

Functional Interactions of the AF-2 Activation Domain Core Region of the Human Androgen Receptor with the Amino-Terminal Domain and with the Transcriptional Coactivator TIF2 (Transcriptional Intermediary Factor 2)

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Previous studies in yeast and mammalian cells showed a functional interaction between the amino-terminal domain and the carboxy-terminal, ligand-binding domain (LBD) of the human androgen receptor (AR). In the present study, the AR subdomains involved in this *in vivo* interaction were determined in more detail. Cotransfection experiments in Chinese hamster ovary (CHO) cells and two-hybrid experiments in yeast revealed that two regions in the NH₂-terminal domain are involved in the functional interaction with the LBD: an interacting domain at the very NH₂ terminus, located between amino acid residues 3 and 36, and a second domain, essential for transactivation, located between residues 370 and 494. Substitution of glutamic acid by glutamine at position 888 (E888Q) in the AF-2 activation domain (AD) core region in the LBD, markedly decreased the interaction with the NH₂-terminal domain. This mutation neither influenced hormone binding nor LBD homodimerization, suggesting a role of the AF-2 AD core region in the functional interaction between the NH₂-terminal domain and the LBD. The AF-2 AD core region was also involved in the interaction with the coactivator TIF2 (transcriptional intermediary factor 2), as the E888Q mutation decreased the stimulatory effect of TIF2 on AR AF-2 activity. Cotransfection of TIF2 and the AR NH₂-terminal domain expression vectors did not result in synergy between both factors in the induction of AR AF-2 activity. TIF2 highly induced AR AF-2 activity on a complex promoter [mouse mammary

tumor virus (MMTV)], but it was hardly active on a minimal promoter (GRE-TATA). In contrast, the AR NH₂-terminal domain induced AR AF-2 activity on both promoter constructs. These data indicate that both the AR NH₂-terminal domain and the coactivator TIF2 functionally interact, either directly or indirectly, with the AF-2 AD core region in the AR-LBD, but the level of transcriptional response induced by TIF2 depends on the promoter context. (Molecular Endocrinology 12: 1172–1183, 1998)

INTRODUCTION

The human androgen receptor (hAR) mediates physiological effects of testosterone and dihydrotestosterone (DHT), which are essential for development and functional maintenance of male reproductive and accessory sex tissues. As a ligand-dependent transcription factor, its structural organization shows a high level of molecular identity to other members of the nuclear receptor superfamily. For these receptors, separate functional domains have been characterized. The variable NH₂-terminal domain is involved in transcription activation and contains the transactivation function AF-1 (see Ref. 1 for review). The centrally located, highly conserved DNA-binding domain (DBD) mediates the interaction with hormone-response elements on the DNA (2). The carboxy-terminal (C-terminal) region contains the ligand binding domain (LBD), which is involved in receptor dimerization (3–6), and can functionally interact with transcriptional intermediary factors (TIFs) (for review see Ref. 7).

For the hAR, two separate transcription activation units (TAUs) in the NH₂-terminal domain were defined

(8). One of these transactivation units [TAU-1; amino acids (aa) 100–370] is only active in the full-length ligand-activated AR. In a truncated hAR, which lacks the LBD, another region (TAU-5, aa 360–485) functions as a constitutively active transactivation domain. The role of TAU-5 in the full-length AR is not yet clear.

The LBDs of various nuclear receptors contain a ligand-dependent transactivation function, AF-2 (9–11). An autonomous activating domain (AD) in this AF-2 region, AF-2 AD, is conserved among many nuclear receptors and is located in the C-terminal part of the LBD. A core region in the AF-2 AD, α -helix 12, appeared to be important for transcriptional activity (9, 10, 12, 13) and the hormone-dependent interaction with TIFs. These TIFs or coactivators can modulate the transcriptional activity of a broad range of nuclear receptors (14–17). Mutations in the AF-2 AD core abolish the *in vitro* association of the receptor with these coactivators. The recent finding that a coactivator displays histone acetyltransferase activity has provided further insights into the molecular events occurring at the chromatin level during transcription activation (18). Wurtz *et al.* (19) proposed a general mechanism for nuclear receptor activation, in which the AF-2 AD core, present in helix 12, plays a central role in the generation of an interaction surface, allowing binding of TIFs to the LBD. The functional role of the AF-2 AD core in the AR is not yet well understood.

Recently, for the estrogen receptor (ER) a functional, ligand-dependent, *in vivo* association between the NH₂-terminal domain and the LBD was described (20). Also for the AR a functional *in vivo* interaction between the NH₂-terminal domain and the LBD was demonstrated (21, 22). In the study presented here, we determined in more detail AR subdomains involved in the functional interaction of the LBD with the NH₂-terminal domain. The data indicated that two NH₂-terminal regions, together with the AF-2 AD core region in the LBD, are important for a functional *in vivo* interaction. Induction of AR AF-2 activity by the transcriptional coactivator TIF2 also required an intact AF-2 AD core. This enhancement of AR AF-2 activity by TIF2 appeared to be promoter dependent.

RESULTS

Two Regions in the AR NH₂-Terminal Domain Are Involved in the Functional Interaction with the AR-LBD

Studies in CHO Cells A functional *in vivo* association between the NH₂-terminal domain and LBD of the hAR was found previously (22). Experiments performed in yeast and mammalian cells showed that this interaction was hormone dependent and could be blocked by antiandrogens. The present study focused on AR subdomains involved in this NH₂-terminal domain/LBD protein-protein interaction.

To determine regions in the NH₂-terminal domain

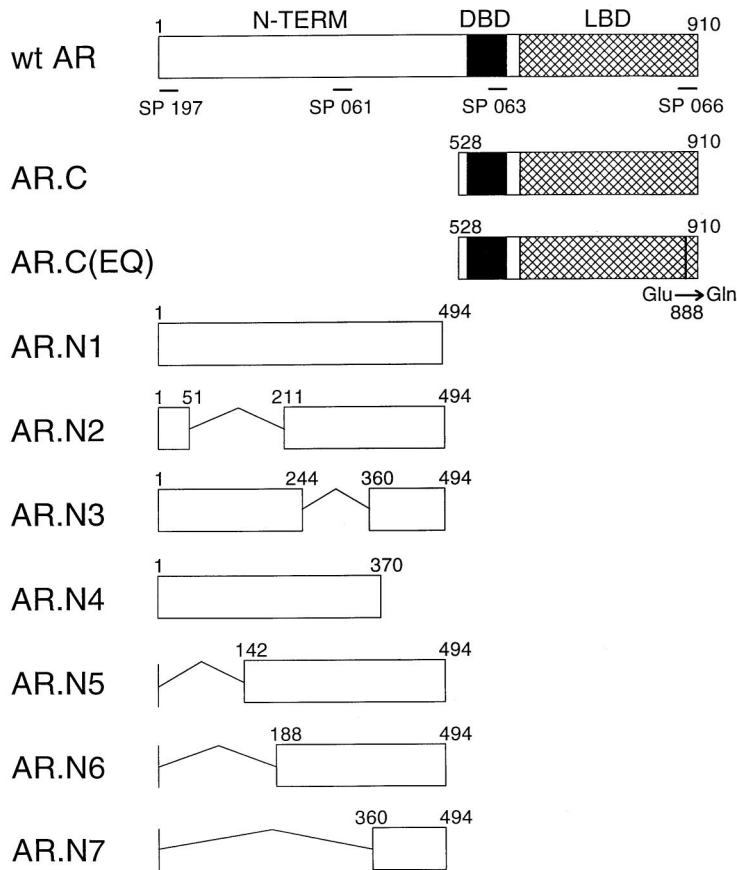
involved in the functional interaction with the AR-LBD, different deletion mutants of the NH₂-terminal domain were constructed, as shown in Fig. 1A (AR.N1–AR.N7). The deletion mutants were transiently transfected into CHO cells, and the appropriate expression of the proteins was assessed by immunoaffinity purification and Western blot analysis of cytosolic fractions (Fig. 1B, lanes 1–7). Constructs (AR.N1–AR.N6) were expressed as proteins of expected molecular mass and at comparable expression levels. The expression level of AR.N7 could not be determined because this protein lacks an epitope for the available antibodies.

The ability of these mutants to induce transcription activation through interaction with the AR-LBD (Fig. 1A; AR.C), using mouse mammary tumor virus (MMTV)-LUC as a reporter gene, is shown in Fig. 2. As shown previously (22), neither AR.N1 nor AR.C could, when expressed separately in CHO cells, activate transcription from the reporter in response to androgens. The lack of activity of AR.C is not due to the experimental setup, because a similar glucocorticoid receptor (GR) fragment, GR.C (aa 369–777), highly induced luciferase activity in CHO cells (24-fold) upon addition of 10 nM dexamethasone (data not shown). Coexpression of AR.N1 and AR.C resulted in an androgen-dependent induction (18-fold) of luciferase expression (Fig. 2; AR.N1), which is comparable to full-length AR activity. Deletion mutants AR.N2 and AR.N3 showed similar responses as seen with the intact NH₂-terminal domain. However, deletion of amino acid residues 371–494 markedly reduced the androgen-induced LUC-activity (AR.N4). Deletions in the first part of the NH₂-terminal domain (AR.N5, AR.N6, AR.N7) almost completely abolished the R1881-mediated response. These data indicate that, for a functional interaction of the NH₂-terminal domain with the LBD of the AR in CHO cells, amino acid residues 1–51 and 371–494 are important. This suggests that the TAU-5 domain, located in the latter region, is involved in this functional *in vivo* interaction. The region between residues 51 and 360, harboring TAU-1, appears not to be involved.

Studies in Yeast To extend the CHO cell experiments, functional NH₂-terminal domain/LBD interactions were studied in yeast, using a two-hybrid system. The high sensitivity of the yeast two-hybrid assay made it also possible to detect weak associations. Furthermore, because of the presence of the Gal4 transactivation domain, the *in vivo* interaction does not solely depend on an intact AR-transactivating domain.

Truncated forms of the AR NH₂-terminal domain were fused to the transactivation domain of GAL4 (Gal4-TAD), in the high-expression vector pACT2 (Fig. 3A). AR-LBD was fused to the DBD of GAL4 (Gal4-DBD). Figure 3B shows the protein expression in yeast Y190 cells, as assessed by immunoblot analysis using specific antibodies directed against either the AR (lanes 1–4) or Gal4-TAD (lanes 5–10). The immunoblot shows expression of most fusion proteins of the appropriate length. However, the expression of GalAD-

A



B

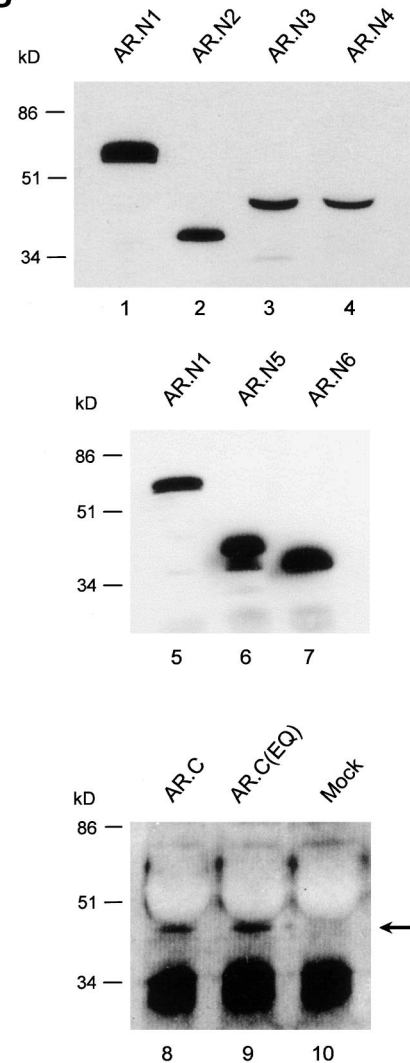


Fig. 1. Structure and Protein Expression Patterns of AR Mutants Used in the Transfection Studies in CHO Cells

A, The full-length hAR is shown with its NH₂-terminal domain (N-TERM), DBD, and LBD. The total number of amino acid residues in the full-length hAR is 910 and is based on amino acid stretches of 20 Gln and 16 Gly residues. Synthetic peptide sequences in the AR to which antibodies were raised and that were used in this study are depicted (SP). The various NH₂-terminal constructs (AR.N1 to AR.N7) and C-terminal constructs [AR.C and AR.C(EQ)] are shown. B, The AR proteins were expressed in CHO cells and immunopurified from cytosolic fractions, as described in *Materials and Methods*. The proteins were visualized by immunoblotting with polyclonal antibodies against SP197 (lanes 1–4), SP061 (lanes 5–7), or SP066 (lanes 8–10). The molecular mass marker values are indicated on the left of each blot.

AR.N9 (lane 2) appeared to be somewhat lower. GalAD-AR.N8 became visible only at an extended exposure time (lane 1a), indicating a very low protein expression level.

Activation of the integrated UAS_{GAL1}-lacZ reporter gene, indicative for the functional interaction of GalAD-AR.N with the promoter-bound GalDBD-AR.C, was assessed in a β-galactosidase (β-GAL) assay (Fig. 4A). The observed reporter gene activity for GalAD-AR.N8 was lower as compared with GalAD-AR.N9, GalAD-AR.N10, and GalAD-AR.N12, most likely due to the very low protein expression level of GalAD-AR.N8. The constructs showing a high β-GAL activity (GalAD-

AR.N8, -N9, -N10, and -N12) all share one common region, spanning residues 3–36. Deletion of this region from constructs GalAD-AR.N12 and GalAD-AR.N15, resulting in constructs GalAD-AR.N13 and GalAD-AR.N16, respectively, caused an intense drop in β-GAL activity. Therefore, in accordance with the CHO results, the data imply an important role of NH₂-terminal amino acid residues 3–36 in the functional interaction with the AR-LBD.

Mutant GalAD-AR.N11, missing residues 245–494, was reduced in its activity as compared with the intact NH₂-terminal domain. A decrease in activity was also seen when residues 330–494 were deleted from

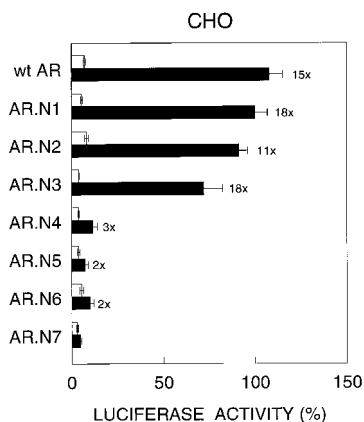


Fig. 2. Transcriptional Activities of AR NH₂-Terminal Deletion Mutants Cotransfected with AR.C in CHO Cells

Transcriptional activities were determined by transfecting full-length AR (3 ng/well) or by cotransfection of AR.C (50 ng/well) with each of the NH₂-terminal constructs (100 ng/well), together with a MMTV-LUC reporter plasmid (200 ng/well), into CHO cells. Cells were incubated either with vehicle (*open bars*) or with 10 nM R1881 (*closed bars*). Luciferase activity was determined as described in *Materials and Methods*. The interexperimental variation of the mean maximal luciferase activities ranged between 2,000 and 9,000 light units. For each experiment the mean AR.N1 activity was set at 100%, and all individual points were calculated relative to this value. Each *bar* represents the mean (\pm SEM) luciferase activity for three experiments. Fold induction is shown at the *top of each bar* and represents the ratio of activity determined in the presence or absence of R1881.

GalAD-AR.N12, resulting in GalAD-AR.N15. This implies that a second region, between residues 244 and 494, is involved in the functional interaction with the LBD. More detailed mapping within this region did not clearly reveal distinctive interaction sites, because neither deletion of residues 245–360 (GalAD-AR.N9), nor deletion of residues 371–494 (GalAD-AR.N10), resulted in a distinct decrease of β -GAL activity. However, interpretation of these data is complicated because of the difference in expression levels of the different constructs (Fig. 3B).

As reported previously (22), the measured β -GAL activities are partly due to Gal4-TAD transactivity, but could also be influenced by the intrinsic transactivating activity of the AR NH₂-terminal domain. The contribution of the intrinsic AR activity to the total measured β -GAL activity could vary between different constructs and thereby affect the mapping of interacting regions. To discriminate between AR transactivity and AR interaction potential, the Gal4-TAD part was deleted from these GalAD-AR.N constructs that showed a considerable interaction with GalDBD-AR.C (as depicted in Fig. 4A). This resulted in constructs AR.N8-AR.N12 and AR.N15. Protein expression of these constructs, as determined by immunoblot analysis, is shown in Fig. 3B (lanes 11–16).

The activities of the AR.N constructs, in the two-hybrid assay with GalDBD-AR.C, are shown in Fig. 4B.

AR.N11 and AR.N15 did not induce a functional interaction, although the corresponding Gal4-TAD chimeric constructs, as shown in Fig. 4A, were active. This indicates that AR.N11 and AR.N15 only contain interaction potential and not transactivity. These constructs have the 3–36 fragment in common. Deletion of the region between residues 244 and 360 (AR.N9) did not reduce β -GAL activity; AR.N12 (missing residues 37–243) also showed a clear response. This indicates that both these proteins still have transactivating activity and interaction potential. However, by deleting residues 371–494 (AR.N10), β -GAL activity dropped by approximately 80%, a decrease comparable to the data obtained in CHO cells (Fig. 2; AR.N4). These results narrow the second interacting region (aa 244–494) to involvement of residues 371–494 as a major transactivation domain in the functional interaction with the LBD.

Taken together, the yeast two-hybrid data imply a prominent role of amino acid residues 3–36 of the AR NH₂-terminal domain in the interaction with the LBD. A second region seems to be mainly constrained to residues 371–494 and further supports the involvement of TAU-5, as a transactivating region, in the functional *in vivo* interaction with the LBD.

A Mutation in the LBD Affects the Functional Interaction with the NH₂-Terminal Domain, but not LBD Dimerization

To determine regions in the AR-LBD involved in the functional interaction with the NH₂-terminal domain, mutational analysis was constrained to the conserved amphipathic helix 12 in the LBD. This helix constitutes the AF-2 activation domain (AD) core region, of which the charged residues might generate a protein-protein interacting surface (19). A single amino acid substitution in the AF-2 AD core was introduced: glutamic acid at position 888, which is highly conserved among nuclear receptors, was replaced by glutamine (E888Q).

Both the wild-type and the mutated LBD construct (Fig. 1A; AR.C and AR.C(EQ), respectively) were transfected into CHO cells. The appropriate expression of both proteins is shown in Fig. 1B (lanes 8 and 9). To determine whether the AF-2 AD mutation had an effect on hormone binding, an *in vivo* androgen binding assay in transfected CHO cells was performed. Scatchard analysis of the data revealed that both proteins had similar K_d values for R1881: AR.C, 0.37 nM, and AR.C(EQ), 0.33 nM, respectively, indicating that the E888Q mutation did not affect hormone binding.

The effect of the E888Q mutation on the functional *in vivo* interaction of the LBD with the NH₂-terminal domain was investigated by cotransfecting AR.N1 with increasing amounts of either AR.C or AR.C(EQ) and the MMTV-LUC reporter into CHO cells (Fig. 5A). As expected, no response to 1 nM R1881 was observed in the absence of the LBD constructs. Coexpression of AR.C and AR.N1 resulted in a high induction (to 24-fold) of luciferase activity. The E888Q mutation

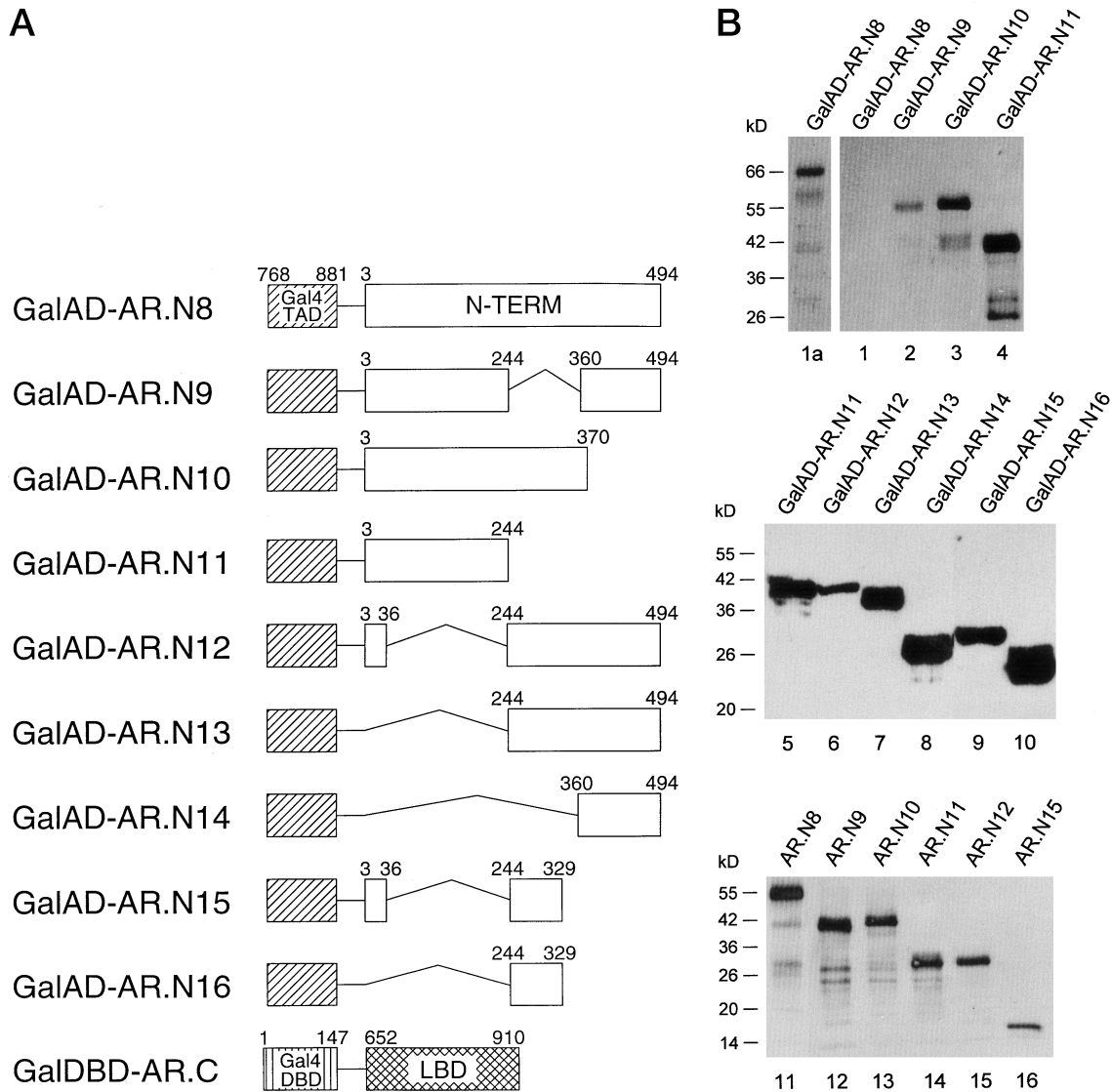


Fig. 3. Structure and Expression Patterns of AR-Gal4 Fusion Proteins Used in the Two-Hybrid Studies in Yeast
 A, Shown are AR NH₂-terminal mutants fused to the Gal4 transactivating domain (TAD) and the LBD fused to the Gal4 DBD.
 B, The appropriate expression of the AR constructs in yeast was determined by immunoblotting of lysates of transformed *S. cerevisiae* Y190 cells with specific antibodies against the AR (SP197, lanes 1–4 and lanes 11–16) or against Gal4-TAD (lanes 5–10). Lane 1a represents GalAD-AR.N8 at a 5-fold longer exposure time of the immunoblot during chemiluminescence detection. The molecular mass marker values are indicated on the left side of each blot.

strongly reduced the response to R1881 (to maximally a 7-fold induction) as compared with the wild-type LBD.

The effect of the E888Q mutation on the functional interaction between the two AR domains was also studied in yeast, using the two-hybrid system. Both C-terminal domains (wild-type and mutant), fused to Gal4-DBD, were transformed together with GalAD-AR.N8 into Y190 (Fig. 5B; NH₂/LBD). The effect of the E888Q mutation appeared to be even more pronounced than in CHO cells, as β -GAL activity measured for the interaction of both AR domains (set at 100% for the intact LBD) dropped more than 90% upon introduction of the E888Q mutation. The data

from CHO cells and from yeast indicate that the AF-2 AD core region in the AR-LBD is important for a functional interaction with the NH₂-terminal domain.

Previously, homodimerization of the AR-LBD was demonstrated in yeast (22, 23). To determine whether the E888Q mutation had an effect on LBD dimerization, both LBDs (wild-type and mutant) were coupled to Gal4-TAD and transformed together with the corresponding LBD domains coupled to Gal4-DBD (Fig. 5C). In this LBD/LBD interaction assay, the wild-type AR.C and the mutant AR.C(EQ) showed similar β -GAL levels, indicating that the E888Q mutation had no effect on LBD dimerization.

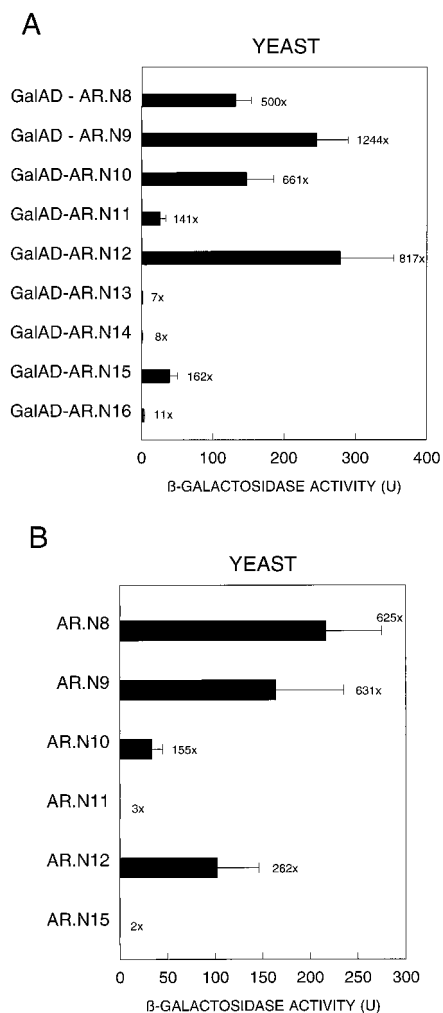


Fig. 4. Two-Hybrid Assay of AR NH₂-Terminal Deletion Mutants and GalDBD-AR.C in Yeast

Yeast Y190 cells, containing the integrated UAS_{Gal1}-lacZ reporter gene, were transformed with the expression plasmid GalDBD-AR.C, together with each of the Gal4-TAD fusion proteins (GalAD-AR.N8 to GalAD-AR.N16) (A), or with the AR proteins without the Gal4-TAD part (AR.N8 to AR.N12 and AR.N15) (B). β -GAL activity was determined after incubation of the cells in the absence (*open bars*, not visible) or presence (*solid bars*) of 1 μ M DHT. Fold induction is shown at the *top of each bar* and represents the ratio of activity determined in the presence and absence of DHT. β -GAL values represent the mean (\pm SEM) of three experiments.

TIF2-Induced Activity of AR AF-2 in CHO Cells Is Affected by the E888Q Mutation

The functional role of the AR AF-2 AD core region was further characterized by comparing the functional *in vivo* interaction of the AR-LBD and the AR NH₂-terminal domain with the interaction of the AR-LBD and the coactivator TIF2. The coactivator TIF2 is able to enhance the AF-2 activity of several steroid receptors including the AR (17). The involvement of the AR AF-2 AD core region in the functional *in vivo* interaction

of TIF2 and the AR-LBD was investigated by examining the effect of the E888Q mutation.

TIF2 was cotransfected with the MMTV-LUC reporter and increasing amounts of the wild-type (AR.C) or the mutant LBD (AR.C(EQ)) into CHO cells (Fig. 6). In the absence of the LBD constructs, no response to 1 nM R1881 was observed. As expected, coexpression of TIF2 and AR.C highly induced luciferase activity (up to 26-fold). The E888Q mutation reduced luciferase activity by approximately 70%, compared with AR.C. This decrease in functional interaction of AR-LBD with TIF2, caused by the E888Q mutation, was comparable to the negative effect of the mutation on the interaction with the NH₂-terminal domain (as shown in Fig. 5A).

TIF2 Does Not Promote the Functional Interaction between the AR NH₂-Terminal Domain and the LBD

To determine whether TIF2 enhances the functional interaction between the NH₂-terminal and C-terminal domains of the AR, coexpression studies with different concentrations of TIF2 and the NH₂-terminal domain were performed (Fig. 7). The luciferase activities obtained were compared with the transactivating activities measured after separate transfection of the different plasmids. Individual expression of TIF2 and the NH₂-terminal domain induced the transcriptional activity of AR.C on the MMTV promoter up to 24-fold (panels a and d, respectively). When coexpressed with 50 ng/well AR.N1 (panel b), TIF2 slightly enhanced the luciferase activity induced by the functional interaction of AR.N1 and AR.C (up to 34-fold). When higher amounts of AR.N1 were transfected, no additional effects of TIF2 could be observed (panels c and d). These data most likely exclude synergy between TIF2 and the NH₂-terminal domain for the interaction with the AR-LBD, indicating that TIF2 is not necessary for the functional interaction between the AR domains.

TIF2-Induced Activity of AR AF-2 in CHO Cells Is Promoter Dependent

To further examine the induction of AR AF-2 activity by the NH₂-terminal domain and by TIF2, *in vivo* interaction assays in CHO cells were performed using different promoter constructs (Fig. 8). AR.C was cotransfected into CHO cells with increasing amounts of either AR.N1 (Fig. 8, A and C) or TIF2 (Fig. 8, B and D), together with either MMTV-LUC (Fig. 8, A and B) or GRE-TATA-LUC (Fig. 8, C and D) as reporter constructs. In the absence of either AR.N1 or TIF2, no significant response to 1 nM R1881 could be detected. AR.N1 as well as TIF2 induced AF-2 activity on the MMTV-LUC promoter (maximally 39- and 44-fold, respectively). Luciferase activity was also highly induced by AR N.1 on the minimal, GRE-TATA, promoter (Fig. 8C). In contrast, the TIF2-induced luciferase activity appeared to be much more affected on this promoter (Fig. 8D). This indicates that the level of transcriptional

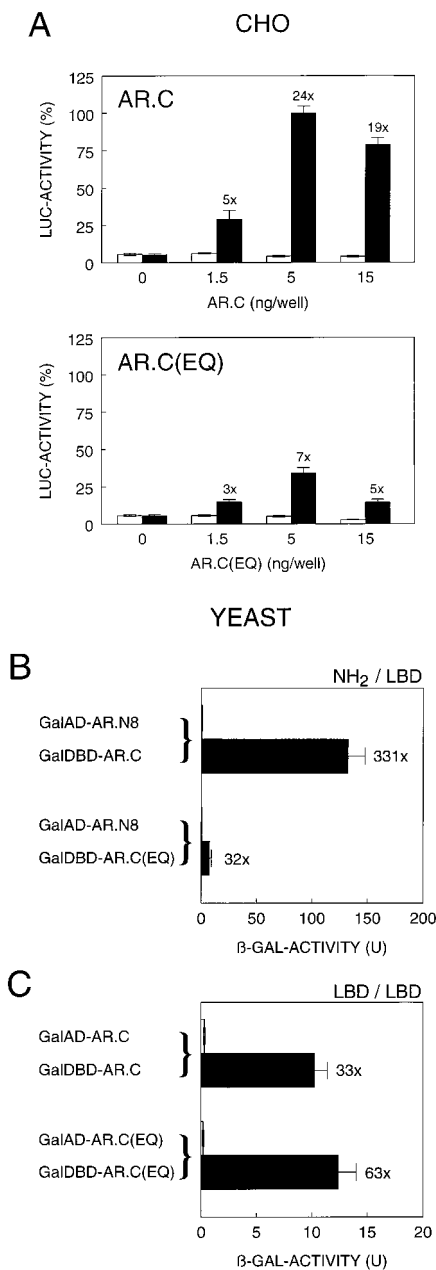


Fig. 5. Differential Effects of the AF-2 AD Core Mutation E888Q on Functional *in Vivo* NH₂-Terminal Domain/LBD and LBD/LBD Interactions

A, CHO cells were transfected with AR.N1 (100 ng/well) and increasing amounts of AR.C or AR.C(EQ) together with the MMTV-LUC reporter plasmid. Cells were incubated with vehicle (*open bars*) or 1 nM R1881 (*solid bars*). LUC activity was determined as described in *Materials and Methods*. The interexperimental variation of the mean maximal luciferase activities ranged between 8,000 and 13,000 light units. For each experiment the mean AR.C activity (5 ng/well) was set at 100%, and all individual points were calculated relatively to this value. Each *bar* represents the mean (\pm SEM) luciferase activity for three experiments. Fold induction represents the ratio of activity determined in the presence and absence of R1881. B, Yeast Y190 cells, containing the integrated UAS-Gal₁-lacZ reporter gene, were transformed with the expression plasmid GalAD-AR.N8 together with GalDBD-AR.C or

response induced by TIF2 depends on the promoter context.

DISCUSSION

Upon hormone binding, steroid receptors undergo conformational changes that result in a cascade of events leading toward DNA binding and transcription activation. Protein-protein interactions play a prominent role in these processes. After releasing associated heatshock proteins, the receptor homodimerizes, and subsequently interacts with TIFs and basal transcription factors, to induce transcription of specific target genes (3–7). For the hAR, two separate transcription activation units in the NH₂-terminal domain were defined: TAU-1 and TAU-5 (8). TAU-1 is active in the full-length, hormone-bound AR, whereas TAU-5 is active in a constitutive receptor that lacks the LBD. This shift in TAU in the NH₂-terminal domain, depending on the absence or presence of the LBD, suggested a functional interaction between the LBD and the NH₂-terminal domain.

It was described recently, for the ER and AR, that functional *in vivo* interactions between the separate NH₂-terminal domain and the LBD can occur (20–22). Whether this association is direct or requires additional factors is unknown. Neither is it clear which regions in these AR domains are involved in this functional interaction. For a better understanding of AR action, we determined the subdomains in the AR involved in the functional interaction between the NH₂-terminal domain and the LBD.

Cotransfection experiments of the AR-LBD with NH₂-terminal deletion mutants in CHO cells and in yeast imply a major role for amino acid residues 3–36 of the NH₂-terminal domain in the functional *in vivo* interaction with the LBD. A previous report by Langley *et al.* (21) indicated residues 14–150 to be involved, suggesting that the interaction domain might be constrained to residues 14–36. In this respect it is interesting that region 16–36 is potentially capable of forming an amphipathic α -helix, if projected on a helical wheel diagram.

Experiments in CHO cells revealed a second region, amino acid residues 371–494, important for a functional *in vivo* interaction with the AR-LBD. The involve-

GalDBD-AR.C(EQ). β -GAL activity was determined after incubation of the cells in the absence (*open bars*) or presence (*solid bars*) of 1 μ M DHT. Fold induction represents the ratio of activity determined in the presence and absence of DHT. β -GAL values represent the mean (\pm SEM) of three experiments. C, Yeast Y190 cells, containing the integrated UAS-Gal₁-lacZ reporter gene, were transformed with GalDBD-AR.C (low) and GalAD-AR.C (high) or GalDBD-AR.C(EQ) (low) and GalAD-AR.C(EQ) (high). β -GAL activity was determined as described in panel B.

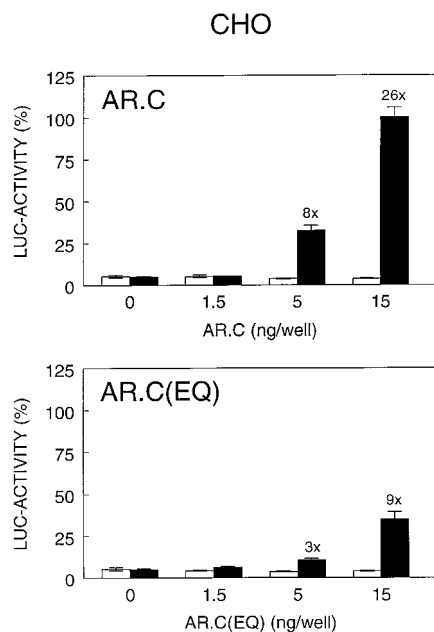


Fig. 6. E888Q Mutation Affects TIF2-Enhanced Activation of AR.C in CHO Cells

TIF2 (100 ng/well) was cotransfected into CHO cells with increasing amounts of AR.C or AR.C(EQ) and MMTV-LUC as a reporter. Cells were incubated with vehicle (*open bars*) or 1 nM R1881 (*solid bars*). LUC activity was determined as described in *Materials and Methods*. The interexperimental variation of the mean maximal luciferase activities ranged between 2,800 and 5,900 light units. For each experiment the mean AR.C activity (15 ng/well) was set at 100%, and all individual points were calculated relative to this value. Each *bar* represents the mean (\pm SEM) luciferase activity of three experiments. Fold induction represents the ratio of activity determined in the presence and absence of R1881.

ment of this NH₂-terminal region was confirmed by data obtained with the two-hybrid system in yeast. This region harbors TAU-5 (8), postulating a role for this transcription activation unit in the functional interaction with the LBD. A recent report by McEwan and Gustafsson (24) described the interaction of the AR NH₂-terminal domain (aa 142–485) with the general transcription factor TFIIF. It remained unclear which of the transactivation units (TAU-1 or TAU-5) in the AR NH₂-terminal domain is involved in the interaction with TFIIF, as both TAUs are present in the AR domain used in this study. Therefore, the role of TAU-5 in the interaction with the AR-LBD might be, at least in part, by recruiting the transcriptional machinery to the target promoter.

The *in vivo* experiments described here imply an important role for the AR AF-2 AD core in the functional interaction with the NH₂-terminal domain. The E888Q mutation markedly decreased the interaction between the two AR domains, without altering hormone binding. As the E888Q mutation did not completely reduce the interaction, it is likely that also other residues or regions in the LBD are involved in the

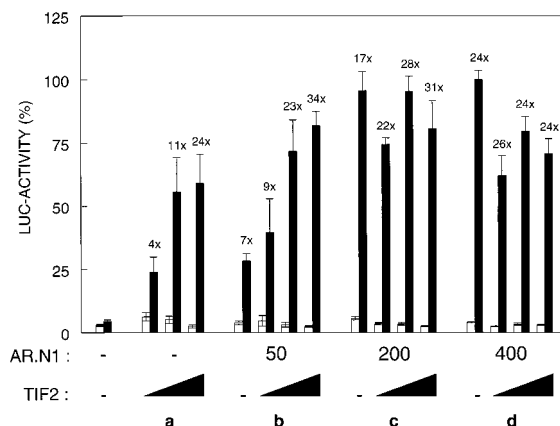


Fig. 7. The Effect of Cotransfecting TIF2 on the Functional Interaction of AR.N1 and AR.C

CHO cells were transfected with AR.C (25 ng/well) together with increasing amounts of either TIF2 (50–400 ng/well, panel a) or AR.N1 (50–400 ng/well, represented by – in panels b–d;) and with combinations of both (panels b, c, and d). MMTV-LUC was used as a reporter. Cells were incubated with vehicle (*open bars*) or 1 nM R1881 (*solid bars*). LUC activity was determined as described in *Materials and Methods*. The interexperimental variation of the mean maximal luciferase activities ranged between 120,000 and 375,000 light units. For each experiment the mean AR.N1 activity (400 ng/well) was set at 100%, and all individual points were calculated relative to this value. Each *bar* represents the mean (\pm SEM) luciferase activity of three experiments. Fold induction represents the ratio of activity determined in the presence and absence of R1881.

formation of a proper interacting surface. Wurtz *et al.* (19) proposed a general mechanism for nuclear receptor activation, in which hormone-induced conformational changes within the LBD result in a close contact of helix 12 and helix 4, thereby creating an interaction surface that allows binding of coactivators to the AF-2 activation domain. As the formation of this interaction surface might also be important for the functional interaction of the LBD with the NH₂-terminal domain, this would suggest that helix 4 is involved. Two-hybrid experiments in yeast showed that the E888Q mutation affected the functional interaction with the NH₂-terminal domain but did not influence LBD homodimerization. This implies that different regions in the AR-LBD are involved in the interaction with the NH₂-terminal domain and in LBD dimerization.

The E888Q mutation also decreased the stimulatory effect of the coactivator TIF2 on AR AF-2 activity. This suggests that the AF-2 AD core region of the hAR is involved in interacting with both the NH₂-terminal domain and the coactivator TIF2. A possible mechanism of TIF2 action could be as a bridging factor in the process of transcription activation by the AR. McInerney *et al.* (25) demonstrated that the steroid receptor coactivator SRC-1 enhanced the functional interaction between the NH₂-terminal and C-terminal domain of the ER. From these data it was suggested that SRC-1 may act as an adaptor protein that promotes the in-

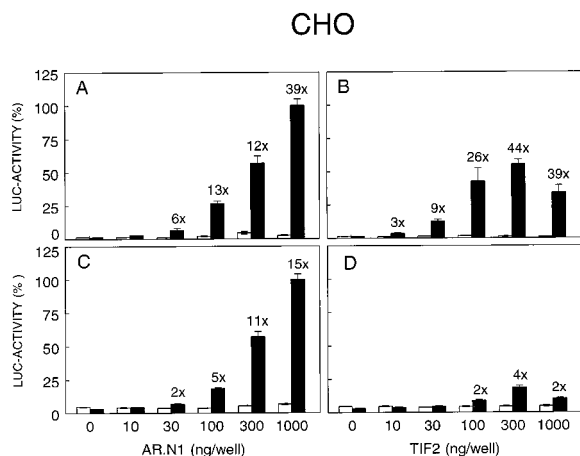


Fig. 8. Enhancement of the Transcriptional Response by TIF2 in CHO Cells Is Promoter-Dependent

CHO cells were transfected with AR.C (50 ng/well) and increasing amounts of AR.N1 (panels A and C) or TIF2 (panels B and D) together with the reporter plasmids MMTV-LUC (panels A and B) or GRE-TATA-LUC (panels C and D). Cells were incubated with vehicle (open bars) or 1 nM R1881 (solid bars). LUC activity was determined as described in *Materials and Methods*. The interexperimental variation of the mean maximal luciferase activities ranged between 4,800 and 6,400 light units for panels A and B, and between 11,500 and 13,500 light units for panels C and D. For each experiment the mean AR.N1 activity (1000 ng/well) was set at 100%, and all individual points were calculated relative to this value. Each bar represents the mean (\pm SEM) luciferase activity of three experiments. Fold induction represents the ratio of activity determined in the presence and absence of R1881.

tegration of NH₂-terminal and C-terminal ER domains. However, cotransfection experiments with TIF2 and the AR NH₂-terminal domain in CHO cells did not show synergy between TIF2 and the AR NH₂-terminal domain in the induction of AR AF-2 activity (Fig. 7). Therefore, it is unlikely that TIF2 functions as a bridging factor for the functional interaction of the NH₂-terminal domain and the LBD of the AR.

The AR appears to be a unique member in the nuclear receptor superfamily, because the AR C-terminal domain, containing the AF-2 AD core region, is inactive in the absence of the NH₂-terminal domain or TIF2. This lack of activity is not due to the experimental setup or the cell type used (CHO), because a similar C-terminal construct of the GR is active. The most likely explanation of this difference is that interactions of the AR-AF2 AD with endogenous coactivators are very weak and therefore undetectable. Overexpression of coactivators (e.g. TIF2) might enhance the interaction and consequently transcriptional activity. Another explanation for the absence of AR AF-2 activity in CHO cells is that endogenous levels of TIF2 are not sufficient for activation of the MMTV promoter in the absence of AR.N1.

A signature motif in transcriptional coactivators that mediated binding to liganded nuclear receptors was described recently (26–28). Most coactivators, includ-

ing TIF2, harbor several of these leucine-rich motifs (LXXLL). Two-hybrid experiments in yeast revealed that the interaction of the ER-LBD with LXXLL motifs was abolished by mutating the AF-2 AD core region in the LBD (26). Despite the demonstration that the NH₂-terminal domain of the AR also interacts with the AF-2 AD, it does not harbor such a motif, suggesting a different type of cooperation between the two AR domains.

The induction of AF-2 activity by TIF2 appeared to be promoter dependent. TIF2 strongly stimulated AR AF-2 activity on a MMTV-LUC reporter but much less on a minimal GRE-TATA promoter. The AR NH₂-terminal domain could, in contrast to TIF2, induce AF-2 activity on both promoters. Possibly, TIF2 not only affects the AR but also interacts with other transcription factors that bind to the complex MMTV promoter and not to the minimal promoter.

From the data presented here, together with previously obtained results (22), the following model for functional interactions between AR domains is proposed. Androgen binding induces a conformational change in the receptor that facilitates the AF-2 AD core in the LBD to interact with the NH₂-terminal domain. This interaction might be either direct or indirect, requiring additional factors, and results in AR transcription activation. Enhancement of AR activity by the coactivator TIF2 also involves the AF-2 AD core. In addition to a functional NH₂-terminal domain/LBD interaction, a LBD/LBD interaction is proposed that requires a region different from the AF-2 AD core.

MATERIALS AND METHODS

Plasmid Construction

Plasmid constructions were performed according to standard methods (29) and where denoted, rendered blunt ended with Klenow. All blunt-ended fusion constructs, and constructs including a PCR amplification step for preparation, were sequenced to verify the correct reading frame and the absence of random mutations. AR.N1 and AR.C were described previously as pSVAR(TAD_{1–494})(22) and pSVAR-104 (8), respectively. AR.N2, AR.N3, AR.N5, AR.N6, and AR.N7 were obtained by modification of AR Δ 51–211 and AR Δ 244–360 (30), AR124, AR127, and AR106 (8) respectively: plasmids were digested with *KpnI* and *EcoRI* and religated by a *KpnI-EcoRI* linker. AR.N4 was obtained by insertion of a *RsrII-EcoRI* linker into AR.N1. pSVAR(EQ) was constructed by site-directed mutagenesis of pSVAR (31), using the following oligonucleotides (the modified codon is shown *underlined*):

AR/EQ1: 5'-ACAGCCAGTGTGTCCGAATG-3';
 AR/EQ2: 5'-CTTGACACAGAGATGATTGTGCCATC-3';
 AR/EQ3: 5'-GATGGCACAAATATCTCTGTGCAAG-3';
 AR/EQ4: 5'-CAAGGGGCTTCATGATGTC-3'.

AR.C(EQ) was prepared by digestion of pSVAR(EQ) with *EcoRI* and ligating the 465-bp fragment into AR.C. MMTV-LUC reporter plasmid was kindly provided by Dr. Dijkema (Organon, Oss, The Netherlands) and was described previously (32). GRE-TATA-LUC, containing the TATA-box and a Sp1-site derived from the Oct-6 gene promoter, has been described previously as pJH4-(ARE)₂-OCT-LUC (33). GR.C (aa 369–777) was constructed by digestion of RshGR α (34)

with *Bst*XI and religation with a compatible DNA fragment obtained by annealing the following oligos: 5'-AGCCCGG-GACCATGGGAT-3' and 5'-CATGGTCCCGGGCTATCC-3'. The obtained plasmid was digested with *Sma*I and *Dra*I, and the 1074-bp fragment, containing the GR DBD and LBD, was ligated into *Sma*I-digested pSV328A (35).

The GAL4(DBD₁₋₁₄₇) two-hybrid cloning vector pGBT9 and the parental GAL4(TAD₇₆₈₋₈₈₁) cloning vector pGAD424 (low expression), or the high-level expression derivative pACT2 (all from Clontech, Palo Alto, CA), were used to generate all yeast fusion protein constructs. GalAD-AR.N8 (low) was described previously as pGAL4(TAD)AR(TAD) (22). GalAD-AR.N11 (low) was generated by digesting GalAD-AR.N8 (low) with *Nco*I and *Sa*I, followed by religation of the blunted vector. The previously described pTZ19NAR (22), containing an additional *Bam*HI site by addition of a *Bam*HI linker to the blunt-ended *Eco*RI site in the polylinker, was digested with *Sma*I and *Nco*I, and the resulting vector was blunt ended and religated, yielding pTZ19AR.N12. The AR fragment was excised with *Bam*HI and cloned in frame into the corresponding site in pGAD424, yielding GalAD-AR.N12 (low). The 0.4-kb *Eco*RI-Acc651 (blunt ended) fragment from the previously described pSVAR-123 (8), containing an additional *Eco*RI site by addition of an *Eco*RI linker to the blunt-ended *Xba*I site, was cloned in frame into pGAD424 via *Eco*RI and *Sma*I compatible ends, yielding GalAD-AR.N14 (low). GalDBD-AR.N8 (low) [previously described as pGAL4(DBD)AR(TAD) (22)] was digested with *Rsr*II and *Acc*651, and the blunted vector was religated, yielding GalDBD-AR.N10 (low). The AR fragment was excised with *Bam*HI and cloned in frame into the corresponding site in pGAD424, yielding GalAD-AR.N10 (low). GalAD-AR.N15 (low) was generated by deletion of the internal *Pst*I fragment from GalAD-AR.N12 (low). GalAD-AR.N16 (low) was constructed by digesting GalAD-AR.N15 (low) with *Eco*RI and *Nco*I, followed by religation of the blunted vector. GalAD-AR.N13 (low) was prepared by integration of the 0.5-kb GalAD-AR.N12 (low) *Pst*I fragment into the homologous GalAD-AR.N16 (low) site. AR.N8, containing the AR fragment cloned in frame to the SV40 large T antigen nuclear localization signal in pGAD424, was described previously as pAR(TAD₃₋₄₉₄) (22). AR.N12 (low) was generated by exchanging the *Acc*651 (blunt ended)-*Eco*RI fragment of pGAD424 with the 0.9-kb *Bam*HI (blunt ended)-*Eco*RI fragment of pTZ19AR.N12. AR.N15 (low) was prepared by digesting GalAD-AR.N15 (low) with *Acc*651 and *Bam*HI, followed by religation of the blunt-ended vector. All high expression derivatives were constructed by exchanging the internal *Hind*III fragments with the internal pACT2 *Hind*III fragment. AR.N9 (high) was generated by exchanging the internal 0.9-kb *Stu*I-*Eco*RI fragment of AR.N8 (high) with the 0.5-kb *Stu*I-*Eco*RI fragment of AR.N3 (described above). AR.N11 (high) was generated by digesting AR.N8 (high) with *Nco*I and *Eco*RI, followed by religation of the blunt-ended vector. Similarly, the high expression level AR.N10 was generated by digesting AR.N8 (high) with *Rsr*II and *Eco*RI, followed by religation of the blunt-ended vector. Only the high expression AR.N constructs were used in the two-hybrid assays. The expression vectors GalDBD-AR.C (low expression) and GalAD-AR.C (high expression) were previously described as pGAL4(DBD)AR(LBD) and pGAL4(TAD)AR(LBD), respectively (22). GalDBD-AR.C(EQ) was prepared by integration of the 0.7-kb *Stu*I-*Sa*I fragment of pSVAR(EQ) (as described above) into the homologous GalDBD-AR.C sites. Similarly, the high-expression GalAD-AR.C(EQ) derivative was generated by integration of the 0.45-kb *Eco*RI fragment of pSVAR(EQ) into the homologous GalAD-AR.C site.

CHO Cell Culture, Transfection, and LUC Assay

Chinese hamster ovary (CHO) cells were maintained in DMEM/F12 culture medium, supplemented with 5% dextran-coated charcoal-treated FCS (Life Technologies, Gaithersburg, MD). For transcription activation experiments, CHO

cells were plated in 12-well plates at a density of 0.6×10^5 cells per well (7 cm²) and grown overnight. Cells were transfected using the calcium phosphate precipitation method as described previously (36), with AR expression plasmids and where indicated with TIF2, reporter plasmids (200 ng/well), and pTZ19 carrier plasmid to a total DNA concentration of 2 μ g/well. After an overnight incubation, the cells were washed and R1881 (methyltrienolone; New England Nuclear, Boston, MA) or vehicle (0.1% ethanol) was added. After overnight incubation the cells were harvested for the luciferase (LUC) assay, as described previously (37). For the GR experiments, GR.C (50 ng/well) was cotransfected with MMTV-LUC and pTZ19 carrier plasmid as described above. After overnight incubation, dexamethasone (10 nM) was added, and luciferase activity was measured the next day. For the coexpression studies of TIF2 and AR.N1, the total amount of vector, added to each well, was equalized by the addition of empty vector, to a total concentration of 800 ng/well. In addition, pTZ19 carrier plasmid was added to a total concentration of 2 μ g DNA/well.

Yeast Growth and Methods

All yeast studies were performed in strain Y190 (*MATa*, *ura3-52*, *his3- Δ 200*, *ade2-101*, *trp1-901*, *leu2-3*, *leu2-112*, *GAL4 Δ* , *GAL80 Δ* , *URA3::GAL-lacZ*, *cyh^r*, *LYS2::GAL-HIS3*), which was purchased from CLONTECH. Yeast cells were grown in the appropriate selective minimal medium [0.67% (wt/vol) yeast nitrogen base without amino acids and 2% (wt/vol) dextrose, pH 5.8] supplemented to the nutritional requirements of the yeast transformants. Yeast transformations were carried out according to the lithium acetate method (38).

Quantitative β -GAL activity assays, indicative of AR domain interactions, were performed as described previously (22).

Immunoprecipitation and Immunoblotting

CHO cells were plated in 80-cm² culture flasks (1×10^6 cells per flask), grown overnight, and transfected with 4 μ g AR-plasmid and 16 μ g pTZ19 as carrier plasmid. The next day, medium was refreshed and the cells were grown for another day. Cytosol of the transfected cells was prepared as described before (22). Immunoprecipitation was performed as described previously (39), using monoclonal antibodies F112.1.1 [directed against synthetic peptide SP197; AR aa 1-20 (40)], F39.4.1 [directed against SP061; aa 301-320; (41)], or F52.24.4 [directed against SP063; aa 593-612; (39)]. Subsequent to SDS-PAGE and Western blotting, the membrane was blocked with 5% nonfat dry milk and incubated with polyclonal antisera against SP197, SP061, or SP066 [directed against AR aa 892-910 (40)]. The proteins were visualized by chemiluminescence detection.

AR proteins in yeast were isolated as described previously (22). Yeast-lysate samples (2.5 μ l) were run on SDS-polyacrylamide gels, after which the gels were electroblotted under semidry conditions. The blots were blocked overnight with 5% nonfat dry milk and incubated with GAL4AD monoclonal antibody (CLONTECH) or the polyclonal AR-antibody SP197. Proteins were visualized by chemiluminescence detection.

In Vivo Hormone Binding Assay

CHO cells were plated in six-well plates at a density of 1×10^5 cells per well, grown overnight, and transfected as described above with AR-C or AR-C(EQ) (0.75 μ g/well). The whole-cell binding assay was performed 48 h later. Cells were washed once with PBS and incubated with various [³H]R1881 (New England Nuclear) concentrations (0.01-30

nm) in the presence or absence of a 200-fold molar excess of unlabeled R1881 in DMEM/F12 for 1 h at 37 C. The cells were washed four times with PBS, collected in PBS, and transferred to a centrifuge tube. After centrifugation (10 min, 800 × g) the pellet was lysed in 0.5 M NaOH (15 min at 56 C), and radioactivity was determined by liquid scintillation counting.

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REFERENCES

- Gronemeyer H 1992 Control of transcription activation by steroid hormone receptors. *FASEB J* 6:2524–2529
- Glass CK 1994 Differential recognition of target genes by nuclear receptor monomers, dimers and heterodimers. *Endocr Rev* 15:391–407
- Evans RM 1988 The steroid and thyroid hormone receptor superfamily. *Science* 240:889–895
- Beato M 1989 Gene regulation by steroid hormones. *Cell* 56:335–344
- Green S, Chambon P 1988 Nuclear receptors enhance our understanding of transcription regulation. *Trends Genet* 4:309–314
- Parker MG 1993 Steroid and related receptors. *Curr Opin Cell Biol* 5:499–504
- Horwitz KB, Jackson TA, Bain DL, Richer JK, Takimoto GS, Tung L 1996 Nuclear receptor coactivators and corepressors. *Mol Endocrinol* 10:1167–1177
- Jenster G, van der Korput HA, Trapman J, Brinkmann AO 1995 Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. *J Biol Chem* 270:7341–7346
- Danielian PS, White R, Lees JA, Parker MG 1992 Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. *EMBO J* 11:1025–1033
- Barettino D, Vivanco Ruiz MM, Stunnenberg HG 1994 Characterization of the ligand-dependent transactivation domain of thyroid hormone receptor. *EMBO J* 13:3039–3049
- Durand B, Saunders M, Gaudon C, Roy B, Losson R, Chambon P 1994 Activation function 2 (AF-2) of retinoic acid receptor and 9-cis retinoic acid receptor: presence of a conserved autonomous constitutive activating domain and influence of the nature of the response element on AF-2 activity. *EMBO J* 13:5370–5382
- Lanz RB, Rusconi S 1994 A conserved carboxy-terminal subdomain is important for ligand interpretation and transactivation by nuclear receptors. *Endocrinology* 135:2183–2195
- Montano MM, Ekena K, Krueger KD, Keller AL, Katzenellenbogen BS 1996 Human estrogen receptor ligand activity inversion mutants: receptors that interpret antiestrogens as estrogens and discriminate among different antiestrogens. *Mol Endocrinol* 10:230–242
- Cavaillès V, Dauvois S, L'Horset F, Lopez G, Hoare S, Kushner PJ, Parker MG 1995 Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. *EMBO J* 14:3741–3751
- LeDouarin B, Zechel C, Garnier JM, Lutz Y, Tora L, Pierrat P, Heery D, Gronemeyer H, Chambon P, Losson R 1995 The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18. *EMBO J* 14:2020–2033
- Vom Baur E, Zechel C, Heery D, Heine MJ, Garnier JM, Vivat V, Le Douarin B, Gronemeyer H, Chambon P, Losson R 1996 Differential ligand-dependent interactions between the AF-2 activating domain of nuclear receptors and the putative transcriptional intermediary factors mSUG1 and TIF1. *EMBO J* 15:110–124
- Voegel JJ, Heine MJ, Zechel C, Chambon P, Gronemeyer H 1996 TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *EMBO J* 15:3667–3675
- Jenster G, Spencer TE, Burcin MM, Tsai SY, Tsai M-J, O'Malley BW 1997 Steroid receptor induction of gene transcription: a two-step model. *Proc Natl Acad Sci USA* 94:7879–7884
- Wurtz JM, Bourguet W, Renaud JP, Vivat V, Chambon P, Moras D 1996 A canonical structure for the ligand-binding domain of nuclear receptors. *Nature Struct Biol* 3:87–94
- Kraus WL, McInerney EM, Katzenellenbogen BS 1995 Ligand-dependent, transcriptionally productive association of the amino- and carboxyl-terminal regions of a steroid hormone nuclear receptor. *Proc Natl Acad Sci USA* 92:12314–12318
- Langley E, Zhou ZX, Wilson EM 1995 Evidence for an anti-parallel orientation of the ligand-activated human androgen receptor dimer. *J Biol Chem* 270:29983–29990
- Doesburg P, Kuil CW, Berrevoets CA, Steketee K, Faber PW, Mulder E, Brinkman AO, Trapman J 1997 Functional *in vivo* interaction between the amino-terminal, transactivation domain and the ligand binding domain of the androgen receptor. *Biochemistry* 36:1052–1064
- Nemoto T, Ohara-Nemoto Y, Shimazaki S, Ota M 1994 Dimerization characteristics of the DNA- and steroid-binding domains of the androgen receptor. *J Steroid Biochem Mol Biol* 50:225–233
- McEwan IJ, Gustafsson J-Å 1997 Interaction of the human androgen receptor transactivation function with the general transcription factor TFIIF. *Proc Natl Acad Sci USA* 94:8485–8490
- McInerney EM, Tsai MJ, O'Malley BW, Katzenellenbogen BS 1996 Analysis of estrogen receptor transcriptional enhancement by a nuclear hormone receptor coactivator. *Proc Natl Acad Sci USA* 93:10069–10073
- Heery DM, Kalkhoven E, Hoare S, Parker MG 1997 A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature* 387:733–736
- Li H, Gomes PJ, Chen JD 1997 RAC3, a steroid receptor-associated coactivator that is related to SRC-1 and TIF2. *Proc Natl Acad Sci USA* 94:8479–8484
- Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK, Rosenfeld MG 1997 The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. *Nature* 387:677–684
- Sambrook J, Fritsch EF, Maniatis T 1989 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Jenster G, van der Korput HA, van Vroonhoven C, van der Kwast TH, Trapman J, Brinkmann AO 1991 Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. *Mol Endocrinol* 5:1396–1404
- Brinkmann AO, Faber PW, van Rooij HC, Kuiper GG, Ris

- C, Klaassen P, van der Korput JA, Voorhorst MM, van Laar JH, Mulder E, Trapman J 1989 The human androgen receptor: domain structure, genomic organization and regulation of expression. *J Steroid Biochem* 34:307–310
32. de Ruiter PE, Teuwen R, Trapman J, Dijkema R, Brinkmann AO 1995 Synergism between androgens and protein kinase-C on androgen-regulated gene expression. *Mol Cell Endocrinol* 110:R1–6
33. Blok LJ, Themmen AP, Peters AH, Trapman J, Baarends WM, Hoogerbrugge JW, Grootegoed JA 1992 Transcriptional regulation of androgen receptor gene expression in Sertoli cells and other cell types. *Mol Cell Endocrinol* 88:153–164
34. Giguere V, Hollenberg SM, Rosenfeld MG, Evans RM 1986 Functional domains of the human glucocorticoid receptor. *Cell* 46:645–652
35. Van Heuvel M, Bosveld IJ, Mooren ATA, Trapman J, Zwarthoff EC 1986 Properties of natural and hybrid murine alpha interferons. *J Gen Virol* 67:2215–2222
36. Veldscholte J, Berrevoets CA, Ris-Stalpers C, Kuiper GG, Jenster G, Trapman J, Brinkmann AO, Mulder E 1992 The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens. *J Steroid Biochem Mol Biol* 41:665–669
37. Kuil CW, Berrevoets CA, Mulder E 1995 Ligand-induced conformational alterations of the androgen receptor analyzed by limited trypsinization. Studies on the mechanism of antiandrogen action. *J Biol Chem* 270:27569–27576
38. Gietz D, St. Jean A, Woods RA, Schiestl RH 1992 Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res* 20:1425
39. Veldscholte J, Berrevoets CA, Zegers ND, van der Kwast TH, Grootegoed JA, Mulder E 1992 Hormone-induced dissociation of the androgen receptor-heat-shock protein complex: use of a new monoclonal antibody to distinguish transformed from nontransformed receptors. *Biochemistry* 31:7422–7430
40. Kuiper GG, de Ruiter PE, Trapman J, Boersma WJ, Grootegoed JA, Brinkmann AO 1993 Localization and hormonal stimulation of phosphorylation sites in the LNCaP-cell androgen receptor. *Biochem J* 291:95–101
41. Zegers ND, Claassen E, Neelen C, Mulder E, van Laar JH, Voorhorst MM, Berrevoets CA, Brinkmann AO, van der Kwast TH, Ruizeveld de Winter JA, Trapman J, Boersma WJA 1991 Epitope prediction and confirmation for the human androgen receptor: generation of monoclonal antibodies for multi-assay performance following the synthetic peptide strategy. *Biochim Biophys Acta* 1073:23–32

