The proto-oncoprotein c-Jun forms as a heterodimer with c-Fos, the transcription factor AP-1. AP-1 regulates transcription through transactivation, a process requiring DNA binding. Here we report an indirect mechanism by which c-Jun can regulate transcription via the androgen receptor. In this process, c-Jun is able to support androgen receptor-mediated transactivation in the absence of an interaction with c-Fos or any apparent DNA binding. This positive effect of c-Jun was dose-dependent. Both exogenously added and endogenously induced c-Jun are able to act on the androgen receptor. Transactivation by the androgen receptor can undergo self-squelching, and this was relieved by transfected c-Jun. Using a time-course experiment, we provide evidence that the c-Jun effect is primary. c-Fos is able to block human androgen receptor activity in both the absence and presence of transfected c-Jun. Using a modified form of the yeast two-hybrid system, we show in Cos cells that c-Jun can interact with the DNA binding domain/hinge region (CD regions) of the androgen receptor. Therefore, we propose that c-Jun functions as a mediator for androgen receptor-induced transactivation.

Interaction between what were once considered distinct signal transduction pathways is beginning to emerge as the rule of the intracellular events leading to cellular growth and differentiation. A paradigm of such an interaction has surfaced that interconnects two families of transcription factors, which are the principal pathways used by the cell to convey signals into the nucleus: the signals transmitted by nuclear receptors and the nuclear proto-oncoproteins c-Jun and c-Fos. Nuclear receptors comprise one of the largest families of transcription factors, which, upon binding of their cognate ligand, stimulate (in some cases, inhibit) the rate of transcription of their target genes and, in this way, mediate cellular differentiation (reviewed in Refs. 1 and 2). The nuclear receptor superfamily includes the receptors for steroid and thyroid hormones, retinoids, vitamin D3, and an ever-growing number of other identified and unidentified ligands (reviewed in Refs. 1–4). Like most nuclear receptors, the proto-oncoproteins c-Jun and c-Fos can synergistically activate with the GR on unknown cell-specific factors, since the c-Jun effect is not apparent (15, 18).

In contrast to these inhibitory effects, there have been several reports suggesting cooperative interactions between nuclear receptors and AP-1. Cotransfection of expression plasmids for c-Jun, c-Fos, and ER causes synergistic activation of the ovalbumin gene (20). Similarly, the GR has been shown to potentiate c-Jun-activated transcription from the proliferin promoter (21). Recently, Harrison et al. (22) have found that both c-Jun and c-Fos can synergistically activate with the GR the neurotensin/neuromedin gene promoter. The mechanism of cooperation on both the proliferin and neurotensin/neuromedin promoters appears to be at the level of DNA binding, since both promoters contain and require functional glucocorticoid response element and AP-1 elements for the synergistic interaction between GR and c-Jun and/or c-Fos.

Contrasting this requirement for DNA binding, c-Jun is also known to positively affect the transcriptional activity of GR (23) and AR (16) without binding to DNA. However, the specific outcome of the c-Jun interaction with GR appears to be dependent on unknown cell-specific factors, since the c-Jun effect is negative in HeLa and Cos cells (9–12) and positive in several T-cell lines (23). On the other hand, c-Jun stimulates AR-mediated transactivation without any apparent dependence on cell- and/or promoter-specific factors (16). This general effect of c-Jun on AR activity opened the possibility that this proto-oncoprotein may mediate AR-induced transcription and perhaps act as a coactivator.

* This work was supported by a grant from the American Cancer Society, Ohio Division. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The Journal of Biological Chemistry Vol. 271, No. 40, Issue of October 4, pp. 24583–24589, 1996

Printed in U.S.A.
**c-Jun Mediates Androgen Receptor Activity**

We provide in this paper evidence that c-Jun is able to support AR-mediated transactivation. Using transient transfection studies, we show here that AR is stimulated by both exogenous and endogenous c-Jun, c-Jun can relieve AR self-squeezing, the c-Jun effect is primary, and that c-Fos counteracts the positive effect of c-Jun. Finally, we demonstrate that c-Jun and hAR can interact in Cos cells, as measured using a form of the yeast two-hybrid system adapted for mammalian cells.

**MATERIALS AND METHODS**

**Plasmids**—Human forms of AR and c-Jun were all expressed from the mammalian expression plasmid pSG5 (24). The GAL4 DNA binding domain (DBD) was polymerase chain reaction-amplified using the upstream oligo 5'-GATCCAGTTGATTACTTCAAGCTGCTTCATGAAGCT-3' and the downstream oligo 5'-GATCCAGCTTCAAGCTGCTCCATGGTGCTG-3' and inserted into pG4AB, a derivative of pSG5 (24), to make the plasmid pG4AB. The hAR CD region was polymerase chain reaction-amplified using the upstream oligo 5'-GATCCAGTTGATTACTTCAAGCTGCTTCATGAAGCT-3' and the downstream oligo 5'-GATCCAGCTTCAAGCTGCTCCATGGTGCTG-3' and inserted into pG4AB, yielding GAL-hAR(CD). The hAR DE region was polymerase chain reaction-amplified using the upstream oligo 5'-gatcGGATCCTCACTGGGTGTGGAAATA-3' and the downstream oligo 5'-gatcGGATCCTCACTGGGTGTGGAAATA-3' and inserted into pG4AB, yielding GAL-hAR(DE). GAL4-c-Fos(Δ1–114) was constructed by digesting c-Fos(Δ1–114) (16) with EcoRI, filling in with Klenow and then digesting with BamHI, and inserted into pG4AB. All clones have been verified by DNA sequencing (available upon request).

The reporter plasmids have the gene for chloramphenicol acetyl transferase (CAT) driven by the MMTV, TRE-θk, or 17k-θk promoters. The AR-inducible reporter placid MMTV-CAT and the GAL4-inducible reporter 17k-θ CAT have been described previously (16). The AP-1-inducible reporter TRE-θk-CAT was constructed by inserting an oligonucleotide (CTAGTCCGACTCTGACTCGATGTCG) containing a single copy of the TRE from the collagenase gene promoter into the plasmid pBl-CAT, which has multiple androgen-responsive elements (also called glucocorticoid response elements or GREs) that can respond to AR, GR, and PR. The ability of DHT-activated hAR to stimulate transcription from MMTV-CAT in cultured Cos cells was weak (Fig. 1A, compare lanes 1 and 7). However, when c-Jun (also expressed from pSG5) was coexpressed with hAR, there was strong stimulation of hAR activity (5–15-fold above the activity measured in the absence of transfected c-Jun) that was dependent on the amount of c-Jun expression plasmid transfected (Fig. 1A, compare lane 7 with lanes 8–10). The effect of c-Jun on ligand-activated hAR, since there was no c-Jun effect on hAR in the absence of DHT (Fig. 1A, compare lanes 4–6 with lanes 8–10). Furthermore, c-Jun had no significant effect on the MMTV reporter in the absence of transfected hAR (Fig. 1A, compare lanes 1 and 2). The positive effect of c-Jun on hAR activity could be due to enhanced levels of hAR protein expression in the presence of transfected c-Jun. However, Western blot analysis showed that c-Jun, at various plasmid concentrations, does not significantly affect the expression of hAR protein nor its mobility on SDS-polyacrylamide gel electrophoresis (Fig. 1B, compare lane 3 with lanes 4–6), suggesting that exogenous c-Jun does not cause a change in posttranslational modifications of hAR. In addition, stability of hAR is not altered by transfected c-Jun.2

The weak hAR activity observed in the absence of transfected c-Jun (see Fig. 1A, lane 3) might be due to endogenous c-Jun in Cos cells. Endogenous c-Jun was measured by both Western blot analysis and transcriptional activity. The Western blot indicated that endogenous c-Jun is in low but detectable con-

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2 A. Babulya, S. Wise, and L. Shemshedini, unpublished data.
centrations in Cos cells (Fig. 1C, lanes 1 and 3), and that this is greatly enhanced in a dose-dependent manner by transfecting the c-Jun plasmid (Fig. 1C, compare lanes 1 and 2 and lanes 3–6). Coexpression of hAR does not affect the expression nor the mobility on SDS-polyacrylamide gel electrophoresis of transfected c-Jun (Fig. 1C, compare lanes 2 and 6). In support of the Western blot results, measuring c-Jun by monitoring transcriptional activation of TRE-tk-CAT, an AP-1-inducible reporter, showed low levels of endogenous AP-1 in Cos cells, and CAT expression was greatly enhanced by transfecting c-Jun. These results clearly show that Cos cells have low but significant levels of endogenous c-Jun and that this is greatly enhanced by transiently transfecting c-Jun into these cells, corresponding to a concomitant increase in hAR transcriptional activity.

Endogenous c-Jun Enhances Transcriptional Activation by the Androgen Receptor—Previous studies indicate that RA can induce in F9 cells the expression of endogenous c-Jun, without affecting the expression of c-Fos (29). Using a derivative of F9 cells, P19 cells (30), we tested whether endogenous c-Jun can have the same positive effect on hAR as does transfected c-Jun. We first tested for the RA effect on c-Jun expression in P19 cells. 9C-RA (10^{-7} M), which is known to activate both kinds of RA receptors, RARs and RXRs (26–28), induction of c-Jun was measured by monitoring transcriptional activation from the TRE-tk-CAT. 9C-RA treatment of P19 cells resulted in a strong (5-fold) stimulation of TRE-tk-CAT (Fig. 2A, compare lanes 3 and 4). The 9C-RA activation of TRE-tk-CAT was comparable to that by transfected c-Jun (Fig. 2A, compare lanes 2 and 4), indicating that RA can induce the expression of endogenous c-Jun in P19 cells, as it can in F9 cells (29). Western blot analysis performed with extracts from P19 cells treated with 9C-RA confirmed that the RA induction of AP-1 activity was due to an increase in endogenous c-Jun expression.

Since RA can induce expression of endogenous c-Jun in P19 cells, we tested the possibility that RA can indirectly, via its induction of c-Jun, stimulate hAR activity. Therefore, we repeated the transient transfection experiment described above, but this time measured hAR activity on MMTV-CAT. Importantly, 9C-RA treatment of P19 cells resulted in enhanced hAR transcriptional activity that was at least as strong as that stimulated by transfected c-Jun (Fig. 2A, compare lanes 6 and 9). 9C-RA had no effect on MMTV-CAT in the absence of transfected hAR. It is significant that RA, like transfected c-Jun, does not affect the expression of hAR. These results strongly suggest that the effect transfected c-Jun has on hAR can be reproduced with endogenous c-Jun and is consistent with c-Jun mediating the transcriptional activity of hAR.

c-Jun Can Relieve Self-squelching by the Androgen Recep-

![Fig. 2. Endogenously expressed c-Jun in P19 cells can stimulate hAR transactivation.](image-url)

![Fig. 3. hAR undergoes self-squelching that can be relieved by c-Jun.](image-url)
Figs. 1 and 2), we wanted to test whether this proto-oncoprotein could relieve hAR self-squelching. Thus, the squelching experiment above was repeated in the presence of 5 μg of transfected c-Jun plasmid. c-Jun was not only able to relieve hAR self-squelching (Fig. 3A, lanes 9–12) but resulted in a stronger hAR transactivation (90-fold) than the maximal activity measured in the absence of transfected c-Jun (23-fold) (compare lanes 3 and 12). These results suggest that endogenous c-Jun is limiting in Cos cells and argue strongly that c-Jun is a mediator for hAR-induced transactivation.

c-Jun Has a Primary Effect on Transcriptional Activation by the Androgen Receptor—To obtain evidence for c-Jun having a primary effect on hAR-mediated transactivation, a time-course experiment was performed to determine at what point during the course of a transient transfection experiment c-Jun action on hAR activity is first detected. This was done by measuring CAT activity and comparing this to the first appearance of hAR and c-Jun proteins, as measured by Western blot analysis. Thus, Cos cells were transfected with hAR plasmid minus/plus the c-Jun plasmid and harvested at six different times: 6, 12, or 24 h after the DNA was added (Post-DNA) or 6, 12, or 24 h after the cells were washed and fresh medium was added (Post-Wash) following a prior 24-hr incubation with DNA and then exposed to a CAT assay (Fig. 4A). Initial transactivation by hAR was observed 6 h Post-Wash (Fig. 4B, lanes 3–10), and it was only seen in the presence of transfected c-Jun (compare lanes 9 and 10). Western blot analysis indicated that this time corresponded exactly with the time when expressed hAR (Fig. 4C, lanes 3–10) and c-Jun (Fig. 4D, lanes 3–10) proteins were first detected. Moreover, hAR transcriptional activity increased with increasing incubation period (Fig. 4B, lanes 9–14), which corresponded with a concomitant increase in hAR and c-Jun protein levels (Figs. 4, C and D, lanes 9–14). Little, if any, hAR transactivation was seen prior to washing the cells (that is, during the Post-DNA stage) (Fig. 4B, lanes 3–8). Therefore, the c-Jun effect on hAR transactivation is observed as soon as exogenous c-Jun is expressed, ruling out a possible secondary effect of c-Jun. More importantly, this result is consistent with c-Jun having a primary, and perhaps direct, effect on hAR.

c-Fos Blocks c-Jun Stimulation of Transactivation by the Androgen Receptor—The time-course results above suggest that c-Jun has a primary effect on hAR, a contention supported by our finding that c-Fos has an antagonistic effect on hAR-induced transactivation. c-Fos is able to repress hAR activity both in the absence (Fig. 5, compare lane 4 with lanes 5–7) and presence of transfected c-Jun (Fig. 5, compare lane 8 with lanes 9–11). These results suggest that: 1) the c-Jun-c-Fos heterodimer is unable to support hAR-induced transcription; and 2) the weak activity observed with hAR in the absence of transfected c-Jun is probably due to low levels of endogenous c-Jun present in Cos cells (Fig. 1C).

c-Jun Can Interact with the CD Regions of the Androgen Receptor in Cos Cells—The effect of c-Jun on hAR transcriptional activity could be mediated by protein-protein interactions between the two proteins. To test this, we have used a form of the yeast two-hybrid system adapted for mammalian cells. A similar system has been used previously to show interactions between the C and N termini of the AR (92). In this system, which we call a mammalian one-hybrid assay, protein-protein interactions are studied in cultured mammalian cells using mammalian plasmids. This system consists of two expression plasmids, both based on the mammalian plasmid pSG5 (24) and the reporter plasmid 17M-th-CAT. One expression plasmid encodes the GAL4-DBD fused to our “bait,” the CD regions of hAR; the other plasmid encodes c-Jun, which contains its own activation functions. We elected to use the CD regions of hAR since previous results with the GR, whose DBD (C region) is highly homologous to that of AR, have shown a protein-protein interaction between GR and c-Jun, which is mediated by the GR C region (9–12). Activation of the 17M-th-

"Fig. 4. c-Jun stimulation of hAR transactivation is a primary effect. In A, Cos cells were transfected with 1 μg of MMTV-CAT, 3 μg of hAR, or 5 μg of c-Jun as indicated in the presence of 100 nM DHT. Some cells were harvested 6, 12, or 24 h after adding the DNA (Post DNA). Other cells were incubated with DNA for 24 h and washed; then fresh medium was added. These cells were then harvested 6, 12, or 24 h after changing the medium (Post Wash). B, CAT assay from experiments described in A. Lanes 1 and 2 are control cells transfected and harvested as in Fig. 1. In C and D, using cell extracts from B standardized according to β-gal activity, protein expression of either hAR or c-Jun was determined by Western blot analysis."
CAT reporter results when hAR(CD) interacts stably with c-Jun, bringing the c-Jun activation functions to the tk promoter and causing the expression of the CAT gene.

Cos cells were transfected with various combinations of hybrid plasmids (described in Fig. 6A), and CAT activity was measured (Fig. 6B). As expected, GAL-VP16 was a strong activator of 17M-tk-CAT (compare lanes 1 and 2). There was no observable CAT activity with GAL(DBD) (compare lanes 1 and 3), hAR(CD)-GAL (compare lanes 1 and 4), or GAL-c-Fos(D1–114) (compare lanes 1 and 5), eliminating the presence of any possible cryptic activation functions in these proteins. c-Jun alone was also inactive (compare lanes 1 and 7), indicating that there is no unexpected binding of c-Jun to the 17M-tk-CAT reporter plasmid. However, when GAL-c-Fos(D1–114), which contains the bZIP region, and c-Jun were coexpressed, there was strong stimulation of CAT activity (lane 6), clearly showing the expected result that the c-Fos bZIP region and c-Jun are able to interact. Importantly, c-Jun was also able to activate the 17M-tk-CAT reporter in the presence of hAR(CD)-GAL (lane 9). Since c-Jun had no effect on the reporter in the presence of the GAL(DBD) (lane 8), these results suggest that c-Jun is able to interact, either directly or indirectly, with the CD regions of hAR. c-Jun did not show a significant interaction with the hAR DE regions (compare lanes 15 and 16), suggesting that the target site for c-Jun interaction is the hAR C region. Interestingly, when c-Fos was added to the system, the c-Jun-hAR(CD) interaction was inhibited (compare lane 9 with lanes 12 and 13), consistent with the idea that c-Fos blocks the interaction between c-Jun and hAR and thereby provides a mechanistic explanation of how c-Fos blocks c-Jun stimulation of hAR transactivation (Fig. 5).

**DISCUSSION**

In this study, we provide evidence for c-Jun playing a direct role in hAR-induced transactivation. Our findings that: 1) c-Jun stimulates hAR activity without affecting hAR expression or DNA binding in vitro; 2) both exogenous and endogenous c-Jun can affect hAR activity; 3) c-Jun can relieve hAR self-squelching; and 4) c-Jun has a primary effect on the hAR, all consistent with this evidence. In addition, we found that c-Fos counteracts the enhancing activity of c-Jun on hAR, suggesting that the c-Jun-c-Fos heterodimer is unable to support hAR activity. Moreover, this negative effect of c-Fos was seen in both the absence and presence of transfected c-Jun, indicating a common mechanism for hAR transactivation with and without transfected c-Jun. Furthermore, these results suggest that the low hAR activity observed in Cos cells without transfected c-Jun is most likely due to endogenous c-Jun.

The enhancing activity of c-Jun on hAR has been seen on several different promoters, including those for the androgen-regulated genes PSA (33) and hKLK2 (34), and in various cell lines, including the prostate line PC-3 (34). These results make the positive activity of c-Jun a general effect on hAR-mediated transactivation and different from its role in synergistically activating transcription with the GR, a phenomenon that appears to be cell- and promoter-specific. Although c-Jun inhibits transactivation of most glucocorticoid-dependent promoters, it stimulates transactivation of the proliferin (21) and neurotensin/neuromedin (22) promoters. In the case of a minimal promoter containing a TATA box driven by a glucocorticoid response element, GR activity is repressed by c-Jun in various cell lines except T cells, where the effect is positive (23). A similar minimal promoter has also exhibited a positive c-Jun effect on hAR-mediated transactivation, but this has been seen in several different cell lines (16). This finding, together with our results with the MMTV promoter showing that c-Jun has no effect in the absence of hAR, suggests that c-Jun can affect hAR in the absence of specific DNA binding. Our attempts to directly test this by using c-Jun mutants deficient in DNA binding have been unrevealing, since the bZIP region of c-Jun has previously been shown to be essential for interaction with nuclear receptors (reviewed in Ref. 35). Extensive mutational

**FIG. 5. c-Fos inhibits c-Jun-stimulated hAR transactivation.** Cos cells were transfected with 1 μg of MMTV-CAT, 1 μg of hAR, or 1 μg of c-Jun and increasing amounts of c-Fos (1, 3, or 5 μg) as indicated. Cells were exposed to 100 nm DHT in all cases.

**FIG. 6. c-Jun can interact with the hAR CD regions in Cos cells.** A. description of the hybrid proteins used in the assay. GAL4-DBD (amino acids 1–147) were fused to VP16 (413–489) to give GAL-VP16, to hAR (556–665) to give hAR(CD)-GAL, to hAR (624–919) to give GAL-hAR(DE), or to c-Fos (115–380) to give GAL-c-Fos(D1–114). B, Cos cells were transfected with 1 μg of the 17M-tk-CAT reporter and 1 μg of each expression plasmid except for c-Fos, of which was transfected either 1 or 3 μg as indicated.

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studies of the bZIP region are necessary to confirm that c-Jun DNA binding is not necessary for c-Jun induction of hAR transactivation.

Previous work has implicated the involvement of protein kinase C (PKC) on androgen-regulated transcription. This was based on transfection studies in which AR-induced transactivation was enhanced by treating cells with the PKC activator phorbol 12-myristate 13-acetate (PMA) (36, 37). Since there were no observable changes in AR expression levels, DNA binding, or phosphorylation, the effect of PMA has been attributed to the activation by PKC of proteins in the transcription machinery (36, 37). Two well studied proteins that are activated by PKC are c-Jun and c-Fos (5). In view of our finding that c-Jun is able to stimulate hAR activity without altering hAR expression or DNA binding, we suggest that PKC activation of c-Jun may be responsible for the PMA effect on AR transactivation. The role of c-Fos in PKC stimulation of hAR activity is unclear. Our results show that c-Fos is a negative regulator of c-Jun action on the AR, suggesting that PMA treatment of cells may result in a possible imbalance in activation of c-Jun and c-Fos. Such differential activation of these two proto-oncoproteins has previously been seen after 12-O-tetradecanoylphorbol-13-acetate treatment of HeLa cells, which respond by expressing higher levels of c-Jun than c-Fos (23). Therefore, it is possible that PMA may in some cells preferentially induces c-Jun, and thereby, results in enhanced AR activity.

Interaction between nuclear receptors can occur at different levels. The best studied example of this is heterodimerization of a growing number of nuclear receptors with RXRs, leading to gene-specific expression (reviewed in Ref. 38). Some receptors are known to regulate the expression of other receptors, such as the ER directly controlling expression of PR in MCF-7 breast cancer cells (39). Finally, early studies on squelching (26, 40, 41) suggested the existence of common coactivators for different kinds of nuclear receptors, something that has been substantiated by the recent identifications and cloning of putative coactivators for various classes of receptors (42–46). The data reported in this study suggest a fourth level of nuclear receptor interaction via regulation by one receptor of the expression of a putative coactivator for another receptor. We have shown here that RA can enhance hAR-mediated transduction by inducing the expression of endogenous c-Jun in P19 cells. However, since RA treatment causes differentiation of P19 cells (30), it is possible that RA-inducible factors other than, or in addition to, c-Jun might be responsible for enhancement of hAR transactivation. Interestingly, a recent study reported that RA can also antagonize androgen signaling by reducing the expression levels of AR in LNCaP cells (47). Therefore, the retinoid receptors RARs/RXRs can have either a positive or negative effect on AR activity and thus provide another example of diversity in nuclear receptor interactions.

With respect to the mechanism involved in c-Jun stimulation of hAR transactivation, our results suggest that c-Jun may be acting as a coactivator, bridging the hAR to the transcription machinery (reviewed in Refs. 48). Consistent with this, we were able to detect a protein-protein interaction between hAR and c-Jun using a one-hybrid system in mammalian cells. Moreover, Franklin et al. (49) have shown previously that c-Jun can directly interact with the two general transcription factors, TBP and TFIIB. The one-hybrid results suggest that the target of c-Jun interaction with the hAR is the DBD of the receptor, which is found in the C region. This is not surprising in view of previous work showing that the receptor DBD is required for the GR (9–12), thyroid receptor (19), and RAR (14–15, 18) to functionally interact with c-Jun. An amino acid comparison of the DBDs of steroid receptors shows that the AR is more closely related to the PR than to the GR (50). Interestingly, this is paralleled by the activity of c-Jun on these three receptors, with the proto-oncoprotein having exclusively a positive effect on AR, no effect, or positive on PR (16), and primarily a negative effect on GR (9–12). These results suggest that the nature of the receptor DBD can determine the transcriptional outcome of the c-Jun-steroid receptor interaction.

Although this one-hybrid assay provides strong evidence for a c-Jun-hAR interaction, it does not allow us to distinguish between a direct and an indirect interaction. It is conceivable that a c-Jun-hAR complex is indirect and/or stabilized by other proteins, including the general transcription factors, or perhaps c-Jun cooperates with other proteins, in addition to the general transcription machinery, to mediate hAR transactivation. Possible candidates are the various proteins identified recently shown to function as coactivators in nuclear receptor-mediated transactivation, including TAF₁₃₀ (46), SP7 (45), hSNF2 (43), TIF1 (42), Sre-c1 (44), or AR₈ (51), a protein shown to mediate AR activity. Whether the effect is direct or indirect, the findings in this study unveil the prospect that c-Jun induces the expression of not only AP-1-responsive genes but also androgen-responsive genes and, hence, may play an important role in androgen regulation of male sexual differentiation.

Acknowledgments—We are grateful to P. Chambo for providing plasmids and cells and S. Liao for providing antibodies against the androgen receptor. We thank X. F. Zhou for his help with protein purification and S. Leisner, M. Wheelock, and K. Johnson for helpful discussions.

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