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c-Jun Targets Amino Terminus of Androgen Receptor in Regulating Androgen-Responsive Transcription

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The human androgen receptor (hAR) is a member of the nuclear receptor superfamily and functions as a ligand-inducible transcription factor. We have previously proposed that c-Jun mediates the transcriptional activity of this receptor. The modular nature of hAR was used in this study to generate several fusions with the heterologous DNA-binding domain of the yeast transcription factor GAL4 in an attempt to identify the c-Jun-responsive domains within the receptor. Our results suggest that the target of c-Jun action is the amino terminus (AB region) of the receptor and that hAR amino acids 502–521 are critical for the c-Jun response. Additionally, amino acids 503–555 were shown to harbor an autonomous transactivation that is stimulated by c-Jun. Furthermore, we demonstrated that transcription intermediary factor-2 (TIF-2), a coactivator that acts on the activation function-2, stimulates the full-length hAR. These results suggest that c-Jun and TIF-2 can work together as coactivators on the hAR by targeting distinct portions of the receptor.

Key Words: Androgen receptor; c-Jun; TIF-2; Transcription; nuclear receptors.

Introduction

Steroid hormones are small cholesterol-based lipophilic molecules that are important for many biological processes, including development, differentiation, and homeostasis. The cellular responses to steroid hormones are mediated by intracellular hormone receptors that belong to the superfamily of nuclear receptors that is distinguished from other families of transcription factors by the presence of a conserved ligand-binding domain (LBD) that is essential for transcriptional activity *(1)*. This family of receptors includes the retinoic acid receptor; retinoid X receptor; thyroid hormone receptor; the subfamily of steroid receptors including the glucocorticoid receptor (GR), progesterone receptor, and androgen receptor (AR); and numerous orphan receptors, ligands for which remain unidentified. Nuclear receptors primarily influence cellular differentiation, a process essential for embryonic development *(1–4)*.

Androgens are steroid hormones that are essential for both male sexual development and virilization during puberty. Two physiological androgens, testosterone and dihydrotestosterone (DHT), are involved in development and puberty, respectively, and exert their biological effects through the androgen receptor (AR). Like other nuclear receptors, the human AR (hAR) is a modular protein, consisting of a 555-amino-acid N-terminal AB region containing activation function-1 (AF-1), and the E region containing both the LBD and a putative AF-2 that is conserved in many other nuclear receptors *(4,5)*. Initially, using deletion analysis, Simental et al. *(6)* and others *(7)* identified the amino terminus of the hAR as essential for full transcriptional activity and postulated that the amino-terminal transactivation domain is inhibited by the LBD in the absence of ligand. Using AR amino-terminal regions fused to the GAL4 DNA-binding domain (DBD), Jenster et al. *(8)* suggested that several regions within the amino terminus of the AR are required for full activity of the A/B region. In a similar study, Chamberlain et al. *(9)* characterized the amino-terminal transactivation function in the context of the full-length rat receptor. From these studies, it appears that the amino terminus of the AR contains a bipartite transactivation domain with the two regions designated as AF-1a (amino acids 154–167 in the rat receptor and 169–182 in the human receptor) and AF-1b (amino acids 295–359 in the rat receptor and 293–357 in the human receptor) *(8,9)*. Interestingly, the amino terminus of the hAR has been shown to interact with both the TATA-box binding protein and TFIIF *(10)*, which may explain the strong transcriptional activity detected from this region of the receptor.

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Amino-terminal deletions of the hAR have been difficult to interpret because there is very little transcriptional activity detected from the carboxy terminus of the receptor in mammalian cells. However, Snoek et al. *(11)* have shown that the CDE region, encoding the DBD, hinge, and LBD, respectively, of the rat receptor, which contains the putative AF-2, expressed as a fusion protein with glutathione-*S*-transferase, exhibits strong transcriptional activity in vitro. In addition, the LBD of the hAR has been shown to be transcriptionally active in yeast, and this activity is inhibited by the hinge region *(12)*. More recently, it has been reported that the AR AF-2 can exhibit significant activity in mammalian cells when coactivators are overexpressed *(13–15)*. These results together suggest that the AF-2 of the hAR is functional both in vitro and in vivo.

Coactivators, proteins that bridge the AFs to the basic transcription machinery *(16)*, appear to target both AFs of nuclear receptors. The hAR has been shown to interact with several coactivators, including TIF-2 *(13,17)*, steroid receptor coactivator-1 (SRC-1) *(14)*, CREB-binding protein (CBP) *(15)*, ARA-70 *(18)*, and ARA160 *(19)*. In addition, hAR has been shown to functionally interact with c-Jun, the bZIP-containing protein that can heterodimerize with c-Fos to form AP-1 (reviewed in ref. *20*). In this interaction, c-Jun was shown to positively affect hAR transactivation apparently independent of promoter- or cell-specific factors *(21,22)*. We have further shown that both exogenous and endogenous c-Jun can carry out this activity *(23)*, that the c-Jun effect is primary *(23)*, that multiple regions of c-Jun are required for the activity *(24)*, and that c-Fos dimerization with c-Jun blocks the latter's positive activity on hAR *(21)*.

In the present study, we provide evidence that c-Jun acts on the amino terminus (AB region) of hAR, similar to ARA160 *(19)* but different from the SRC-1 family members, which have been shown to target both the amino terminus and the carboxy-terminal AF-2 of AR *(13–15,17)*. Furthermore, the target of c-Jun action appears to be hAR amino acids 502–521, which, together with amino acids 522–555, form an autonomous activation domain distinct from the previously identified activation domains. Finally, we show that c-Jun and TIF-2 can act together on hAR by targeting different regions of the receptor.

Results

Experimental Design

The hAR is a modular protein with functionally distinct and separable domains. Figure 1A shows the diagrammatic representations and expression of all hAR mutants and fusion proteins used in this study. As shown in Fig. 1B, all hAR proteins used in this study were expressed in transient transfection experiments in Cos cells.

c-Jun Acts on Amino Terminus of hAR

The AR, like many nuclear receptors, has a highly variable AF-1 sequence in the amino terminus and a conserved AF-2 in the carboxy terminus (*see* Fig. 1A). To determine whether either of these AFs alone is a target of c-Jun action, hAR was divided into two halves, one encompassing the ABC region (amino acids 1–623) and the other the CDE region (amino acids 556–918). As shown in Fig. 2A, both hAR and hAR(ABC) were able to activate the MMTV-CAT reporter, although hAR(ABC) was significantly less active than the full-length receptor. As we have shown previously *(23)*, hAR responded positively and strongly to transfected c-Jun (Fig. 2A). Interestingly, hAR(ABC) was also stimulated by c-Jun, but more weakly than full-length receptor. By contrast, no activity could be detected from the hAR(CDE) region in either the absence or presence of ligand, in agreement with previous studies *(6)*, and there was no observable influence of exogenous c-Jun (Fig. 2A). Note that hAR(ABC) was indeed expressed in the transient transfection experiment (*see* Fig. 1B) and that transfected c-Jun alone has no significant activity on the reporter (data not shown; *[23,24]*). These results suggest that the amino terminus and potentially the AF-1 of the hAR is targeted by c-Jun.

Because the activity of hAR(ABC) without or with c-Jun was substantially weaker than that of full-length receptor (Fig. 2A) and to rule out the possible involvement of the hAR DBD in the c-Jun stimulatory effect, the AB and DE regions of the hAR were fused to the heterologous GAL4 DBD. These fusions are designated hAR(AB)-GAL, containing hAR amino acids 1–555, and GAL-hAR(DE), harboring hAR amino acids 624–918 (*see* Fig. 1A). The activities of these proteins were assessed on the synthetic GAL4-responsive promoter 17mer-tk-CAT. hAR(AB)-GAL was able to activate the reporter, whereas the GAL-hAR(DE), with or without ligand, was unable to significantly activate this reporter (Fig. 2B). When c-Jun was cotransfected, the activity of hAR(AB)-GAL was markedly enhanced by c-Jun whereas GAL-hAR(DE) was unaffected (Fig. 2B). These results further support the idea that c-Jun is acting on hAR via the receptor's AB region.

Novel Region of AR Amino Terminus Mediates c-Jun Effect on AR-Induced Transcription

To map further the region within the amino terminus of the hAR that is essential for c-Jun stimulation, truncations of hAR(AB)-GAL were made using convenient restriction sites. Two truncations were tested in a transient transfection experiment, hAR(1-502)-GAL and hAR(1-330)-GAL. hAR(1-502)-GAL was less active than the intact hAR(AB)-GAL and hAR(1-330)-GAL had no detectable activity (Fig. 3A). Interestingly, transfected c-Jun was unable to stimulate either hAR(1-502)-GAL or hAR(1-330)-GAL (Fig. 3A). These results strongly suggest that within the hAR(AB)-GAL, amino acids 503–555 are essential for the c-Jun response.

A series of smaller truncation mutants of hAR(AB)-GAL were constructed to more finely map the c-Jun-responsive domain. These are designated hAR(1-538)-GAL and hAR(1-521)-GAL. As shown in Fig. 3B, hAR(1-538)-GAL

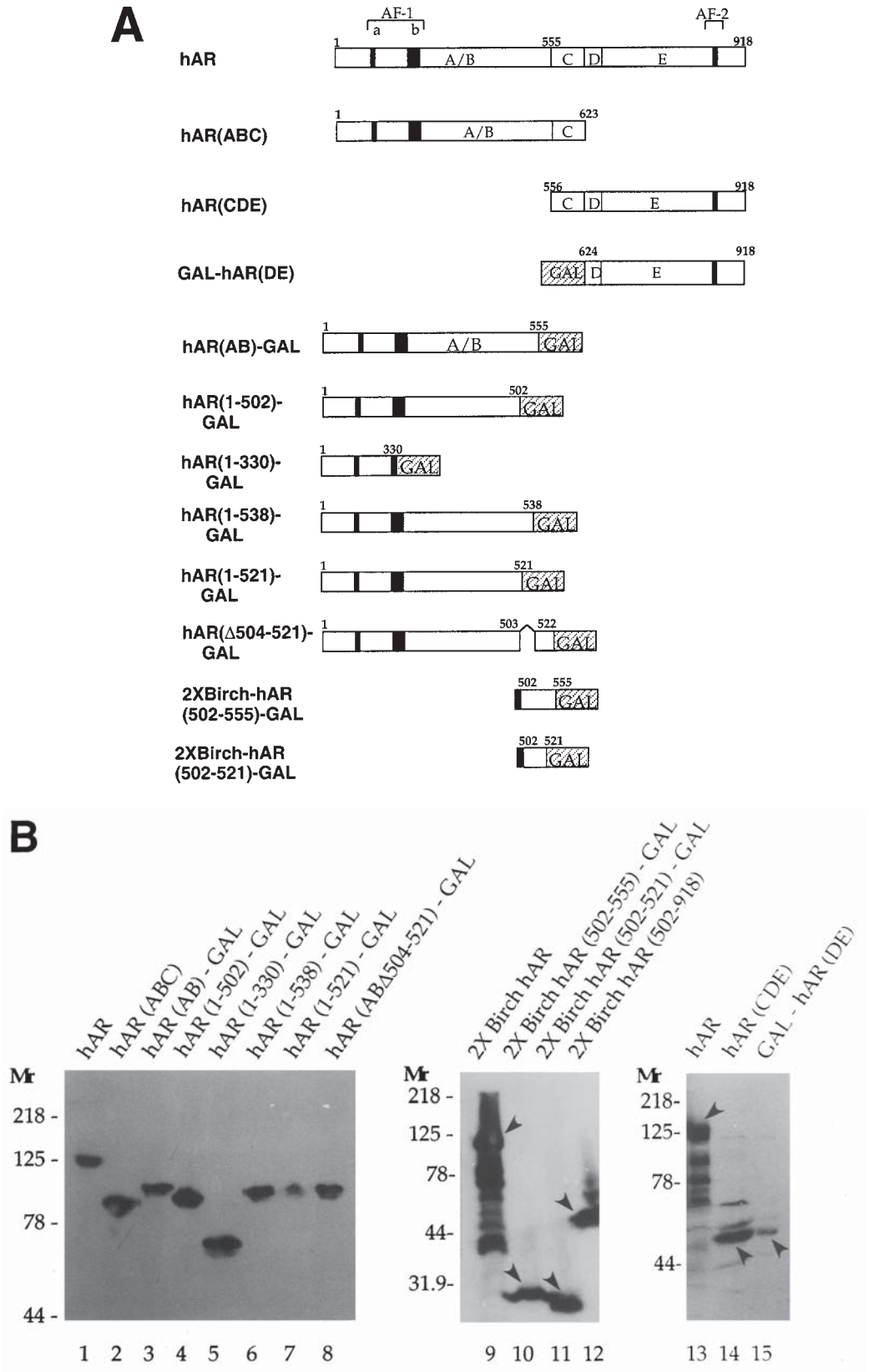

Fig. 1. **(A)** Schematic diagram of hAR truncation mutants and fusions with the GAL4 DBD. The positions of the bipartite AF-1 (amino acids 169–182 and 293–357) and the AF-2 (amino acids 883–889) are shown as black boxes. Note that the GAL DBD is depicted as a stippled box and the 2XBirch epitope tag as a gray box at the amino terminus of the fusion proteins. **(B)** Expression of hAR truncation mutants and GAL chimeras in transfected cells. Cos cells were transfected with 5 μg of each of the hAR plasmids and subjected to Western blot analysis. The positions of the various hAR proteins are indicated by arrowheads. Molecular weight markers are given in kilodaltons. Note that three different antibodies were used to detect the various proteins.

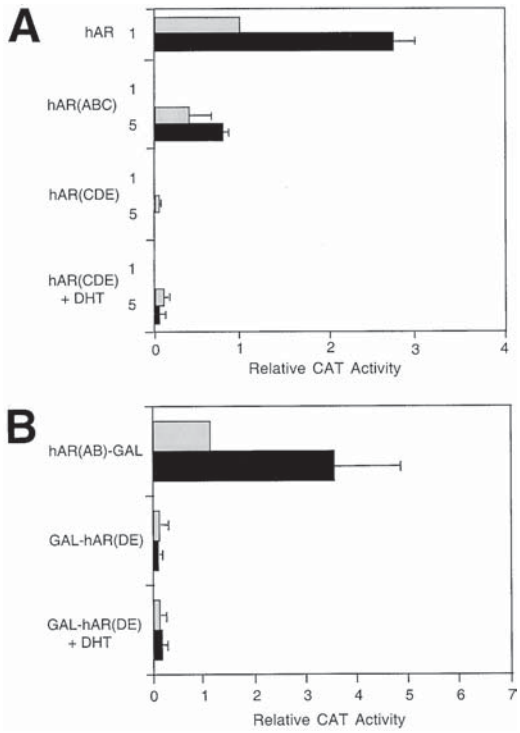

Fig. 2. c-Jun targets the amino terminus of hAR. Cos cells were transfected with **(A)** 1 μg of the MMTV-CAT reporter, 1 μg of hAR, or 5 μg of either hAR(ABC) or hAR(CDE), with or without 1 μg of c-Jun as indicated, or **(B)** with 1 mg of the 17mer-tk-CAT reporter, 5 μg of either hAR(AB)-GAL or GAL-hAR(DE), with or without 1 μg of c-Jun as indicated. In both cases 100 n*M* DHT was used as indicated. Gray bars are without and black bars are with transfected c-Jun. Note that in both cases CAT activity is represented relative to activity of the first condition, which was set to 1. Error bars represent standard deviations of (SDs) three to five independent replicates.

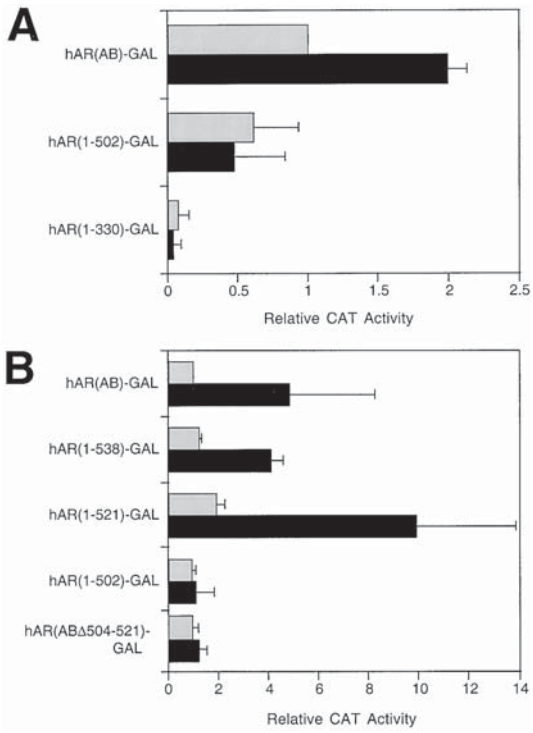

Fig. 3. hAR amino acids 502–521 are important for the c-Jun response of hAR(AB)-GAL. Cos cells were transfected with 1 μg of 17mer-tk-CAT, with or without 1 μg of c-Jun as indicated, and 5 μg of **(A)** hAR(AB)-GAL, hAR(1-502)-GAL, or hAR (1-330)-GAL, or **(B)** hAR(AB)-GAL, hAR(1-538)-GAL, hAR(1-521)-GAL, hAR(1-502)-GAL, or hAR(ABΔ504-521)-GAL. Gray bars are without and black bars are with transfected c-Jun. Note that in both cases CAT activity is represented relative to activity of the first condition, which was set to 1. Error bars represent SDs of three independent replicates.

and hAR(1-521)-GAL were able to activate transcription to a similar extent as hAR(AB)-GAL (Fig. 3B). Transfected c-Jun stimulated all three GAL chimeras (Fig. 3B). Interestingly, hAR(1-521)-GAL was a stronger activator in both the absence and presence of c-Jun. Because hAR(1-521)-GAL still responded to c-Jun, we tested the internal deletion mutant hAR(ABΔ504-521)-GAL, which contains hAR amino acids 1–503 and 522–555 (*see* Fig. 1A). hAR(ABΔ504-521)-GAL activated transcription almost as well as full-length hAR(AB)-GAL in the absence of transfected c-Jun (Fig. 3C). However, and interestingly, the presence of c-Jun had no significant effect on hAR(ABΔ504-521)-GAL, suggesting that hAR amino acids 503–521 are important in the c-Jun stimulation of hAR(AB)-GAL.

AR Amino Acids 502–555 Comprise Autonomous and c-Jun-Responsive Activation Function

The data in Fig. 3 suggest that amino acids 503–521 of hAR are required for the c-Jun positive effect. To determine whether these amino acids alone are sufficient for c-Jun action, they were fused to the GAL4 DBD to make 2XBirch-hAR(502-521)-GAL (*see* Fig. 1). The 2XBirch denotes an epitope tag (a kind gift from Dr. Martin Rothkegel) *(25)* that can be used to monitor the expression of these fusion proteins. More important, the presence of the epitope tag does not affect transactivation or c-Jun-induced stimulation of the hAR (data not shown). 2XBirch-hAR(502-521)-GAL was found to have significant transcriptional activity in Cos cells, but it was about threefold less active than the full-length hAR(AB)-GAL (Fig. 4A). However, transfected c-Jun had no positive activity on 2XBirch-hAR(502-521)-GAL (Fig. 4A). c-Jun responsiveness was restored, however, when amino acids 522–555 were added, making 2XBirch-hAR(502-555)-GAL (*see* Fig. 1). Indeed, c-Jun gave 2XBirch-hAR(502–555)-GAL the same activity as hAR(AB)-GAL in the absence of transfected c-Jun (Fig. 4A). hAR(501–555) also exhibited transcriptional activity in yeast, as a fusion with the LexA DBD, although this activity was much lower than that of the full-length hAR(AB) (Fig. 4B), paralleling what was observed in Cos cells (Fig. 4A). These results strongly suggest that hAR

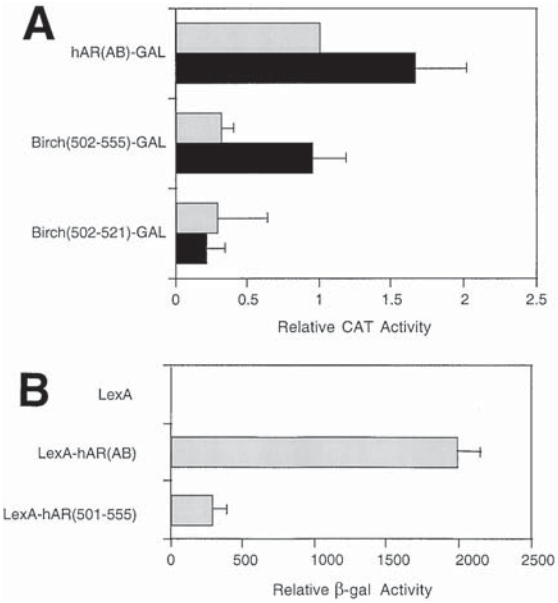

Fig. 4. hAR amino acids 503–555 form an autonomous and c-Jun-responsive activation domain. **(A)** Cos cells were transfected with 1 μg of 17mer-tk-CAT and 5 μg of hAR(AB)-GAL, Birch(502-555)-GAL, or Birch(502-521)-GAL with or without 1 μg of c-Jun as indicated. **(B)** Yeast cells were transformed with 1 μg of pEG202, hAR(AB)/pEG202, or hAR(501-555)/pEG202. Gray bars are without and black bars are with transfected c–Jun. Note that in both cases CAT activity is represented relative to activity of the first condition, which was set to 1. Error bars represent SDs of three independent replicates.

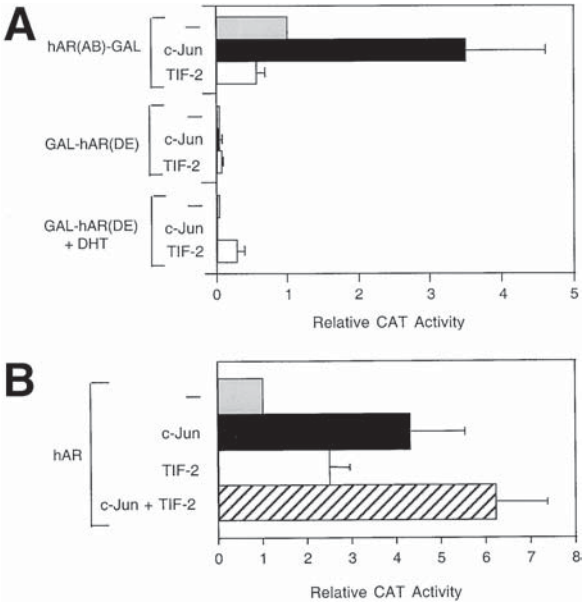

Fig. 5. c-Jun and TIF-2 work cooperatively on the hAR. Cos cells were transfected with **(A)** 1 μg of 17mer-tk-CAT, or 5 μg of hAR(AB)-GAL, or GAL-hAR(DE), with or without 1 μg of c-Jun and/or 5 μg of TIF-2 or **(B)** 1 μg of MMTV-CAT and 1 μg of hAR, with or without 1 μg of c-Jun and/or 5 μg of TIF-2. In both cases, DHT was added to a final concentration of 100 n*M* as indicated. Note that in both cases CAT activity is represented relative to activity of the first condition, which was set to 1. Error bars represent SDS of five independent replicates.

amino acids 502–555 harbor an autonomous transactivation domain that is stimulated by c-Jun.

TIF-2 Acts on Carboxy Terminus of hAR and Cooperates with c-Jun for Androgen-Induced Transcription

In addition to c-Jun, other cellular factors referred to as coactivators, including TIF-2, have been shown to enhance hAR transcriptional activity *(13–15,17–19)*. We obtained TIF-2 (a kind gift from Dr. Pierre Chambon), which was originally shown to act on the DE region of hAR *(14)*, and tested it on the hAR fusions with GAL4. TIF-2 was able to stimulate the activity of ligand-bound GAL-hAR(DE) but not hAR(AB)-GAL, as measured on the 17mer-tk-CAT reporter (Fig. 5A). By contrast, c-Jun was able to stimulate the hAR(AB)-GAL but not GAL-hAR(DE) (Fig. 5A). These results suggest that c-Jun and TIF-2 may target different regions of the receptor.

Because c-Jun and TIF-2 act on different regions on the AR, these two proteins were examined for a cooperative effect on full-length receptor. Exogenous TIF-2 was able to significantly stimulate full-length ligand-bound hAR, but this activity was weaker than that of exogenous c-Jun (Fig. 5B). When c-Jun and TIF-2 were tested together, stimulation of the receptor was reproducibly greater than with either protein alone, suggestive of an additive rather than synergistic effect by the two proteins (Fig. 5B). These results together suggest that c-Jun and TIF-2 can act together on the hAR by targeting different portions of the receptor.

Discussion

Coactivators are proteins that enhance the transcriptional activity of transcription factors by bridging activators to the basic transcription machinery (reviewed in ref. *16*). Onate et al. *(26)* identified the first nuclear receptor coactivator, SRC-1. Since then, many other nuclear receptor coactivators have been identified, including TIF-1, TIF-2, GRIP-1, $TAF_{II}30$, hSNF2, SPT6, CBP/p300, and AIB-1 (*[27–33]*; reviewed in ref. *34*). An initial hallmark of these coactivators was that they targeted the conserved AF-2 region of nuclear receptors *(5)*. However, more recent work has deomonstrated that several of the SRC-1 family members, including TIF-2 *(13)* and SRC-1 *(14)*, can also act on the amino terminus of the AR and other nuclear receptors. Regarding the AR, Voegel et al. *(17)* showed that TIF-2 has a positive activity on this receptor's ligand-dependent AF-2, providing the first demonstration of AR AF-2 activity in mammalian cells and the most convincing evidence for the existence of a functional AF-2 in the AR. Further work with TIF-2 and the DE region of the AR has identified amino acid 888E, which lies within the AF-2 core of the

AR, as essential for the positive effect of TIF-2 on the AR *(13)*. Another coactivator specific for the AR is ARA-70, which interacts with the carboxy terminus of the receptor in a ligand-dependent manner and enhances AR transcriptional activity *(15)*.

In contrast to the aforementioned coactivators, which target either selectively the AF-2 or both the amino and carboxy termini of AR, c-Jun appears to be acting as a coactivator via the hAR amino terminus, which has, in the case of the AR, the significantly more active AF-1. However, because the specific boundaries of the hAR AF-1 are still in dispute, it is not possible to determine whether c-Jun is indeed targeting the hAR AF-1. Our evidence shows that hAR amino acids 503–521, which are found just amino terminal of the DBD, are important for c-Jun activity. Although these amino acids are important for the c-Jun response in the context of the GAL fusion protein, they are not sufficient on their own, suggesting that the amino acids flanking 503–521 may play a significant role in the transcriptional activity of this region. Interestingly, although amino acids 503–521 are outside the bipartite transactivation domain, designated AF-1a and AF-1b, which Chamberlain et al. *(9)* identified, they are contained within another transactivation function, TAU5 *(8)*, which encompasses amino acids 360–550. Thus, it is conceivable that TAU5 might be the target of c-Jun action. Although this may be the case, our data clearly demonstrate that hAR amino acids 502–555, at least within the context of a GAL chimera, are not only essential but also sufficient for a c-Jun positive effect. Furthermore, this region exhibits strong transcriptional activity in both mammalian and yeast cells. Accordingly, hAR amino acids 502–555 represent a novel autonomous activation function that positively responds to c-Jun. The nature of this activation function is not understood. However, a potential phosphorylation site on serine 508 may influence hAR transactivation (reviewed in ref. *35*). Moreover, a somatic mutation found in prostate cancer at amino acid 527, which changes an aspartic acid to a glycine, may influence the hAR transcriptional activity (reviewed in ref. *35*).

Interestingly, the TAU5 region, when included with the DBD and hinge region (D region), appears to respond to the coactivator CBP *(36)*. CBP has been shown to be required for transactivation by both nuclear receptors and AP-1 *(32)*, thereby making it possible that CBP might mediate the c-Jun response through hAR residues 503–555. However, several lines of evidence argue against this possibility. First, we have been unable to detect any response of hAR to CBP under our experimental conditions (data not shown). Second, Wise et al. *(24)* have shown that the double mutant c-Jun Ala63/73, which is unable to interact with CBP *(37)* and therefore activate transcription, is still able to stimulate hAR. Finally, the CBP effect requires the receptor's native DBD, which has been replaced with the DBD of GAL4 in our c-Jun-responsive hAR-GAL chimeric proteins. Together, these findings suggest that c-Jun may be stimulating hAR independently of CBP. However, they do not rule out the possibility that c-Jun and CBP may be acting together by interacting with different portions of the hAR amino terminus, not with each other.

Although c-Jun and CBP may be targeting the amino terminus of hAR, the SRC-1 family member TIF-2 has previously been seen to act on on this region and the DE region of this receptor *(13,14,17)*. In the present study, we provide evidence that the E region, fused to the GAL4 DBD, is sufficient for TIF-2 activity on hAR. More important, TIF-2 was shown to enhance positively the transcriptional activity of full-length hAR and act additively with c-Jun on hAR-induced transcription. Because no protein–protein interaction has been detected between c-Jun and TIF-2 (data not shown), it is likely that these two coactivator proteins act on hAR by targeting different regions of the receptor. In view of the documented interaction between SRC-1 family members and CBP *(38)*, it is possible that the CBP effect on hAR might be mediated by TIF-2 or the reverse. It is nevertheless clear that multiple regions of hAR are used for multiple interactions with coactivator proteins in the induction of androgen-responsive transcription. Future work should shed more light on this complex of proteins that work together with the AR, and perhaps other nuclear receptors.

Materials and Methods

Plasmids

To construct hAR(AB)/pTLI, hAR amino acids 502–563 were polymerase chain reaction (PCR) amplified using the upstream oligo 5'-GATCTGGTACCCTGGCGGCATG-3' and the downstream oligo 5'-GATCAAGCTTACTCGA GGTGGGGTGGAAAGTAATA-3'. This PCR fragment was digested with *Kpn*I and *Hind*III and inserted into the same restriction sites of hARI/pSG5 *(21)*. This placed an artificial in-frame *Xho*I site after hAR amino acid 555. The resulting clone, hARI LS48/49, was subsequently digested with *Bam*HI and *Xho*I and inserted into the same restriction sites of pTLI.

To generate hAR(ABC)/pTLI, hAR amino acids 502–623 were PCR amplified using the upstream oligo 5'–GATCTGGTACCCTGGCGGCATG-3' and the downstream oligo 5'-GATCGGATCCTCACATCCCTGCTT CATAACA-3'. This PCR fragment was digested with *Kpn*I and *Bam*HI inserted into the same restriction sites of hAR(AB)/pTLI.

To construct hAR(CDE)/pTLI, hAR amino acids 556–918 were PCR amplified using the upstream oligo 5'-GATC CTCGAGATGAAGACCTGCCTGATCTGT-3' and the downstream oligo 5'-GATCGGATCCTCACTGGGT GTGGAAATA-3'. This PCR fragment was digested with

*Xho*I and *Bam*HI and inserted into the same restriction sites of pTLI.

To construct hAR(AB)-GAL, the GAL4 DBD amino acids 1–148 were PCR amplified using the upstream oligo 5'-GATCGTCGACGGGTACCTGAGCTCAATG CTACTGTCT-3' and the downstream oligo 5'-GATCA GATCTTCAAATCGATACAGT CAA-3'. This PCR fragment was digested with *Sal*I and *Bgl*II and inserted into the *Xho*I and *Bgl*II sites of hAR(AB)/pTLI. Note that artificial *Kpn*I and *Sac*I sites were created between the 3' end of the hAR sequence and the 5' end of the GAL4 DBD that are in frame with naturally occurring sites in the hAR(AB) region.

To generate hAR(1-502)-GAL, hAR(AB)-GAL was digested with *Kpn*I and religated. To generate hAR(1-330)-GAL, hAR(AB)-GAL was digested with *Sac*I and religated. To generate hAR(1-502)-New Linker-GAL, hAR(1-502)-GAL was digested with *Kpn*I. Oligos 5'-CTGAAGCTT TCGTAC-3' and 5'-GAAAGCTTCAGGTAC-3' were annealed to create an in-frame *Hind*III site and inserted into hAR(1-502)-GAL.

To generate hAR(1-538)-GAL, hAR amino acids 502–538 were PCR amplified using the upstream oligo 5'-GATCTGGTACCCTGGCGGCATG-3' and the downstream oligo 5'-GATCAAGCTTCAAACGCATGTC CCC-3'. This fragment was digested with *Kpn*I and *Hind*III and inserted into the same restriction sites of hAR(1-502)-NL-GAL.

To generate hAR(1-521)-GAL, hAR amino acids 502–521 were PCR amplified using the upstream oligo 5'-GATCTGGTACCCTGGCGGCATG-3' and the downstream oligo LS 155 5'-GATCAAGCTTTTCGCTTT TGACACA-3'. This PCR fragment was digested with *Kpn*I and *Hind*III and inserted into the same restriction sites of hAR(1-502)-NL-GAL. To generate hAR(Δ504-521), hAR amino acids 522–555 were PCR amplified using the upstream oligo 5'-GATCGGTACCCTATGGGCCCCT GGATGGAT-3' and the downstream oligo 5'-GAT CAAGCTTCTGGGGTGGAAAGTA-3'. This PCR fragment was digested with *Kpn*I and *Hind*III and inserted into the same restriction sites of hAR(1-502)-NL-GAL.

To generate 2XBirch(502-555)-GAL, hAR amino acids 502–555 were PCR amplified with the upstream oligo 5'-GATCAAGCTTCTGGGGTGGAAAGTA-3' and the downstream oligo 5'-GATCGGATCCATGTACCCT GGCGGCATGGTG-3'. This PCR fragment was digested with *Bam*HI and *Hind*III and inserted into the same restriction sites of hAR(1-502)-NL-GAL. This construct was subsequently digested with *Bam*HI and *Bgl*II and inserted into the same restriction sites of 2XBirch/pTLI.

To generate 2XBirch(502-521)-GAL, hAR amino acids 502–521 were PCR amplified with upstream oligo 5'-GATCAAGCTTCTGGGGTGGAAAGTA-3' and downstream oligo 5'-GATCAAGCTTTTCGCTTTTGACACA-3'. This PCR fragment was digested with *Bam*HI and *Hind*III and inserted into the same restriction sites of hAR(1-502)-NL-GAL. This construct was subsequently digested with *Bam*HI and *Bgl*II and inserted into the same restriction sites of 2XBirch/pTLI.

Cell Transfections and CAT Assays

Cos cells were grown in Dulbecco's Modified Eagle's (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone). Cells were grown in 60-mm dishes and transiently transfected using the calcium phosphate precipitation method *(39)*. The hAR was activated by the addition of 100 n*M* DHT. Chloramphenicol acetyltransferase (CAT) assays were performed and standardized according to the measured β-galactosidase activity as previously described *(39)*. For all transfections, we used different amounts of expression plasmid, 1 μg of reporter plasmid (either MMTV-CAT or 17M-tk-CAT), 2 μg of pCH110, and enough pTLI to bring the final plasmid amount to a maximum of 15 μg per dish.

CAT assay results were quantified by densitometric scanning of autoradiograms (420 oe scanner; PDI) of at least three repeats for each transfection, and, hence, each value represents the average of three to five repetitions plus SD.

Yeast Transformation and Quantification of β-Galactosidase Activity

To determine transcriptional activity of hAR in yeast, hAR amino acids 1–555 or 501–555 were fused in-frame to the LexA DBD to make LexA-hAR(AB) or LexA-hAR(501-555), respectively. Both constructs were confirmed by DNA sequencing and Western blot analysis using an LexA antibody (data not shown). These plasmids were transformed together with the LacZ reporter pSH18-34 into the *Saccharomyces cerevisiae* strain EGY48 *(40)*. Activities were quantified by measuring β-galactosidase activity with *o*-nitrophenyl-β-D-galactoside as described *(41)*.

Sodium Dodecyl Sulfate Polyacryamide Gel Electrophoresis and Western Blot

To prepare cell extracts for Western blot analysis, transfected Cos cells were extracted by boiling for 5–10 min in sodium dodecyl sulfate (SDS) sample buffer (63 m*M* Tris, pH 6.8; 20% glycerol; 2% SDS; 5% β-mercaptoethanol). The amount of extract used was standardized according to β-galactosidase activity. Proteins were separated by SDS-polyacrylamide gel electrophoresis and were electrotransferred onto nitrocellulose (Micron Separations). After blocking with nonfat dry milk, the nitrocellulose blots were probed with either the amino-terminal anti-AR antibody PG-21 (Affinity Bioreagents), the carboxy-terminal anti-AR antibody AR(C-19) (Santa Cruz Biotechnology), or the monoclonal anti-Birch prophilin antibody 4A6 (a kind gift from Martin Rothkegel). The blots were developed using a chemiluminescence detection kit from Amersham.

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