MOLECULAR AND CELLULAR BIOLOGY, Jan. 1993, p. 399–407
0270-7306/93/010399-09$02.00/0

Vol. 13, No. 1

Direct Interaction of the τ_1 Transactivation Domain of the Human Glucocorticoid Receptor with the Basal Transcriptional Machinery

IAIN J. McEWAN,* ANTHONY P. H. WRIGHT, KARIN DAHLMAN-WRIGHT, JAN CARLSTEDT-DUKE, AND JAN-ÅKE GUSTAFSSON

Centre for Biotechnology and Department of Medical Nutrition, Karolinska Institute, NOVUM, Huddinge University Hospital, Huddinge S-141 57, Sweden

Received 24 August 1992/Accepted 26 October 1992

We have used a yeast (*Saccharomyces cerevisiae*) cell free transcription system to study protein-protein interactions involving the τ_1 transactivation domain of the human glucocorticoid receptor that are important for transcriptional transactivation by the receptor. Purified τ_1 specifically inhibited transcription from a basal promoter derived from the *CYC1* gene and from the adenovirus 2 major late core promoter in a concentration-dependent manner. This inhibition or squelching was correlated with the transactivation activity of τ_1. Recombinant yeast TATA-binding protein (yTFIID), although active in vitro, did not specifically reverse the inhibitory effect of τ_1. In addition, no specific interaction between τ_1 and yTFIID could be shown in vitro by affinity chromatography. Taken together, these results indicate that the τ_1 transactivation domain of the human glucocorticoid receptor interacts directly with the general transcriptional apparatus through some target protein(s) that is distinct from the TATA-binding factor. Furthermore, this assay can be used to identify interacting factors, since after phosphocellulose chromatography of a whole-cell yeast extract, a fraction that contained an activity which selectively counteracted the squelching effect of τ_1 was found.

The effects of glucocorticoid steroid hormones are mediated by an intracellular soluble receptor protein. The glucocorticoid receptor is a member of a large family of ligand-dependent, sequence-specific transcription factors that apart from classical steroids have thyroid hormones and vitamins as known ligands (1, 12, 17). In transient transfection studies, the major transactivation activity of the human glucocorticoid receptor was localized to the N-terminal part of the molecule between amino acids 77 and 262 and was termed τ_1 (16, 20, 21). This region has also been shown to activate reporter genes in yeast cells when fused to the DNA-binding domain (61). A weaker transactivation function (τ_2) was mapped to the C-terminal half of the molecule, between amino acids 526 and 556 (16, 20, 21). The τ_1 domain has a relatively high proportion of glutamic and aspartic amino acids and therefore may be a member of the acidic class of transactivators (20). The net negative charge of this region may be further increased by phosphorylation on serine residues (4, 19). However, there has been no direct correlation of negative charge and transactivation function for the receptor.

Initiation of transcription by RNA polymerase II requires the ordered assembly of a number of general factors, some of which have been identified (e.g., TFIIA, -IIB, -IID, -IIE, -IIF, -IIH, and -IIJ), and the polymerase at or near the transcription start site (5, 14, 46, 49). Upstream factors are thought to work by recruiting or modifying the activity of one or more of the general transcription factors, resulting in the formation of a more stable preinitiation complex (for reviews, see references 40 and 41). However, the mechanism by which regulatory factors transactivate transcription is poorly understood.

Two possible models have been suggested to explain how upstream transcription factors could interact with the general transcriptional apparatus. DNase I footprinting (22), coimmunoprecipitation (27), and affinity chromatography (23, 24, 51) studies have shown that E1A and the acidic activators GAL4 and VP16 can contact the general transcriptional apparatus directly through the TATA box-binding factor TFIID. In a recent series of experiments, Lin and coworkers (29, 30) demonstrated that VP16 also acts via contacts with another general transcription factor, TFIIB. Others have suggested a second model in which a new class of factors, termed coactivators or adaptors, mediates the effects of some upstream factors on the general transcriptional apparatus (for review, see reference 41). The existence of these intermediate proteins was demonstrated in studies in which endogenous but not cloned preparations of TFIID could respond to sequence-specific activators (39, 42) and in squelching studies in vivo (33) and in vitro (2, 13, 26). An intriguing possibility from this work is that different activator classes may function via different coactivator proteins (33, 54).

There is now compelling evidence from a number of different studies demonstrating that the mechanism of transactivation has been conserved between yeast and mammalian cells (6, 8, 18, 25, 35, 50, 57, 59). We have previously used yeast cells as a model system to investigate the mechanism of transactivation by the human glucocorticoid receptor and have shown that overexpression of the τ_1 transactivation domain in vivo inhibited both gene expression (squelching) and cell growth. The effect on gene expression preceded that on cell growth, consistent with specific inhibition of transcription by τ_1, and could be observed by using a basal promoter, suggesting direct interaction of τ_1 with one or more basal factor (61). However, toxicity of τ_1 to a process other than transcription within the yeast cells or an indirect effect of τ_1 on the expression of basal factor genes could not rigorously be excluded as an alternative explana-

* Corresponding author.

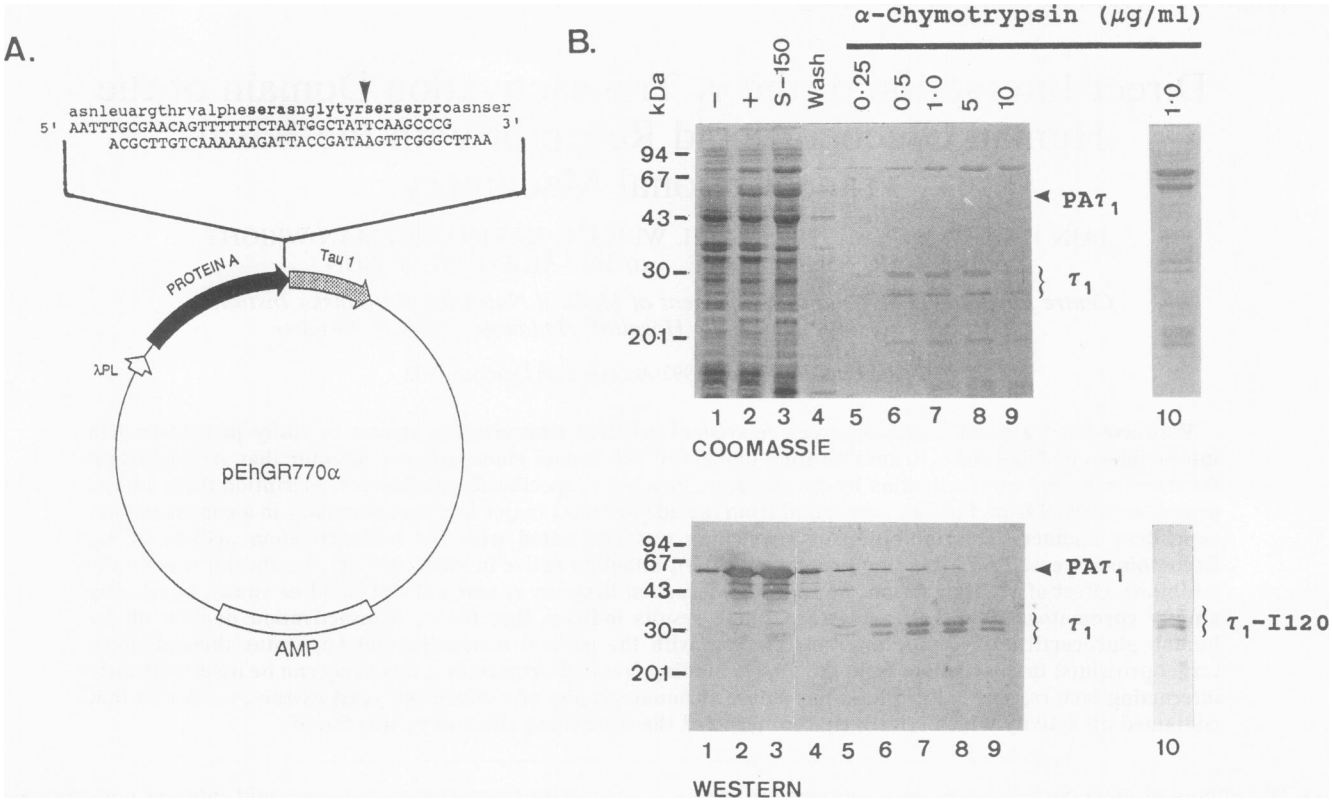

FIG. 1. Expression and purification of τ_1 and τ_1-I120 polypeptides. (A) The expression plasmid pEhGR770α, showing the arrangement of the PAτ_1 fusion protein, and the synthetic α-chymotrypsin cleavage site (solid arrowhead; boldface letters) derived from amino acids 390 to 396 of the human glucocorticoid receptor (see Materials and Methods [7, 10]) are represented schematically. (B) Purification of τ_1 analyzed by Coomassie staining (top panels) and Western blotting (bottom panels). Results for total cell extract before (−) and after (+) heat induction of bacteria (lanes 1 and 2), high-speed supernatant fraction (lane 3), IgG-Sepharose column wash (lane 4), cleavage of immobilized fusion protein with α-chymotrypsin (0.25, 0.5, 1.0, 5.0, and 10 μg/ml) and elution from column (lanes 5 to 9, respectively) are shown. The 1.0-μg/ml enzyme elution of τ_1-I120 is shown in lane 10. The fusion protein (PAτ_1) and isolated proteins (τ_1 and τ_1-I120) are indicated. Molecular mass markers are shown to the left of each panel.

tion. We have now used a yeast cell free transcription assay to study the interaction of the glucocorticoid receptor transactivation domain with the basal transcriptional apparatus. Our in vitro results demonstrate that the τ_1 transactivation domain interacts directly with the general transcriptional apparatus. In addition, we demonstrate that, unlike the case for E1A, GAL4, or VP16 activation domains, the TATA-binding protein (TBP) does not appear to interact with τ_1, but a fraction derived from a whole-cell yeast extract did contain the target factor(s). This assay system therefore provides a functional test for target protein activity and can be used to identify and isolate the factor(s) in the general transcriptional apparatus that is contacted by τ_1.

MATERIALS AND METHODS

Construction of plasmids. The τ_1 region of the human glucocorticoid receptor (amino acids 77 to 262) was removed as a 555-bp *Bgl*II fragment from plasmid pRSVhGR and cloned into the *Bam*HI site of the protein A (PA) expression plasmid pERAT318 (36) to give plasmid pEHGR770. The τ_1 region was then taken from pEHGR770 as a 582-bp *Eco*RI-*Xba*I fragment and cloned into the corresponding sites in pRIT33(chym) (60) to give plasmid pEhGR770α (Fig. 1A). The isolated τ_1 polypeptide was predicted to have the following additional amino acids (in the one-letter code) after cleavage with α-chymotrypsin: SSPNSSSVPGD and PLEST KRHASLSK at the N and C termini, respectively. The reporter plasmid p(GRE)^{2}CG− was constructed by cloning two adjacent glucocorticoid response elements (GREs) (60) into the *Xho*I site of plasmid pΔCG− (32).

In vitro mutagenesis of the τ_1 transactivation domain. A 4-amino-acid insertion (RGSA [16]) was introduced into the τ_1 domain by oligonucleotide-directed mutagenesis (Amersham) by following the manufacturer's instructions. The mutation was checked by sequencing (Sequenase; United States Biochemical Corp.), and the mutant τ_1-I120 was cloned into pRIT33(chym) as a *Sac*I fragment.

Expression and purification of fusion proteins. PA fusion proteins were expressed in *Escherichia coli* MZ1 and purified on immunoglobulin G (IgG)-Sepharose as described previously (10, 36, 60). Briefly, the fusion protein was heat induced and the soluble protein was loaded directly onto an IgG-Sepharose column (Fig. 1B, lanes 1 to 4). τ_1 (or τ_1-DBD or τ_1-I120) was released from PA by treatment with 0.5 to 10 μg of α-chymotrypsin per ml for 30 min at 4°C and then eluted from the column (Fig. 1B, lanes 5 to 10). Receptor polypeptides were identified after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by Coomassie blue staining and Western blot (immunoblot) analysis. The isolated protein ran as a doublet with an apparent molecular mass of about 30 kDa, which was higher

than expected. The N terminus of the released τ_1 was checked by N-terminal sequence analysis (Applied Biosystems 470 protein sequencer with an on-line 120 PTH analyzer) and found to be a mixture of the expected N terminus and MGETETKVMGNDLGFPQQ. The latter corresponded to the sequence starting at Met-90 within the τ_1 domain and was thought to be the result of further proteolytic activity after the initial cleavage, since a fusion protein lacking the α-chymotrypsin site did not give isolated τ_1 under identical conditions (data not shown). The τ_1 protein fractions shown in Fig. 1B, lanes 6 to 8, were judged to be 60 to 70% pure after densitometry of the Coomassie blue-stained gel and were used in subsequent experiments. The isolated proteins were dialyzed against 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.6)–10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid]–5 mM dithiothreitol (DTT)–10 mM $MgSO_4$–20% glycerol–50 mM $(NH_4)_2SO_4$; the volume of the τ_1-I120 fractions was reduced by using Centricon-10 filters (Amicon) prior to dialysis. Total protein was determined by the method of Bradford; τ_1 and τ_1-I120 concentrations were estimated to be about 0.12 mg/ml (60 to 70% of total protein) and 0.08 mg/ml (10 to 15% of total protein), respectively.

Preparation of yeast whole-cell and nuclear extracts. Whole-cell extracts from *Saccharomyces cerevisiae* BJ3501 were prepared as described by Olesen et al. (37), except that a 70% $(NH_4)_2SO_4$ fractionation step was made and the precipitated proteins were resuspended in 25 mM HEPES (pH 7.9)–1 mM EDTA–10% glycerol–2 mM DTT and dialyzed against the same buffer containing 50 mM KCl. Yeast nuclear extracts were prepared from a 6-liter culture of cells (strain BJ926) essentially as described by Lue et al. (31) with appropriate adjustments of volume. The final ammonium sulfate precipitate was resuspended in buffer C (31), snap frozen, and stored at −80°C. Extracts for transcription reactions were prepared by dialyzing small volumes of this material against the same buffer until the salt concentration was between 50 and 100 mM; protein concentrations ranged from 10 to 15 mg/ml.

In vitro transcription and squelching reactions. Transcription reactions were carried out as described by Lue et al. (31, 32), except that 3'-*O*-methyl-GTP (100 μM) was present in all reactions and 20 U of RNase T_1 (Boehringer Mannheim) was needed to reduce background signals. Transcription reactions routinely contained 100 ng of template DNA (3.33 μg/ml) unless stated otherwise, 55 to 60 μg of protein (1.7 to 2.0 mg/ml), and 5 μCi of α-^{32}P-UTP (>400 Ci/mmol). The activator proteins τ_1 and τ_1-I120 were added to transcription reactions prior to extract and nucleotides at the concentrations indicated in the figure legends. τ_1-DBD was preincubated with template DNA and nuclear extract for 25 min at 4°C before the addition of nucleoside triphosphates. Transcription products were resolved on 7% polyacrylamide–7 M urea gels and visualized by autoradiography. Transcription levels were quantitated by densitometry of exposed X-ray film (model 301; X-rite Co., Grand Rapids, Mich.).

Expression and purification of yTFIID from bacteria. The yeast TATA-binding factor was expressed in BL21 cells from the bacteriophage T7 promoter in plasmid pKA9 (constructed by S. Buratowski, Massachusetts Institute of Technology). After induction with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 40 min, yTFIID was isolated and partially purified by DEAE-Sephacryl (Pharmacia) and heparin-Sepharose (Pharmacia) chromatography essentially as described by Lieberman et al. (28). The last fraction of the 0.6 M KCl-heparin-Sepharose elution, used in subsequent in vitro transcription and squelching studies, was dialyzed against nuclear extract dialysis buffer, and yTFIID was estimated to be 40 to 50% of the total protein (40 μg/ml).

τ_1 affinity chromatography. τ_1 affinity chromatography was performed as follows. Affinity (1-ml) and control columns were prepared by immobilizing PAτ_1 from the plasmid pEHGR770 or PA only from the plasmid pERAT318 on IgG-Sepharose (Pharmacia), respectively (0.5 to 1.0 mg of protein per ml of resin). A bacterial lysate (4 mg of total protein) containing yTFIID was diluted with affinity (AC) buffer (20 mM HEPES, pH 7.6, 0.2 mM EDTA and EGTA, 20% glycerol, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride) to give 100 mM final KCl concentration and was loaded directly onto the affinity or control columns previously equilibrated with AC-100 buffer (AC buffer plus 100 mM KCl). The flowthrough plus 1 ml of wash fraction was collected, and the columns were washed with a further 9 ml of AC-100 and then eluted with 2 ml of AC-300 and AC-1,000 buffer. Eluted fractions were precipitated with 10 to 15% trichloroacetic acid, and the recovered proteins were analyzed by SDS-PAGE.

P11 chromatography. Whole-cell yeast extract (50 mg of protein) was adjusted to 100 mM KCl and chromatographed on a P11 (Whatman) column (ca. 10 mg of protein per ml of bed volume), equilibrated with 25 mM HEPES (pH 7.6)–1 mM EDTA–10% glycerol–1 mM DTT–100 mM KCl and protease inhibitors. The flowthrough material was collected (fractions 1 to 4), and the column was eluted successively with 0.5 M KCl (fractions 5 to 8) and 0.8 M KCl (fractions 9 to 12). Fractions used in subsequent experiments were dialyzed against 20 mM HEPES (pH 7.6)–20% glycerol–1 mM EDTA–1 mM DTT–100 mM potassium acetate and protease inhibitors. The presence of yeast TBP was assayed for by Western blotting by using anti-yTFIID rabbit antiserum (a gift from A. Berk, University of California, Los Angeles).

RESULTS

The τ_1 transactivation domain of the glucocorticoid receptor activates transcription in vitro. Previously we have shown that the τ_1 domain can transactivate transcription in yeast cells in vivo from a reporter gene containing a GRE binding site (61). To test whether τ_1 would also activate transcription in vitro, a protein consisting of the τ_1 domain (amino acids 77 to 262) fused to the receptor DNA-binding domain (amino acids 415 to 500) (Fig. 2A) was expressed in bacteria and purified as described previously (60). Transcription in yeast nuclear extracts was assayed by using reporter genes consisting of a G-free cassette (47) downstream of sequences from the *CYC1* promoter with or without upstream DNA response elements (Fig. 2A). This promoter is transcribed efficiently in vitro and gives rise to two RNase T_1-resistant transcripts (Fig. 2B) (32). RNA polymerase II-dependent transcription in vitro was demonstrated by α-amanitin sensitivity (data not shown) (32). The transcription start sites for the *CYC1* promoter construct have been mapped and shown to be the same in vivo and in vitro (32). Figure 2B shows that nuclear extracts supported both basal (lanes 1 and 3) and induced (lanes 2 and 4) levels of transcription. The bacterially produced τ_1-DBD was competent to induce transcription at levels comparable to those induced by an endogenous activator binding to the DED48 response elements.

Therefore, the τ_1 domain when coupled to a DNA-binding domain will activate transcription in a yeast cell free assay; thus this system is appropriate for isolation of the factors

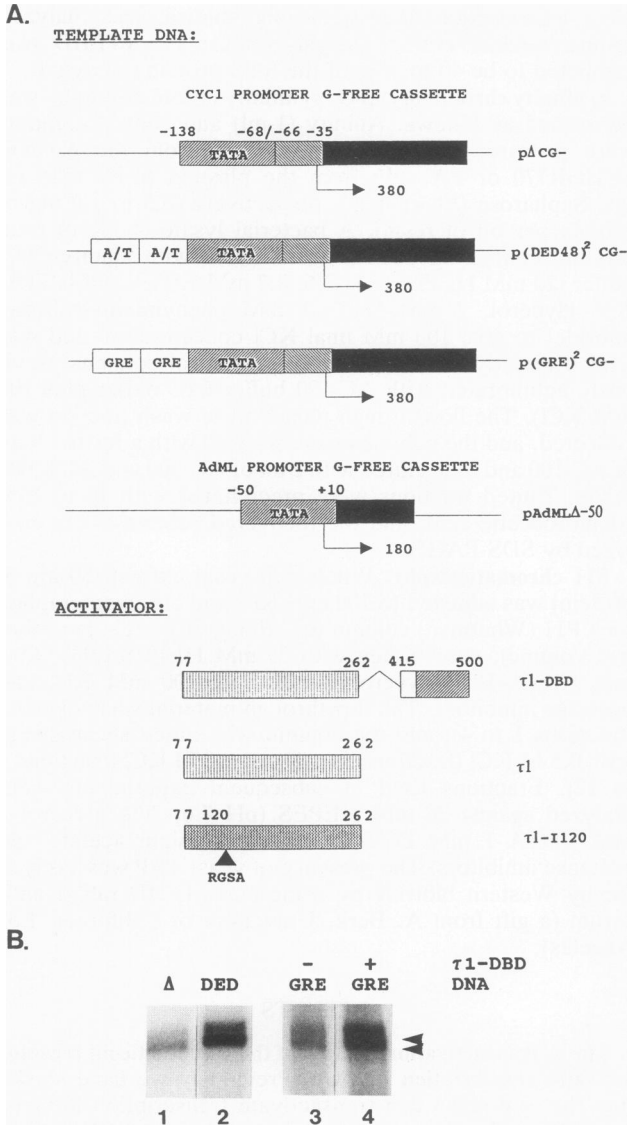

FIG. 2. τ_1 activation of transcription in a cell free transcription system. (A) Schematic representations of G-free cassette reporter constructs pΔCG− (32), p(DED48)²CG− (32), p(GRE)²CG−, and pAdMLΔ−50 (48) and the activator proteins τ_1-DBD, τ_1, and τ_1-I120. (B) Typical levels of transcription seen in yeast nuclear extracts with 100 ng of pΔCG− (lane 1), p(DED48)²CG− (lane 2), and 400 ng of p(GRE)²CG− (lanes 3 and 4). The absence (−) or presence (+) of 5 pmol of purified τ_1-DBD activator protein is indicated. RNA products were analyzed by urea-PAGE and visualized by autoradiography. RNase T1-resistant transcripts are indicated by the solid arrowheads.

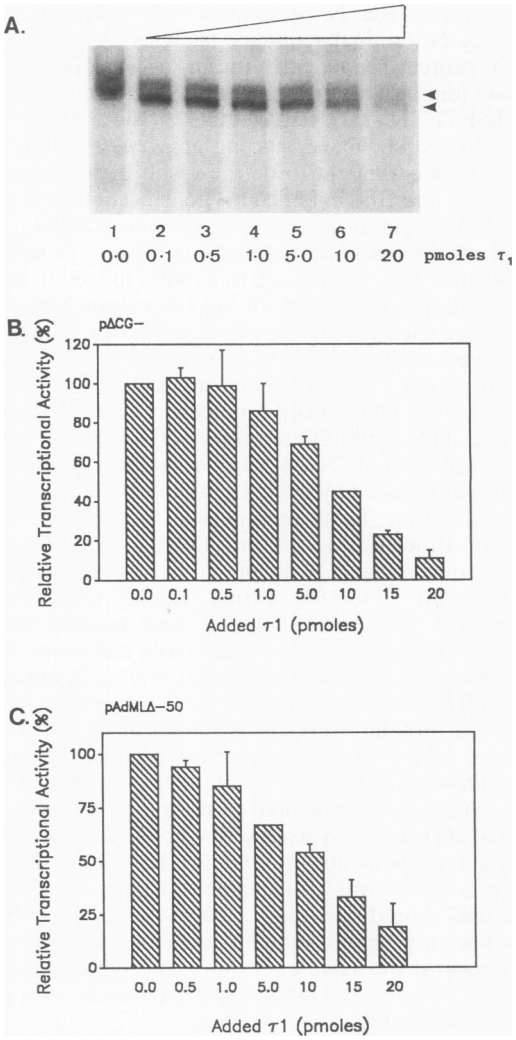

FIG. 3. Squelching of basal transcription by τ_1. (A) Transcription of pΔCG− (100 ng) in the absence (lane 1) or presence (lanes 2 to 7) of increasing amounts of τ_1. The two specific RNase T1-resistant transcripts are shown (arrowheads). (B) Transcription activity in four experiments quantitated by densitometry and the mean relative levels of transcription (± standard deviation) plotted against the amount of τ_1 added (0 pmol of τ_1 = 100%). (C) Increasing amounts of τ_1 (0 to 20 pmol) added to transcription reactions primed with 800 ng of pAdMLΔ−50 DNA and assayed as described. Transcription activity in three experiments was quantitated by densitometry, and the mean relative levels of transcription (± standard deviation) were plotted against the amount of τ_1.

involved. Similar results have been reported for the corresponding region of the rat glucocorticoid receptor in *Drosophila melanogaster* embryo nuclear extracts (15) and the intact human glucocorticoid receptor in HeLa cell nuclear extracts (56).

Isolated τ_1 inhibits transcription of a basal promoter in vitro. To test whether τ_1 interacts directly with the general transcription machinery, isolated τ_1 was added to the cell free transcription system primed with the pΔCG− plasmid. Addition of increasing amounts of τ_1 resulted in a progressive inhibition of transcription (Fig. 3A, lanes 2 to 7). In the presence of 10 pmol of τ_1, transcription was only 45 to 50% of control values, and this dropped to 10 to 20% in the presence of 20 pmol of τ_1 (Fig. 3B). Thus, the isolated τ_1 domain can squelch transcription of a basal promoter, presumably through direct protein-protein interactions with a factor(s) in the general transcription apparatus.

The τ_1 domain squelches transcription from the adenovirus major late core promoter. The generality of the squelching effect was investigated by using the adenovirus 2 major late core promoter (48). All known upstream activating sequences have been removed, leaving only the TATA box and initiator site downstream of position −50 linked to a shortened form of the G-free cassette (Fig. 2A). Figure 3C

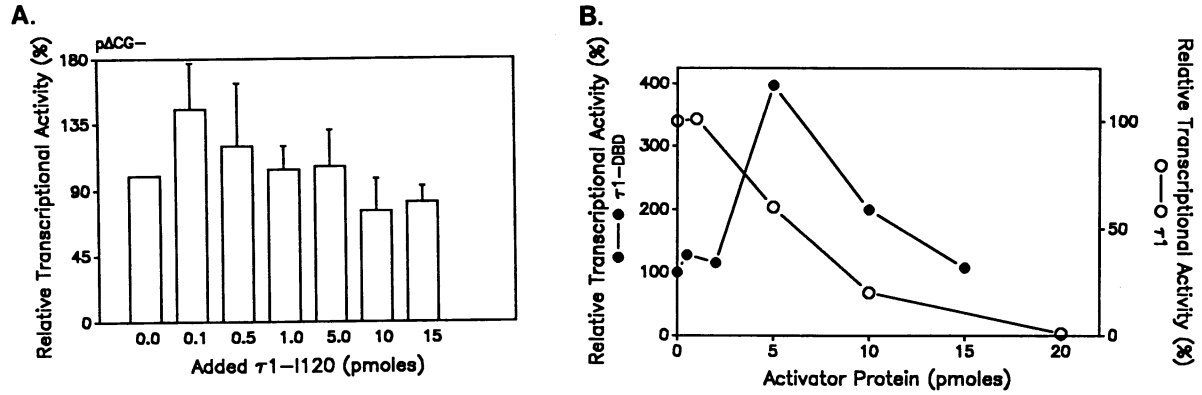

FIG. 4. Correlation of the squelching activity of τ_1 with transactivation activity. (A) Levels of basal transcription in the absence or presence of increasing amounts (0.1 to 15 pmol) of τ_1-I120 protein, quantitated as described. The mean values (± standard deviation) from up to four experiments were plotted relative to control values (100%). (B) Comparison of the transactivation and squelching activities of τ_1-DBD [p(GRE)^{2}CG−] (●) and τ_1 (pΔCG−) (○), respectively. Note the difference in scales on the *y* axis.

shows that increasing amounts of τ_1 led to a dose-dependent inhibition of transcription similar in magnitude to that seen with the basal *CYC1* promoter. Thus, the ability of τ_1 to inhibit transcription from two different basal promoters suggests a direct interaction with a general transcription factor(s) that is required for the activity of both promoters.

Squelching activity correlates with the transactivation activity of the τ_1 domain. To determine whether the ability to squelch transcription correlates with the transactivation activity of τ_1, a 4-amino-acid insertion (RGSA) was introduced into the τ_1 domain after position Lys-120 by oligonucleotide site-directed mutagenesis (Fig. 2A). This mutation has been reported to reduce the transactivation activity of τ_1 to 2% of the wild-type level (16). The mutant protein τ_1-I120 was expressed and isolated as described above (Fig. 1B, lane 10). Figure 4A shows the effect of adding increasing amounts of the mutant protein on the level of transcription from the pΔCG− template. Although there was generally increased variability in the levels of basal transcription, probably resulting from the addition of higher levels of nonspecific protein to the reactions, the mutant protein's behavior was clearly different from that seen with the wild-type τ_1 (compare Fig. 3B with 4A). Thus, it was concluded that the mutant protein had little or no inhibitory effect on basal transcription and that the squelching activity of τ_1 correlates with its activity as an activator of transcription. In addition, comparison of the activity of τ_1 with that of τ_1-DBD revealed that under conditions where τ_1 alone was already squelching transcription, τ_1-DBD gave maximum stimulation (Fig. 4B). Up to 5 pmol of τ_1-DBD gave a level of activation three- to fourfold higher than that of basal transcription, while the same concentration of τ_1 resulted in a reduction of basal transcription by 30 to 40%. Increasing the concentration of each protein to 10 pmol resulted in a decrease in τ_1-DBD activation by at least twofold (self-squelching) and a further reduction of basal transcription by τ_1 to 20 to 40%. Thus, inhibition of basal transcription (pΔCG−) by τ_1 occurred progressively at concentrations higher than 1 pmol, indicating the sequestering of one or more general factors. Self-squelching [p(GRE)^{2}CG−], on the other hand, was seen only when the concentration of τ_1-DBD exceeded the concentration giving maximum activation, indicating competition between τ_1-DBD bound at specific sites and the excess τ_1-DBD for target factor(s) binding. It should be noted that when the pΔCG− template, lacking GREs, was used, τ_1-DBD inhibited basal transcription at least as well as τ_1 alone (data not shown).

The TATA-binding factor TBP is not the target for τ_1. Considering the acidic nature of τ_1, a likely candidate for the τ_1 target protein was TBP (see the introduction). Recombinant yeast TBP (yTFIID) was expressed in *E. coli* and partially purified. Addition of yTFIID to transcription reactions containing the pΔCG− template and 10 to 15 pmol of τ_1 resulted in the dose-dependent recovery of transcription to control levels (Fig. 5A, lanes 1 to 5 and hatched bars). However, addition of the same amounts of yTFIID to transcription reactions in the absence of τ_1 showed clear stimulatory activity, of up to three times control values (Fig. 5A, lanes 6 to 10 and open bars). The results demonstrate that while the concentration of TFIID is below saturating levels in the in vitro assay, squelching of the *CYC1* basal promoter by τ_1 remains unaltered at 39 to 43% by the addition of yTFIID and thus TBP is unlikely to be the target for interaction with τ_1.

The possibility of a direct interaction between TBP and τ_1 in vitro was further tested by affinity chromatography. Figure 5B shows that yTFIID present in a crude bacterial lysate appeared mainly in the flowthrough fractions from both a PAτ_1 affinity column and a PA-only control column. However, small amounts of yTFIID (Fig. 5B, solid arrowhead) together with bacterial proteins were retained on both columns. Under similar conditions, at least three proteins from a yeast whole-cell extract were specifically retained on the τ_1 affinity column (34). Interestingly, two *E. coli* proteins with molecular weights of 41,000 and 45,000 were selectively retained and eluted from the PAτ_1 affinity column with 1.0 M KCl (Fig. 5B, open arrowheads). The functional significance of these interactions remains to be determined. Taken together, the results of the in vitro functional and binding studies strongly suggest that TBP is not contacted directly by the τ_1 transactivation domain.

Squelching by τ_1 is selectively reversible. To demonstrate that this squelching assay could be used to identify the τ_1 target factor(s), it was important to show that the inhibitory effects of τ_1 can be specifically reversed. Therefore, a whole-cell yeast extract was fractionated by phosphocellulose (P11) chromatography, and fractions representing the flowthrough (fractions 2 and 3), the 0.5 M KCl elution (fractions 5 to 7), and the 0.8 M KCl elution (fractions 9 and

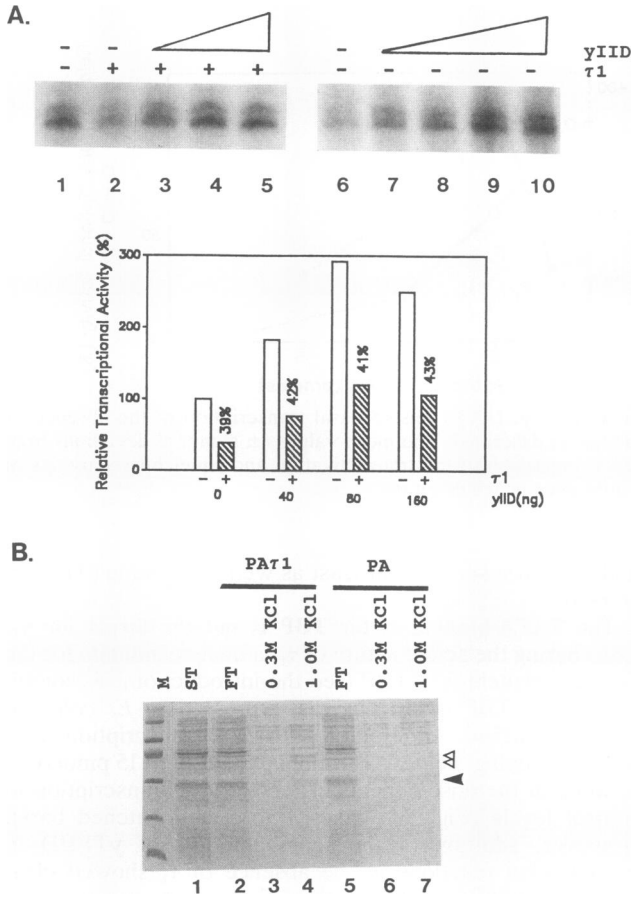

FIG. 5. TATA-binding factor yTBP is not the target for τ_1. (A) Partially purified yTFIID (20 ng, lane 7; 40 ng, lanes 3 and 8; 80 ng, lanes 4 and 9; and 160 ng, lanes 5 and 10) was added to transcription reactions containing 100 ng of pΔCG− template with or without 10 to 15 pmol of τ_1 (lanes 2 to 5 and 7 to 10, respectively). The data for 0, 40, 80, and 160 ng of yTFIID, with (hatched bars) or without (open bars) τ_1, were quantitated and plotted relative to basal levels (100%; lanes 1 and 6, respectively). The autoradiograph and calculated relative levels of transcription for representative experiments are shown, and similar results were seen in at least two other experiments. (B) Affinity chromatography was used to test for direct interaction between τ_1 and yTFIID. Crude bacterial supernatant (lane 1; ST) was fractionated on either a PAτ_1 affinity column (lanes 2 to 4) or a PA control column (lanes 5 to 7), and the proteins retained were analyzed by SDS-PAGE. yTFIID is indicated (solid arrowhead). Two *E. coli* proteins selectively bound to the PAτ_1 (open arrowheads; compare lanes 4 and 7) are also marked. Molecular mass markers (from top to bottom, lane M; Pharmacia) were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). FT, flowthrough fraction.

10) were dialyzed and assayed for their effect on transcription in the presence or absence of τ_1.

These fractions were analyzed for their ability to overcome τ_1 specific inhibition of transcription from the basal *CYC1* promoter. Fractions 5, 7, and 9 were all able to reproducibly increase the level of basal transcription in the presence of τ_1 (Fig. 6A). However, in the absence of τ_1, fraction 5 had no significant effect on basal transcription, while fractions 7 and 9 both showed a modest stimulatory

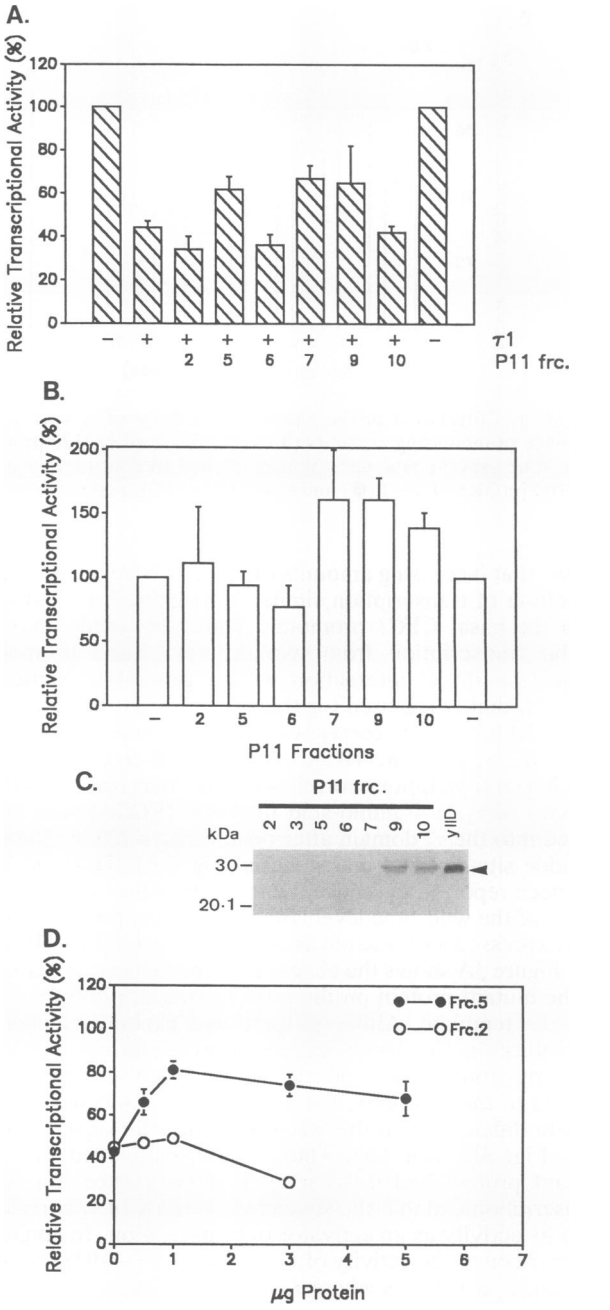

FIG. 6. The τ_1 target protein is present in a P11 column fraction. (A) Fractions (frc.) from a P11 column were tested for their ability to overcome the inhibitory action of 10 to 15 pmol of τ_1 on the *CYC1* promoter. One microgram of protein was added to transcription reactions prior to addition of nuclear extract. The mean relative levels of transcription (± standard deviation) from three to four experiments are shown (control, − = 100%). (B) Same conditions as in panel A except that τ_1 was omitted from all reactions. The mean values (± standard deviation) from two experiments are shown. (C) The presence of TBP in P11 column fractions, determined by Western blotting using anti-yTFIID rabbit antiserum. Lane yIID represents ca. 20 ng of recombinant yTFIID, and lanes 2 to 10 represent 2 μg of each phosphocellulose column fraction. (D) Dose-response curve for increasing amounts of fraction 5 (0.5 to 5.0 μg of total protein) in the presence of 10 to 15 pmol of τ_1. The relative levels of transcription (mean ± standard deviation of two experiments) are shown (●); increasing amounts (0.5 to 3.0 μg of total protein) of fraction 2 are also shown (○).

activity (Fig. 6B). Thus, fraction 5 is the only fraction that specifically reverses squelching by τ_1. The general increase in transcription stimulated by fraction 9 was probably due to the yeast TBP, which was present in this fraction but was not present in fraction 5 (Fig. 6C).

Figure 6D shows the dose-response curve for the antisquelching activity in fraction 5. Addition of 0 to 1.0 μg of fraction 5 resulted in about a twofold recovery of basal transcription, from 40 to 80%. Increasing the amount of total protein added above 1 μg did not result in any further increase in the relative level of transcription (Fig. 6D, solid circles). Increasing amounts of fraction 2 (flowthrough) are shown for comparison (open circles). Over the same concentration range, fraction 5 had no apparent effect on basal transcription in the absence of τ_1 (data not shown). Thus, by using P11 chromatography, it was possible to separate an activity that specifically reversed the squelching effect of τ_1 from general stimulatory activities, such as TBP, and so the squelching assay can be used to isolate factors which interact with τ_1.

DISCUSSION

Previously, we have shown that overexpression of the τ_1 transactivation domain of the human glucocorticoid receptor in yeast cells inhibits both gene expression and cell growth (61). This was thought to be the result of τ_1 squelching a factor needed for basal promoter activity. In the present study, an in vitro transcription assay was used to directly study the effect of τ_1 on basal transcription. When added to nuclear extracts, the isolated τ_1 domain inhibited transcription from two different basal promoters in a concentration-dependent manner. This effect was correlated with the activity of the τ_1 transactivation function, since a mutation known to knock out transactivation activity also reduced the ability of τ_1 to squelch transcription in vitro. In addition, the τ_1 domain fused to the DNA-binding domain (τ_1-DBD) activated transcription from the *CYC1* promoter in the presence of two upstream receptor binding sites [p(GRE)^{2}CG−]. Thus, extracts contain the components needed for activation by the receptor. This induction was seen at concentrations at which τ_1 alone squelched basal transcription from the *CYC1* promoter (i.e., at 5 pmol; Fig. 4B). Increasing the concentration of τ_1-DBD above 5 pmol resulted in self-squelching of p(GRE)^{2}CG− activity (Fig. 4B). This behavior has previously been seen for the chimeric activator GAL4-VP16 and was taken as evidence for the same factor(s) being the target for activation and squelching (26). From studies in vitro with the intact receptor (56), it would seem likely that the target factor(s) is part of the preinitiation complex, although we cannot exclude the possibility that τ_1 is acting at some step subsequent to preinitiation complex formation.

General transcription factors versus coactivators. Interference or squelching of transcription by the overexpression of a transactivator was originally demonstrated for the yeast transcriptional activator GAL4 (for reviews, see references 40 and 41 and the references therein). The interference is thought to result from protein-protein interactions between the activator and a limiting pool of target factors so that the latter are no longer available to mediate the action of DNA-bound activators. Recently, squelching studies carried out in an in vitro system similar to the one described here revealed the involvement of coactivator or adaptor proteins in the activity of a sequence-specific transcription factor (2, 26). However, in both of these studies, the addition of the chimeric activator GAL4-VP16 inhibited activated levels of transcription but not basal activity. Berger et al. (2) did observe squelching of basal activity, but this was due to nonspecific DNA binding by GAL4-VP16 and was not seen in the presence of competing GAL4 binding sites. In the present study, squelching of basal transcription by the transactivation domain of the glucocorticoid receptor occurred in the absence of a DNA-binding domain; it was therefore concluded that this was the result of a direct interaction with at least one of the general transcription factors.

An alternative explanation is that τ_1 was squelching a cofactor which itself was tightly associated with one of the basal transcription factors, analogous to recent reports of TBP-associated factors (TAFs [11, 43, 53]). However, this cofactor, if it exists, is unlikely to be a TAF, since purified TBP from yeast, in contrast to that from higher eukaryotes, behaves as a monomer (5, 43, 44). Furthermore, if τ_1 was contacting a TBP-associated factor and indirectly squelching TBP, then addition of the recombinant yTFIID should have overcome the effect of τ_1 on basal transcription, which was clearly not the case. Taken together, the simplest explanation for the present findings is that the τ_1 transactivation domain is interacting directly with one or more of the general transcription factors subsequent to the template commitment step involving TBP-DNA binding. It may seem paradoxical in the absence of a DNA-binding domain that τ_1 should squelch basal transcription since the interaction with the target factor(s) is presumably the same as that which occurs during activation. We conclude from this that while free τ_1 can interact with its target, it cannot form part of a productive transcriptional complex, a process that appears to require the tethering of τ_1 to DNA. The explanation for this remains unclear but may involve stearic hindrance by the free τ_1.

Recently a class of yeast genes that are required for the function of a number of sequence-specific DNA-binding transcription factors has been described (references 38 and 52 and the references therein). These genes, *SWI1*, *SWI2*, and *SWI3*, encode for large proteins (1,314, 1,704, and 825 amino acids, respectively) that do not appear to bind DNA but may instead act together as accessory proteins or coactivators with DNA-binding proteins such as GAL4 and ADR1 (38). In addition, it was reported that glucocorticoid receptor function in yeast cells was dependent upon these *SWI* genes (38, 52). However, since the SWI1, 2, and 3 gene products are not required for basal transcription (38, 52), it would seem likely that they act by a mechanism distinct, although not necessarily mutually exclusive, from the one suggested by the above squelching studies. The SWI proteins could, for example, act at the level of receptor DNA binding either directly or via effects on chromatin structure (reference 38 and the references therein). Alternatively, they could function as coactivators at a step subsequent to the interaction of the receptor with one of the basal factors by a mechanism similar to that suggested previously in the case of VP16 (41). Neither of these possibilities for SWI function can be distinguished by using the present squelching assay since it is specifically aimed at identifying direct protein-protein interactions involving the τ_1 domain and the general transcriptional machinery.

Mechanisms of transactivation by members of the nuclear receptor superfamily. The transactivation and squelching activity of the human estrogen receptor, glucocorticoid receptor, and acidic activator GAL4-VP16 in mammalian cells have been compared (54, 55). From these studies, it

was concluded that the N and C termini of the estrogen and glucocorticoid receptors contain complex constitutive and hormone-dependent transactivation functions, respectively, which function by different mechanisms (54, 55). Others have shown that the glucocorticoid receptor can squelch the transactivation activity of the lymphoid-specific Oct 2A factor, when both were cotransfected into HeLa cells (58). This interference of Oct 2A factor activity requires, at least in part, sequences within the DNA-binding domain of the receptor. However, the nature of possible target proteins for the estrogen receptor, glucocorticoid receptor, Oct-2A factor, and GAL4-VP16 in these systems remains to be identified.

More recently, a functional synergism between the retinoic acid receptor and recombinant human (h)TFIID when both proteins were cotransfected into embryonal carcinoma cells has been described (3). This cooperativity requires the core domain of hTFIID and an activity absent from COS cells that can be substituted by the adenovirus E1A protein. Thus, in contrast to the above model for the glucocorticoid receptor, the interaction of the retinoic acid receptor with the basal transcription apparatus is apparently indirect and involves an E1A-like activity. Current data therefore support the idea that not all members of the nuclear receptor superfamily will activate transcription by the same mechanism (54).

The glucocorticoid receptor can regulate transcription of target promoters at several levels, including indirect effects on chromatin structure allowing the binding of other sequence-specific activators (9, 45) and direct effects on the formation of the preinitiation complex (56). In the present study, by using an in vitro squelching assay, we have described direct contact between the receptor transactivation domain (τ_1) and the general transcriptional apparatus. Furthermore, the value of the squelching assay as a functional test for target factor activity was demonstrated by the ability of a phosphocellulose column fraction to specifically reverse the inhibitory effect of τ_1. Therefore, this assay will be useful in studies to further define the interactions between the glucocorticoid receptor and basal transcription factors and to identify the factor(s) involved.

ACKNOWLEDGMENTS

We thank members of the Steroid Receptor Unit at the Centre for Biotechnology for helpful discussions and comments during this work and L. Poellinger for critical reading of the manuscript. We are grateful to A. J. Berk (University of California, Los Angeles), R. D. Kornberg (Stanford University), L. Poellinger (Department of Medical Nutrition, Karolinska Institute), and B. L. West (University of California, San Francisco) for the gifts of plasmids pKA9, pΔCG−, p(DED48)^{2}CG−, pAdMLΔ−50, and pRSVhGR, respectively. In addition, we thank P. M. Flanagan, N. F. Lue, and R. J. Kelleher III from the Kornberg Laboratory (Stanford) for help and advice in setting up the yeast cell free transcription assay. We are also grateful to Anne Peters (CBT, NOVUM) and Margaret Warner (Department of Medical Nutrition, Karolinska Institute) for help with N-terminal sequence analysis and Marika Rönnholm and Ann-Charlotte Wikström (Department of Medical Nutrition, Karolinska Institute) for generously supplying monoclonal antibodies raised against the glucocorticoid receptor.

This work was supported in part by a Swedish Cancer Society guest scientist fellowship to I.J.M. (project no. 3025-B91-01V) and grants 13X-2819 from the Swedish Medical Research Council, 89-00521P from the Swedish National Board for Technical Development, and K-KV 9756-301 from the Swedish National Science Research Council. J.C.D. is supported by a research fellowship from the Swedish Medical Research Council.

REFERENCES

1. **Beato, M.** 1989. Gene regulation by steroid hormones. Cell **56:**335–344.
2. **Berger, S. L., W. D. Cress, A. Cress, S. J. Triezenberg, and L. Guarente.** 1990. Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors. Cell **61:**1199–1208.
3. **Berkenstam, A., D. M. M. Vivanco Ruiz, D. Barettino, M. Horikoshi, and H. G. Stunnenberg.** 1992. Cooperativity in transactivation between retinoic acid receptor and TFIID requires an activity analogous to E1A. Cell **69:**401–412.
4. **Bodwell, J. E., E. Orti, J. M. Coull, D. J. C. Pappin, L. I. Smith, and F. Swift.** 1991. Identification of phosphorylation sites in the mouse glucocorticoid receptor. J. Biol. Chem. **266:**7549–7555.
5. **Buratowski, S., S. Hahn, L. Guarente, and P. A. Sharp.** 1989. Five intermediate complexes in transcription initiation by RNA polymerase II. Cell **56:**549–561.
6. **Buratowski, S., S. Hahn, P. A. Sharp, and L. Guarente.** 1988. Function of a yeast TATA element-binding protein in a mammalian transcription system. Nature (London) **334:**37–42.
7. **Carlstedt-Duke, J., P. E. Strömstedt, Ö. Wrange, T. Bergman, and J.-Å. Gustafsson.** 1987. Domain structure of the glucocorticoid receptor protein. Proc. Natl. Acad. Sci. USA **84:**4437–4440.
8. **Cavallini, B., J. Huet, J. L. Plassat, A. Sentenac, J. M. Egly, and P. Chambon.** 1988. A yeast activity can substitute for the HeLa cell TATA box factor. Nature (London) **334:**77–80.
9. **Cordingley, M. G., A. T. Riegel, and G. L. Hager.** 1987. Steroid-dependent interaction of transcription factors with the inducible promoter of mouse mammary tumor virus *in vivo*. Cell **48:**261–270.
10. **Dahlman, K., P. E. Strömstedt, C. Rae, H. Jörnvall, J. I. Flock, J. Carlstedt-Duke, and J.-Å. Gustafsson.** 1989. High level expression in *Escherichia coli* of the DNA-binding domain of the glucocorticoid receptor in a functional form utilizing domain specific cleavage of a fusion protein. J. Biol. Chem. **264:**804–809.
11. **Dynlacht, B. D., T. Hoey, and R. Tjian.** 1991. Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation. Cell **66:**563–576.
12. **Evans, R. M.** 1988. The steroid and thyroid hormone receptor superfamily. Science **240:**889–895.
13. **Flanagan, P. M., R. J. Kelleher III, M. H. Sayre, H. Tschochner, and R. D. Kornberg.** 1991. A mediator required for activation of RNA polymerase II transcription *in vitro*. Nature (London) **350:**436–438.
14. **Flores, O., H. Lu, and D. Reinberg.** 1992. Factors involved in specific transcription by mammalian RNA polymerase II. J. Biol. Chem. **267:**2786–2793.
15. **Freedman, L. P., S. K. Yoshinaga, J. N. Vanderbilt, and K. R. Yamamoto.** 1989. *In vitro* transcription enhancement by purified derivatives of the human glucocorticoid receptor. Science **245:**298–301.
16. **Giguere, V., S. M. Hollenberg, M. G. Rosenfeld, and R. M. Evans.** 1986. Functional domains of the human glucocorticoid receptor. Cell **46:**645–652.
17. **Green, S., and P. Chambon.** 1988. Nuclear receptors enhance our understanding of transcription regulation. Trends Genet. **4:**309–314.
18. **Hahn, S., S. Buratowski, P. A. Sharp, and L. Guarente.** 1989. Identification of a yeast protein homologous in function to the mammalian general transcription factor, TFIIA. EMBO J. **8:**3379–3382.
19. **Hoeck, W., and B. Groner.** 1990. Hormone-dependent phosphorylation of the glucocorticoid receptor occurs mainly in the amino-terminal transactivation domain. J. Biol. Chem. **265:**5403–5408.
20. **Hollenberg, S. M., and R. M. Evans.** 1988. Multiple and cooperative *trans*-activation domains of the human glucocorticoid receptor. Cell **55:**899–906.
21. **Hollenberg, S. M., V. Giguere, P. Segui, and R. M. Evans.** 1987. Colocalization of DNA-binding and transcriptional activation functions in the human glucocorticoid receptor. Cell **49:**39–46.

22. **Horikoshi, M., M. F. Carey, H. Kakidani, and R. G. Roeder.** 1988. Mechanism of action of a yeast activator: direct effect of GAL4 derivatives on mammalian TFIID-promoter interactions. Cell **54**:665–669.
23. **Horikoshi, N., K. Maguire, A. Kralli, E. Maldonado, D. Reinberg, and R. Weinmann.** 1991. Direct interaction between adenovirus E1A protein and the TATA box binding transcription factor IID. Proc. Natl. Acad. Sci. USA **88**:5124–5128.
24. **Ingles, C. J., M. Shales, W. D. Cress, S. J. Triezenberg, and J. Greenblatt.** 1991. Reduced binding of TFIID to transcriptionally compromised mutants of VP16. Nature (London) **351**:588–590.
25. **Kakidani, H., and M. Ptashne.** 1988. GAL4 activates gene expression in mammalian cells. Cell **52**:161–167.
26. **Kelleher, R. J., III, P. M. Flanagan, and R. D. Kornberg.** 1990. A novel mediator between activator proteins and the RNA polymerase II transcription apparatus. Cell **61**:1209–1215.
27. **Lee, W. S., C. C. Kao, G. O. Bryant, X. Liu, and A. J. Berk.** 1991. Adenovirus E1A activation domain binds the basic repeat in the TATA box transcription factor. Cell **67**:365–376.
28. **Lieberman, P. M., M. C. Schmidt, C. C. Kao, and A. J. Berk.** 1991. Two distinct domains in the yeast transcription factor IID and evidence for a TATA box-induced conformational change. Mol. Cell. Biol. **11**:63–74.
29. **Lin, Y. S., and M. R. Green.** 1991. Mechanism of action of an acidic transcriptional activator in vitro. Cell **64**:971–981.
30. **Lin, Y. S., I. Ha, E. Maldonado, D. Reinberg, and M. R. Green.** 1991. Binding of general transcription factor TFIIB to an acidic activating region. Nature (London) **353**:569–571.
31. **Lue, N. F., P. M. Flanagan, R. J. Kelleher III, A. M. Edwards, and R. D. Kornberg.** 1990. RNA polymerase II transcription *in vitro*. Methods Enzymol. **194**:545–550.
32. **Lue, N. F., P. M. Flanagan, K. Sugimoto, and R. D. Kornberg.** 1989. Initiation by yeast RNA polymerase II at the adenoviral major late promoter *in vitro*. Science **246**:661–664.
33. **Martin, K. J., J. W. Lillie, and M. R. Green.** 1990. Evidence for interaction of different eukaryotic transcriptional activators with distinct cellular targets. Nature (London) **346**:147–152.
34. **McEwan, I. J.** Unpublished observation.
35. **Metzger, D., J. White, and P. Chambon.** 1988. The human oestrogen receptor functions in yeast. Nature (London) **334**:31–36.
36. **Nilsson, B., L. Abrahamsen, and M. Uhlen.** 1985. Immobilisation and purification of enzymes with staphylococcal protein A gene fusion vectors. EMBO J. **4**:1075–1080.
37. **Olesen, J., S. Hahn, and L. Guarente.** 1987. Yeast HAP-2 and HAP-3 activators both bind to the *CYC1* upstream activation site, UAS2 in an interdependent manner. Cell **51**:953–961.
38. **Peterson, C. L., and I. Herskowitz.** 1992. Characterization of the yeast *SWI1*, *SWI2* and *SWI3* genes, which encode a global activator of transcription. Cell **68**:573–583.
39. **Peterson, M. G., N. Tanese, B. F. Pugh, and R. Tjian.** 1990. Functional domains and upstream activation properties of cloned human TATA binding protein. Science **248**:1625–1630.
40. **Ptashne, M.** 1988. How eukaryotic transcriptional activators work. Nature (London) **335**:683–689.
41. **Ptashne, M., and A. A. F. Gann.** 1990. Activators and targets. Nature (London) **346**:329–331.
42. **Pugh, B. F., and R. Tjian.** 1990. Mechanism of transcriptional activation by Sp1: evidence for coactivators. Cell **61**:1187–1197.
43. **Pugh, B. F., and R. Tjian.** 1992. Diverse transcriptional functions of the multisubunit eukaryotic TFIID complex. J. Biol. Chem. **267**:679–682.
44. **Reinberg, D., M. Horikoshi, and R. G. Roeder.** 1987. Factors involved in specific transcription by mammalian RNA polymerase II. J. Biol. Chem. **262**:3322–3330.
45. **Rigaud, G., J. Roux, R. Pictet, and T. Grange.** 1991. In vivo footprinting of rat TAT gene: dynamic interplay between the glucocorticoid receptor and a liver-specific factor. Cell **67**:977–986.
46. **Saltzman, A. G., and R. Weinmann.** 1989. Promoter specificity and modulation of RNA polymerase II transcription. FASEB J. **3**:1723–1733.
47. **Sawadogo, M., and R. G. Roeder.** 1985. Factors involved in specific transcription by human RNA polymerase II: analysis by a rapid and quantitative *in vitro* assay. Proc. Natl. Acad. Sci. USA **82**:4394–4398.
48. **Sawadogo, M., and R. G. Roeder.** 1985. Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. Cell **43**:165–175.
49. **Sawadogo, M., and A. Sentenac.** 1990. RNA polymerase B (II) and general transcription factors. Annu. Rev. Biochem. **59**:711–754.
50. **Schena, M., and K. R. Yamamoto.** 1988. Mammalian glucocorticoid receptor derivatives enhance transcription in yeast. Science **241**:965–967.
51. **Stringer, K. F., C. J. Ingles, and J. Greenblatt.** 1990. Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. Nature (London) **345**:783–786.
52. **Tamkun, J. W., R. Deuring, M. P. Scott, M. Kissinger, A. M. Pattatucci, T. C. Kaufman, and J. A. Kennison.** 1992. *brahma*: a regulator of Drosophila homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. Cell **68**:561–572.
53. **Tanese, N., B. F. Pugh, and R. Tjian.** 1991. Coactivators for a proline-rich activator purified from the multisubunit human TFIID complex. Genes Dev. **5**:2212–2224.
54. **Tasset, D., L. Tora, C. Fromental, E. Scheer, and P. Chambon.** 1990. Distinct classes of transcriptional activating domains function by different mechanism. Cell **62**:1177–1187.
55. **Tora, L., J. White, C. Brou, D. Tasset, N. Webster, E. Scheer, and P. Chambon.** 1989. The human estrogen receptor has two independent nonacidic transcriptional activation functions. Cell **59**:477–487.
56. **Tsai, S. Y., G. Srinivasan, G. F. Allan, E. B. Thompson, B. W. O'Malley, and M. J. Tsai.** 1990. Recombinant human glucocorticoid receptor induces transcription of hormone response genes *in vitro*. J. Biol. Chem. **265**:17055–17061.
57. **Webster, N., J. R. Jin, S. Green, M. Hollis, and P. Chambon.** 1988. The yeast UAS_G is a transcriptional enhancer in human HeLa cells in the presence of the GAL4 trans-activator. Cell **52**:169–178.
58. **Wieland, S., U. Döbbeling, and S. Rusconi.** 1991. Interference and synergism of glucocorticoid receptor and octamer factors. EMBO J. **10**:2513–2521.
59. **Wright, A. P. H., J. Carlstedt-Duke, and J.-Å. Gustafsson.** 1990. Ligand-specific transactivation of gene expression by a derivative of the human glucocorticoid receptor expressed in yeast. J. Biol. Chem. **256**:14763–14769.
60. **Wright, A. P. H., and J.-Å. Gustafsson.** 1991. Mechanism of synergistic transcriptional transactivation by the human glucocorticoid receptor. Proc. Natl. Acad. Sci. USA **88**:8283–8287.
61. **Wright, A. P. H., I. J. McEwan, K. Dahlman-Wright, and J.-Å. Gustafsson.** 1991. High level expression of the major transactivation domain of the human glucocorticoid receptor in yeast cells inhibits endogenous gene expression and cell growth. Mol. Endocrinol. **5**:1366–1372.