

Inhibition of NF κ B Activity through Maintenance of I κ B α Levels Contributes to Dihydrotestosterone-mediated Repression of the Interleukin-6 Promoter*

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Androgens repress expression of many genes, yet the mechanism of this activity has remained elusive. The cytokine, interleukin-6, is active in a variety of biological systems, and its expression is repressed by androgens. Accordingly we dissected the mechanism of androgen's ability to inhibit interleukin-6 expression at the molecular level. In a series of co-transfection assays, we found that 5 α -dihydrotestosterone, through the androgen receptor, repressed activation of the interleukin-6 promoter, in part, by inhibiting NF κ B activity. It did not appear that 5 α -dihydrotestosterone inhibited NF κ B by activating the androgen receptor to compete for the NF κ B response element as we could not detect androgen receptor binding to the IL-6 promoter by DNase I footprinting assay. However, by electrophoretic mobility shift assay we found that 5 α -dihydrotestosterone repressed formation of NF κ B-NF κ B response element complex formation. In LNCaP prostate carcinoma cells, 5 α -dihydrotestosterone achieved this effect through maintenance of I κ B α protein levels in the face of phorbol ester, a stimulus that results in I κ B α degradation. Finally, we confirmed that I κ B α inhibits NF κ B-mediated activation of the interleukin-6 promoter. These data suggest that maintenance of I κ B α levels may represent the first identified mechanism for androgen-mediated repression of a natural androgen-regulated gene.

Androgen hormones modulate expression of many genes, and both androgen-inducible (reviewed in Refs. 1, 2) and androgen-repressed (3–18) genes have been described. Androgen induction of transcription is mediated through the androgen receptor (AR),¹ a member of the steroid hormone receptor family. The AR is a 110-kDa nuclear protein (19, 20) that consists of trans-

activation, DNA binding, nuclear localization, dimerization, and ligand binding domains (reviewed in Ref. 21). The AR activates gene transcription by specific binding to a DNA sequence, the androgen response element (ARE), in a ligand-dependent manner. The consensus ARE is similar to the glucocorticoid response element (GRE) (22) and as such can be stimulated by glucocorticoid receptor (GR). However, there are AREs that favor AR-induced transcriptional activation over that of GR (23–26). Although the ARE confers AR-mediated transcriptional activation on a gene, to date there has been no documentation of its ability to play a role in androgen-mediated repression of transcription.

Transcriptional repression of genes is a vital component for modulation of gene expression in eukaryotes. Major mechanisms responsible for negative regulation of eukaryotic gene expression include cytoplasmic sequestration of transcription factors, blocking of transcription factor response elements, direct inhibition of transcription factors by protein-protein interactions, interference with *trans*-activation of DNA-bound transcription factors, and direct inhibition of transcription by binding to the promoter (reviewed in Refs. 27–33). Whether one of these mechanisms or some other mechanism accounts for AR's ability to repress transcriptional activity is currently not known.

The cytokine interleukin-6 (IL-6) gene provides an excellent model system in which to study androgen-mediated repression. Serum androgen levels decrease with age (34–36) and serum IL-6 levels increase (37) raising the possibility that androgens influence IL-6 expression. This contention is further supported by the observation that in a murine orchietomy model, gonadal hormone loss induced by orchietomy results in elevated serum IL-6 levels that are reversible by testosterone administration (38, 39). IL-6 gene expression is regulated by complex arrangement of 5'-flanking elements. Several *cis*-acting response elements mediate activation of the IL-6 promoter including AP-1, nuclear factor IL-6 (NF-IL6) response element, NF- κ B response element, and the multiple response element (MRE). The MRE confers induction of the IL-6 promoter to phorbol 12-myristate 13-acetate (PMA), serum, forskolin, interleukin-1 α (IL-1 α), and tumor necrosis factor (40). Repression of the IL-6 promoter can be mediated by various *trans*-acting factors including Fos (40), retinoblastoma protein (41), estrogen receptor (42, 43), and GR (44). Androgens also mediate repression of IL-6 promoter activity (Ref. 38 and current report), but how they do so has not been characterized.

This report examines the mechanism of androgen-mediated repression of IL-6 transcription. We have found that 5 α -dihydrotestosterone (DHT) inhibits IL-6 gene expression through activation of the AR, which in turn represses NF κ B-induced stimulation of the IL-6 promoter through maintenance of I κ B α levels.

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¹ The abbreviations used are: AR, androgen receptor; ARE, androgen response element; β -gal, β -galactosidase; bp, base pair(s); CMV, cytomegalovirus; DHT, 5 α -dihydrotestosterone; DBD, DNA binding domain; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; GR, glucocorticoid receptor; GRE, glucocorticoid response element; IL, interleukin; LNGFR, low affinity nerve growth factor receptor; MRE, multiple response element; NF-IL6, nuclear factor IL-6; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; TAT, tyrosine aminotransferase; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol.

EXPERIMENTAL PROCEDURES

Plasmids—The luciferase-encoding plasmids pGL2-Basic, pGL3-Promoter, and pGL2-Control that contain either no promoter, a SV40 promoter, or a SV40 promoter and enhancer, respectively, and pSP73, an *in vitro* transcription vector, were purchased from Promega (Madison, WI). pGL2-IL6p(-1200) was constructed by using *SacI* and *XhoI* to excise the 1200-bp IL-6 5'-untranslated region from a pGEM4 plasmid containing the human IL-6 gene (45) (kindly provided by T. Kishimoto, Osaka, Japan), gel-purifying the fragment, and directionally subcloning the fragment into the *SacI* and *XhoI* sites of pGL2-Basic. pGL2-IL6p(-225) and pGL2-IL6p(-160) were created by excising the *SacI*-*NheI* or *SacI*-*AatII* fragments, respectively, from pGL2-IL6p(-1200) followed by blunt ending the plasmid with Klenow and religation with T4 DNA ligase. Inserts were confirmed by partial DNA sequencing with Sequenase (U. S. Biochemical Corp.). The expression plasmids, CMV-neo and CMV-AR, contain the neomycin resistance cDNA or full-length human AR cDNA, respectively, driven by the cytomegalovirus (CMV) promoter. The expression plasmids p50, p65, and pNF-IL-6 contain cDNA for NF κ B-p50, NF κ B-p65, and NF-IL6, respectively, driven by the SV40 virus early promoter (kindly provided by S. Akira and T. Kishimoto, Osaka, Japan). The expression plasmid, SV40- β gal contains the cDNA for β -galactosidase (β -gal) driven by the SV40 virus early promoter. The expression plasmid c-Jun, contains cDNA for c-Jun driven by the SV40 virus early promoter (kindly provide by R. Tjian, Berkeley, CA). The expression plasmid containing the cDNA for human I κ B α (46) driven by a CMV promoter was kindly provided by A. Baldwin, Jr. (Chapel Hill, NC).

Cell Culture—HeLa cells and LNCaP cells were obtained from American Type Cell Culture (Rockville, MD). The cell lines were maintained at 37 °C, 5% CO₂ in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin (complete media). For experiments, cells were placed in phenol red-free DMEM supplemented with 10% that had been charcoal stripped (stripped media). For addition to culture media, DHT (Sigma) was first diluted to 10⁻³ M in 100% ethanol and then serially diluted in stripped media as required. PMA (Sigma) was first diluted to 160 \times 10⁻⁴ M with 100% ethanol prior to further dilution with media. Ethanol alone was added to those cultures that did not receive DHT or PMA.

Measurement of IL-6 Protein Concentration and RNA Levels—After treatment of cells with DHT as indicated below, cell supernatants were collected and frozen at -80 °C until assayed. IL-6 concentration was measured in the supernatants by enzyme-linked immunosorbent assay (ELISA) (Quantikine Immunoassay Kit, R&D Systems, Minneapolis, MN) according to the manufacturer's directions. The ELISA demonstrated a <5% coefficient of variation for intra-assay precision. For IL-6 RNA levels, RNA was extracted after DHT treatment and quantified by competitive polymerase chain reaction (PCR) as we have previously described (47) with minor modifications. Briefly, after RNA levels were deduced to within 10-fold of IL-6 MIMIC levels by competitive PCR, we narrowed the comparison by performing 2-fold dilutions of IL-6 MIMIC and spiking the PCR reactions with [³²P]dCTP. The PCR products were electrophoresed on a 1.8% agarose gel; the gel was imaged by ultraviolet light, dried at 80 °C for 2–3 h, and individual band's cpm were quantified on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The concentration of IL-6 mRNA in the sample was deduced by plotting the ratio of IL-6 mRNA cpm/MIMIC cpm versus the input MIMIC concentration. Where the ratio of IL-6 mRNA cpm/MIMIC cpm = 1 indicates the concentration of IL-6 mRNA = the concentration of input MIMIC.

Cell Transfection and Reporter Assays—Cells were grown to 80% confluence in 100-mm plates, trypsinized, washed in phosphate buffered saline (PBS), and replated at 5 \times 10⁵ cells/60-mm plate in 3 ml of complete media. Twenty-four hours after plating, cells were transfected with the plasmids as indicated under "Results" by standard calcium phosphate transfection (48). Briefly, plasmid DNA in transfection buffer was added to the cells. A total of 8–10 μ g of plasmid was used for each plate. To keep total plasmid DNA consistent between treatment groups, pSP73, an *in vitro* transcription vector with no action *in vivo*, was added. After incubation for 16 h at 37 °C, 5% CO₂ the media containing the DNA was replaced with stripped media. DHT or ethanol alone was added as required. At 24 h after addition of DHT, more DHT, PMA, or ethanol alone was added as required. At 24 h after PMA addition, total cell extracts were harvested for reporter assays. Briefly, cells were washed twice with 2 ml of PBS, scraped using a rubber policeman into 1 ml of PBS, and microcentrifuged at 12,000 rpm for 10 s. After draining the PBS, the cell pellet was resuspended in 50 μ l of Tris buffer (pH 7.8) and subjected to three cycles of freezing in dry ice and ethanol and thawing at 37 °C in a water bath. Finally, the lysed

cells were microcentrifuged at 14,000 rpm for 10 min at 4 °C, and the supernatant was used for reporter assays. To determine β -gal activity, we evaluated the ability of a 15- μ l aliquot of cell extract to hydrolyze *o*-nitrophenyl- β -galactopyranoside (48). Luciferase activity present in 10 μ l of extract was evaluated with the Luciferase Reporter Assay System (Promega). Luminescence was quantitated on a luminometer (Lumat LB9501, Berthold). Luciferase activity was normalized between samples by β -gal activity.

Nuclear Extracts—LNCaP cells were treated in one of the following ways: no treatment, 160 nM PMA for 30 min, or 10 nM DHT for 2 h followed by 160 nM PMA for 30 min. Nuclear extracts were obtained by standard methods (49). Adherent cells were then scraped with a rubber policeman into 5 ml of ice-cold PBS and centrifuged at 250 \times *g* for 10 min at 4 °C twice. The resulting cell pellet was resuspended in 5 volumes of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride), incubated on ice for 10 min, centrifuged at 250 \times *g* for 10 min, resuspended in 3 volumes of buffer A with Nonidet P-40 (Nonidet P-40) added to 0.05% (v/v). Nuclei were then released by homogenizing the cells with 30 strokes in a tight-fitting Dounce homogenizer. Successful lysis was confirmed by phase-contrast microscopy. Nuclei were pelleted by centrifugation at 250 \times *g* for 10 min, resuspended in 500 μ l buffer C (5 mM HEPES (pH 7.9), 26% glycerol (v/v), 1.5 mM MgCl₂, 300 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride), and incubated on ice for 30 min. After incubation, the lysed nuclei were centrifuged at 24,000 \times *g* for 20 min at 4 °C, and the supernatant was snap-frozen in dry ice and ethanol and frozen at -80 °C until use. Protein concentration was determined with the BCA protein assay kit (Pierce).

Electrophoretic Mobility Shift Assay (EMSA)—Two oligomers consisting of the sequences 5'-TCGACATGTGGGATTTCCCATGAC-3' and 5'-TCGAGTCATGGGAAAATCCCATG-3' were annealed to form the NF κ B response element present within the IL-6 promoter from -43 to -61 bp (based on numbering system in Ref. 50). The probe was ³²P-end-labeled with T4 kinase and purified on a Chromaspin 10 column (Clontech, Palo Alto, CA). 10,000 cpm of probe was incubated with 10 μ g of LNCaP nuclear extract in EMSA binding buffer 1 (10 mM HEPES (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 2.5 mM DTT, 10% glycerol (v/v), 0.05% Nonidet P-40 (v/v)) for 30 min at room temperature. For competition studies, prior to addition of labeled probe, nuclear extracts were incubated for 10 min at room temperature with 100 \times unlabeled specific probe consisting of the consensus NF κ B response element (Promega), with nonspecific probe consisting of consensus Oct 1 response element (Promega), or with 20 μ l of protein purified from DHT-treated Sf9 cells transduced with a baculovirus vector encoding human AR as we have previously described (20). Protein-DNA complexes without the addition of loading dye were resolved on a 7% nondenaturing polyacrylamide gel (37.5:1 acrylamide:bisacrylamide) run in TG buffer (5 mM Tris, 38 mM glycine). The gels were then imaged after autoradiography at -80 °C for 1–4 h.

DNase I Footprinting—PCR primer IL6p(-280)-U consisting of 5'-AAAAGAAGTAAAGGAAGAGTGGTT-3' was ³²P-end-labeled with T4 kinase and purified on a Chromaspin 10 column (Clontech). The end-labeled primer was used with PCR primer pGL2b(594)-L consisting of 5'-AGGTAGATGAGATGTGACGAAC-3' to amplify an 854-bp fragment from pGL2-IL6p(-1200). PCR cycling parameters were the same as we have previously reported (47). The resulting PCR product consisting of the IL6 promoter from -280 to +13 fused to pGL2b from +33 to +594 (numbering based on sequence deposited in Genbank) and ³²P-end-labeled at the -280-bp site of the IL-6 promoter was purified using Wizard PCR DNA Purification System (Promega). Approximately 50,000 cpm of the end-labeled PCR product was incubated on ice for 30 min with 0 to 20 μ g of a synthesized peptide consisting of the AR DNA binding domain (DBD) in a total volume of 50 μ l consisting of 25 μ l of 2 \times binding buffer (20% glycerol (v/v), 0.2 mM EDTA, 120 mM KCl, 1 mM DTT, 20 mM HEPES (pH 7.9), 4% polyvinyl alcohol (w/v)). An equal volume of a mixture of 10 mM MgCl₂ and 5 mM CaCl₂ was added at the end of incubation, and samples were digested for 2 min at room temperature with DNase I (5 μ l of dilutions ranging from 0.001 to 0.05 Kunitz units/ μ l) (Sigma). Digestion was stopped by addition of 100 μ l of stop solution (200 mM NaCl, 20 mM EDTA (pH 8.0), 1% sodium dodecyl sulfate (SDS), 0.025% glycogen (w/v) (Boehringer Mannheim)). The samples were then extracted with 1 volume of TNE (10 mM Tris-HCl (pH 8.3), 0.3 M NaCl, 1 mM EDTA)-saturated phenol and chloroform/isoamyl alcohol, precipitated with 100% ethanol at -80 °C for 30 min, washed with 80% ethanol, and dried in a vacuum desiccator (Speed Vac, Savant Instruments, Farmingdale, NY). The samples were resuspended in 5 μ l of loading buffer (7 M urea, 0.1 \times TBE, 0.05% (w/v) each

of xylene cyanol and bromphenol blue), heated at 95 °C for 2 min, chilled on ice, and resolved on a 6% polyacrylamide sequencing gel. The gels were then imaged by autoradiography at 80 °C for 2–8 h. To identify the size of bands, we simultaneously resolved the sequencing reactions consisting of the M13 primer and template provided in the Sequenase kit (United States Biochemical Corp.). Additionally, an end-labeled 200-bp DNA fragment encompassing two tandem copies of the rat tyrosine aminotransferase (TAT) ARE (51) was used as a positive footprint control.

Western Analysis—LNCaP cells were grown in complete media to 80% confluency in 225-cm² flasks at which time the media were changed to stripped media. After being incubated in stripped media for 48 h, the cells were treated with vehicle alone or DHT at 10 and 100 nM for 2 h, followed by vehicle alone or PMA (160 nM) for 30 min at which time the cells were harvested to obtain protein. Total protein was obtained by washing the cells with PBS, scraping the cells into and lysing them in 3 ml of ice-cold RIPA buffer (9.1 mM Na₂PO₄, 1.7 mM NaHPO₄, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μ g/ml, aprotinin 30 μ l/ml, 1 mM sodium orthovanadate (pH 7.4)) with a 20-gauge needle, followed by centrifuging the lysate at 14,000 rpm in a microcentrifuge at 4 °C for 20 min. The supernatant was collected and protein concentration was determined by BCA protein assay (Pierce).

To evaluate for I κ B α , 100 μ g of protein was mixed with an equal volume of SDS-gel loading buffer, boiled for 90 s, cooled on ice and electrophoresed in 1 \times TGS buffer (0.025 M Tris (pH 8.3), 0.192 M glycine, 0.1% (w/v) SDS) on a 10% acrylamide-SDS gel, with a 4% stacking gel overlay. The proteins were transferred onto a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Marlborough, MA) in a modified Towbin transfer buffer (25 mM Tris (pH 8.3), 192 mM, 10% (v/v) methanol, 3.5 mM SDS). The membrane was probed with 0.1 μ g/ml polyclonal rabbit antihuman-I κ B α (I κ B α /MAD-3 (C-21), Santa Cruz Biotechnology, Santa Cruz, CA), and bound antibodies were detected by modified chemiluminescence with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody and luminol as recommended by the manufacturer (ECL Western, Amersham Corp.). Bands were quantified by video densitometry (Zeineh Video Densitometer, Biomedical Instruments, Fullerton, CA).

Statistical Analysis—Where appropriate, analysis of variance or Student's *t* test was used to analyze for differences between various treatments or constructs. Statistical significance was determined at *p* \leq 0.05.

RESULTS

DHT Represses IL-6 Protein and mRNA Expression in LNCaP Cells—Androgens have been implicated to mediate down-regulation of IL-6 gene expression (38, 52). Prior to elucidating a molecular mechanism for DHT's inhibitory action, we first characterized an *in vitro* experimental system by evaluating for DHT's effect on IL-6 protein and mRNA expression in LNCaP cells. Accordingly, LNCaP cells were plated at a density 5 \times 10³ cells/100 μ l-well in stripped media in 96-well plates. Cells were then treated with either 0, 10, or 100 nM DHT for 24 h followed by 160 nM PMA treatment for an additional 24 h. After incubation, IL-6 protein levels in the supernatants were evaluated by ELISA. We observed that DHT reduced IL-6 protein expression in a dose-dependent fashion (Fig. 1) with an effective dose₅₀ (ED₅₀) of 7 nM. In parallel experiments, 1 \times 10⁶ cells/10 ml in 100-mm tissue culture dishes were treated with either 0 or 10 nM DHT for 24 h. After incubation, total RNA was collected and evaluated for IL-6 mRNA levels by competitive PCR. Incubation of LNCaP cells with DHT resulted in a 9-fold decrease of IL-6 mRNA levels (Fig. 2). That we observed both androgen-induced down-regulation of IL-6 protein and steady state mRNA levels demonstrated that the LNCaP cells were an appropriate *in vitro* model for exploring androgen's inhibitory effect on IL-6 expression.

DHT Represses Transcriptional Activation of the IL-6 Promoter—Having confirmed that DHT can decrease IL-6 mRNA expression at the cellular level, we next asked if this was due to modulation of transcriptional events. To examine this question LNCaP cells were transfected with either pGL2-Basic, pGL2-IL6p(-1200) that contains the proximal 1200 bp of the IL-6

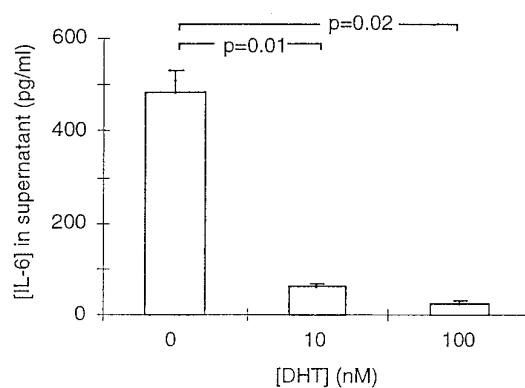


FIG. 1. DHT inhibits IL-6 production from LNCaP cells. LNCaP cells were plated in 96-well plates at a density of 5 \times 10³ cells/100- μ l well. The cells were incubated in DMEM supplemented with 10% charcoal-stripped FBS and either vehicle alone or DHT in ethanol vehicle at the indicated concentrations. After 24 h, 160 nM PMA was added, and cell supernatants were collected after an additional 24 h. IL-6 concentration in the supernatant was measured by ELISA. Results are shown as mean \pm 1 S.E. from three individual samples each measured in duplicate.

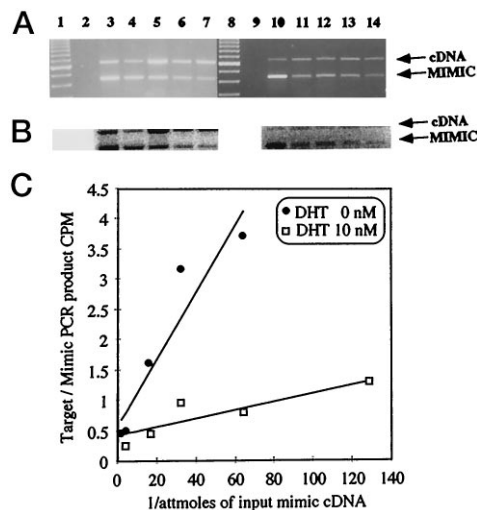


FIG. 2. DHT decreases steady state IL-6 mRNA levels in LNCaP cells. LNCaP cells were plated at a density of 1 \times 10⁶/10 ml in 100-mm dishes. They were incubated in DMEM supplemented with charcoal-stripped FBS and either 10 nM DHT or vehicle alone. After 24 h, total RNA was extracted and reverse-transcribed. IL-6 mRNA levels were semi-quantitated by competitive PCR. A, ethidium bromide-stained 1.8% agarose gel for PCR products from control (lanes 3–7) and DHT-treated (lanes 10–14) cell's cDNA. IL-6 mimic template was input in a series of 2-fold dilutions consisting of 0.5, 0.25, 0.125, 0.0625, and 0.0312 attomol (lanes 3–7) or 0.25, 0.125, 0.0625, 0.0312, and 0.0156 attomol (lanes 10–14). Kilobase ladder (lanes 1 and 8) and negative controls in which water was substituted for cDNA (lanes 2 and 9) are shown. B, phosphorimage of gel from A. Bands correspond to bands above them in A. C, linear regression analysis of cpm for PCR products.

promoter, or pGL2-Control followed by incubation of cells in the presence or absence of 10 nM DHT for 24 h followed by the addition of PMA or vehicle. Basal activity of the IL-6 promoter was negligible (Fig. 3, compare pGL2-IL6p(-1200) to pGL2-Basic), and DHT did not appear to repress this activity. However, upon PMA stimulation, a 3-fold rise in IL-6 promoter activity was observed that was completely abrogated by DHT (Fig. 3). However, DHT had no effect on the SV40 promoter present in pGL2-Control. Together, these results demonstrate that DHT was capable of inhibiting PMA-induced activation of the 1200-bp IL-6 promoter region in a promoter-specific fashion.

Androgen Receptor Is Required for DHT's Inhibitory Effect on

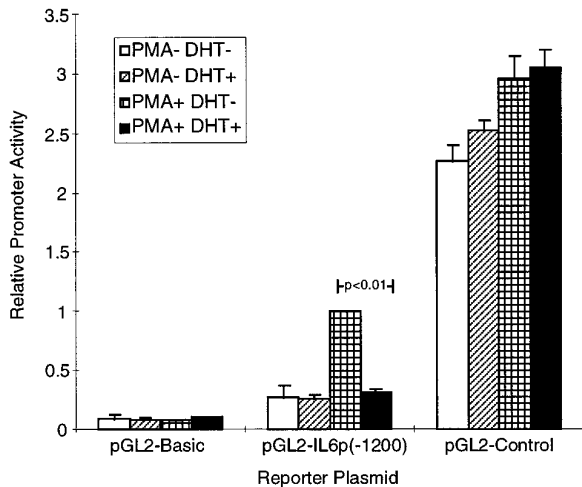


FIG. 3. DHT inhibits transcriptional activation of the IL-6 promoter in LNCaP cells. The plasmid pGL2-IL6p(-1200) consists of a 1200-bp fragment of the IL-6 promoter subcloned into a promoterless luciferase reporter vector (pGL2-Basic). LNCaP cells were co-transfected by calcium phosphate precipitation with 6 μ g of reporter plasmid as indicated, 2 μ g of the inactive plasmid pSP73, and 1 μ g SV40- β -gal. After transfection, cells were incubated in DMEM supplemented with 10% charcoal-stripped FBS in the presence or absence of 10 nM DHT for 24 h. At this time point, DHT was replenished and either PMA (160 nM final concentration) or ethanol vehicle alone was added. After an additional 24 h cells were collected by scraping; cell extracts were obtained by three freeze-thaw cycles; and β -gal and luciferase values were obtained. Luciferase activity was normalized for β -gal activity. Results are reported as promoter activity relative to pGL2-IL6p(1200) with PMA in the absence of DHT. Data are shown as the mean \pm 1 S.E. from two or more independent experiments each performed with duplicates.

IL-6 Promoter Transcriptional Activation—The AR in LNCaP cells contains a point mutation in its ligand binding domain that confers the ability to be responsive to anti-androgens and a variety of steroids besides DHT (53–55). To confirm that DHT was functioning through a classical steroid-steroid receptor interaction, we co-transfected HeLa cells, which do not contain AR, with pGL2-IL6p(-1200) and various concentrations of CMV-AR, which encodes wild-type human AR. In the absence of the CMV-AR expression vector (*i.e.* CMV-Neo only), DHT did not inhibit PMA-induced IL-6 promoter activity (Fig. 4, compare [AR] = 0 μ g, DHT 0 *versus* 10 nM). However, an AR dose-dependent inhibition of IL-6 promoter activity was noted. Additionally, CMV-AR did not mediate inhibition in the absence of DHT (Figs. 3 and 5B, compare PMA+, DHT- *versus* DHT+, both groups have AR, yet repression is noted when DHT is added). These data demonstrate that activated AR was capable of inhibiting PMA-induced IL-6 promoter activity and was required for DHT's effect.

The IL-6 Promoter Fragment between -225 and -160 Is Necessary but Not Sufficient for AR-mediated Inhibition of IL-6 Promoter Activity—The proximal 1200 bp of the IL-6 promoter is a complex region with many putative and confirmed *cis*-acting sites for transcription factor activity (Fig. 5A). To define *cis*-acting regions through which AR mediates its effect, the 5' region of the IL-6 promoter was progressively deleted and tested for its ability to be repressed by DHT. PMA induced the truncated 225-bp promoter fragment 17.5-fold compared with its basal activity. This was 6.7-fold the activity observed for PMA induction of the 1200-bp promoter fragment. DHT repressed the PMA-mediated activation by 52 and 62% for pGL2-IL6p(-1200) and pGL2-IL6p(-225), respectively (Fig. 5B). These results suggested that the region between -1200 and -225 contains a negative regulatory element that is independent of DHT-mediated inhibition. Further deletion to -160 resulted in an overall 82% decrease in basal activity of the pro-

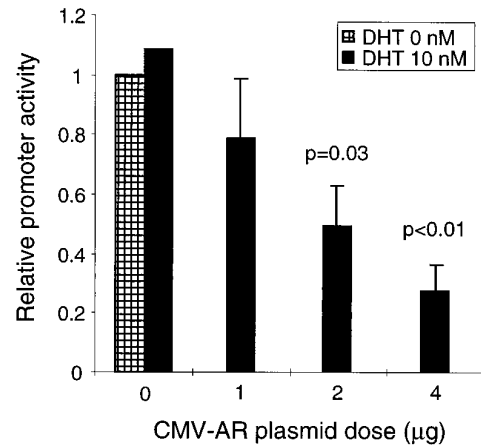


FIG. 4. The androgen receptor is required for DHT-mediated inhibition of IL-6 promoter activation. HeLa cells were co-transfected with 1 μ g of SV40- β -gal, 4 μ g of pGL2-IL6p(-1200), and either 4 μ g of CMV-Neo (AR = 0 μ g) or the indicated concentrations of CMV-AR by calcium phosphate precipitation and then treated with 10 nM DHT and 160 nM PMA. The inactive plasmid, pSP73, was added, as required, to keep total plasmid DNA levels equal between treatment groups. After 24 h, the cells were harvested as described in Fig. 3. Results are reported as luciferase activity relative to the AR = 0 μ g and DHT = 0 nM treatment group. The data represent the mean \pm 1 S.E. from three independent experiments each containing duplicate plates.

motor compared with the -225 fragment of the IL-6 promoter. Nonetheless, PMA induced 4-fold activation over basal activity of the -160 fragment, but DHT did not inhibit this activation. This observation suggests that the region between -225 and -160 is important for DHT-mediated inhibition of the IL-6 promoter. Accordingly, we subcloned this fragment into pGL3-Promoter (creating pGL3-IL6p(-225/-160)) and tested for its ability to confer AR-mediated inhibition on the heterologous SV40 promoter (Fig. 5C). The basal promoter activity of SV40 was unaltered by inclusion of the -225/-160 fragment. Furthermore, PMA did not induce promoter activity in either construct, and DHT did not reduce basal promoter activity of this construct. These results suggested that while the -225/-160 fragment was important in AR-mediated inhibition of IL-6 activation, it did not contain all the elements required to independently perform this activity.

On the surface, these data appear confusing, but based on recent work by other investigators, we feel we can offer an explanation for these results. Several investigators have noted the NF-IL6 site is required for strong NF κ B-mediated induction of the IL-6 promoter (56, 57). Therefore, when we delete the promoter to -160, which alters the region of the NF-IL6 response element, we lose the synergistic effect of NF-IL6 necessary for NF κ B-mediated activation of the IL-6 promoter. Thus, the -160 fragment is not strongly activated even though the NF κ B site is present; in fact, the activation is relatively weak compared with the other promoter constructs (Fig. 5). We hypothesize that because the minimal activation observed is not mediated (or minimally so) by NF κ B (due to the absence of NF-IL6's influence) the inhibitory action of androgen is not observed. We feel this accounts for why the -160 fragment, which contains the NF κ B site, is not repressed by DHT. Alternatively, DHT may repress the activity of other transcription factors, in addition to that of NF κ B, which activate the IL-6 promoter.

AR Inhibits Rel Family Member-induced Activation of the IL-6 Promoter—PMA is known to induce a variety of factors for which there are response elements on the IL-6 promoter (40, 58). To explore which transcription factors DHT may mediate its repressive effect on, we co-transfected HeLa cells with AR,

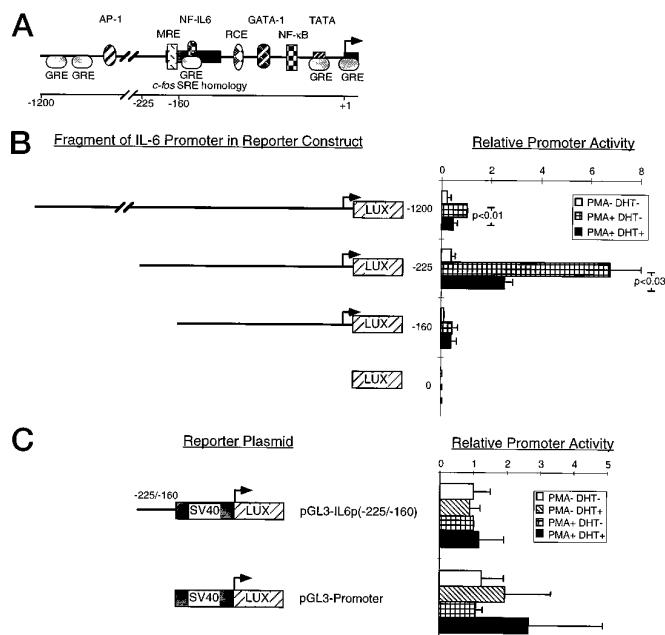


FIG. 5. A cis-acting region of the IL-6 promoter is necessary but not sufficient for AR-mediated inhibition. The 5'-region of the IL-6 promoter was progressively deleted by selective restriction digests of either pGL2-IL6(1200) followed by religation or the -225 to -165 fragment was excised and subcloned into pGL3-Promoter. To assess for activity, 4 μ g of these constructs, 4 μ g of CMV-AR, and 1 μ g of SV40- β -gal were used to co-transfect HeLa cells that were treated and harvested as described in Fig. 3. *A*, the IL-6 promoter region. Several response elements (confirmed and putative) are indicated. *Numbers* on the map indicate the digestion sites used to create deletions. *GRE*, glucocorticoid receptor response element; *MRE*, multiple response element; *NF-IL6*, nuclear factor IL-6. *B*, promoter activity in 5'-deletion mutants. Reporter plasmids contained promoter region indicated on left of the graph. These are aligned to their respective IL-6 promoter region above in *A*. Results are shown as promoter activity relative to that of pGL2-IL6p(-1200) in the presence of PMA. The data represent the mean \pm 1 S.E. from duplicates of two or more independent experiments. *C*, the -225 to -160 fragment of the IL-6 promoter was subcloned into pGL3-Promoter resulting in pGL3p-IL6p(-225/-160). Results are shown as promoter activity relative to pGL3p-IL6p(-225/-160) in the presence of PMA. Data are shown as the mean \pm 1 S.E. from two independent experiments each performed with duplicates.

pGL2-IL6p(-1200), and expression plasmids for several transcription factors (as indicated in Fig. 6). We found that all transcription factors were able to induce IL-6 promoter activity (data not shown). Yet, DHT did not inhibit NF-IL6 or c-Jun-mediated activation of the IL-6 promoter. However, DHT did inhibit the induction mediated by NF κ B p50, p65, and NF κ B (p50-p65 combination) by 47, 35, and 52%, respectively. These findings prompted us to explore how DHT modulates NF κ B-mediated activation of the IL-6 promoter.

The AR Does Not Bind to the IL-6 Promoter—One mechanism through which DHT may inhibit NF κ B's ability to stimulate IL-6 promoter activity is by activating AR to bind to the IL-6 promoter and occlude the NF κ B response element. To explore this possibility, we performed DNA footprinting using an AR DNA binding domain (DBD) peptide. We first confirmed that the AR DBD peptide was active in our experimental system by footprinting to the TAT ARE (Fig. 7). However, we did not observe a footprint in either the proximal 120 bases of the IL-6 promoter, which contains the NF κ B response element, or the -120 to -225 fragment of the IL-6 promoter.

DHT Inhibits NF κ B Complex Formation on the IL-6 NF κ B Response Element—To further define the mechanism by which DHT inhibits NF κ B-induced IL-6 promoter activity, we questioned if DHT could affect the ability of NF κ B to bind to the NF κ B response element present in the IL-6 promoter. We as-

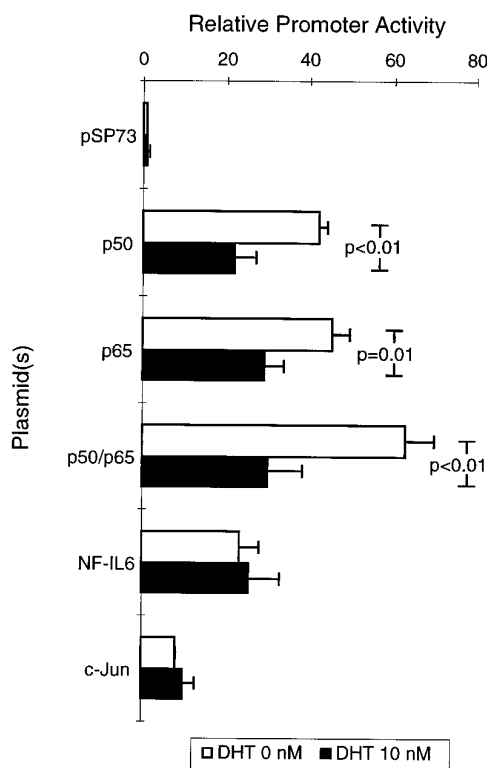


FIG. 6. DHT inhibits Rel family member-induced transcriptional activation of the IL-6 promoter. HeLa cells were co-transfected with 1 μ g of the indicated transcription factor expression plasmid or pSP73, 4 μ g of pGL2-IL6p(-1200), 4 μ g of CMV-AR, and 1 μ g of SV40- β -gal. The cells were then incubated in charcoal-stripped FBS with DHT (10 nM) or vehicle alone for 24 h followed by harvesting and reporter gene expression analysis as described in Fig. 3. Results are shown as promoter activity relative to that of pGL2-IL6p(-1200) with pSP73 in the absence of DHT. The data represent the mean \pm 1 S.E. from two or more independent experiments each containing duplicate plates.

sessed this by EMSA. LNCaP cells were either untreated, treated with PMA for 30 min, or incubated with DHT for 2 h followed by PMA treatment for 30 min. Nuclear extracts were obtained from these cells and incubated with radiolabeled NF κ B response element that is encoded within the IL-6 promoter. PMA treatment induced marked complex formation (Fig. 8, compare lanes 2 and 3). Preincubation of the PMA-treated nuclear extract with human AR (20) did not alter complex formation (Fig. 8, compare lanes 3 and 4). In contrast, DHT treatment of cells prior to PMA treatment resulted in marked attenuation of complex formation (Fig. 8, compare lanes 3 and 5). Preincubation of the nuclear extract from PMA-treated cells with 100-fold unlabeled consensus NF κ B competed away the complex (Fig. 8, lane 6), whereas 100-fold unlabeled nonspecific Oct 1 response element did not (Fig. 8, lane 7), thus demonstrating the specificity of the complexes for NF κ B. Furthermore, neither AR nor DHT pretreatment altered the degree of AP-1 or NF-IL6 complex formation (Fig. 8), thus demonstrating that DHT has some degree of specificity for blocking NF κ B binding activity.

DHT Maintains I κ B α Protein Expression—One mechanism to account for DHT's ability to decrease NF κ B complex formation, as observed in the EMSA, is the sequestration of NF κ B in the cytoplasm. Such a phenomenon has recently been reported for the glucocorticoid, dexamethasone, which induces I κ B α protein expression in HeLa cells thus resulting in sequestration of NF κ B in the cytoplasm (59, 60). To explore if DHT may work by a similar mechanism, we examined DHT's influence on I κ B α protein expression. In the absence of PMA, 10 nM DHT resulted

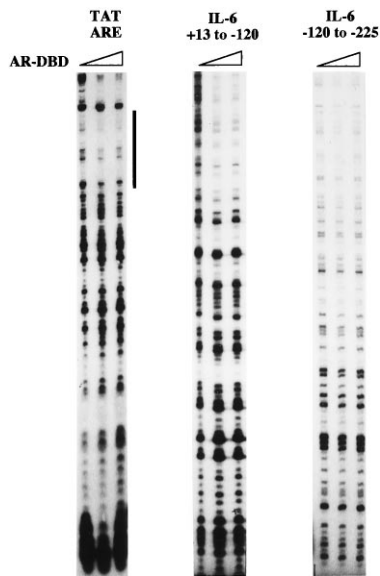


FIG. 7. Androgen receptor does not bind to the IL-6 promoter *in vitro*. The tyrosine aminotransferase (*TAT*) androgen response element (*ARE*) and the proximal (+13 to -120) and distal (-120 to -225) IL-6 promoter were subjected to DNase I footprinting with 0, 1, or 5 μ g (as indicated by wedge) of a synthesized AR DNA binding domain (*DBD*) peptide. The bar indicates the protected TAT ARE.

in approximately a 69% increase of I κ B α protein expression (Fig. 9, *A* and *B*, compare lane 1 with lane 3). More importantly, upon PMA stimulation even though I κ B α levels decreased, those cells pretreated with 10 nM DHT had approximately 80% greater I κ B α protein expression than cells not treated with DHT (Fig. 9, *A* and *B*, compare lane 2 with lane 4). These results demonstrate that DHT maintains I κ B α protein expression at a level comparable with that of nonstimulated cells. This finding raises the possibility that the higher level of I κ B α could result in increased cytoplasmic sequestration of NF κ B culminating in less activation of the IL-6 promoter.

I κ B α Inhibits NF κ B-induced IL-6 Promoter Activation—In order to confirm that I κ B α can mediate inhibition of the IL-6 promoter, we co-transfected HeLa cells with AR, pGL2-IL6p(-225), expression vectors for p50 and p65, and an expression vector encoding I κ B α followed by incubation of the transfected cells with DHT or vehicle alone (Fig. 9C). Additionally, CMV-Neo was used in treatment groups not receiving the I κ B α expression plasmid to control for the possible sequestration of transcription factors secondary to the further addition of CMV promoter that is present on the I κ B α expression plasmid. When the NF κ B subunits, p50 and p65, were used to co-transfect HeLa cells in the absence of I κ B α or DHT, we observed an average 22-fold increase over basal promoter activity (even in the presence of CMV-Neo). In the absence of exogenous I κ B α , DHT reduced this activation by approximately 48%. However, I κ B α reduced induction of the IL-6 promoter by approximately 80% in the presence or absence of DHT. We conclude based on these results that I κ B α can inhibit NF κ B-mediated IL-6 promoter activity.

DISCUSSION

In this report we demonstrate a novel mechanism through which an androgen inhibits *trans*-activation of a promoter. Specifically, DHT maintains steady state levels of a second protein that down-regulates expression of the IL-6 gene. Initially, we demonstrated that DHT inhibited IL-6 protein and mRNA expression in LNCaP cells. Furthermore, we have shown by a combination of transient transfection assays and EMSA that PMA and NF κ B-induced IL-6 gene expression were

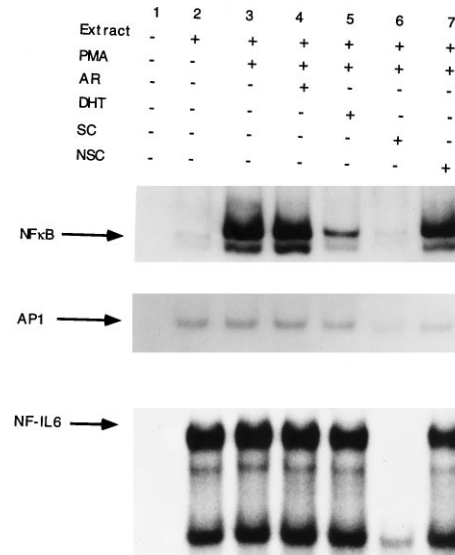


FIG. 8. DHT inhibits NF κ B complex formation on the IL-6 promoter. LNCaP cells were either untreated, treated with PMA (160 nM) for 30 min, or treated with DHT (10 nM) for 2 h followed by PMA (160 nM) for 30 min. After treatment, nuclear extracts were obtained and incubated with the 32 P-labeled IL-6 promoter NF κ B, AP-1, or NF-IL6 response element. Protein-DNA complexes were resolved by gel electrophoresis on a 12% native polyacrylamide gel. Bands were imaged by autoradiography for 6 h. In some cases, extract was preincubated with 20 μ l of protein purified from Sf9 cells transfected with a baculovirus vector encoding human AR (lane 4), 100 \times unlabeled specific competitor probe (lane 6), or 100 \times unlabeled nonspecific competitor Oct 1 response element probe (lane 7). No complex is noted when the probe is incubated in the absence of nuclear extract (lane 1).

inhibited at the transcriptional level through inhibition of NF κ B-DNA complex formation. Finally, we have determined that DHT maintained I κ B α protein expression that is capable of inhibiting IL-6 promoter activity thus accounting for a mechanism by which DHT inhibits NF κ B activity. This work contributes to the observations by several others regarding the role that steroids play in modulating IL-6 expression (44, 61, 62). For example, dexamethasone has been shown to inhibit IL-6 expression (44). Additionally, 17- β -estradiol has been demonstrated to inhibit IL-6 expression in bone-derived cells and endometrial cells (61, 62).

Several mechanisms have been demonstrated for glucocorticoid-mediated inhibition of the IL-6 gene *in vitro*. These include 1) occlusion of the MRE, TATA box, and transcription initiation site by GR (44), and 2) direct antagonism of NF κ B through protein-protein interaction between GR and NF κ B (p65) (63). The mechanism of estradiol-mediated inhibition of IL-6 gene expression has been demonstrated to occur at the level of transcription in the absence of high affinity binding of the estrogen receptor to the IL-6 promoter (42, 43). This has been confirmed by a recent study that demonstrates that estrogen receptor mediates *trans*-repression of the IL-6 promoter by binding to C/EPB β (NFIL-6) and both p50 and p65 subunits of NF κ B (64).

Mechanisms of transcriptional inhibition for a variety of steroids and their nuclear receptors have been well described (reviewed in Refs. 31, 65, 66). Androgens down-regulate expression of a variety of genes. These include carboxyglutamic acid (67), testosterone-repressed prostatic message 2 (12), *c-fos* and *c-myc* (13), low affinity nerve growth factor receptor (LNGFR) (9), and AR (17). However, reports on mechanisms of AR-mediated inhibition are sparse. DHT can mediate down-regulation of basal and forskolin-induced activity of a 1300-bp fragment from the 5'-flanking region of the murine AR (17). Deletion of the only consensus ARE present in this fragment

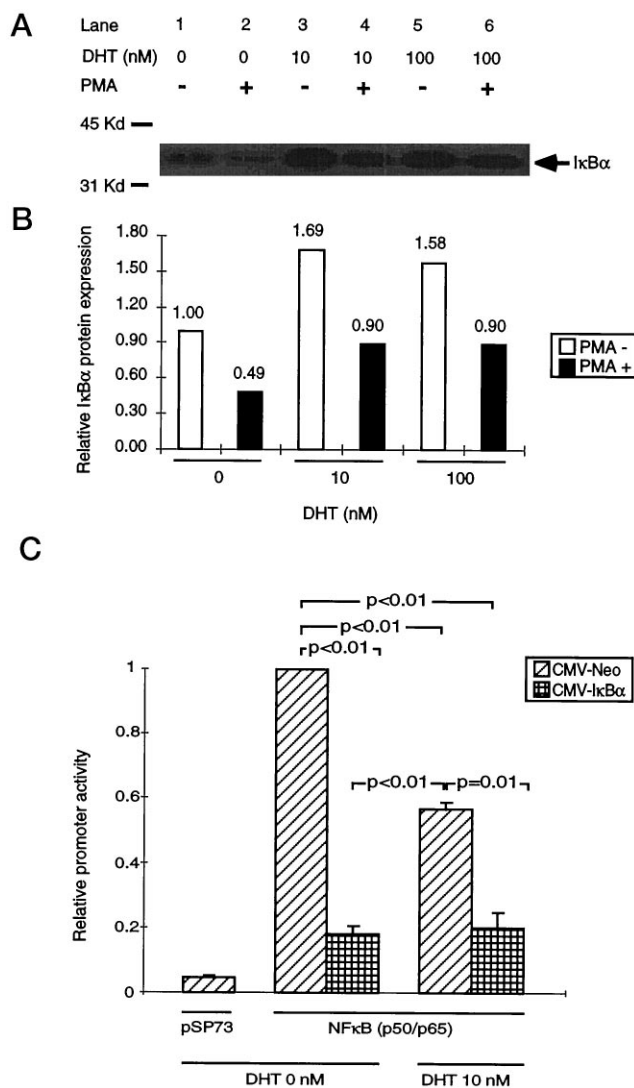


FIG. 9. I κ B α protein levels are maintained by DHT and I κ B α can inhibit IL-6 promoter activation. *A*, LNCaP cells in DMEM supplemented with 10% charcoal-stripped FBS were treated for 2 h with the indicated dose of dihydrotestosterone (DHT) followed by treatment with either vehicle or 16 α phorbol myristate acetate (PMA) for 30 min. Total cell lysates were obtained as described under "Experimental Procedures." Lysates, containing 100 μ g of protein/lane, were electrophoresed on a 10% SDS-polyacrylamide gel and electrotransferred to a polyvinylidene fluoride membrane. The membrane was probed with rabbit anti-human I κ B α . Bound antibody was detected with goat anti-rabbit alkaline phosphatase-conjugated secondary antibody followed by detection with chemiluminescence. *B*, bands from *A* were quantified by video densitometry. Columns correspond to bands above them in *A*. Numbers above columns indicate protein level relative to untreated cells (PMA $^-$, DHT 0 nM). Results are reported as average of duplicates. *C*, HeLa cells were transfected, as described in Fig. 3, with 4 μ g of pGL2-IL6p(-1200), 4 μ g of CMV-Neo or CMV-I κ B α , and 1 μ g of p50 and 1 μ g of p65, or 2 μ g of pSP73 as indicated. Twenty-four hours after transfection, 10 nM DHT or vehicle alone was added, and cells were incubated for another 24 h, then harvested, and processed for β -gal and luciferase assays as described in Fig. 3. Results are reported as promoter activity relative to p50-p65-stimulated cells in the absence of I κ B α and DHT. The data represent the mean \pm 1 S.E. from two independent experiments each containing duplicate plates.

did not alter DHT's ability to repress the promoter suggesting that 1) protein-protein inhibition, 2) the presence of a cryptic ARE site in the promoter, or 3) induction of a secondary protein were possible mechanisms for DHT's action.

A fragment between -860 and -394 bp from the translation initiation site of the LNGFR gene conferred androgen-induced

repression on the thymidine kinase promoter (10). Two palindromic inverted repeats that share some identity to hormone receptor-responsive elements were identified within this fragment. However, their role in mediating the androgen-induced repression has not been established to date. When examined in context of the reporter vector pBLCAT₃ the LNGFR promoter was again shown to be down-regulated by androgen (11). However, the presence of *cis*-acting elements within the promoter was not confirmed, and in fact it was determined that down-regulation was occurring through an AP-1-like element present within the vector. Further examination revealed that AR inhibited the interaction of c-Jun with the AP-1 site if AR was present prior to c-Jun binding the AP-1 site. Interestingly, c-Jun did not inhibit AR-ARE interaction. These data suggest that AR induces a secondary protein that inhibits c-Jun activity. However, a direct interaction between c-Jun and AR has not been ruled out. While in the current study the possibility of direct protein-protein interactions was not eliminated, we demonstrated increased levels of a protein known to inhibit NF κ B activity, namely I κ B α .

Our first clue that DHT inhibited NF κ B activity was in the transfection experiments in which the activation of the IL-6 promoter by NF κ B subunits and NF κ B but not NF-IL6 or c-Jun was inhibited. We noted that the inhibition in these experiments was between 32 and 52% which does not account entirely for the amount of inhibition (62%) noted for the PMA-stimulated pGL2-IL6p(-225) (Fig. 5*B*). One possible explanation for this discrepancy may be that PMA induces other transcription factors, which we have not evaluated, and DHT inhibits their activity also. This would result in an overall greater inhibition of IL-6 promoter activity than we observe for the NF κ B proteins alone. Carefully planned plasmid dose titration experiments may help address some of these unresolved issues.

Our finding that incubation of PMA-induced nuclear extract with wild-type AR did not alter NF κ B-DNA complex formation (Fig. 8) suggests that direct protein-protein inhibition by AR of NF κ B does not occur in the nucleus. This finding in combination with the observation that incubation of LNCaP cells with DHT prior to PMA treatment did inhibit NF κ B-DNA complex formation (Fig. 8) suggests that NF κ B was sequestered in the cytoplasm. This hypothesis is further supported by our observation of DHT-maintained I κ B α protein expression. Whether AR maintains I κ B α levels through induction of protein expression or inhibits degradative phosphorylation of I κ B α was not determined in the current study. However, observations are consistent with the recent reports (59, 60) that glucocorticoids induce I κ B α expression and inhibit NF κ B p65 nuclear translocation. Dexamethasone increased I κ B α steady state mRNA levels, but whether this is due to increased mRNA stability or increased transcriptional activity is not clear. Examination of the human I κ B α promoter (68, 69) by computer analysis did not reveal steroid response elements.^{22;10} This finding suggests that if activated AR or glucocorticoid receptor stimulate transcription from the I κ B α gene, then they do so indirectly.

I κ B α (also known as MAD3) is a member of the I κ B family of proteins that are characterized by the presence of ankyrin repeat domains (reviewed in Refs. 70, 71). The I κ B ankyrin repeat domains are a series of approximately 30 amino acid motifs that provide an interaction site for I κ B and transcriptional activators of the Rel family. I κ B proteins demonstrate subunit specificity for inhibition of Rel family member transcription factor complexes (summarized in Ref. 72). In our experiments, we observed that DHT inhibited activation of the IL-6 promoter when expression plasmids encoding p50 or p65 alone or in combination were used for transfection. Previous reports that I κ B α is capable of inhibiting either DNA binding of

or activation by the p50-p65 and p65-p65 complexes (73–76) combined with our observation that I κ B α repressed NF κ B-induced activation of the IL-6 promoter (Fig. 9B) suggest that the I κ B α induced by DHT in our system accounts for the inhibition of either p65 homodimers or p50-p65 heterodimers that may have formed. Additionally, the observation that DHT did not increase the repression mediated by I κ B α (Fig. 9B) is consistent with our hypothesis that DHT mediates repression of the IL-6 promoter through I κ B α . An alternative explanation is that transfection induced a high level of expression of I κ B α that resulted in squelching, thus not allowing for DHT's effects to be realized.

In contrast to its interaction with p65, I κ B α does not appear to bind to p50 (75); thus, its induction does not account for DHT-mediated inhibition of p50 homodimers that may have formed in those cells transfected with the p50 plasmid alone. A possible explanation for the DHT-mediated inhibition observed in this treatment group is that p50 homodimers did not form or were not active in our system, rather activation of the IL-6 promoter was induced by p50-p65 heterodimers induced by overexpression of p50. In this situation, I κ B α would be capable of inhibiting the IL-6 promoter activation. An alternative possibility is that DHT induced other proteins, such as I κ B γ , Bcl3, or p105, which can inhibit p50 heterodimers (summarized in Ref. 72). Evaluation for changes in the expression of these proteins may provide clues to this problem. Finally, in addition to induction of the I κ B proteins that sequester NF κ B, inhibition of I κ B α phosphorylation, and thus its degradation, may play a role in DHT's action.

The promoter mapping data depicted in Fig. 5 is difficult to interpret. Our results show that the region between –225 and –160 contributes to DHT-mediated repression of the IL-6 promoter, yet does not confer this property onto a heterologous promoter. Together, these observations suggest that the component within the –225/–160 region that mediates DHT's inhibitory effect interacts with a component within the –160 promoter fragment (*e.g.* NF- κ B response element) to achieve this action. Additionally, the observation that the –160 construct demonstrates only a weak response to PMA *versus* the response observed for the –225 construct suggests that there is some component of the –225/–160 fragment that contributes to NF κ B-induced IL-6 activation of the IL-6 promoter. The sum of these data support the contention that the –225/–160 fragment contributes to both activation and DHT-mediated repression of the IL-6 promoter. However, this would appear to contradict the claim that DHT mediates inhibition through the NF κ B site located in the –160 fragment. This apparent contradiction can be resolved if we assume that the contribution from the –225/–160 fragment involves synergistic interaction with NF κ B and that DHT's major effect is to inhibit this synergistic interaction. In fact, it has been demonstrated that NF-IL6, whose response element is located immediately adjacent to –160, synergizes with NF κ B to amplify the response mediated by the NF κ B site on the IL-6 promoter (56, 57, 77). This would be consistent with the observation that deletion of the –225/–160 fragment, which alters the context of the NF-IL6 site, results in marked diminution of the PMA-induction of the promoter. Alternatively, DHT may inhibit the action of other transcription factors that activate the IL-6 promoter.

The observation that the –160 construct, which still responds to PMA, albeit weakly, is not inhibited by DHT even though the NF κ B response element is found in this region suggests that PMA stimulates the –160 fragment through a mechanism in addition to NF κ B activation. Thus, we may be observing induction of the –160 fragment unrelated to NF κ B, which, accordingly, would be unresponsive to DHT-mediated

inhibition. This would be consistent with our observation that DHT does not repress either the –1200 or the –225-containing constructs 100%, because stimuli, other than those repressed by DHT, are effective.

That we did not observe an area of protection on the IL-6 promoter by DNase protection assay (Fig. 7) was somewhat surprising in light of the fact that GR has been reported to bind several sites on this promoter (44). The previous reports that isolated AR DBD can effectively produce a footprint protection pattern of C3(1) and C3(2) ARE (78, 79) and our finding that it can protect the TAT-ARE support the contention that the assay was effective. Perhaps the fact that the GREs in the IL-6 promoter are not consensus GRE and in fact represent sites of imperfect palindromic sequence results in the inability of AR to bind to these sites. Furthermore, two of the sites, the MRE II and the TATA box, possess only two spacer nucleotides between the response element half-sites (44) as opposed to the three observed in all currently recognized ARE (reviewed in Ref. 21) which suggests that a less than optimal interaction may occur. Based on our results we cannot discount that either full-length AR is required to observe binding to the promoter or that there is a weak interaction between the AR DBD and the IL-6 promoter that may require accessory factors to stabilize the interaction. Regardless of the reason for a lack of protection by AR DBD on the IL-6 promoter, our hypothesis that DHT results in the sequestration of NF κ B in the cytoplasm does not invoke the requirement of AR binding to the IL-6 promoter.

IL-6 is a multifunctional cytokine with impact on a wide variety of body systems (reviewed in Ref. 80). Dysregulation of its expression may result in a variety of disorders including osteoporosis, B-cell lymphoma, hot flashes, and autoimmune disease. Although its role in prostate cancer is not clear several lines of evidence suggest it may be important in either progression of prostate cancer (81–85) or in mediating morbidity associated with prostate cancer (86). Often the first line of treatment in prostate cancer is androgen deprivation therapy by chemical-induced androgen blockade or orchiectomy. Our results suggest that androgen deprivation therapy may result in overexpression of IL-6 and thus initiate a variety of pathophysiologic states. This finding is supported by our observation that orchiectomy in mice results in increased serum IL-6 levels (39) and the observation that orchiectomy can induce osteoclastogenesis that is inhibited by IL-6-neutralizing antibody (38). Furthermore, bone mineral density and bone mineral content were significantly decreased following orchiectomy in a group of men treated for prostate cancer (87). Intriguingly, if orchiectomized men were supplemented with estrogen, which has been shown to inhibit IL-6 promoter activation (42, 43), the bone loss was not observed (87). Because of IL-6's effects on bone metabolism, its overexpression by prostate cancer cells in the androgen-deprived state may create in the bone microenvironment changes that are conducive for metastatic growth or the development of osteoporosis. Further studies will be required to elucidate these issues.

In conclusion, our data support that DHT, through the AR, maintains I κ B α protein levels, resulting in continued sequestration of NF κ B in the cytoplasm. This effectively contributes to the suppression of IL-6 promoter activation. The mechanism by which DHT maintains I κ B α protein expression remains to be determined but may involve either increased transcription of I κ B α mRNA or decreased degradation of I κ B α protein, perhaps through inhibition of I κ B α phosphorylation.

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