear receptor function (28) prompted us to analyze in further details the function of TFTC in activated transcription on chromatin templates. We describe herein the direct physical interactions between TFTC and the two distinct activation regions (H1 and H2) of the VP16 activation domain, and we show that p300 and CBP interact only with the H2 subdomain of VP16. Using cell transfection experiments we demonstrate the requirement of both CBP/p300 and TFTC for efficient transcriptional activation. Moreover, we report that on an *in vitro* reconstituted chromatin template human TFTC mediates the transcriptional activity of the VP16 activation domain (AD) in concert with p300 and in an acetyl-CoA-dependent manner. Altogether our results suggest that the two HAT-containing co-activators, p300/CBP and TFTC, play complementary roles during transcriptional activation.

EXPERIMENTAL PROCEDURES

Plasmid Constructions, Cell Transfections, and GST Pull-down Assays—The eukaryotic expression plasmids for wild type E1A, E1AΔN mutant, and the E1A-CR2mut, AS-TRRAP, have been described previously (28, 32–34). The 17M/ERE-Glob-Luciferase reporter plasmid has been described elsewhere (35). The hGCN5 cDNA was cloned into the pcDNA3 vector (Invitrogen) in an antisense orientation to generate pcDNA-AS-GCN5. The expression plasmids to produce either the different GST-VP16 fusion proteins (see Fig. 1*C*) or the mammalian expression plasmids producing GAL-VP16 and its derivatives have been described previously (36).

2×10^6 HeLa cells were cotransfected by calcium phosphate precipitation in 6-well dishes. Total amounts of DNA were adjusted by supplementing with an empty vector up to 5 μg/well. Routinely 300 ng reporter plasmid was used with 20 ng of GAL-VP16 expression vector, or with its derivatives, and with the indicated amounts of the other expression vectors (see also the legend to Fig. 2). Cell culture and growth conditions as well as the luciferase assay have been described (37). For the luciferase assays, the same amount of protein was taken from each transfection. Similar results were obtained in at least three independent transfections. GST-pull down assays were carried out as described (36).

Western Blot Analysis—Routinely proteins were boiled in SDS sample buffer and separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with the indicated primary antibodies. Chemiluminescence detection was performed according to manufacturer's instructions (Amersham Biosciences, Inc.). The anti-TRRAP antisera (25, 38), anti-SPT3, anti-GCN5, and anti-SAP130 antibodies (27), anti-TBP, anti-TAF10, anti-TAF5, and anti-TAF6 monoclonal antibodies (4), anti-Med-6 (kind gift from R. Kornberg), anti-Med 7 (kind gift from D. Reinberg), anti-CBP, anti-p300, anti-TRAP240, and anti-TRAP95, anti-SPT6 (Santa Cruz) have been described previously.

Chromatin Template Assembly—For the chromatin assembly the pIC-2085S/G5-E4R plasmid (39) was digested with *Hae*III and Asp^{718} to generate a 1241-bp fragment and incubated with HeLa histones octamers (1:1 molar ratio between one histone octamer and one nucleosome binding site). The chromatin template was assembled by salt dilution (39). Nucleosomal assembly was confirmed by MNase digestion. Chromatin template (250 ng of DNA) was digested with 2 milliunits of micrococcal nuclease (Sigma) in buffer B (10 mM Hepes, pH 7.6, 50 mM KCl, 5% glycerol, 10 mM sodium butyrate, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.25 mg/ml bovine serum albumin) containing 3 mM $CaCl_2$ for 20 s to 3 min at 25 °C. DNA was precipitated, run on a 1.3% agarose gel, and either visualized by ethidium bromide staining or transferred to a nylon membrane (Hybond, Amersham Biosciences, Inc.) and hybridized with a ^{32}P-labeled probe (Fig. 3*B*).

In Vitro Transcription—Nuclear extract preparations, in vitro transcription reactions, and primer extensions were carried out as described previously (40, 41). GAL1–147, GAL-VP16, TFTC, and p300 were purified as described previously (4, 42, 43). About 20 ng of chromatin-assembled E4 template was preincubated with the indicated factors (Fig. 3) in the presence or absence of 2 μM acetyl-CoA in a 20-μl volume of buffer B. After 40 min of preincubation at 30 °C, 30 μl of buffer T (30 mM Hepes, pH 7.8, 60 mM KCl, 12 mM $MgCl_2$, 4% PVA (30–70), 10 mM sodium butyrate, 12 ng/μl poly(dI·dC)) was added to the reactions together with 2 ng of pHIV-1 plasmid as an internal control (41) and 30 μg of HeLa nuclear extract. Transcription reactions were started by the addition of the four rNTPs (10 mM) at time 0. Reactions were stopped after a 45-min incubation at 30 °C with the S buffer (300 mM NaCl, 20 mM EDTA, 1% SDS, 50 ng/μl tRNA). Correctly initiated transcripts were detected by primer extension using a ^{32}P-labeled probe corresponding to the complementary positions of +86 to +110 of the E4 transcript (39).

RESULTS AND DISCUSSION

TFTC Is Recruited by Both Subregions of the VP16 AD, whereas p300/CBP Interact Specifically Only with the H2 Region—First we wanted to examine whether VP16 AD could directly recruit TFTC. Thus, we tested whether TFTC would bind to the activation domain of VP16 when fused to GST. The GST-VP16 fusion protein was immobilized on glutathione-agarose resin in parallel with GST alone, as a control (Fig. 1*A*). Highly purified TFTC was able to bind to the GST-VP16 fusion protein but not to the GST alone (compare *lanes 2* and *3*). This is in accordance with the fact that a TFTC-like complex was previously purified by its direct association with the liganded estrogen receptor (28) and that the related STAGA complex was able to interact directly with the VP16 activator (24).

As it has also been shown that the VP16 AD functions in *in vitro* transcription systems through direct interactions with p300/CBP and the Mediator complex (TRAP/DRIP/SMCC/ARC) (36, 43, 44) and that these factors can interact directly with the VP16 AD from crude extracts, we tested whether TFTC could also bind directly to the GST-VP16 in parallel with the Mediator complex and/or p300/CBP from a HeLa cell nuclear extract. Similar to the results obtained with purified TFTC (Fig. 1*A*), TFTC components such as TRRAP, GCN5, SAP130, TAF5 (formerly TAF$_{II}$100 (3), TAF6 (formerly TAF$_{II}$80), and TAF10 (formerly TAF$_{II}$30) were detected in the high salt elution from the VP16 column (Fig 1*B*, *lane 6*). Moreover, as previously reported, other known VP16-interacting proteins such as CBP, p300, MED6 and MED7, TRAP240, and TRAP95 were also present in the elution, whereas hSPT6,

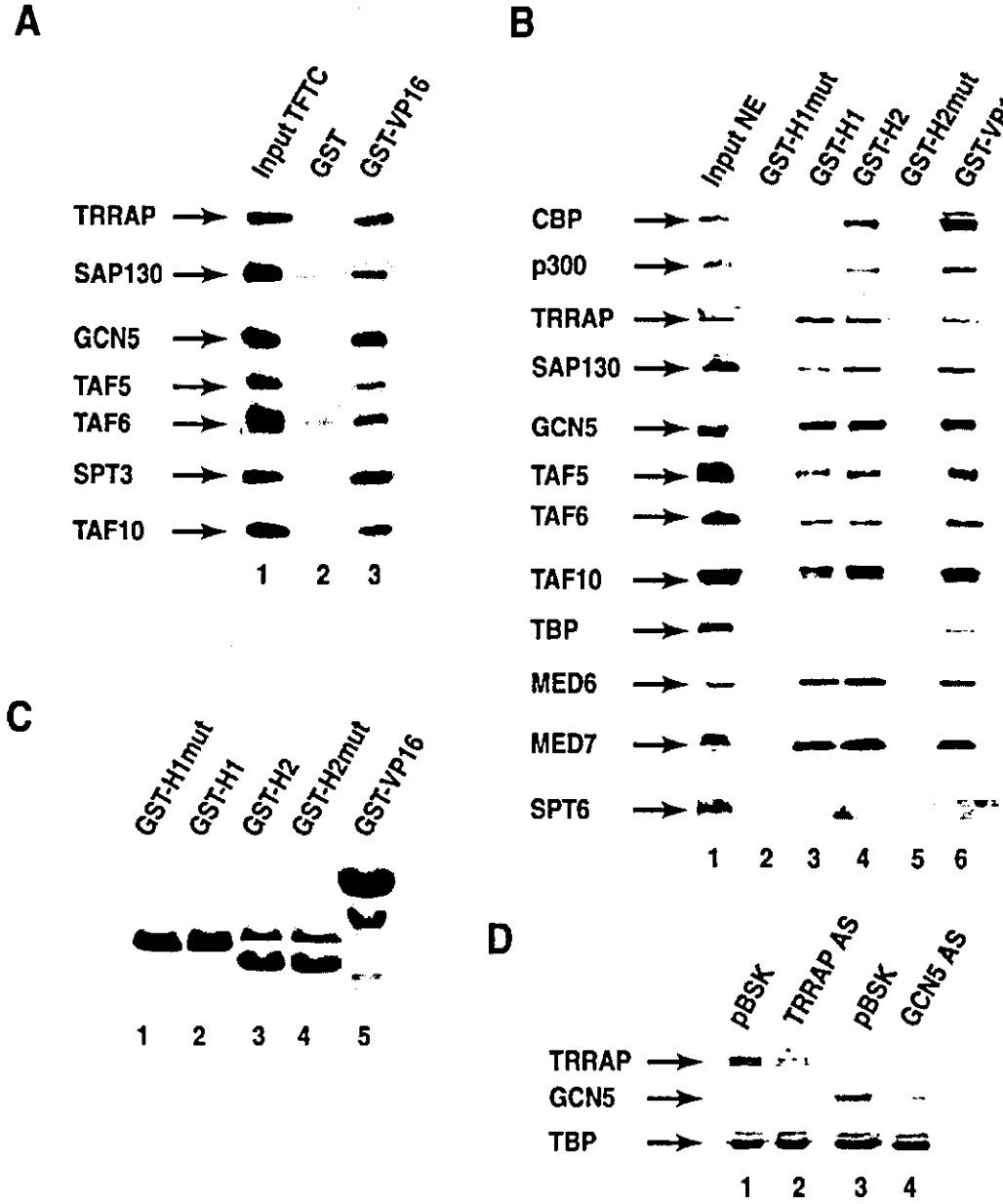

FIG. 1. **Specific binding of the TFTC complex to VP16 AD and to its H1 and H2 subdomains.** Immunoblot analyses of 500 mM salt eluates from the GST-VP16 column or its derivatives (as indicated), using either highly purified TFTC (*A*) or crude HeLa nuclear extracts (NE) (*B*), are shown. The blots were probed with the indicated antibodies. The functional mutations in H1 (F442P) and H2 (F473A, F475A, F479A) precluded binding of TFTC and the Mediator complex. *C*, 10 μl from the indicated GST fusion protein-containing beads were boiled and separated by SDS-PAGE, and the fusion proteins were Coomassie-stained. *D*, 10^7 HeLa cells were transfected with the indicated antisense (*AS*) expression vectors or an empty vector (*BSK*). 48 h after transfection whole cell extracts were prepared by three cycles of freeze-thawing. 150 μg of total protein was separated by SDS-PAGE and analyzed by immunoblot with the indicated antibodies.

another nuclear protein, did not bind to the VP16 column (Fig. 1*B*, *lane 6*, and data not shown).

It has also been reported that the activation domain of VP16 can be subdivided into two regions, the N-terminal region, H1 (amino acids 411–452), and the C-terminal, region H2 (amino acids 453–490), both of which independently activate transcription (45, 46). The Mediator complex binds to both regions, whereas CBP only binds to the H2 subregion (36). These observations prompted us to further analyze the regions of VP16 that interact with TFTC and p300. The VP16 H1 and H2 regions were expressed separately as GST fusion proteins, and as controls functional mutations of VP16/H1 (F442P) and VP16/H2 regions (F473A, F475A and F479A) were also expressed as GST fusions (called *GST-H1mut* and *GST-H2mut* in Fig. 1*C*). TFTC subunits, *i.e.* TRRAP, GCN5, SAP130, TAF5, TAF6, and TAF10, were specifically recruited by both the GST-H1 and the GST-H2 regions, similar to the Mediator complex subunits MED6, MED7, TRAP240, and TRAP95. These interactions are specific because none of these factors were bound to either the GST-H1mut or GST-H2mut columns (Fig. 1*B*, *lanes 2–4*). Interestingly, p300 was recruited by the GST-H2 region, but not by GST-H1, in good agreement with the previous finding that CBP binds only to the VP16 H2 region (Fig. 1*B*, *lanes 2–4*) (36). Strikingly, under the same conditions,

TBP did not interact significantly, or only very weakly, with either the H1 or H2 subdomain of VP16. Altogether these results show that the two subdomains of VP16 AD are able to specifically recruit the TFTC and the Mediator complexes from nuclear extracts, whereas CBP and p300 interact only with the H2 region. The fact that p300 and CBP bind only to the H2 subdomain but TFTC and the Mediator bind to both H1 and H2 subdomains, suggests also that the binding of p300 and/or CBP to the H2 subdomain can occur in the absence of TFTC and/or the Mediator, and *vice versa*, TFTC and the Mediator binding to an activation (sub)domain does not necessarily require p300 and/or CBP.

The Decrease in Either the TRRAP or GCN5 Subunits of TFTC or Inhibition of p300/CBP Activity Reduces Stimulation of Transcription by VP16—To study the role of TFTC and p300/CBP in the activation of transcription mediated by GAL-VP16, we used mammalian cell-based transfection experiments. Consistent with previous reports (47), GAL-VP16 strongly stimulated transcription from the rabbit β-globin promoter, which contains one GAL4 binding site fused to a luciferase reporter in HeLa cells (Fig. 2*A*). When a vector expressing an antisense region of either TRRAP or GCN5 mRNA (TRRAP AS and GCN5 AS, respectively), we observed an important (dose-dependent) decrease in the GAL-VP16 activation potential (Fig. 2*A*, and data not shown). This decrease was paralleled by the reduction of the amount of endogenous cellular TRRAP or GCN5 protein levels as detected by Western blot (Fig. 1*D*). These results indicate that in HeLa cells normal levels of TRRAP or GCN5 proteins are needed for full activation by GAL-VP16. Interestingly, the co-transfection of TRRAP AS with GCN5 AS did not further reduce activation by GAL-VP16, suggesting that both AS constructions inhibit the same step in the activation pathway.

The expression of the transcriptional repressor E1A (12S) (48–50), which has been proposed to inhibit the activity of CBP/p300, PCAF, or GCN5, also efficiently diminished the transcriptional activation by GAL-VP16 (Fig. 2*A*), in accordance with Ikeda *et al.* (36). In contrast to the results obtained by co-transfection of TRRAP AS and GCN5 AS, co-transfection of TRRAP AS together with the E1A expression plasmid cooperatively reduced activation by GAL-VP16, suggesting that TRRAP AS and E1A affect independent interactions (or processes) in the mechanism of VP16-mediated activation of transcription.

As TFTC binds to both the H1 and H2 VP16 activation domains (Fig. 1*C*), and as it has been described that E1A inhibits only the activation by the H2 activation domain of VP16 (36), we tested whether TRRAP AS and GCN5 AS would inhibit the activity of H1 or H2 domains of VP16. As shown in Fig. 2, *B* and *C*, both TRRAP AS and GCN5 AS inhibit activation by H1 as well as by H2; however, again no cooperativity in the inhibition was observed. These data further underline the above observations, suggesting that both AS constructions may impair the function of the same complex and thus inhibit the same step in the VP16 activation pathway.

The N-terminal 20 amino acids of E1A and a portion of conserved region 1 (CR1) where shown to be responsible for p300/CBP and PCAF binding (11, 48–51), whereas other N-terminal regions interact with the pocket-containing protein family, the most characterized of which is the retinoblastoma (Rb) tumor supressor protein (48). Despite the high structural similarity between hGCN5 and hPCAF, GCN5 does not interact with the N-terminal end of E1A and/or the CR1 region, but its interaction domain has been localized primarily to the CR2 region of E1A (34). CBP-, p300-, PCAF-, and GCN5-mediated activity of different transcription factors can be abrogated by E1A (11, 32, 34, 49, 50).

Thus, we wanted to examine whether the inhibitory effect of E1A on VP16-mediated activation of transcription (which acts in cooperation with the inhibitory effect of TRRAP AS) is due to the effect of E1A on p300/CBP and/or GCN5. To investigate this point, we took advantage of previously described E1A mutants that were shown to be defective in either interactions with p300/CBP (E1AΔN) (32) or GCN5 (E1A-CR2mut) (34). Thus we tested whether they would affect GAL-VP16 activation. Overexpression of wild type E1A efficiently inhibited VP16-dependent activation (Fig. 2*D*). A similar degree of inhibition was obtained with E1A-CR2mut but not with E1AΔN (Fig. 2*D*). Thus, in our system E1A seems to inhibit mainly the CBP/p300 activity without a detectable effect on GCN5 activity. Indeed, the E1AΔN mutant, which has been shown to be unable to interact with p300/CBP, has lost its inhibitory effects, whereas the E1A-CR2mut, which is defective in its interaction with GCN5, has been as efficient in inhibiting the VP16 activity as the wild type E1A (Fig. 2*D*). This result, together with the previously observed inhibition of transcription by both E1A and TRRAP AS (Fig. 2*A*), suggests that p300 and TRRAP (a subunit of the TFTC complex) contribute to transactivation of the promoters via at least partially independent pathways or that their activities are complementary.

E1A could affect the HAT activity of p300; however, the effect of E1A on the HAT activity of different co-factors is at present contradictory. E1A was shown to (i) inhibit the HAT activity of p300, PCAF, and GCN5 (34, 49, 52); (ii) not influence the HAT activity of CBP and PCAF (30, 32); or (iii) stimulate the HAT activity of CBP (53). As the transactivation capability of these co-factors seems to be promoter- and transactivation domain-dependent, the effect of E1A on the activity these HAT-containing cofactors therefore likely depends on the system analyzed.

On a Reconstituted Chromatin Template, Purified p300 and TFTC Cooperate in the Activation of Transcription-mediated by VP16—To confirm more directly the complementary roles of the transcriptional coactivators p300 and TFTC, and to avoid the possibility of studying effects due to incomplete chromatinization of transiently transfected DNA (54), we decided to use a transcription system consisting of an *in vitro* chromatinized template reconstituted from DNA and purified histone octamers (39). The DNA fragment used for the reconstitution of the nucleosome array consists of a central dinucleosome-length sequence containing five GAL4 binding sites (17M/5) upstream of the adenovirus E4 promoter, flanked on either side by five repeats of a nucleosome positioning sequence from the sea urchin 5S rDNA (18). Core nucleosomes were purified from HeLa cells (55) and assembled on the above described DNA fragment by salt dilution (39). The efficiency of assembly was confirmed by micrococcal nuclease digestion (Fig. 3*B*). The VP16 AD stimulated transcription from the naked 17M/5-E4 promoter very efficiently in the presence of 30 μg of HeLa cell nuclear extract, whereas the GAL4 DNA binding domain alone (GAL1–147) did not (Fig. 3, *C* and *D*). In accordance with previous results (17), VP16-activated transcription was abolished from the chromatinized 17M/5-E4 template under the same conditions (Fig. 3, *A* and *C*, compare *lanes 2* and *4*). The addition of TFTC to this transcription system in the presence of acetyl-CoA did not relieve the repressive effect of chromatin on the GAL-VP16-mediated activation (Fig 3*C*, *lanes 13* and *14*). We first investigated the effect of p300 on GAL-VP16-activated transcription from our chromatin-assembled template in the presence of acetyl-CoA. A limited amount of purified p300 (1.6 ng, as estimated by Coomassie staining; data not shown) effi-

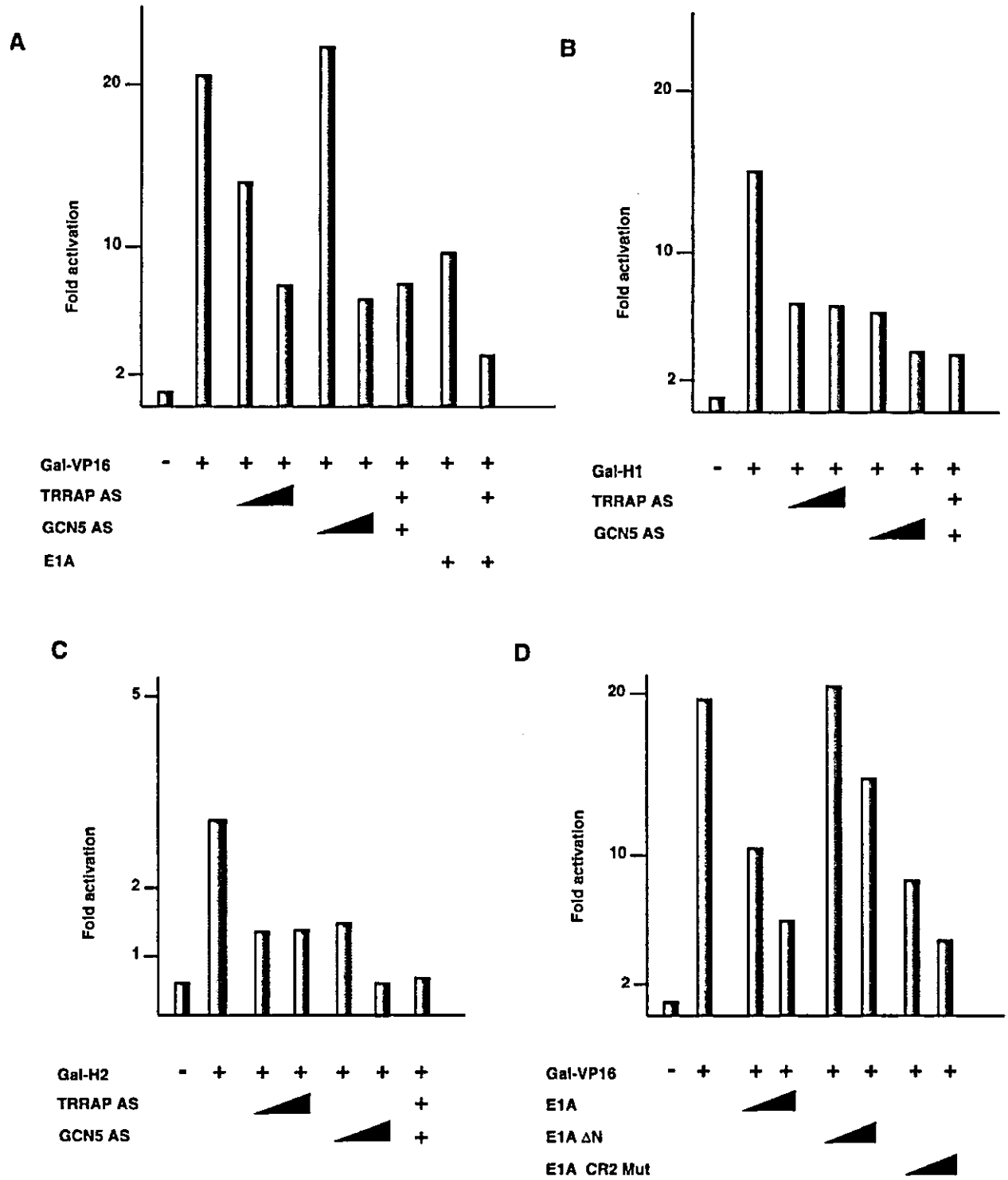

FIG. 2. **Inhibition of TFTC subunit expression or CBP/p300 activity reduces VP16-driven transactivation in HeLa cells.** The luciferase reporter plasmid containing the rabbit β-globin proximal promoter and one GAL4 binding site, the expression plasmids for GAL-VP16, and its activation subdomain derivatives (GAL-H1 and GAL-H2) were transiently transfected. Where indicated 300 ng or 1 μg of antisense (*AS*) as well as wild type or mutated E1A 12S expression vectors (per/well) were co-transfected (as indicated). Where either TRRAP AS and GCN5 AS or TRRAP AS and E1A vectors were co-transfected, the higher 1-μg expression vector concentrations were used.

ciently mediated GAL-VP16-stimulated transcription, whereas the GAL4 DNA binding domain alone did not, or it was much less efficient (Fig. 3, *C* and *D*, *lanes 5* and *6*). Interestingly, the activation of transcription mediated by p300 in this system was greatly stimulated by the addition of increasing amounts of purified TFTC (Fig. 3*C*, compare *lane 6* with *lanes 8*, *10*, and *12*). The cooperative effect of TFTC with p300 on VP16 activation was the highest at 2 μl (200 ng) of TFTC (*lane 10*), whereas either 0.5 or 5 μl of TFTC had a weaker effect but still higher than p300 alone (compare *lane 6* with *lanes 8* and *12*). This

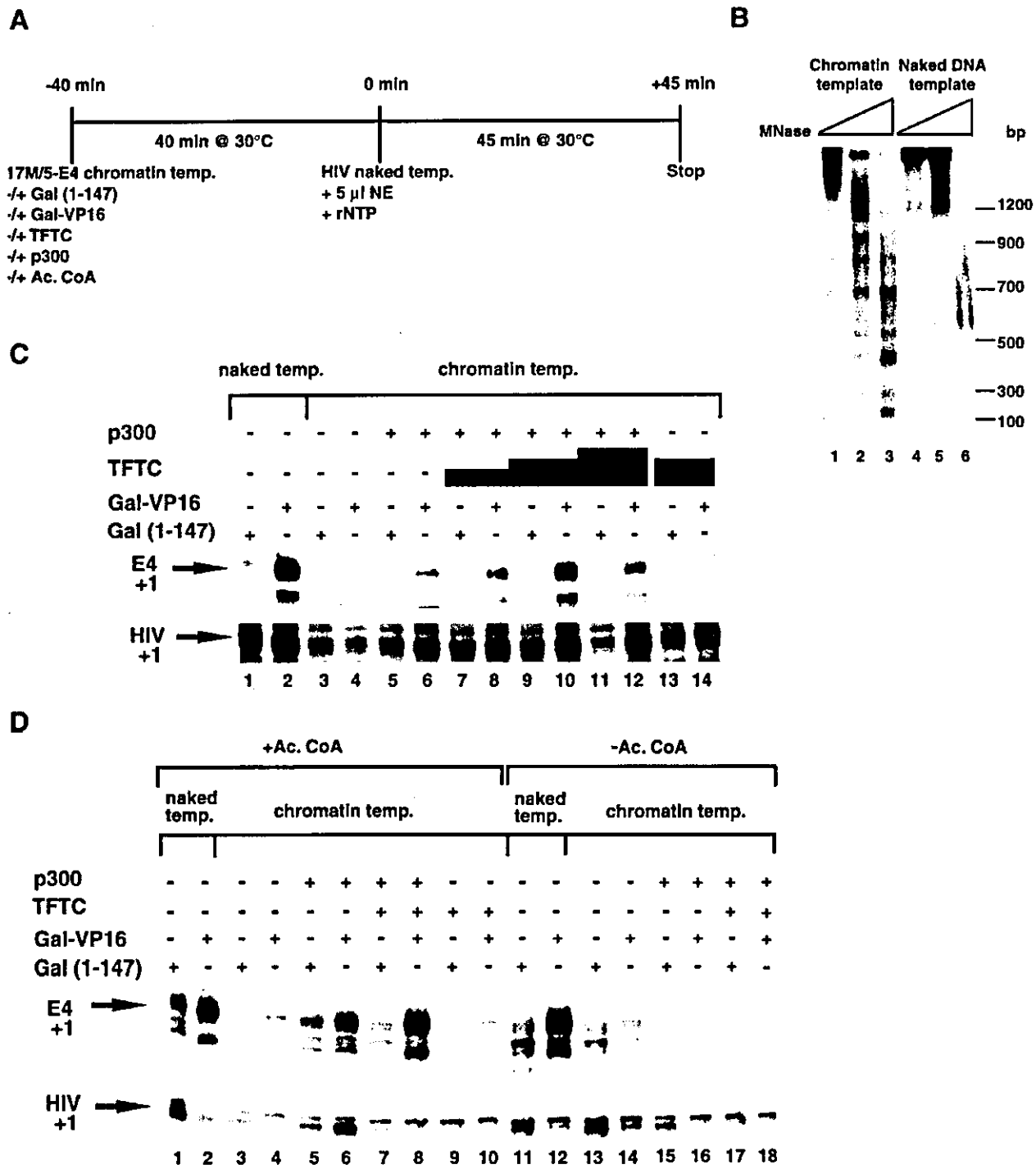

FIG. 3. **TFTC mediates the transcriptional activity of the VP16 activation domain in the presence of p300 from chromatin templates.** *A*, an outline of the chromatin and transcription assembly. *NE*, nuclear extract. *B*, for chromatin assembly, the 1241-bp fragment containing the adenovirus E4 promoter flanked on either side by five repeats of a nucleosome positioning sequence from the sea urchin 5S rDNA was incubated with histone octamers prepared from HeLa cells (1:1 molar ratio between one histone octamer and one nucleosome binding site). The chromatin template was assembled by salt dilution, and nucleosomal assembly was confirmed by micrococcal nuclease (*MNase*) digestion. The chromatin template (250 ng of DNA) was digested with 2 milliunits for 20 (*lane 1*), 60 (*lane 2*), and 180 s (*lane 3*) at 25 °C. The naked DNA template was digested with 10-fold less MNase for the same periods of time (*lanes 4–6*). The positions of the migration of some fragments (in bp) from the DNA ladder mix are indicated. *C* and *D*, the adenovirus E4 promoter-containing template (*E4 + 1*) was either assembled in chromatin or not, and equivalent gel shift units of GAL-(1–147) or GAL-VP16 were bound to these templates in the presence of highly purified endogenous TFTC (200 ng) or baculovirus overexpressed p300 (1.6 ng) as indicated. After 40 min of preincubation in the presence or absence of acetyl-CoA (*Ac.CoA*; 2 µM, as indicated) nuclear extract; 30 µg) was added together with the naked HIV template (*HIV + 1*) as an internal control for transcription efficiency. Transcription was initiated by the addition of rNTPs.

indicates that despite the fact that in our system TFTC alone is not sufficient to relieve the repressive effect of chromatin on GAL-VP16-mediated activation of transcription (*lanes 13* and *14*), it can cooperate with p300 to further increase p300-mediated transcriptional activation by VP16 AD. Furthermore, this cooperative effect is acetyl-CoA-dependent, because when acetyl-CoA was omitted from the reactions no more VP16 stimulation was observed either in the presence of p300 alone or of p300 and TFTC together (Fig. 3*D*, compare *lanes 3–10* with *13–18*). This suggests that the HAT activities of p300 and perhaps TFTC together or individually are required for the efficient stimulation of the VP16 AD from chromatin template. The fact that TFTC alone did not (or only weakly) allow transcriptional activation by VP16 AD on the chromatin template seems to be in apparent contradiction with the finding that the related human STAGA complex functioned as a cofactor on the chromatin template without the addition of p300 (24). This difference can be explained in several ways: (i) the correct nucleosome positioning on the chromatin templates was achieved by using different systems; (ii) different promoters were used; (iii) the nuclear extract used by Martinez *et al.* (24) contained more p300; and/or (iv) the reported differences in the polypeptide composition of the two complexes may be important for their respective cooperative function with p300.

CONCLUSION

In this study we have shown a direct physical interaction between TFTC and the two separable subdomains of the strong activation domain of VP16, presenting evidence that only subdomain H2 is able to bind either p300 or CBP. Our cell transfection experiments have shown that these interactions are functional because when the endogenous level of TRRAP or GCN5 proteins was decreased by antisense mRNA expression, the activation of transcription mediated by GAL-VP16 was inhibited significantly. Moreover, cotransfection experiments suggested that in the cells both CBP/p300 and TFTC may be necessary for fully activated transcription at least on certain promoters. This suggestion was then verified by using *in vitro* transcription experiments on chromatin templates, where we showed that p300 and TFTC can mediate transcriptional activation by VP16 in a synergistic manner. Thus, our results, together with recent findings that a TFTC-type TRRAP-GCN5-containing complex acts as a co-regulator for c-Myc, E2F, STAT2, and several nuclear receptors (25, 28, 34, 56, 57), strongly suggest that the recruitment of the TFTC complex, containing an acetyltransferase activity (and possibly other activities), is critical for the regulation of transcription in general. In addition, it has been demonstrated *in vitro* that activators recruit p300 to nucleosomal templates by direct interactions and that bound p300 stimulates transcription, at least in part, by localized histone acetylation (44). Our results have demonstrated that TFTC is required in addition to p300 in order to achieve efficient transcriptional activation.

We have shown that TFTC is able to bind to both TATA-containing and TATA-less promoters *in vitro* on naked DNA templates (4). Moreover, it has been demonstrated *in vivo* that transcription initiation can occur without TBP on certain promoters (58) and that TFTC may mediate preinitiation complex formation and transcriptional activation on some promoters in the absence of TBP (34),[2] independently of whether they are TATA-less or TATA box-containing. Nevertheless, further chromatin immunoprecipitation experiments will determine whether TFTC activity is needed before that of TFIID (or other TBP-containing complexes), and later in the activation process TFIID/TBP can replace TFTC at most promoters for mediating preinitiation complex (PIC) formation *in vivo*; or whether TFTC can entirely replace TFIID at a certain subset of promoters in both mediation of transcriptional activation and PIC formation.

The exact sequential mechanism by which p300, CBP, the p160 family of coactivators (including TIF2/GRIP1, SRC-1, RAC3/ACTR/AIB1/pCIP (Ref. 59 and references therein)), the ATP-dependent chromatin remodeling complexes (including SWI/SNF, ISWI, ACF, and CHRAC complexes (Ref. 60 and references therein)), the Mediator complexes (including the DRIP, TRAP, SMCC, PC12, CRSP, ARC complexes (Ref. 61 and references therein)), the TFTC-type complexes (see the Introduction), and TFIID complexes mediate transcriptional activation on chromatin templates *in vivo* is not yet clear. Furthermore, it is also unknown whether all of these factors need to be recruited by every activator at each promoter to achieve efficient transcription activation. All of these transcription cofactors contain several well defined domains and/or subunits that have been shown to contact a large variety of transcription factors, including sequence-specific activators as well as basal transcription factors. Moreover, chromatin immunoprecipitation experiments suggest that *in vivo* the CBP, p300, p160, co-activators, Mediator, SWI/SNF, and TFTC complexes are recruited to promoters at different time points (probably depending on the given promoter context) in the cascade of events following the binding of an activator to its cognate response element (28, 59, 62–66). The simultaneous recruitment by a given activation domain (*i.e.* VP16 or nuclear receptors) of TFTC-type complexes with the Mediator complexes (this study and Refs. 24 and 28) and the fact that in chromatin immunoprecipitation experiments TFTC subunits and Mediator subunits are recruited to promoters with similar kinetics, but after p300 or CBP (28), suggest a possible functionally important cross-talk between TFTC and the Mediator complexes and that their action requires the preliminary action of CBP/p300. It is also conceivable that TFTC and the Mediator complex are not recruited at exactly the same time by a given AD, and thus they play nonredundant roles during the sequential events of the activation process. Our present *in vitro* and *in vivo* experiments, together with those of Yanagisawa *et al.* (28), demonstrate that in this complicated network of interactions, which leads to the opening of the chromatin and the subsequent transcriptional activation, TFTC plays the role of a general transcriptional coactivator and is at least partially dependent on the recruitment of p300. Thus, our results suggest that *in vivo* the co-activators p300 and TFTC are both required for transcriptional activation and that they have rather complementary roles during the transcriptional activation process.

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Ligand-Selective Potentiation of Rat Mineralocorticoid Receptor Activation Function 1 by a CBP-Containing Histone Acetyltransferase Complex

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The rat mineralocorticoid receptor (MR) has two activation functions in distinct regions of the A/B domain, designated activation function 1a (AF-1a; amino acids 1 to 169) and AF-1b (amino acids 451 to 600). Since the p160 family protein TIF2, a known component of the AF-2 coactivator complex, potentiates the transactivation function of AF-1b but not that of AF-1a, it is likely that some other, novel protein complex interacts with the AF-1a region. Therefore, we attempted to identify such coactivator complexes from HeLa nuclear extracts by biochemical purification using a glutathione *S*-transferase–MR AF-1a fusion protein. Purified AF-1a region-interacting proteins were found to contain RNA helicase A (RHA) and CBP. Further analysis showed that RHA interacted with the AF-1a region directly and then recruited a complex with histone acetyltransferase (HAT) activity that contained CBP. For full-length MR, aldosterone, but not hydrocortisone, was found to induce the binding of RHA/CBP complexes to the AF-1a region, as well as to allow the cooperative potentiation of MR transcriptional activity by RHA and CBP. In addition, a chromatin immunoprecipitation assay showed that aldosterone-bound MR, but not hydrocortisone-bound MR, recruited RHA/CBP complexes to native MR target gene promoters. Our results suggested that an altered conformation of the A/B region induced by aldosterone, but not hydrocortisone, might determine the accessibility of MR AF-1a to RHA/CBP complexes.

Mineralocorticoid receptor (MR) is a member of the steroid/thyroid hormone nuclear receptor superfamily and acts as a ligand-inducible transcription factor responsible for ion homeostasis by regulating ion channel expression in epithelial cells (1, 5, 11, 14). Recently, it was shown that MR participates in a wide range of biological functions in nonepithelial tissues, including the heart and brain (5, 17, 32). MR has two native ligands, aldosterone (mineralocorticoid) and hydrocortisone (glucocorticoid), both of which bind MR and induce its transactivational function (26). However, the ligands mediate distinct physiological actions via MR (10, 20, 21, 40, 58). While these different physiological actions may partially depend on the enzymatic activity of 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2), which inactivates hydrocortisone (glucocorticoid) and results in the preferential activation of MR by aldosterone in target tissues (15), this does not explain the distinct physiological actions of the two hormones in nonepithelial cells, where 11βHSD is not present (13, 34).

Nuclear receptors have two functional domains in their N-terminal A/B and C-terminal E/F domains. While autonomous activation function 2 (AF-2) in the C-terminal E/F domain is ligand binding dependent (49), autonomous activation function 1 (AF-1) in the N-terminal A/B domain is constitutively active, and unliganded nuclear receptors are able to block the transactivational function of AF-1 (31). Compared with the well-conserved E/F domains, the A/B domains, which contain AF-1, display no regions that are highly conserved between different nuclear receptors. We have previously detected two transactivational functions in MR, AF-1a and AF-1b, in the N-terminal A/B domain (16). Although previous studies have suggested a possible role for the A/B domain in ligand-selective functions of MR (26, 61), the physiological role of the A/B domain at the molecular level remains unclear.

DNA binding activators, like nuclear receptors, require basic transcription factors and coactivators for RNA polymerase II-mediated gene activation. RNA helicase A (RHA) is reported to be one of the components of the RNA polymerase II holoenzyme complex (36) and is a member of the DExH family of ATPase/helicases (48). Recent studies have shown that RHA interacts directly with coactivators and activators such as CBP/p300, BRCA1 (breast cancer-specific tumor suppressor protein), and SMN (survival motor neuron) to stabilize complex formation (2, 36, 39). These studies indicated that RHA might be a common coactivator acting as a bridging factor between basal transcription factors and activators.

For the ligand-induced transactivation function of nuclear receptors, two classes of coactivator complexes for AF-2 have been identified so far (11, 19). One is a histone acetyltransferase (HAT) complex thought to contain CBP/p300, p160

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protein family members (SRC-1 [steroid receptor coactivator 1], TIF2 [transcriptional intermediate factor 2], AIB1 [amplified in breast and ovarian cancer protein 1]), and an RNA coactivator, SRA, along with other, unknown components (3, 30, 38, 50, 53). Histone acetylation by intrinsic HAT activity of complexes converts the nucleosome into a transcriptionally active state by facilitating the access of activators to DNA binding (37). The other AF-2 coactivator complex is the recently reported DRIP (vitamin D receptor-interacting protein)/TRAP (thyroid hormone receptor-associated protein) complex, which is composed of at least 12 proteins but has no HAT activity (23, 41, 42). Although these coactivator complexes are reported to interact directly with the A/B domains of some nuclear receptors (8, 25, 27, 54, 55), little is known regarding AF-1-specific coactivator complexes. For MR, our observations that AF-1b, but not AF-1a, activity was enhanced by p160-family proteins support the existence of novel coactivator complexes for AF-1a (16).

In this study we attempted to identify MR AF-1a-interacting complexes by biochemical purification and mass fingerprinting by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS). One of the purified AF-1a region-interacting protein complexes was found to contain RHA and CBP (referred to below as the RHA/CBP complex). The interaction between RHA/CBP complexes and AF-1a was mediated through direct binding of RHA to the AF-1a region of MR. The finding that RHA did not bind to any MR region except AF-1a supported our previous observation that RHA potentiated the transcriptional activity of AF-1a but not that of AF-1b. For full-length MR, recruitment of the RHA/CBP complex to the AF-1a region was induced by aldosterone rather than hydrocortisone, which suggested that binding of hydrocortisone to MR may make the MR AF-1a region inaccessible to the RHA/CBP complex. Our results also raised the possibility that the differences between the biological activities of aldosterone and hydrocortisone in target tissues may be due to the ligand-selective recruitment of RHA/CBP complexes to MR.

MATERIALS AND METHODS

Plasmids. Rat MR deletion mutants FLAG-MR, FLAG-AF-1a, FLAG-AF-1b, FLAG-AF-2, and pcDNA-TIF2 were constructed as previously described (16). A series of rat MR deletion fragments (consisting of amino acids 1 to 169, 170 to 450, or 451 to 600, or fragment DEF) were inserted in-frame into the pAcG 2T vector (Pharmingen), while the series of RHA deletion mutants (with amino acids 1 to 262, 255 to 664, 649 to 1077, or 1064 to 1270 deleted) and the resultant chimeric proteins fused to glutathione *S*-transferase (GST) were as described previously (12). MREx2-tk-Luc was constructed by inserting two mineralocorticoid-responsive elements (MREs) from the tyrosine aminotransferase gene promoter into the pGL3-Basic vector (Promega) with a thymidine kinase (TK) promoter which was described previously (52, 54). Human CBP cDNA was obtained as several fragments by PCR using HeLa cell cDNA as the template and was reconstructed into pcDNA3, as described previously (36).

Antibodies. Antibodies used were an anti-FLAG M2 monoclonal antibody (F3165; Sigma), an anti-hemagglutinin (anti-HA) antibody (no. 561; Medical and Biological Laboratories Co., Ltd.), an anti-CBP antibody (sc369; Santa Cruz Biotechnology), an anti-MR antibody (sc11412; Santa Cruz Biotechnology), and an anti-RHA antibody (as previously described [36]).

Purification and separation of MR AF1a-interacting proteins. HeLa nuclear extracts were loaded onto a P11 phosphocellulose column. After extensive washes with washing buffer (20 mM Tris-HCl [pH 7.9], 150 mM KCl, 0.2 mM EDTA, 0.05% NP-40, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM dithiothreitol [DTT]), bound proteins were eluted by using elution buffer (20 mM Tris-HCl [pH 7.9], 1 M KCl, 0.2 mM EDTA, 0.05% NP-40, 10% glycerol, 0.5 mM PMSF, 1 mM DTT) and were then dialyzed in GST-binding buffer (20 mM Tris-HCl [pH 7.9], 180 mM KCl, 0.2 mM EDTA, 0.05% NP-40, 0.5 mM PMSF, 1 mM DTT) containing bovine serum albumin (1 mg/ml). After 4 to 5 h of dialysis, immobilized GST-MR AF-1a fusion proteins on the beads were incubated at 4°C for 6 to 10 h with HeLa nuclear extracts. After three washes with GST wash buffer (GST-binding buffer with 0.1% NP-40), the beads were further washed with GST wash buffer containing 0.2% *N*-lauryl sarcosine (Sarkosyl; Sigma). Complexes bound to MR AF-1a were eluted with 15 mM reduced glutathione in elution buffer (50 mM Tris-HCl [pH 8.3], 150 mM KCl, 0.5 mM EDTA, 0.5 mM PMSF, 5 mM NaF, 0.08% NP-40, 0.5 mg of bovine serum albumin/ml, and 10% glycerol). Eluates were then layered on top of a 4.5-ml linear 10-to-40% glycerol gradient in GST-binding buffer and centrifuged for 16 h at 4°C at 40,000 rpm in an SW40 rotor (Beckman). Each fraction (600 μl) was then applied to a Western blot using anti-CBP or anti-RHA antibodies. Protein standards used were vitamin B_{12} (1.3 kDa), myoglobin (17 kDa), ovalbumin (44 kDa), β-globulin (158 kDa), and thyroglobulin (667 kDa) (42, 54).

HAT assay. HAT activity in the glycerol density gradient fractions was assayed essentially as described previously (6). Briefly, the separated fractions were incubated with or without 10 μg of calf thymus histones (type IIA; Sigma) and ^{3}H-labeled acetyl coenzyme A (acetyl-CoA) (4.7 Ci/mmol; Amersham) for 30 min at 30°C, spotted onto Whatman P-81 filters, and washed extensively with sodium carbonate buffer (pH 9.1). Radioactivity remaining on the filter was then quantitated by liquid scintillation counting.

Protein identification by MALDI-TOF MS. Protein bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were excised and in-gel digested with trypsin according to published procedures (24, 43, 45). Eluted peptides were then prepared on the sampling plate for MALDI-TOF MS (Voyager DE-STR; Perseptive Biosystems). After analysis of each protein fragment mass, results were compared by using the MS-Fit program (University of California—San Francisco Mass Spectrometry Facility).

GST pulldown assay. A series of MR and RHA deletion mutants fused to GST were expressed in a baculovirus and in *Escherichia coli*, respectively, as described elsewhere (4). The predicted sizes of the expressed proteins were verified by SDS-PAGE. For GST pulldown assays, baculovirus- and bacterially expressed GST fusion proteins or GST bound to glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) was incubated at 30°C with [^{35}S]methionine-labeled proteins expressed by in vitro translation using the TNT coupled transcription-translation system (Promega). Baculovirus-expressed GST-MR-DEF was preincubated with aldosterone (10^{-6} M) for 15 min at room temperature. After 2 h of incubation, free proteins were removed by washing the beads with NET-N$^+$ buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl [pH 7.5], 0.5% NP-40, 1 mM PMSF, 1 mM DTT). Specifically bound proteins were eluted by boiling in SDS sample buffer and then analyzed by SDS–PAGE. After electrophoresis, radiolabeled proteins were visualized with an image analyzer (BAS1500; Fuji Film, Tokyo, Japan) (56).

Immunoprecipitation. After 293T cells were washed twice with ice-cold phosphate-buffered saline, the collected cells were resuspended in 1 ml of ice-cold lysis buffer (10 mM Tris-HCl [pH 4.7], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% [vol/vol] NP-40) and incubated on ice for 30 min; then they were centrifuged again for 5 min at 500 × *g*. The sedimented nuclear fractions were resuspended in TNE buffer (10 mM Tris-HCl [pH 7.5], 1% NP-40, 0.15 M NaCl, 1 mM EDTA) and incubated for 30 min on ice. After centrifugation, supernatants were used as whole-cell extracts of 293T cells for immunoprecipitation using an anti-FLAG M2 affinity resin (no. A2220; Sigma) after Western blotting with anti-FLAG M2 monoclonal, anti-HA, or anti-CBP antibodies.

Chromatin immunoprecipitation (ChIP) assay. HEK293 cells were cultured for 3 to 4 days in media supplemented with 10% charcoal-dextran stripped serum and were then infected with rat MR and RHA adenovirus expression constructs made by using the Adeno-X Expression System (Clontech Laboratories Inc.). After 2 days, cultures were treated with aldosterone (10^{-8} M) or hydrocortisone (10^{-8} M) for 45 min and immunoprecipitated with specific antibodies for MR, RHA, or CBP. Soluble chromatin was prepared by using an acetyl-histone H4 immunoprecipitation assay kit (Upstate Biotechnology) and was immunoprecipitated with antibodies against the indicated proteins. Extracted DNA samples were amplified with primer pairs Na-K-ATPase α1 (5′-CAGATTCTCAT TTT GGAATCTCGAAG-3′ and 5′-GATCTCCTCTGGGACTCA-3′) and αENaC (5′-TTCCTTTCCAGCGCTGGCCAC-3′ and 5′-CCTCCAACCTTGT CCAGA CCC-3′). Optimized PCR conditions used to allow semiquantitative measurement were 20 cycles of 30 s at 96°C, 15 s at 56°C, and 1 min at 72°C. PCR products were visualized on 2% agarose–Tris-acetate EDTA gels.

Luciferase assay. 293T cells were transfected by using Lipofectin reagent (GIBCO BRL). A luciferase reporter plasmid containing two MREs and the thymidine kinase promoter (MREx2-tk-Luc) was cotransfected with expression

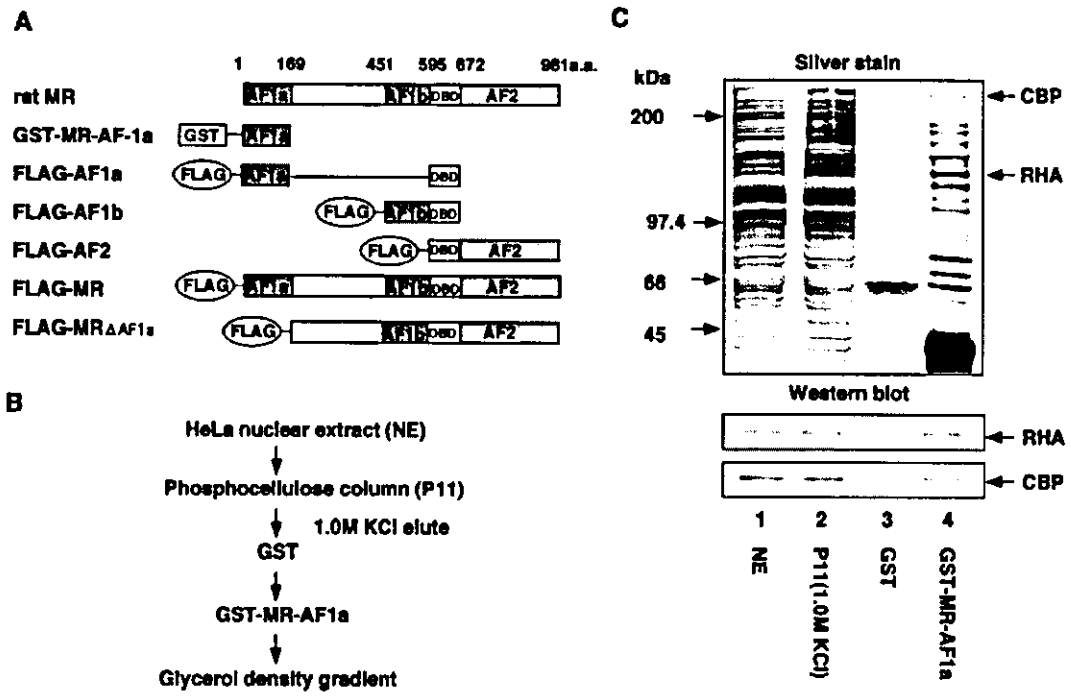

FIG. 1. Purification and identification of MR AF-1a-binding complexes. (A) Schematic representation of rat MR activation domains and deletion mutants. AF-1a and AF-1b are ligand-independent activation regions within the rat MR A/B domain. GST-fused MR AF-1a (GST-MR-AF-1a) was used as bait for binding-complex purification. A DNA binding domain (DBD) was present in all rat MR deletion mutants (FLAG-AF1a, FLAG-AF1b, FLAG-AF2, and FLAG-MRΔAF1a). (B) Purification scheme for MR AF-1a interactants. Nuclear extracts from HeLa S3 cells were applied to P11 phosphocellulose columns. After extensive washes with wash buffer (20 mM Tris-HCl [pH 7.9], 0.2 mM EDTA, 0.05% NP-40, 10% glycerol, 0.5 mM PMSF, 1 mM DTT) containing 0.15 M KCl, bound proteins were eluted with wash buffer containing 1.0 M KCl. Eluted fractions were then incubated with immobilized GST-MR-AF-1a, and MR AF1a-interacting proteins were eluted by using *N*-lauryl sarcosine. (C) Identification of MR AF-1a interactants. The indicated fractions were subjected to SDS-PAGE followed by silver staining. Total HeLa S3 nuclear extracts (NE) (lane 1), a fraction eluted from a P11 column (P11) (lane 2), and eluted fractions from GST and GST-MR-AF-1a (lanes 3 and 4) are shown. Proteins eluted from GST-MR-AF-1a (lane 4) were identified by MS analysis. Lower panels show Western blotting for each fraction using antibodies against RHA or CBP.

vectors as indicated in the figure legends. Six hours after transfection, the medium was replaced with fresh medium containing 0.2% fetal bovine serum. At this time, aldosterone (10^{-9} M) or hydrocortisone (10^{-9} M) ligands were added, and cells were incubated for an additional 12 h. Preparation of cell extracts and dual luciferase assays were performed according to the manufacturer's protocols (Promega). Individual transfections, each consisting of triplicate wells, were repeated at least three times.

RESULTS

A HAT complex containing RHA and CBP interacts with rat MR AF-1a. To identify coactivator complexes for rat MR AF-1a, HeLa cell nuclear extract fractions, prepurified on phosphocellulose columns, were incubated with either GST-fused AF-1a protein or GST protein alone. After extensive washing, bound proteins were eluted by reduced glutathione and subjected to SDS-PAGE analysis. Protein bands which specifically bound to GST-MR-AF-1a protein were subjected to peptide mass fingerprinting by MALDI-TOF MS. Obtained masses and apparent molecular weights of the different polypeptides revealed that the fraction eluted from the AF-1a fusion protein contained RHA and CBP. Mass fingerprinting results were confirmed by Western blotting using specific antibodies against RHA and CBP (Fig. 1C). The presence of CBP in the purified fraction was consistent with our previous findings that CBP/p300 enhanced AF-1a activity (16) and with another study in which interaction between CBP and RHA was observed (36). Thus, the AF-1a region may interact with a coactivator complex containing both CBP and RHA.

AF-1a region-interacting proteins were further fractionated according to molecular mass by using a glycerol density gradient. Since CBP has HAT activity, HAT activity for each fraction was assessed (Fig. 2B). Proteins in each fraction were separated by SDS-PAGE in parallel and were either silver stained or analyzed by Western blotting using specific antibodies against RHA and CBP (Fig. 2A and C, respectively). RHA and CBP were detected in fractions that possessed high HAT activity and were included in a multiprotein complex of more than 670 kDa, which suggested that a CBP-containing HAT multiprotein complex (designated the RHA/CBP complex) bound MR AF-1a (Fig. 2C).

RHA interacts directly with MR AF-1a. To test whether RHA and CBP interacted directly with the AF-1a region, a GST pulldown assay was performed by using a series of GST-fused MR and RHA deletion mutants, together with in vitro-translated proteins. While RHA directly bound the N-terminal region (amino acids 1 to 169) of MR, CBP did not bind to any of the MR A/B domain deletion mutants (Fig. 3A). We then

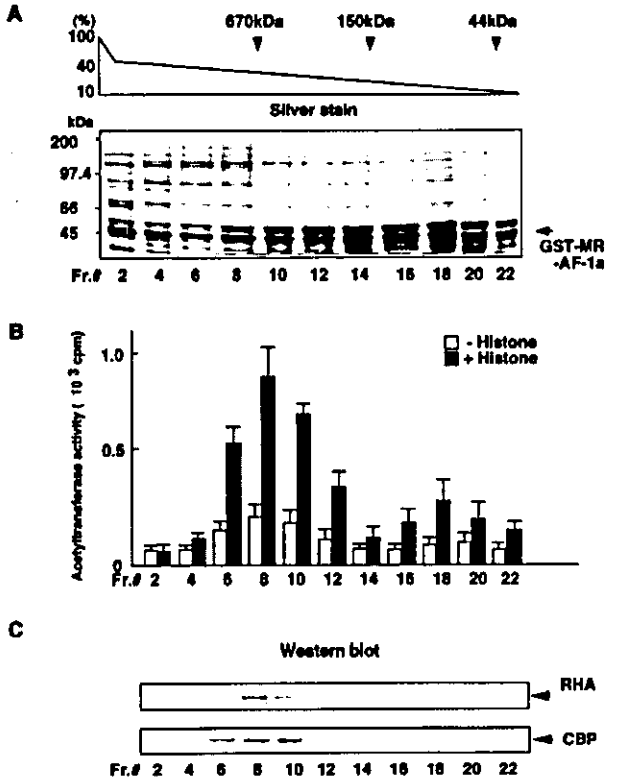

FIG. 2. A HAT complex containing RHA and CBP interacts with MR AF-1a. (A) Glycerol density gradient analysis. Fractions eluted on a P11 column were passed over an immobilized GST-MR-AF-1a column. Protein complexes bound to GST-MR-AF-1a were then dissociated by reduced glutathione and applied to 10-to-40% glycerol density gradients. Each fraction was subjected to SDS-PAGE followed by silver staining. The positions of marker proteins with known molecular masses on the gradient are indicated. (B) HAT activity in the glycerol density gradient fractions. The indicated fractions were incubated with or without free histones, together with ^{3}H-labeled acetyl-CoA, and assayed for HAT activity in a filter-binding assay as described in Materials and Methods. HAT activity is quantitated as counts per minute of ^{3}H-labeled acetate transferred from acetyl-CoA to histones. (C) Western blot analysis of glycerol density gradient fractions. To identify the proteins contained in each gradient fraction, Western blot analysis was performed with specific antibodies against RHA or CBP.

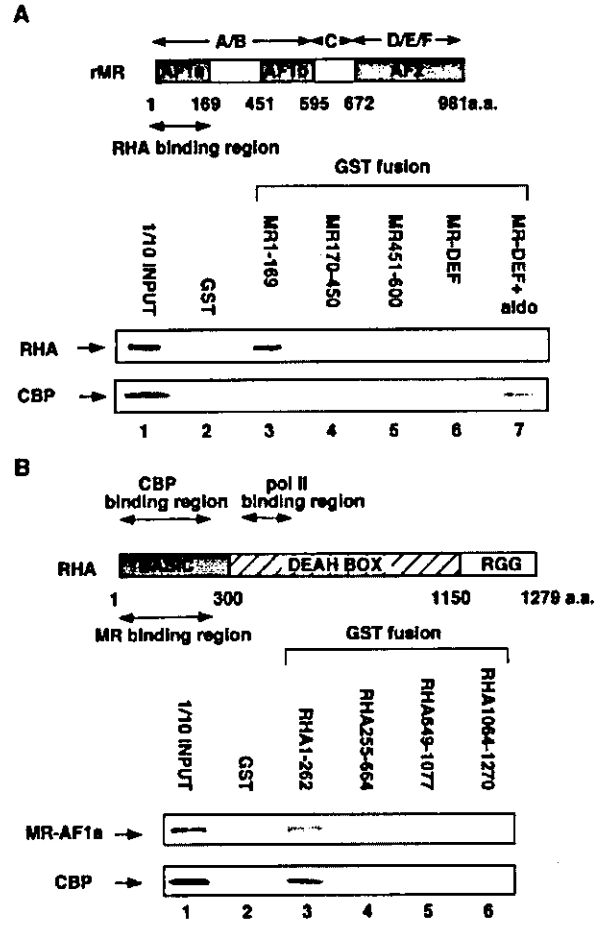

FIG. 3. RHA interacts directly with MR AF-1a. (A) RHA, but not CBP, interacts directly with MR AF-1a. A GST pulldown assay was performed as described in Materials and Methods. A series of MR deletion mutants fused to GST were expressed by a baculovirus, and the GST fusion proteins were immobilized on glutathione-Sepharose beads. In vitro-translated RHA and CBP were incubated with glutathione-Sepharose beads loaded with each MR mutant fused to GST (for GST-MR-DEF, in the absence and presence of 10^{-6} M aldosterone). Bound proteins were subjected to SDS-PAGE followed by autoradiography. rMR, rat MR; aldo, aldosterone. (B) Mapping of the MR binding region to the N-terminal domain of RHA. A series of RHA deletion mutants fused to GST were expressed by *E. coli* and immobilized on glutathione-Sepharose beads. In vitro-translated MR AF-1a and CBP were then incubated with the glutathione-Sepharose beads loaded with each RHA mutant fusion protein. Bound proteins were subjected to SDS-PAGE followed by autoradiography.

tested for interaction between RHA and AF-1a or CBP. The AF-1a region exhibited affinity for the N-terminal region (amino acids 1 to 262) of RHA (Fig. 3B). As expected from previous studies, we observed direct binding of CBP to the basic region of RHA (36). However, we failed with the N-terminal deletion mutants to map the core regions for the interactions, because RHA and CBP could not be distinguished in the interactions. The physical interactions appeared to require that the protein structures of the interacting regions be intact. These results suggested that RHA within the RHA/CBP complex acted as the interface for the AF-1a region.

RHA/CBP complexes are recruited to MR bound on the target gene promoters in the presence of aldosterone but not hydrocortisone. We then used coimmunoprecipitation to test whether interaction between AF-1a and RHA/CBP complexes took place in living cells. By use of antibodies against FLAG and HA, both HA-RHA fusion protein and endogenous CBP were detected by Western blotting in AF-1a immunoprecipitates from extracts of expression vector-transfected 293T cells. Increased amounts of CBP were immunoprecipitated with AF-1a in cells that overexpressed RHA (Fig. 4A, lane 4), which supported our finding that RHA mediated the indirect association of CBP with MR AF-1a. We next tested for in vivo interaction between full-length MR and RHA/CBP complexes. Like the AF-1a region, full-length MR also coimmunoprecipitated with CBP and RHA in an aldosterone-dependent manner, with increased CBP retention in immunoprecipitates from

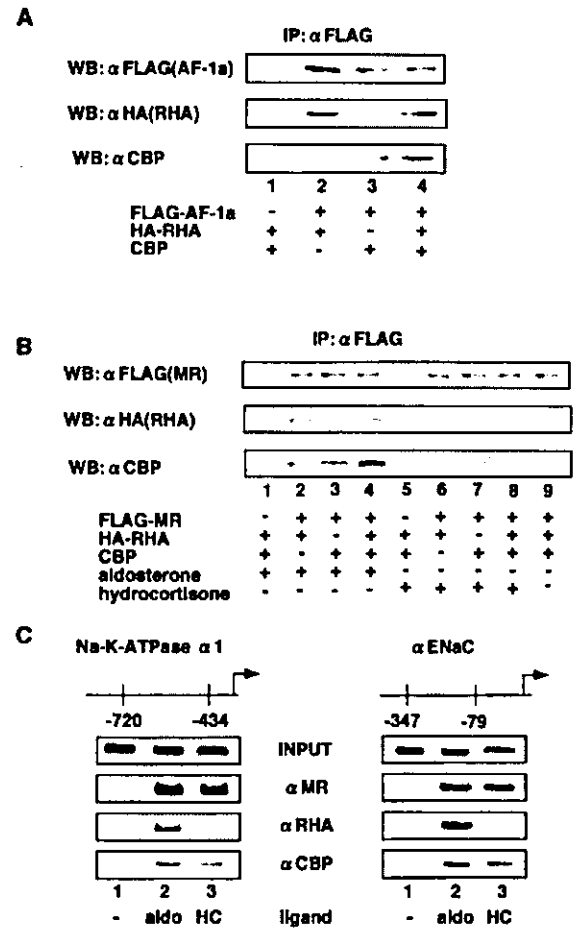

FIG. 4. Ligand-selective recruitment of RHA-CBP complexes to MR AF-1a in vivo. (A) RHA/CBP complexes interact with MR AF-1a in vivo. 293T cells were cotransfected with or without 5 μg of pc-DNA-FLAG-MR-AF-1a, pc-DNA-HA-RHA, or pc-DNA-CBP. Cells were lysed in TNE buffer and subjected to immunoprecipitation (IP) using an anti-FLAG (αFLAG) affinity resin. Precipitates were Western blotted by using antibodies against FLAG, HA, or CBP as indicated. (B) Aldosterone, but not hydrocortisone, induces interaction between RHA/CBP complexes and MR. 293T cells were cotransfected with or without 5 μg of pc-DNA-FLAG-MR, pc-DNA-HA-RHA, or pc-DNA-CBP in the presence or absence of 10^{-8} M aldosterone or hydrocortisone. Cells were lysed in TNE buffer and subjected to immunoprecipitation using an anti-FLAG affinity resin. Precipitates were Western blotted with antibodies against FLAG, HA, or CBP as indicated. (C) RHA/CBP complexes are recruited to the MR target gene promoters Na-K-ATPase α1 and αENaC in an aldosterone-dependent manner. HEK293 cells were transfected with an adenovirus expressing rat MR and RHA. Soluble chromatin was prepared from HEK293 cells treated with 10^{-8} M aldosterone (aldo) or hydrocortisone (HC) for 45 min and was immunoprecipitated with antibodies against MR, RHA, or CBP. (i.e., the ChIP assay was performed). DNA was then extracted and amplified with pairs of primers covering the Na-K-ATPase α1 and αENaC gene promoter regions as indicated.

RHA-overexpressing cells (Fig. 4B, lanes 1 to 4). However, in the presence of hydrocortisone, coimmunoprecipitation of MR and RHA was not detected and no increase in the level of MR-associated CBP was observed when RHA was overexpressed (Fig. 4B, lanes 5 to 8).

To further investigate whether the RHA/CBP complex was indeed recruited to MR in the presence of aldosterone rather than hydrocortisone in living cells, we performed a ChIP assay using promoters from the MR target genes encoding the Na-K-ATPase α1 subunit and the α subunit of the epithelial sodium channel (αENaC) (29, 35, 46). MR was recruited together with RHA/CBP complexes to the promoter regions of both target genes in the presence of aldosterone, but not in the presence of hydrocortisone, supporting the hypothesis that aldosterone selectively induced the recruitment of RHA/CBP complexes to MR (Fig. 4C).

RHA and CBP cooperatively enhance MR AF-1a transactivation. To test whether the aldosterone-induced recruitment of RHA/CBP complexes potentiated MR function, a transient expression assay using a luciferase reporter with MREs in the promoter was performed. Full-length and deletion mutant MR expression plasmids were cotransfected into 293T cells in the presence or absence of aldosterone. The results of this assay showed that the expression of RHA or CBP enhanced both AF-1a region transactivation and the aldosterone-induced transactivation function of full-length MR. RHA and CBP exhibited cooperative activity in transactivational enhancement of both AF-1a and full-length MR, which was consistent with the hypothesis that RHA and CBP act as part of the same coactivator complex. The lack of synergism between RHA and CBP in the AF-1a potentiation may be due to the relative abundances of the two factors. The transactivation functions of the AF-1b and AF-2 regions, which did not bind RHA, were not potentiated by RHA, while CBP and TIF2 potently coactivated AF-2 (Fig. 5).

Interaction with RNA polymerase II is not essential for MR transactivation by RHA. It has been reported previously that RHA directly interacts with RNA polymerase II through the minimal transactivation domain (MTAD) within the RHA molecule (4). However, RNA polymerase II was not detected in the glycerol density gradient fractions that contained the RHA/CBP complexes (data not shown). Therefore, using RHA mutants RHAΔMTAD and RHA W339A, which do not bind RNA polymerase II, we examined whether interaction between RHA and RNA polymerase II was required for the enhancement of AF-1a function by RHA/CBP complexes (4). Since both mutants were still functional, our results suggested that association between RNA polymerase II and RHA was not essential for RHA/CBP complex-mediated potentiation of AF-1a transactivation (Fig. 6).

RHA potentiates MR in a ligand-selective manner. Our results suggested that recruitment of RHA/CBP complexes to MR bound to target gene promoters was ligand selective. Therefore, we next investigated whether RHA/CBP complexes potentiated MR in a ligand-selective manner. In agreement with previous results, RHA was active only for aldosterone-bound MR (Fig. 7A). An MR mutant that lacked the AF-1a region (MRΔAF1a) lost potentiation of aldosterone-induced transactivation by RHA (Fig. 7B). Thus, aldosterone binding to MR may induce an altered conformation of the A/B domain, which would then result in increased access of RHA/CBP complexes to the A/B domain. In contrast, the AF-1a region of hydrocortisone-bound MR may be in a conformation that blocks RHA/CBP complex association.

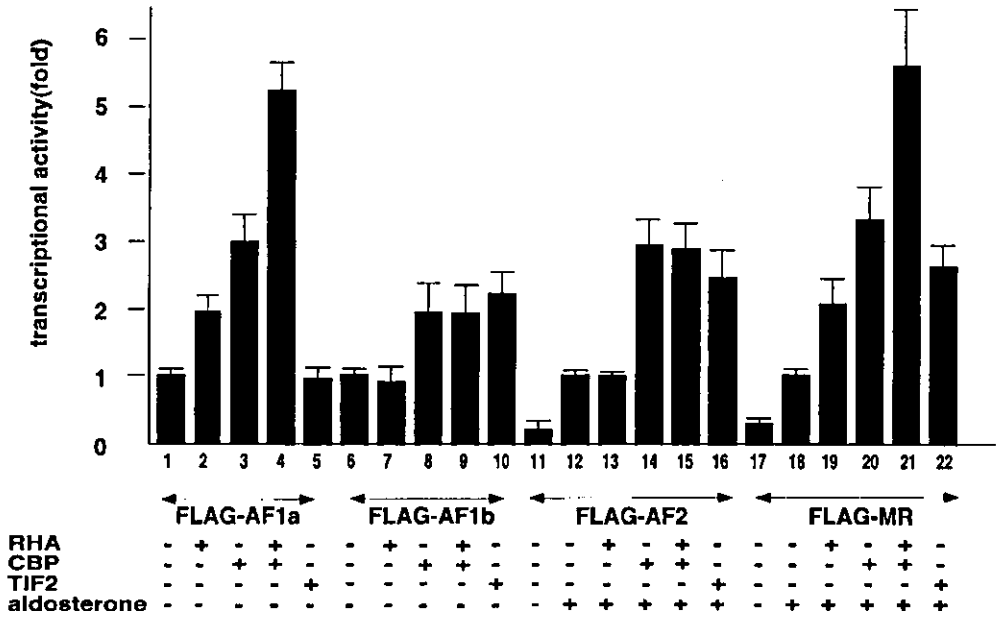

FIG. 5. RHA and CBP cooperatively potentiate AF-1a and aldosterone-bound MR. 293T cells were cotransfected with 0.25 μg of a luciferase reporter plasmid bearing progesterone response elements (PREx2-tk-luc), 50 ng of an expression vector containing an MR deletion mutant tagged with FLAG, and 0.3 μg of either pc-DNA, pc-DNA-HA-RHA, pc-DNA-CBP, or pc-DNA-TIF2 in the presence (+) or absence (−) of aldosterone (10^{-9} M). Bars show the fold change in luciferase activity relative to the activity of the MR deletion mutant (in the presence of 10^{-9} M aldosterone) or the activity of pc-DNA-FLAG-MR-FL (in the presence of 10^{-9} M aldosterone) without transfection of coactivators.

DISCUSSION

The A/B domain is poorly conserved among the members of the nuclear receptor superfamily, and the function of the A/B domain in terms of ligand-induced transactivation and coactivator action is not fully understood. In a previous study, we reported that the rat MR A/B domain harbored two AF-1 regions, designated AF-1a and AF-1b. While AF-1b and AF-2 activities were enhanced by TIF2 and CBP/p300, AF-1a activity was potentiated by CBP/p300 but not by TIF2 (16). Therefore, it was thought likely that AF-1a was potentiated by different coactivator complexes that contained CBP/p300 rather than TIF2. In the present paper, we have shown that the RHA/CBP complex acts as a coactivator of AF-1a activity. Glycerol density gradient analysis showed that RHA and CBP were present in fractions that possessed high HAT activity and were likely to contain multiprotein complexes of more than 670 kDa. Together with our previous observation that TIF2 was unable to potentiate AF-1a function, our present study suggests that RHA/CBP complexes probably contribute, at least in part, to the observed HAT activity of AF-1a-bound protein fractions (37).

RHA has been reported to be a component of the holoenzyme complex with RNA polymerase II, as well as to interact with CBP (36). In agreement with this previous study, RHA cosedimented with CBP in the same glycerol density gradient fractions, and RHA potentiated the interaction between CBP and the AF-1a region, leading to enhanced CBP-stimulated AF-1a activity. However, in contrast to the findings of previous studies (4, 12, 36), RNA polymerase II was not detected in the glycerol density gradient fractions that contained RHA and CBP (data not shown). Furthermore, the potentiation of AF-1a function by RHA mutants that lacked the putative interacting region for RNA polymerase II was comparable to that by wild-type RHA, which indicated that the RHA coactivator function for MR AF-1a required CBP but did not require direct association with RNA polymerase II.

SDS-PAGE analysis revealed that several proteins cosedimented with RHA and CBP. Unfortunately, the mass fingerprinting method we employed was unable to identify these other proteins. The failure to ascertain the identity of these other RHA/CBP complex components may be due to their relatively low abundance. Given the fact that MR AF-1a-associated complexes exhibited a broad spectrum of molecular weights within the glycerol density gradient fractions, it is likely that AF-1a-interacting complexes other than the RHA/CBP complex exist in the nucleus. Given the observed sizes of the HAT complexes on the glycerol density gradient, only some of the AF-1a-associated proteins appear to form the RHA/CBP complex.

Comparison of the crystal structures of ligand-binding domains from several nuclear receptors revealed that cognate ligand binding induced the repositioning of the C-terminal α-helical structure (H12) to form a hydrophobic groove, which then served as an interaction surface for AF-2 coactivators (7, 9, 18). We have previously shown that for the vitamin D receptor, some synthetic ligands can induce different H12 configurations that create recognition surfaces for the ligand-selective recruitment of various coactivators (47). However, GST pulldown and luciferase assays using MR and known AF-2 coactivators failed to detect ligand-selective coactivator re-

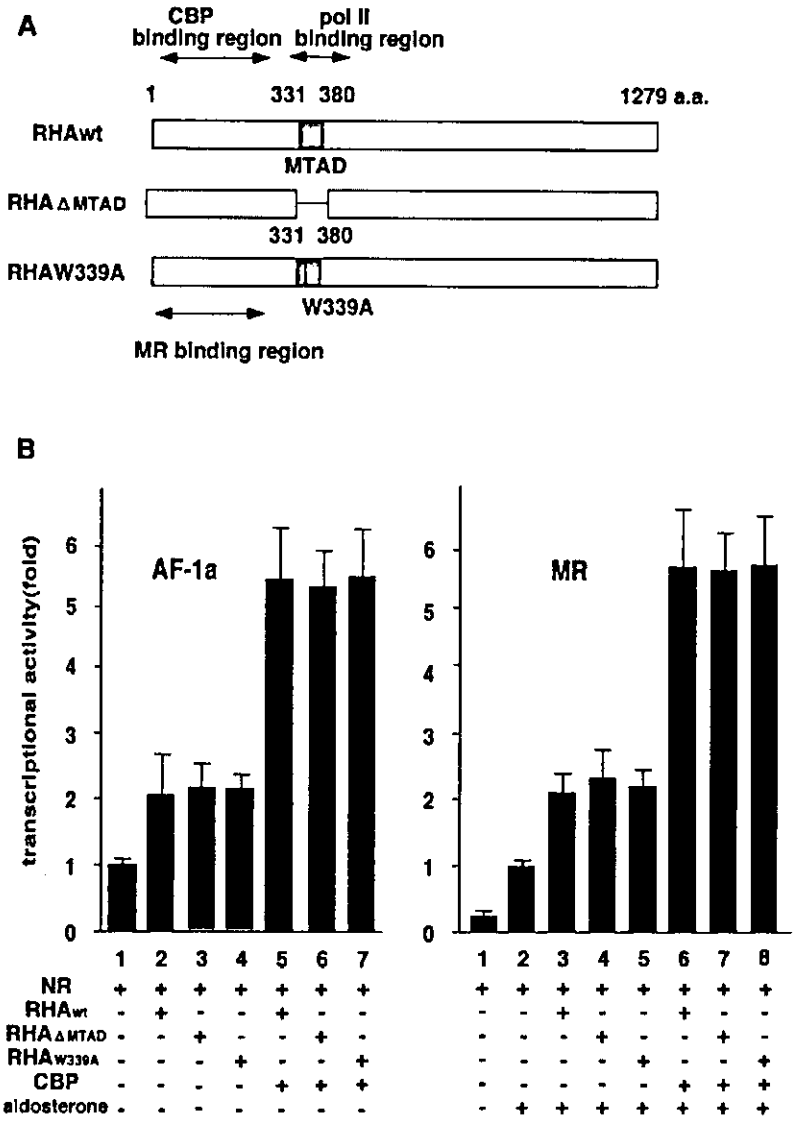

FIG. 6. RHA-RNA polymerase II binding is not necessary for potentiation of MR AF-1 by RHA/CBP complexes. (A) Schematic representation of the RHA mutants. MTAD acts as the RNA polymerase II interacting domain. However, the point mutant RHA W339A prevents this interaction. (B) RHA-RNA polymerase II binding is not necessary for potentiation of MR AF-1 by the RHA/CBP complex. 293T cells were cotransfected with 0.25 μg of a luciferase reporter plasmid bearing progesterone response elements (PREx2-tk-luc), 50 ng of an expression vector containing pc-DNA-FLAG-MR-AF-1a or pc-DNA-FLAG-MR, and 0.3 μg of either pc-DNA-HA-RHA, pc-DNA-HA-RHAΔMTAD, pc-DNA-HA-RHAW339A, or pc-DNA-CBP in the presence (+) or absence (−) of aldosterone (10^{-9} M). Bars show the fold change in luciferase activity relative to the activity of FLAG-MR-AF1a (AF-1a) or FLAG-MR (MR) in the presence of aldosterone (10^{-9} M) without transfection of coactivators. NR, nuclear receptor.

cruitment in MR (data not shown). In this study we showed that full-length MR recruited RHA/CBP complexes through the AF-1a region and that this enhanced transactivation function was ligand dependent. Considering these results together with the results of previous studies showing the different contributions of AF-1 activity to full-length MR activity induced by endogenous aldosterone and hydrocortisone (22, 26), the two different ligands appear to induce different A/B region conformations. Thus, it appears that different nuclear receptor ligands may induce different conformations not only in E/F domains but also in A/B domains (44, 51), so that ligand-selective coactivator recruitment could be a feature of both E/F and A/B domains (28).

It is thought that the specific actions of the two MR ligands observed in vivo are at least partially mediated by the restricted tissue expression of the enzyme 11βHSD2 and the subsequent inactivation of hydrocortisone in MR target tissues. While the balance between MR and glucocorticoid receptor expression levels in a given tissue or cell may lead to ligand-specific actions, these factors appear to be insufficient to fully confer ligand specificity, particularly in nonepithelial mineralocorticoid target tissues (e.g., the heart and central nervous system),

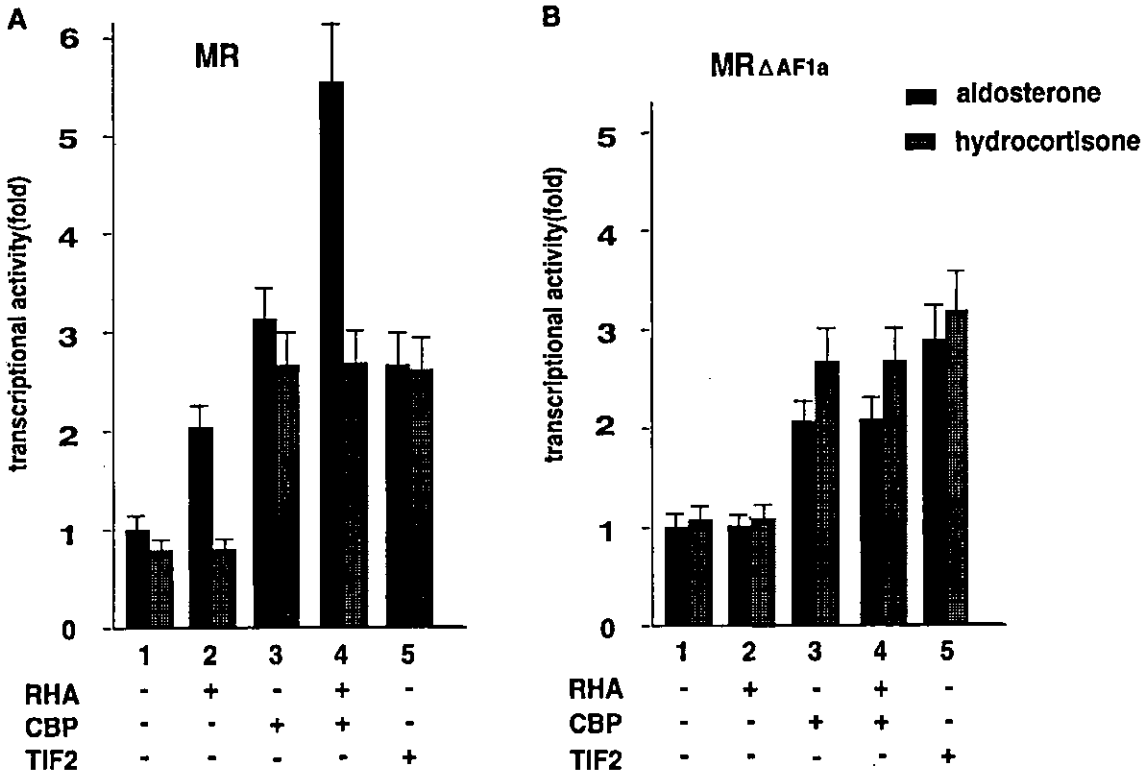

FIG. 7. RHA and CBP cooperatively potentiate MR in a ligand-selective manner. (A) Aldosterone-dependent potentiation of the MR transactivation function by the RHA/CBP protein complex was observed, but hydrocortisone-dependent potentiation was not. (B) AF-1a is essential for the ligand-selective potentiation by the RHA/CBP complex. 293T cells were cotransfected with 0.25 μg of a luciferase reporter plasmid bearing progesterone response elements (PREx2-tk-luc), 50 ng of an MR deletion mutant expression vector, and 0.3 μg of either pc-DNA-HA-RHA, pc-DNA-CBP, or pc-DNA-TIF2 in the presence (+) or absence (−) of either aldosterone (10^{-9} M) or hydrocortisone (10^{-9} M). Bars show the fold change in luciferase activity relative to the activity of FLAG-MR-FL (MR) or FLAG-MRΔAF1a (MRΔAF1a) in the presence of aldosterone (10^{-9} M) without transfection of coactivators.

where 11βHSD2 is not expressed (33). We show here that the recruitment of RHA/CBP coactivator complexes may discriminate between the actions of the two endogenous MR ligands. The tissue-specific activity of the MR ligands suggests the possible existence of tissue-specific components of coactivator complexes (57, 59, 60). A recent report using MR knockout mice shows that MR is essential for the neurogenesis of granule cells in the hippocampus (17). As the ratio between serum aldosterone and hydrocortisone levels varies during neuronal development, it is thought that MR functions at a critical stage during neuronal development. Therefore, to better understand MR ligand-specific activity at the molecular level, it will be of great interest to assess the expression and physiological function of the RHA/CBP complex in different tissues.

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