Role of Important Hydrophobic Amino Acids in the Interaction between the Glucocorticoid Receptor τ1-Core Activation Domain and Target Factors†

Tova Almlöf,* Annika E. Wallberg, Jan-Åke Gustafsson, and Anthony P. H. Wright

Department of Biosciences, Karolinska Institute, Novum, S-141 57 Huddinge, Sweden

Received December 10, 1997; Revised Manuscript Received March 25, 1998

ABSTRACT: In this work, we determined how altered-function mutants affecting hydrophobic residues within the τ1-core activation domain of the human glucocorticoid receptor (GR) influence its physical interaction with different target proteins of the transcriptional machinery. Screening of putative target proteins showed that the τ1-core can interact with the C-terminal part of the CREB-binding protein (CBP). In addition, the previously identified interactions of the τ1-core with the TATA-binding protein (TBP) and the Ada2 adaptor protein were localized to the C- and N-terminal regions of these proteins, respectively. A panel of mutations within the τ1-core that either decrease or increase activation potential was used to probe the interaction of the τ1-core domain with TBP, Ada2, and CBP. We found that the pattern of effects caused by the mutations was similar for each of the interactions and that the effects on binding generally reflected effects on gene activation potential. Thus, the predominant effect of the mutations appears to influence a property of the τ1-core that is common to all three interactions, rather than properties that are differentially required by each of the target factor interactions, individually. Such a property could be the ability of the domain to adopt a folded conformation that is generally necessary for interaction with target factors. We have also shown that TBP, Ada2, and CBP can interact with both the τ1-core and the GR ligand-binding domain, offering a possible mechanism for synergistic interaction between the τ1-core and other receptor activation domains. However, other target proteins (e.g., RIP140, and SRC-1), which interact with the GR C terminus, did not show significant interactions with the τ1-core under our conditions.

The GR[1] is a member of a large family of ligand inducible nuclear receptors (*1, 2*). This receptor mediates the effects of glucocorticoids by regulating transcription of target genes. Binding of glucocorticoids causes dissociation of the receptor from an inactive complex containing heat shock proteins, allowing it to bind to DNA. The receptor binds as a homodimer to specific glucocorticoid response elements (GREs) present within genes regulated in response to glucocorticoids. Transcriptional activation by GR is mediated mainly by two transactivation functions, τ1 (residues 77−262) located in the N-terminal part of the receptor and τ2 (residues 526−556) in the C-terminal part of the receptor (*3, 4*). An additional, highly conserved region located at the very C terminus of the receptor may also be involved in transcriptional activation (*5*). Among these transactivation domains, the τ1 domain constitutes the major transactivation function (*4*). This domain also functions in isolation when fused to the DNA-binding domains of either the GR (*6*) or the LexA repressor protein (*7*). The τ1 domain contains a 58 amino acid peptide, the τ1-core, that retains almost all the τ1 activity when assayed in yeast (*8*). However, structural studies using circular dichroism and nuclear magnetic resonance spectroscopy have shown that the τ1 domain and the τ1-core are largely unstructured in aqueous solution (*9*). Thus, as seen in a number of other proteins (*10*−*13*), a very small segment of the GR protein with poor propensity for structure formation still functions as a powerful mediator of gene activation.

It is possible that unstructured activation domains contribute to gene activation via relatively nonspecific ionic or hydrophobic interactions with target proteins, but, in the case of the GR τ1-core domain, the phenotypes of amino acid substitution mutants that alter activity are critically dependent on their position within the protein (*7*). This observation is more easily interpreted in the context of a structured activation domain. Consistent with this view, the τ1-core does adopt an α-helical conformation in the nonpolar solvent trifluoroethanol (*9*). Proline substitution mutants within the predicted α-helical segments reduce the activity of the τ1-core, suggesting that these putative α-helices may play a role in vivo (*14*). One possibility is that a structured conformation of the τ1-core is induced or stabilized during interaction with target proteins. Three recent reports support this model more directly in the case of the activation domains of the mammalian activator, c-myc (*13*), and the herpes simplex virus protein, VP16 (*15, 16*).

The mechanism by which DNA-bound activator proteins stimulate transcription is poorly understood, but it is thought to involve direct or indirect contacts between activation

† This work was supported by the Swedish Natural Science Research Council (Grants K-AA/KU9756-308 and I-AA/LS9756-307) and the Swedish Medical Research Council (Grant 13x-2819).

* To whom correspondence should be addressed. Telephone: +46-8-608 9158. Fax: +46-8-774 5538. E-mail: tova.almlof@cbt.ki.se.

[1] Abbreviations: GR, glucocorticoid receptor; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; TBP, TATA-binding protein; GREs, glucocorticoid responsive elements; GST, glutathione *S*-transferase; IPTG, isopropyl 1-thio-β-D-galactose; AF-2, activation function-2.

S0006-2960(97)03029-8 CCC: $15.00
Published on Web 06/11/1998

domains and the general transcriptional machinery (RNA polymerase II and a range of general transcription factors) (reviewed in *17*). Evidence for the involvement of the τ1-core in such interactions comes from squelching experiments in which elevated levels of the isolated τ1 or τ1-core domain suppress transcription from minimal promoters in vivo (*6*) and in vitro (*8, 18*). These results suggest that the activation domain titrates limiting factors that are required for basal transcription. At least some of these factors are likely to be involved in formation of the transcription complex because the GR has been shown to facilitate this process in vitro (*19, 20*). However, kinetic experiments, in which monoclonal antibodies were used to block τ1 activity, indicate that the τ1 domain also functions at the stages of initiation and/or reinitiation that follow assembly of the transcription complex (*21*).

During recent years, a number of proteins have been identified that modulate GR activity (reviewed in *22*). Of these, GRIP1 (*23*), RIP 140 (*24*), SRC-1 (*25*), and CBP/p300 (*26*) have been shown to potentiate transcriptional activation and/or bind to the GR (*23*; Windahl, personal communication). Additional coactivators that have been shown to modulate GR activity are the η member of the 14-3-3 protein family (*27*), GRIP170 (*28*), RAP46 (*29*), and hbrm which is the human homologue of the yeast chromatin modulating factor Swi/Snf (*30*). In addition, the yeast ubiquitin protein ligase Rsp5 (homologue of human RPF1) has been shown to potentiate transcriptional activation by the GR and the progesterone receptor in both yeast and mammalian cells. Coexpression of the yeast protein Spt3 (homologue of human $TAF_{II}18$) further increased this activity (*31*). Most coactivator proteins have been isolated as targets for the ligand-binding domain of the GR, and it is not known whether they function exclusively through this part of the receptor, or whether they can also modulate the activity of the N-terminal transactivation domain, the τ1-core.

The only proteins that have been shown to be direct targets for the τ1-core activation domain are TBP and the Ada2 protein (*32, 33*). TBP is the DNA-binding subunit of the TFIID coactivator complex. TFIID has the capacity to phosphorylate other components of the transcription complex (*34*) and to acetylate histones (*35*) in addition to its established role in specifying the site of transcription complex formation (*36*). TFIID is critical for τ1-mediated transcriptional activation in HeLa cell nuclear extracts (*32*). Ada2 is a subunit of the Ada adaptor complex that can enhance interaction between some activation domains and TBP (*37*). This complex also has a histone acetylase activity (*38*). The Ada complex is critical for τ1-core-mediated activation in yeast, and the human Ada2 protein stimulates GR activity in mammalian cells (*33*).

Despite its small size and low propensity for structure conformation, the τ1-core of the GR makes important contacts with at least two target factors (TFIID and the Ada adaptor complex). It is possible that the mutants' influence on the interaction of activation domains with target factors occurs at two levels: (i) activation domain folding and (ii) target factor interaction (recognition and binding). If the major effect of the mutants is on level (i), the interactions with all target proteins would be changed in a similar way. A disruption at level (ii) would give different effects for each target protein, except in the unlikely case where apparently unrelated target proteins are recognized in the same way. The purpose of this work was to (a) attempt to identify new potential targets for the τ1-core domain, (b) use a panel of τ1-core altered-activity mutations to determine whether they cause similar or distinct effects on the interaction with different target factors, and (c) determine whether the different GR activation domains function via the same or distinct sets of target proteins.

MATERIALS AND METHODS

Plasmids: Constructs for in Vitro Translation. pBK-CMV-HA-RIP140 expresses RIP140 fused to the hemagglutinin (HA) epitope. pBK-CMV-CBP-N and pBK-CMV-CBP-C1 express amino acids 1−452 and 1678−2441 of mouse CBP, respectively. Details of cloning will be presented elsewhere. The clone containing the mouse CBP coding sequence (*26*) was a gift from Richard G. Goodman (Vollum Institute, Oregon Health Science University, Portland, OR). pBK-CMV-hTBP was constructed by cloning the coding sequence for human TBP amplified by PCR from pET-6HishTBP (M. Meyer, EMBL, Germany) into pBK-CMV. This plasmid expresses human TBP from the T3 promoter. pBK-CMV-HA-RIP140, pBK-CMV-CBP-N, pBK-CMV-CBP-C1, and pBK-CMV-hTBP were kindly provided by Eckardt Treuter (Karolinska Institute, Sweden). pT7-ADA2 (*39*) was a gift from Leonard Guarente (Massachusetts Institute of Technology), and pBK-CMV-SRC-1 (*25*) was provided by Bert W. O'Malley (Baylor College of Medicine, Houston, TX).

GST-Fusion Constructs. pGEX-KG-TBP, pGEX-KG-TBP-N (residues 1−163) and pGEX-KG-TBP-C (residues 168−339) were kindly provided by Tony Kouzarides (Cambridge, United Kingdom) and have been described previously (*40*). The plasmids pGEX-Ada2, pGEX-Ada2-N, (residues 1−176), pGEX-Ada2-M (residues 176−333), and pGEX-Ada2-C (residues 333−434) (*37*) were gifts from Shelly Berger (Wistar Institute). The plasmid pGEX-h$TAF_{II}18$ (*41*) was kindly provided by Irwin Davidson (Collège de France, France), pGEX-PC4 (*42*) was a gift from Hui Ge (Rockefeller University, New York, NY), and pGEX-14-3-3η (*27*) was kindly provided by Hideki Wakui (Karolinska Institute, Sweden). pGEX-CBP-C1 was constructed by inserting the sequence encoding residues 1678−2441 of CBP as a *Eco*RI−*Not*I fragment into pGEX-5x (Pharmacia). pGEX-CBP-C2 was constructed by deleting a *Nae*I−*Not*I fragment from pGEX-CBP-C1 and filling in the cohesive ends using the Klenow fragment of DNA polymerase I (Amersham) followed by religation. This plasmid expresses residues 1678−1868 of CBP fused to GST. pGEX-RSP5 expresses full-length Rsp5 fused to GST and was kindly provided by Donald P. McDonnell (Duke University Medical Center, Durham, NC). pGEX-τ1-core (*32*) expresses residues 187−244 of the human GR coding sequence fused to GST and was kindly provided by Jacqueline Ford (Karolinska Institute, Sweden). pGEX-GRLBD expresses amino acids 485−777 of the human GR coding sequence and was cloned as a *Bam*HI fragment from pEG202-GR 485-777 (*27*) into *Bam*HI digested pGEX-5x-3 (Pharmacia). This plasmid was kindly provided by Johanna Zilliacus (Karolinska Institute, Sweden).

Histidine-Tagged Proteins. pET-τ1core-LexA and derivatives containing τ1-core mutations were constructed by

inserting *Sac*I fragments from pRS315-LexA and derivatives (*7*) into pET-LexA (*33*). These plasmids express wild-type and mutant τ1-core proteins fused to a six-histidine tag and residues 1−87 from the *Escherichia coli* (*E. coli*) LexA repressor protein DNA-binding domain.

Protein Expression. [^{35}S]Methionine-labeled proteins were synthesized using an in vitro translation kit (Promega, Madison, WI) at 30 °C for 90 min. Protein expression plasmids were transformed into the *E. coli* strain BL21-(DE3) pLys S. The expression of GST, GST-fusion proteins, and histidine-tagged τ1-core-LexA proteins was induced by adding isopropyl 1-thio-β-D-galactose (IPTG) to a final concentration of 0.1 M for 1.5−2 h. The induced GST-fusion proteins were purified from bacterial extracts using glutathione−agarose beads (Sigma) according to standard protocols. Induced histidine-tagged proteins were purified using Ni NTA agarose (Qiagen) according to manufacturer's instructions.

In Vitro Binding Assay. GST-fusion proteins were bound to glutathione−agarose beads (Sigma) at an approximate concentration of 1 mg of protein/mL of beads in GST pull-down buffer [20 mM Hepes (pH 7.9), 10% (v/v) glycerol, 100 mM KCl, 0.2 mM EDTA, 5 mM $MgCl_2$, 0.2 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol]. Purified τ1-core-LexA proteins were added to the beads in 8 bed volumes (200 μL) of GST pull-down buffer containing 0.01% (v/v) Nonidet P-40 and 1 mg of bovine serum albumin/mL (Sigma). Alternatively, 6 μL of in vitro translated [^{35}S]methionine-labeled protein was added in the presence of 1 μM dexamethasone (Sigma) in ethanol or vehicle alone in a total volume of 200 μL of pull-down buffer containing 0.01% (v/v) Nonidet P-40 and 0.2 mg of bovine serum albumin/mL (Sigma). Incubation with rotation was carried out at 4 °C for 15 h. Beads were collected by centrifugation and washed 4 times for 5 min with 400 μL of GST pull-down buffer containing 0.01% (v/v) Nonidet P-40. Washed beads were resuspended in 30 μL of 1× SDS sample buffer, boiled for 10 min, and pelleted in a microfuge. Five microliters of the supernatant was analyzed on SDS−PAGE gels and visualized by autoradiography or by immunoblot analysis as described previously (*7*) using a rabbit polyclonal antibody raised against the *E. coli* LexA repressor DNA-binding domain. Immunoblots were analyzed using the computer imaging software Gel Pro ANALYSER (Media Cybernetics, L. P.).

RESULTS

Screening Known Coactivators To Identify New Proteins That Interact with the τ1-Core. The τ1-core is known to bind to TBP and Ada2 (*32*, *33*). In an attempt to identify additional τ1-core targets, we screened cofactors for interactions with the τ1-core using GST pull-down assays. We selected a set of coactivators [RIP140, SRC-1, CBP, 14-3-3η, Rsp5, and the TBP-associated factor $TAF_{II}18$ (human homologue of Spt3)] that have been shown previously to interact with the GR ligand-binding domain and/or potentiate GR-transcriptional activation. We also included the cofactor PC4 that has been shown to interact with the herpes simplex virus protein VP16 transactivation domain (*42*). First we investigated whether in vitro translated RIP140, SRC-1, and N-terminal and C-terminal parts of CBP could be precipitated by the GST-τ1-core fusion protein coupled to glutathione−agarose beads. The autoradiograph in Figure 1A shows that the GST-τ1-core fusion protein was unable to bind to RIP140, SRC-1 or the N-terminal part of CBP. Interestingly, we detected a weak interaction between GST-τ1-core and the C-terminal part of CBP (Figure 1A, CBP-C1). This is not due to an excess of GST-τ1-core protein, since similar amounts of GST and GST-τ1-core proteins were coupled to the beads (Figure 1B). To further evaluate this result and to screen additional potential target factors, we also screened for interactions between GST-CBP-C1, GST-14-3-3η, GST-$TAF_{II}18$, GST-Rsp5, and GST-PC4 fusion proteins and purified τ1-core-LexA. We incubated similar amounts of these GST-fusion proteins (Figure 1E) coupled to glutathione−agarose beads with purified τ1-core-LexA protein. The presence of the τ1-core-LexA protein in the supernatant or the pellet was detected by immunoblot analysis using an antibody against the LexA DNA-binding domain (Figure 1C). As expected, no τ1-core-LexA protein was detected in the pellet fraction of GST alone. Interestingly, there was a clear interaction between the C-terminal part of CBP and the τ1-core-LexA protein. A weak interaction was detected with GST-Rsp5 (Figure 1C), but it is unclear whether this is significant. 14-3-3η, $TAF_{II}18$, PC4 (Figure 1C), and CBP-N (data not shown) did not interact significantly with the τ1-core-LexA protein.

As expected, the τ1-core protein did interact with TBP and Ada2 that were included as positive controls (Figure 1C). Smaller regions of TBP and Ada2 that can account for the interactions with the τ1-core were localized in order to further examine the specificity of these interactions. The τ1-core interacting region of TBP was mapped to the conserved C-terminal part (residues 168−339; Figure 1D, TBP-C) whereas the N-terminal part (residues 1−163; Figure 1D, TBP-N) did not bind significantly to the τ1-core. The N-terminal part of Ada2 (residues 1−176; Figure 1D, Ada2-N) interacts well with the τ1-core and could account for the interaction involving the intact Ada2 protein. The middle part (residues 177−333; Figure 1D, Ada2-M) interacts but less strongly, and the significance of this interaction is doubtful. No interaction was detected with the C-terminal region (residues 334−434; Figure 1D, Ada2-C). Since CBP-C1 containing the C-terminal part of CBP (residues 1678−2441) was extensively degraded (Figure 1E), a smaller GST-fusion protein (CBP-C2) containing the N-terminal region of CBP-C1 (residues 1678−1868) was made. This protein is much more stable than the larger protein but binds equally well to the τ1-core. Therefore, sequences between residues 1678 and 1868 of CBP can account for the interaction of the τ1-core with the GST-CBP-C1 protein.

To What Extent Does Binding of the τ1-Core Mutant Proteins to TBP, Ada2, and CBP in Vitro Correlate with Their Transactivation Activity in Vivo? To elucidate whether the differences in transactivation activities of the τ1-core mutants are a consequence of changes in their ability to interact with target proteins, we expressed and purified a set of τ1-core mutant proteins fused to the LexA DNA-binding domain (Figure 2B, top panel) and analyzed their ability to bind TBP, Ada2, and CBP-C2 fused to GST in pull-down assays. We selected eight τ1-core mutants containing point mutations in different segments of the τ1-core. As shown in Figure 2A, five of the mutants (F191D, I193D,

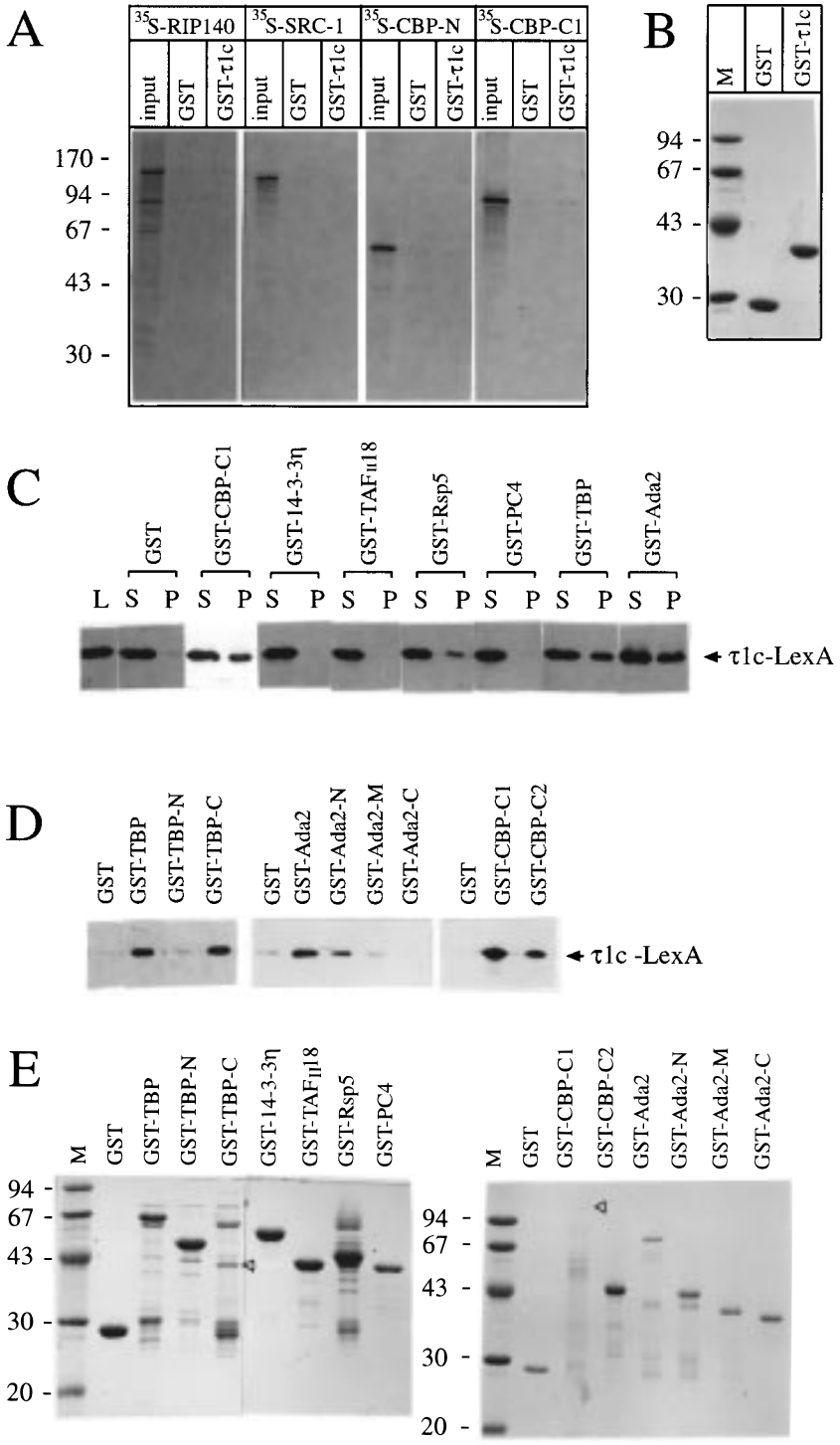

FIGURE 1: Screening coactivator proteins for interaction with the τ1-core in vitro. (A) ^{35}S-Labeled RIP140, SRC-1, CBP-N (residues 1−452), and CBP-C1 (residues 1678−2441) were translated in vitro and incubated at 4 °C for 15 h with GST or GST-τ1-core (GST-τ1c) coupled to glutathione−agarose beads. 10% of input and eluted samples from washed beads was analyzed by 10% SDS−PAGE and visualized by autoradiography. (B) Coomassie Blue-stained SDS−PAGE showing the GST and GST-τ1-core (GST-τ1c) proteins used for precipitation. The migration of the molecular mass markers is shown in kDa (M). (C) Western blots showing coprecipitation or lack of coprecipitation of purified τ1-core-LexA fusion protein (τ1c-LexA) with GST and GST-fusion proteins. The supernatant (S) and pellet (P) fractions obtained after precipitation of the GST-fusion proteins are shown. The pellet fractions (P) are in each case 1.25× concentrated relative to L (load) and S. The τ1-core-LexA protein was detected using an antibody raised against the LexA DNA-binding domain. (D) Further delineation of regions in TBP, Ada2, and CBP required for binding to τ1-core. The data are presented as in Figure 1C except that only pellet fractions (P) are shown. (E) Coomassie Blue-stained SDS−PAGE showing the GST and GST-fusion proteins that were bound to agarose beads and used for the precipitation of putative target proteins. The migration of the intact GST-fusion proteins is indicated in some cases where there is significant degradation of the proteins. An additional higher molecular mass band is present in the TBP-C preparation that corresponds to a copurified but unknown protein. The migration of the molecular mass markers is shown in kDa (M).

L197E, H1ala, and D196Y) have mutations in helical region I. H2ala is mutated in helical region II. W213R contains a mutation in an intervening sequence between helical regions I and II, and F235L,L236V contains two mutations in helical

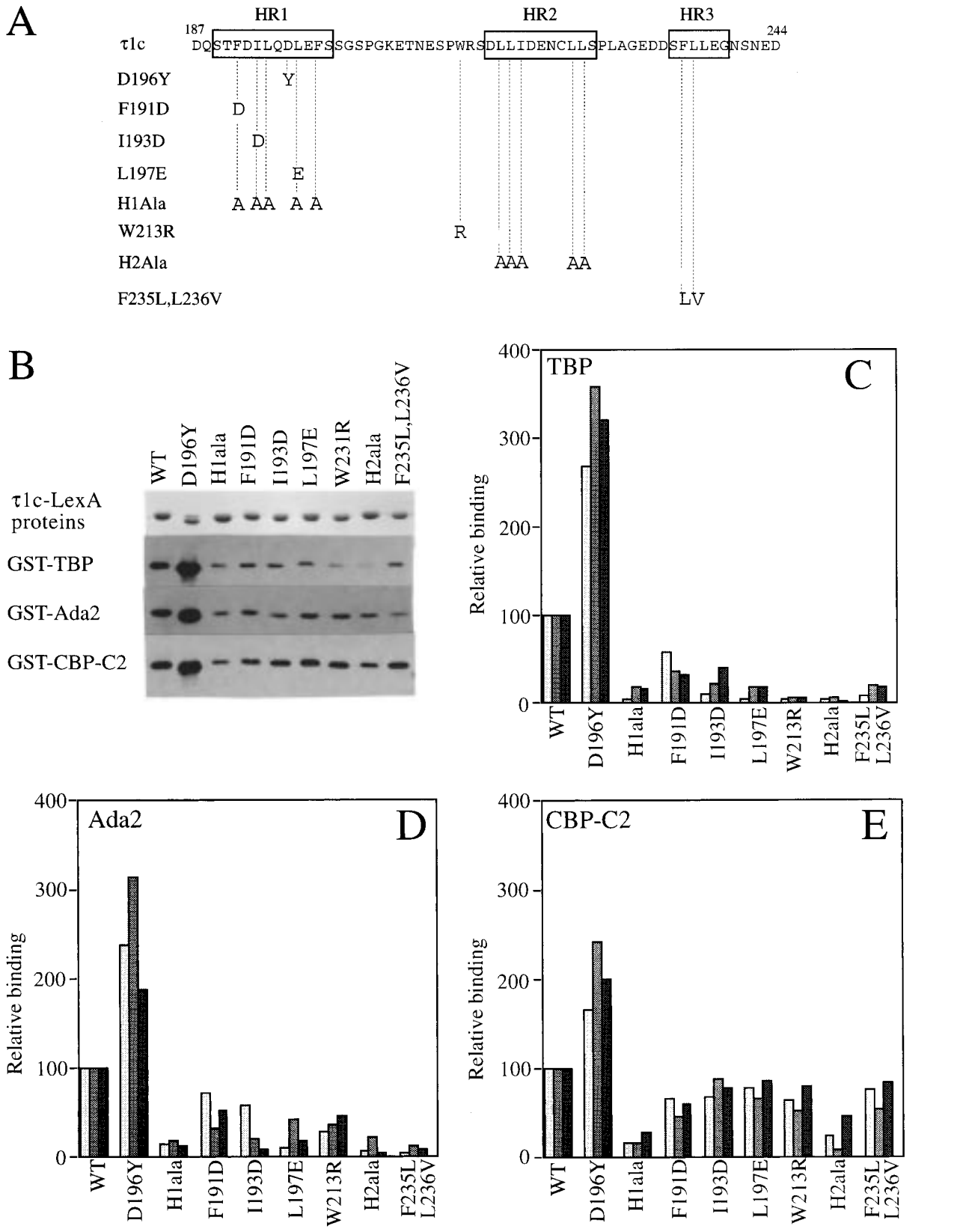

FIGURE 2: Binding of τ1-core mutant proteins to TBP, Ada2, and CBP-C2. (A) Schematic representation showing the amino acid substitutions in the τ1-core mutants used. The locations of putative helical regions I, II, and III are indicated by boxes. (B) Top panel: Coomassie Blue-stained SDS−PAGE showing the migration of wild-type (WT) and mutant τ1-core-LexA proteins used for precipitation. Lower panels: Three representative immunoblots showing coprecipitation of purified τ1-core-LexA proteins with GST-TBP, GST-Ada2, and GST-CBP-C2. The τ1-core-LexA proteins were detected using an antibody raised against the LexA DNA-binding domain. (C) Graph showing the relative binding of the τ1-core mutant proteins to GST-TBP from three independent experiments. The relative binding is expressed as a percentage of wild-type (WT) τ1-core level. The data were obtained by scanning three low-exposure immunoblots and analyzing them using the computer imaging software Gel Pro ANALYSER. (D) and (E) show the relative binding of the τ1-core mutant proteins to GST-Ada2 and GST-CBP-C2, respectively. The data are presented as in Figure 2C.

Table 1: Relative Transactivation Activities and Binding Data of the Mutant τ1-Core Proteins

mutant	relative activity[a]	relative binding[b]		
		TBP	Ada2	CBP-C2
τ1-core (WT)	100	100	100	100
D196Y	281 ± 26	316 ± 45	247 ± 63	203 ± 38
W213R	30 ± 2	6 ± 2	37 ± 9	66 ± 14
L197E	30 ± 1	14 ± 8	24 ± 16	78 ± 10
F191D	28 ± 3	42 ± 14	53 ± 20	57 ± 10
I193D	27 ± 8	24 ± 16	29 ± 26	78 ± 11
H2ala	18 ± 2	4 ± 2	11 ± 9	27 ± 19
F235L L236V	11 ± 2	16 ± 7	9 ± 4	72 ± 16
H1ala	7 ± 1	13 ± 7	15 ± 3	21 ± 7

[a] Mean relative β-galactosidase activity of τ1-core-LexA fusion proteins [percentage of wild-type level ± standard deviation ($n = 3$)]. Taken from Table 1 and Figure 4B (τ1c) in ref 7. [b] Mean relative binding intensity [percentage of wild-type level ± standard deviation ($n = 3$)]. Calculated from Figure 2.

region III. All these mutants display reductions to less than 50% of the wild-type transactivation activity in vivo in yeast, except one mutant, D196Y, which is nearly 3 times as active (281%,Table 1). All mutants behaved similarly during purification and SDS−PAGE except D196Y which migrates as two bands (Figure 2B, top panel), as seen previously (*7*). As shown in Figure 2B, each of the mutations has an effect on binding to TBP, Ada2, and CBP-C2. The τ1-core point mutants F191D, I193D, L197E, H1ala, W213R, H2ala, and F235L,L236V that all display reduced transactivation activities show reduced binding to all target proteins. Conversely, the mutant D196Y that has increased transactivation activity binds more strongly to all the fusion proteins (see D196Y, Figure 2B). These results indicate that all the regions in the τ1-core probed in this analysis play a role in the interaction with all the target proteins (TBP, Ada2, and CBP-C2) tested.

To elucidate further whether any individual τ1-core mutants caused differential effects on any of the three target factor interactions, we measured the amount of τ1-core proteins that had been precipitated by the various cofactors. The levels of the bound mutant τ1-core proteins from three independent experiments were compared to that of the wild-type τ1-core protein in each experiment. Figure 2C,D,E shows that the relative binding of TBP, Ada2, and CBP-C2 to the eight τ1-core mutants follows a similar trend but clearly the TBP interaction is most sensitive to mutations while interaction with CBP-C2 is least sensitive.

TBP and Ada2 Also Interact with the GR Ligand-Binding Domain. As shown in Figure 1A, one of the coactivators, CBP, that has been shown to potentiate transcriptional activation and interact with the GR via the ligand-binding domain (Windahl, personal communication) also interacted with the N-terminal transactivation domain. Since the interaction of different activation domains with common target proteins could provide a mechanism to explain how they collaborate during synergistic gene activation, we decided to determine whether other target proteins were shared by the N- and C-terminal activation domains of the GR. Our inability to detect interactions between the τ1-core and RIP140 and SRC-1 (Figure 1A) was not due to trivial technical problems since the interactions between the coactivators and the GR ligand-binding domain (Windahl, personal communication) can be reproduced in our hands

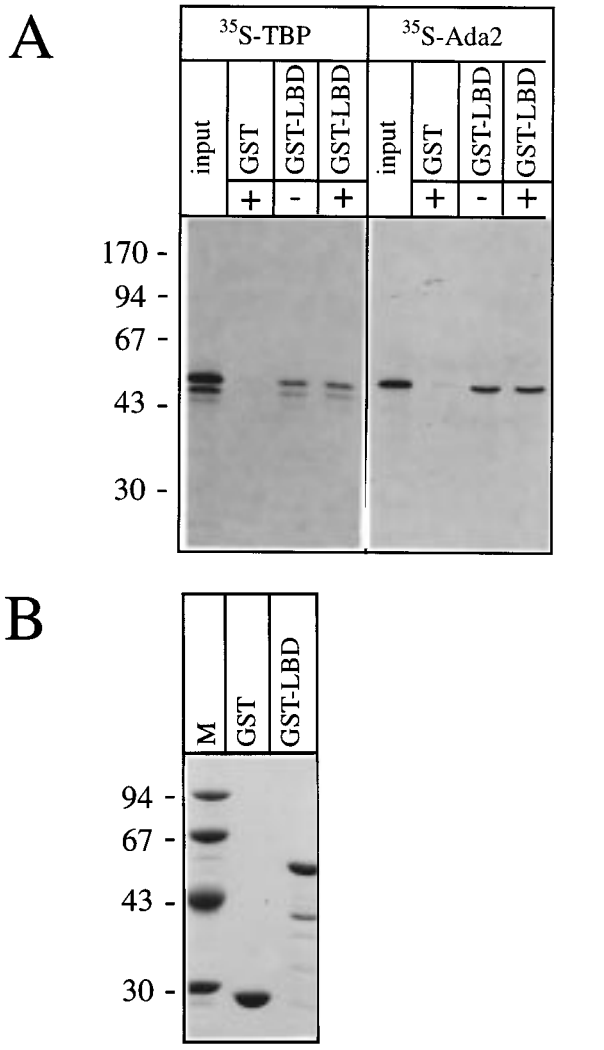

FIGURE 3: TBP and Ada2 interact with the ligand-binding domain of GR. ^{35}S-labeled TBP and Ada2 were translated in vitro and incubated at 4 °C for 15 h with GST or GST-LBD (residues 485−777) coupled to glutathione−agarose beads in the presence (+) of 1 μM dexamethasone in ethanol or vehicle alone (−). The input and eluted samples from washed beads were analyzed on 10% SDS−PAGE and visualized by autoradiography. (B) Coomassie Blue-stained SDS−PAGE showing the migration of the GST and GST-LBD proteins used for precipitation. Approximately equal amounts of GST and GST-LBD were coupled to glutathione−agarose beads. The migration of the molecular mass markers is shown in kDa (M).

(data not shown). This suggests that some target factors for the ligand-binding domain are distinct from those of the τ1-core. To investigate whether τ1-core target proteins could be utilized by both transactivation domains, we tested whether the GR ligand-binding domain can interact with TBP and Ada2. Interestingly, as shown in the autoradiograph in Figure 3A, a GST-fusion of the ligand-binding domain of GR (GST-LBD) could precipitate both in vitro translated TBP and Ada2, and the interactions were specific since no binding to GST was observed in either case. Under the conditions used, the interactions appear to be ligand-independent.

DISCUSSION

CBP Interacts with the τ1-Core. The main aim of this work was to use a panel of mutant τ1-core proteins, selected

because they have altered transactivation activities in vivo, to study determinants required for interaction with different target proteins. We wanted to compare how these mutations affect the interaction of the τ1-core with a number of different target proteins. Our first goal was to attempt to identify further proteins, in addition to TBP and Ada2, that interact with the τ1-core. We have shown that CBP interacts with the τ1-core in vitro. CBP seems to be required for the action of many different activator proteins and has been suggested to function as a cointegrator of different signal transduction pathways. Overexpression of GR has been shown to inhibit AP-1-induced gene expression, which may be through titration of limiting amounts of CBP present in the cell (*43*). CBP has been shown to play an important role in glucocorticoid induction of the MMTV promoter (*44*). The τ1-core interacts with a region of the C-terminal part of CBP (residues 1678−1868) that has been shown previously to contain binding sites for activator proteins such as p/CAF (*45*), STAT1α (*46*), c-Fos (*47*), and TFIIB (*48*). The N terminus of this region (residues 1708−1733) contains a cysteine-rich region that shows some sequence similarity to a segment in Ada2 (*49*). This segment is present in the N terminus of Ada2, which we show here is the part of the Ada2 protein most important for interactions with the τ1-core. Whether these similarities represent structurally similar interaction sites for the τ1-core remains to be shown. The ligand-binding domain of some other nuclear receptors has been demonstrated to interact with the N-terminal part of CBP (*43*). More recently it has been shown that the ligand-binding domain of the GR interacts both with the N terminus and with the C terminus of CBP (Windahl, personal communication). It is possible that CBP exerts its effects by contacting both the GR ligand-binding domain and the τ1 domain simultaneously through different CBP regions.

Effect of τ1-Core Mutations on Interaction with Target Proteins. The general pattern of binding to TBP, Ada2, and CBP-C2 by the panel of τ1-core mutants is quite similar (see Figure 2). All the interactions are influenced by all the mutations. The increased activity mutant, D196Y, interacts more strongly with each of the proteins, and the H1ala and H2ala mutants interact very poorly in all cases. In line with our results, correlations between transactivation activity and binding to target factors have been reported previously with other activator proteins (*50*−*53*). Our results suggest that the property of the τ1-core that is affected by the mutants is common to and important for each of the interactions studied here. The simplest interpretation is that the mutants affect the ability of the τ1-core to fold into a structured form that is competent to interact with any of the target factors. Consistent with this, all the exchanges in the mutants involve hydrophobic residues that could contribute to a hydrophobic core in the folded protein.

If the altered activity mutants affect a common property of the τ1-core, such as folding, that plays an equivalent role in interactions with all target factors, we might expect the activity of the mutants to correlate with their ability to interact with each of the target factors. In Figure 4 the relative binding of the mutant τ1-core proteins to each of the target proteins is plotted as a function of their relative activity. Linear regression analysis shows that binding to TBP and Ada2 by the mutants is almost directly proportional to their activity, such that the regression line has a slope close to unity and intersects the axes near the origin. Notably, however, the interaction with CBP-C2 appears to be less sensitive to the τ1-core mutations (Figure 2). This is also reflected in Figure 4C where the linear regression analysis yields a line with a slope that is less than unity and does not bisect the origin. This suggests that the mutations might affect important aspects of the TBP and Ada2 interactions that are less important for the CBP-C2 interaction in addition to aspects that are common to all three interactions.

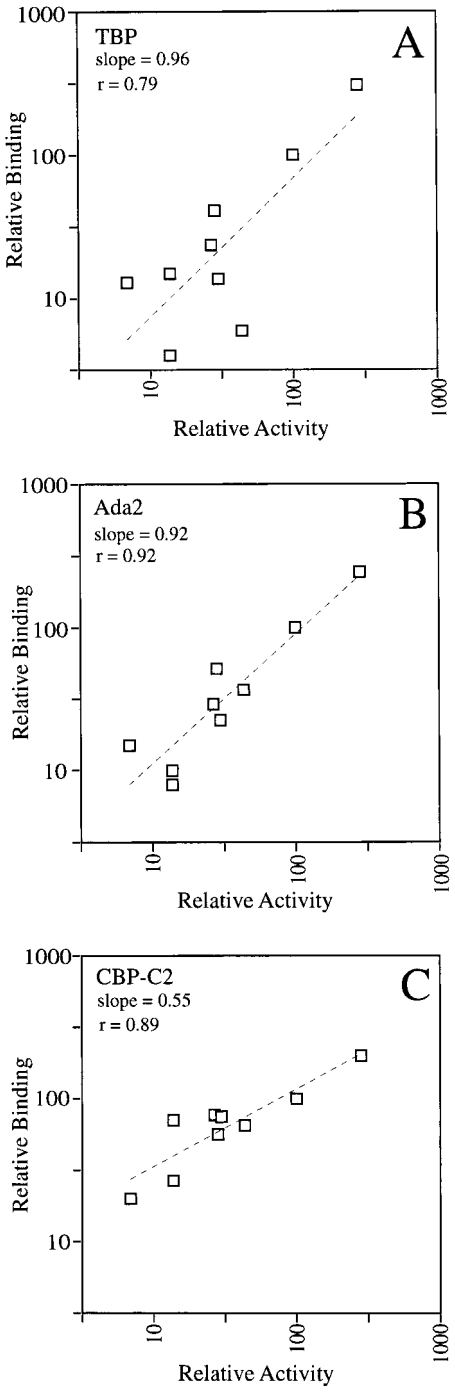

FIGURE 4: Transactivation activity of the τ1-core mutant proteins in vivo correlates with binding to target factors in vitro. The relative binding of the τ1-core mutant proteins to TBP (A), Ada2 (B), and CBP-C2 (C) was plotted as a function of their transactivation activity in vivo (Table 1). The dotted lines represent curves fitted by linear regression analysis. The slope (binding/activity) of the regression line and the regression coefficient (r) are shown.

Closer inspection of the quantitated data in Figure 2C−E reveals that although all mutations affect all the interactions there are some target factor specific effects. Most clearly W213R has a relatively stronger impact on the interaction with TBP compared to Ada2 and CBP-C2. This suggests that in addition to the general structural role which might be attributed to this residue, it could also play a more specific role that is restricted to the TBP interaction. Similar, albeit less clear, trends of this type can be observed in other cases, but interpretation of these changes becomes difficult because the changes are not always much greater than the intrinsic variability of the assay. However, it is possible that the general structural effects that result from the mutations mask target factor specific effects that might reflect unique stereospecific interactions between τ1-core and its individual target factors.

GR N-Terminal and C-Terminal Domains Use both Common and Distinct Coactivators. In contrast to the DNA-binding and ligand-binding domains, the activation domains of nuclear receptors are often present in more than one copy per receptor protein, and it is of interest to know whether the different activation domains interact with the same or distinct subsets of target proteins. Indirect evidence suggests that common targets may be used since the activation domain from the N terminus of receptors can squelch activation by the C-terminal domain and vice versa (*54*).

The data presented here indicate that the GR transactivation regions could utilize both common and distinct coactivators. We demonstrate that the coactivator CBP that has been isolated as a target for the GR ligand-binding domain (*23*; Windahl, personal communication) can also interact with the N-terminal transactivation domain, the τ1-core, in vitro. Furthermore, the τ1-core target proteins, TBP and Ada2, interact with the GR ligand-binding domain in vitro, suggesting that these factors are common targets for both activation regions of the receptor. These interactions could be direct or indirect, mediated by proteins present in the reticulocyte lysate during translation. In a previous study, we showed that Ada2 plays a major role for the activity of the τ1 domain (*33*). However, this does not exclude some contribution of Ada2 to the activity of the ligand-binding domain. In contrast to TBP, Ada2, and CBP, the yeast proteins Rsp5 and $TAF_{II}18$ (human homologue of Spt3) and coactivators such as RIP140, SRC-1, and 14-3-3η that potentiate and/or interact with the ligand-binding domain of GR, did not make detectable contacts with the τ1-core in vitro. 14-3-3η has been shown to interact weakly with the τ1 domain under low stringency conditions (*27*). We detected no such interaction with the τ1-core under these conditions (data not shown), which is not unexpected since the putative 14-3-3η protein-binding motif that has been identified in the τ1 domain (*55*) lies outside the τ1-core region. Similarly, it was recently reported that another nuclear receptor coactivator protein, GRIP1, that interacts with the ligand-binding domain of the rat GR in vitro (*23*), did not interact with the N-terminal transactivation domain under similar conditions (*56*). Neither did GRIP1 potentiate the activity of the rat GR enh2 (which is the equivalent of τ1) in yeast assays, indicating that GRIP1 is a GR AF-2 specific coactivator. Although some of the coactivators analyzed in this study appear to be specific for the ligand-binding domain, we cannot exclude the possibility that some of them play a role in the activity of the τ1-core through indirect interactions with other adaptor proteins. Further analysis of the interactions between target factors and the N-terminal and C-terminal activation domains in vivo will be required for further elucidation of the molecular mechanism of gene activation by the GR.

ACKNOWLEDGMENT

We thank the members of the Receptor Biology Unit at the Department of Biosciences for helpful discussions and commenting during this work and Elizabeth Flinn for critical reading of the manuscript. We thank Sara Windahl for sharing unpublished data. We are also grateful to the following doctors, for plasmid gifts: M. Meyer (EMBL, Germany), Leonard Guarente (Massachusetts Institute of Technology), Richard G. Goodman (Vollum Institute, Oregon Health Science University, Portland, OR), Bert W. O'Malley (Baylor College of Medicine, Houston, TX), Shelly Berger (Wistar Institute), Donald P. McDonnell (Duke University Medical Center, Durham, NC), Hui Ge (Rockefeller University, New York, NY), Tony Kouzarides (Cambridge, United Kingdom), Irwin Davidson (Collège de France, France), and Eckardt Treuter, Sara Windahl, Hideki Wakui, Johanna Zilliacus, and Jacqueline Ford (Karolinska Institute, Sweden).

REFERENCES

1. Beato, M., Herrlich, P., and Schutz, G. (1995) *Cell 83*, 851−857.
2. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) *Cell 83*, 835−839.
3. Giguère, V., Hollenberg, S. M., Rosenfeld, M. G., and Evans, R. M. (1986) *Cell 46*, 645−652.
4. Hollenberg, S. M., and Evans, R. M. (1988) *Cell 2*, 899−906.
5. Danielian, P. S., White, R., Lees, J. A., and Parker, M. G. (1992) *EMBO J. 11*, 1025−1033.
6. Wright, A. P., McEwan, I. J., Dahlman-Wright, K., and Gustafsson, J.-Å. (1991) *Mol. Endocrinol. 5*, 1366−1372.
7. Almlöf, T., Gustafsson, J.-Å., and Wright, A. P. (1997) *Mol. Cell. Biol. 17*, 934−945.
8. Dahlman-Wright, K., Almlöf, T., McEwan, I. J., Gustafsson, J.-Å., and Wright, A. P. (1994) *Proc. Natl. Acad. Sci. U.S.A 91*, 1619−1623.
9. Dahlman-Wright, K., Baumann, H., McEwan, I. J., Almlöf, T., Wright, A. P., Gustafsson, J.-Å., and Härd, T. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 1699−1703.
10. Donaldson, L., and Capone, J. P. (1992) *J. Biol. Chem. 267*, 1411−1414.
11. Van Hoy, M., Leuther, K. K., Kodadek, T., and Johnston, S. A. (1993) *Cell 72*, 587−594.
12. Schmitz, M. L., dos Santos Silva, M. A., Altmann, H., Czisch, M., Holak, T. A., and Baeuerle, P. A. (1994) *J. Biol. Chem. 269*, 25613−25620.
13. McEwan, I. J., Dahlman-Wright, K., Ford, J., and Wright, A. P. (1996) *Biochemistry 35*, 9584−9593.
14. Dahlman-Wright, K., and McEwan, I. J. (1996) *Biochemistry 35*, 1323−1327.
15. Shen, F., Triezenberg, S. J., Hensley, P., Porter, D., and Knutson, J. R. (1996) *J. Biol. Chem. 271*, 4827−4837.
16. Uesugi, M., Nyanguile, O., Lu, H., Levine, A. J., and Verdine, G. L. (1997) *Science 277*, 1310−1313.
17. Zawel, L., and Reinberg, D. (1995) *Annu. Rev. Biochem. 64*, 533−561.

18. McEwan, I. J., Wright, A. P., Dahlman-Wright, K., Carlstedt-Duke, J., and Gustafsson, J.-Å. (1993) *Mol. Cell. Biol. 13*, 399−407.
19. Freedman, L. P., Yoshinaga, S. K., Vanderbilt, J. N., and Yamamoto, K. R. (1989) *Science 245*, 298−301.
20. Tsai, S. Y., Srinivasan, G., Allan, G. F., Thompson, E. B., O'Malley, B. W., and Tsai, M. J. (1990) *J. Biol. Chem. 265*, 17055−17061.
21. McEwan, I. J., Almlöf, T., Wikström, A.-C., Dahlman-Wright, K., Wright, A. P., and Gustafsson, J.-Å. (1994) *J. Biol. Chem. 269*, 25629−25636.
22. McEwan, I. J., Wright, A. P. H., and Gustafsson, J.-Å. (1997) *Bioessays 19*, 153−160.
23. Hong, H., Kohli, K., Trivedi, A., Johnson, D. L., and Stallcup, M. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 4948−4952.
24. Cavaillès, V., Dauvois, S., L'Horset, F., Lopez, G., Hoare, S., Kushner, P. J., and Parker, M. G. (1995) *EMBO J. 14*, 3741−3751.
25. Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1995) *Science 270*, 1354−1357.
26. Chrivia, J. C., Kwok, R. P., Lamb, N., Hagiwara, M., Montminy, M. R., and Goodman, R. H. (1993) *Nature 365*, 855−859.
27. Wakui, H., Wright, A. P., Gustafsson, J.-Å., and Zilliacus, J. (1997) *J. Biol. Chem. 272*, 8153−8156.
28. Eggert, M., Mows, C. C., Tripier, D., Arnold, R., Michel, J., Nickel, J., Schmidt, S., Beato, M., and Renkawitz, R. (1995) *J. Biol. Chem. 270*, 30755−30759.
29. Zeiner, M., and Gehring, U. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 11465−11469.
30. Muchardt, C., and Yaniv, M. (1993) *EMBO J. 12*, 4279−4290.
31. Imhof, M. O., and McDonnell, D. P. (1996) *Mol. Cell. Biol. 16*, 2594−2605.
32. Ford, J., McEwan, I. J., Wright, A. P. H., and Gustafsson, J.-Å. (1997) *Mol. Endocrinol. 11*, 1467−1475.
33. Henriksson, A., Almlöf, T., Ford, J., McEwan, I. J., Gustafsson, J.-Å., and Wright, A. P. (1997) *Mol. Cell. Biol. 17*, 3065−3073.
34. Dikstein, R., Ruppert, S., and Tjian, R. (1996) *Cell 84*, 781−790.
35. Mizzen, C. A., Yang, X. J., Kokubo, T., Brownell, J. E., Bannister, A. J., Owen-Hughes, T., Workman, J., Wang, L., Berger, S. L., Kouzarides, T., Nakatani, Y., and Allis, C. D. (1996) *Cell 87*, 1261−1270.
36. Burley, S. K., and Roeder, R. G. (1996) *Annu. Rev. Biochem. 65*, 769−799.
37. Barlev, N. A., Candau, R., Wang, L., Darpino, P., Silverman, N., and Berger, S. L. (1995) *J. Biol. Chem. 270*, 19337−19344.
38. Brownell, J. E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D. G., Roth, S. Y., and Allis, C. D. (1996) *Cell 84*, 843−851.
39. Marcus, G. A., Silverman, N., Berger, S. L., Horiuchi, J., and Guarente, L. (1994) *EMBO J. 13*, 4807−4815.
40. Hagemeier, C., Walker, S., Caswell, R., Kouzarides, T., and Sinclair, J. (1992) *J. Virol. 66*, 4452−4456.
41. Mengus, G., May, M., Jacq, X., Staub, A., Tora, L., Chambon, P., and Davidson, I. (1995) *EMBO J. 14*, 1520−1531.
42. Ge, H., and Roeder, R. G. (1994) *Cell 78*, 513−523.
43. Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S. C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996) *Cell 85*, 403−414.
44. Chakravarti, D., LaMorte, V. J., Nelson, M. C., Nakajima, T., Schulman, I. G., Juguilon, H., Montminy, M., and Evans, R. M. (1996) *Nature 383*, 99−103.
45. Yang, X. J., Ogryzko, V. V., Nishikawa, J., Howard, B. H., and Nakatani, Y. (1996) *Nature 382*, 319−324.
46. Zhang, J. J., Vinkemeier, U., Gu, W., Chakravarti, D., Horvath, C. M., and Darnell, J. E., (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 15092−15096.
47. Bannister, A. J., Oehler, T., Wilhelm, D., Angel, P., and Kouzarides, T. (1995) *Oncogene 11*, 2509−2514.
48. Kwok, R. P., Lundblad, J. R., Chrivia, J. C., Richards, J. P., Bachinger, H. P., Brennan, R. G., Roberts, S. G., Green, M. R., and Goodman, R. H. (1994) *Nature 370*, 223−226.
49. Candau, R., Moore, P. A., Wang, L., Barlev, N., Ying, C. Y., Rosen, C. A., and Berger, S. L. (1996) *Mol. Cell. Biol. 16*, 593−602.
50. Hagemeier, C., Cook, A., and Kouzarides, T. (1993) *Nucleic Acids Res. 21*, 4998−5004.
51. Silverman, N., Agapite, J., and Guarente, L. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11665−11668.
52. Melcher, K., and Johnston, S. A. (1995) *Mol. Cell. Biol. 15*, 2839−2848.
53. Chiang, Y. C., Komarnitsky, P., Chase, D., and Denis, C. L. (1996) *J. Biol. Chem. 271*, 32359−32365.
54. Tasset, D., Tora, L., Fromental, C., Scheer, E., and Chambon, P. (1990) *Cell 62*, 1177−1187.
55. Muslin, A. J., Tanner, J. W., Allen, P. M., and Shaw, A. S. (1996) *Cell* 84, 889−897.
56. Hong, H., Kohli, K., Garabedian, M. J., and Stallcup, M. R. (1997) *Mol. Cell. Biol. 17*, 2735−2744.

BI973029X