

Brief Report

ANDROGEN-INSENSITIVITY SYNDROME
AS A POSSIBLE COACTIVATOR DISEASE

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ANDROGEN-insensitivity syndromes in 46,XY fetuses result in various degrees of impairment in genital virilization.¹ These syndromes are caused by mutations in the androgen receptor gene that result in decreased binding of androgen to the receptor.²⁻⁹ As a consequence, the transcriptional activity of the androgen-androgen-receptor complex is reduced, and therefore, genital virilization is reduced. The androgen receptor, like other steroid hormone receptors, has two major transactivation domains¹⁰ — activation function 1 (AF-1) in the N-terminal region¹¹⁻¹³ and activation function 2 (AF-2) in the C-terminal ligand-binding domain¹⁴ — that interact with the target genes directly as well as indirectly by means of intermediary coactivators.¹⁵

We describe a patient in whom the complete androgen-insensitivity syndrome was diagnosed on the basis of phenotypic and endocrinologic findings, but who had no mutations in the androgen receptor gene. Detailed studies revealed that transmission of the activation signal from the AF-1 region of the androgen receptor was disrupted, suggesting that a coactivator interacting with the AF-1 region of the androgen receptor was lacking in this patient.

CASE REPORT

A 19-year-old woman reported primary amenorrhea. The patient had normal breast development and normal female external genitalia, but she had no pubic or axillary hair, and the vagina was short (6 cm in length) and ended in a blind pouch. Abdominal exploration revealed no uterus, but testes were present, which were resected. Histologic examination of the testes revealed small numbers of immature Sertoli cells and germ cells and a moderate num-

ber of Leydig cells. Preoperatively, the patient's serum testosterone concentration was 614 ng per deciliter (21.3 nmol per liter) and her serum 5 α -dihydrotestosterone concentration was 49 ng per deciliter (1.7 nmol per liter); both values were within the normal range for men. The karyotype was 46,XY. The patient was given a diagnosis of complete androgen-insensitivity syndrome. Her two older sisters were not affected.

METHODS

Analysis of the Androgen Receptor

The study was approved by the local institutional review committee, and written or oral informed consent for a genital-skin biopsy was obtained from the patient, another patient with complete androgen-insensitivity syndrome, and five normal men. Primary culture of genital-skin fibroblasts, androgen-binding assays, and sequence analysis of the androgen receptor gene were performed as previously described.^{4,9} Tissue concentrations of androgen receptor messenger RNA (mRNA) were determined by a quantitative reverse-transcriptase-polymerase-chain-reaction assay (RT-PCR) as described previously.^{16,17}

Plasmid Construction and Reporter Assay

We constructed a firefly-luciferase-reporter vector (pGL3-MMTV), which was under the control of the mouse-mammary-tumor virus (MMTV) promoter, by inserting the mouse-mammary-tumor virus long terminal repeat promoter¹⁸ into a pGL3 basic vector (Promega). The expression vectors for the human androgen receptor,⁴ the human glucocorticoid receptor,¹⁹ and the C-terminal-truncated glucocorticoid receptor²⁰ were constructed as described previously. We used PCR techniques to assemble the expression vectors for an androgen receptor-glucocorticoid receptor chimera, a glucocorticoid receptor-androgen receptor chimera, a C-terminal-truncated androgen receptor, and an N-terminal-truncated androgen receptor and glucocorticoid receptor. To construct the expression vectors for p300,²¹ transcriptional intermediary factor 2,²² full-steroid-receptor coactivator-1,²³ androgen-receptor-associated protein,²⁴ (also referred to as ELE1 α ²⁵), and steroid-receptor RNA activator,²⁶ the complementary DNAs (cDNAs) of human origin were cloned and inserted into a pCDNA3.1 plasmid (Invitrogen).

Genital-skin fibroblasts cultured in six-well plates (0.3×10^6 cells per well) were transfected with 7 μ l of SuperFect reagent (Qiagen) per well; we used 1.5 μ g of the firefly-luciferase-reporter vector (pGL3-MMTV) per well as the reporter and 3 ng of pRL-CMV vector (a *Renilla* luciferase vector, Promega) per well as the internal control. Depending on the experiment, we added to each well 0.2 μ g of the expression vector for the androgen receptor, the glucocorticoid receptor, or the mutated receptors. Starting 3 hours after transfection, the cells were incubated for 48 hours in Dulbecco's minimal essential medium with 10 percent charcoal-treated fetal-calf serum in the presence or absence of 10^{-7} M 5 α -dihydrotestosterone or 10^{-7} M dexamethasone and were then solubilized with 150 μ l of lysis buffer (Promega). The activities of the reporter gene were determined by a commercial kit (the Dual-Luciferase Reporter Assay System, Promega), and the values were adjusted for the activity of the internal control (*Renilla* luciferase activity). We used one-way analysis of variance followed by Scheffé's test for multigroup comparisons.

Glutathione S-Transferase Assay

We prepared glutathione S-transferase-fused AF-1 proteins (involving amino acid residues 1 to 532 of the androgen receptor and 13 to 438 of the glucocorticoid receptor) using a baculovirus expression vector system (PharMingen). Fibroblasts from the patients and the control subjects, cells from an androgen-independent prostate-cancer line (LNCaP), and CV-1 cells were incubated with 500 μ Ci of [³⁵S]methionine per milliliter for 16 hours at 37°C. The ³⁵S-labeled cellular extracts were then incubated with the glutathione S-transferase-fused AF-1 proteins for 16 hours at 4°C.

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Molecules bound to glutathione *S*-transferase–fused AF-1 protein were purified with glutathione Sepharose 4B (Pharmacia) and subjected to sodium dodecyl sulfate–polyacrylamide-gel electrophoresis. Dried gels were exposed to an imaging plate (Fuji) for three weeks, and the images were analyzed with a fluorescent-sample imager (Storm Fluorimager, Molecular Dynamics).

RESULTS

Characterization of the Androgen Receptor

When the ligand-binding properties of the androgen receptor in primary cultures of genital-skin fibroblasts from the patient were examined, the maximal binding capacity of the labeled androgen analogue [³H]mibolerone was 9210 sites per cell, and the apparent dissociation constant was 0.52 nM, values that were close to the mean (\pm SD) values in the five normal men (mean maximal binding capacity, 9305 ± 2030 sites per cell; dissociation constant, 0.56 ± 0.21 nM).⁴ The androgen receptor was not thermolabile.⁵⁻⁷ The rate of nuclear translocation of the bound androgen receptor and the stability of the transfected androgen receptor were similar in fibroblasts from the patient and fibroblasts from the normal subjects when determined with use of a chimera of the androgen receptor and the green fluorescent protein²⁷ (data not shown). The mRNA concentration of the androgen receptor was also similar in the fibroblasts from the patient and fibroblasts from the normal subjects (data not shown). Direct sequencing of the PCR products for the exons of the androgen receptor gene and the coding region of the androgen-receptor cDNA prepared from the patient's fibroblasts revealed no mutations in the androgen receptor gene.

Activation of Transcription by the Androgen Receptor, the Glucocorticoid Receptor, Their Chimeras, and the Truncated Mutants

The absence of abnormalities in the androgen receptor gene in the patient suggested that the defect must be in the transmission of the transactivation signal from the ligand–androgen-receptor complex to the transcription machinery. To examine this possibility, we studied the ability of the normal androgen receptor to activate an MMTV–luciferase reporter gene in genital-skin fibroblasts (Fig. 1). The degree of transcriptional activation induced by the glucocorticoid receptor was similar in fibroblasts from our patient, the normal subjects, and another patient with complete androgen-insensitivity syndrome, who had a mutation in the androgen receptor gene⁹ (Fig. 1A). The degree of transcriptional activation induced by the normal androgen receptor in our patient's fibroblasts, however, was less than 9 percent of that in the normal fibroblasts, whereas the degree of activation induced by the normal androgen receptor in the fibroblasts from the patient with androgen-insensitivity syndrome caused by a mutation in the androgen receptor gene was similar to that in the normal subjects (Fig. 1B). These results indicated that in the fibroblasts

from the patient with androgen-insensitivity syndrome who had a mutation in the androgen receptor gene, as well as in those from the normal subjects, the system for the transmission of a transactivating signal from the androgen receptor was intact, whereas it was not intact in the fibroblasts from our patient. The degree of dexamethasone-dependent transcriptional activation induced by an androgen receptor–glucocorticoid receptor chimera, which consisted of the N-terminal domain and DNA-binding domain of the androgen receptor and the ligand-binding domain of the glucocorticoid receptor, in fibroblasts from our patient was 12 to 17 percent of that in the fibroblasts from the patient with androgen-insensitivity syndrome who had a mutation in the androgen receptor gene and the normal subjects (Fig. 1C). The degree of 5 α -dihydrotestosterone-dependent transcriptional activation induced by a glucocorticoid receptor–androgen receptor chimera consisting of the N-terminal and DNA-binding domains of the glucocorticoid receptor and the ligand-binding domain of the androgen receptor was similar in the fibroblasts from the patients and the normal subjects (Fig. 1D).

The transactivation function of the AF-1 region is ligand-independent and autonomous,¹²⁻¹⁴ and that of the AF-2 is ligand-dependent.^{14,15} The degree of ligand-independent transcriptional activation induced by the AF-1–containing N-terminal fragment of the androgen receptor in our patient's fibroblasts was approximately 20 percent of that in the fibroblasts from the patient with androgen-insensitivity syndrome who had a mutation in the androgen receptor gene and the normal subjects (Fig. 2A), whereas the degree of activation induced by the N-terminal fragment of the glucocorticoid receptor was similar in the fibroblasts from the patients and the normal subjects (Fig. 2B). The degree of ligand-dependent transcriptional activation by the AF-2–containing C-terminal fragments of the androgen receptor and glucocorticoid receptor was also similar among the three types of fibroblasts (Fig. 2C and 2D). These findings indicated that the transmission of the transactivating signal from the AF-1 region of the glucocorticoid receptor in our patient was normal but the transmission from the AF-1 region of the androgen receptor was impaired, results that strongly suggested the existence of a defect in a coactivator specific for the AF-1 region of the androgen receptor.

The coactivators CREB(cyclic AMP–responsive element)-binding protein, p300, androgen-receptor–associated protein₇₀, full-steroid-receptor coactivator-1, transcriptional intermediary factor 2, and steroid-receptor RNA-activator increase androgen-receptor–induced transactivation.^{13,24-26,28,29} When these coactivators were also transfected in molar quantities that were greater by a factor of 1.5 to 3 than those of the androgen receptor or glucocorticoid receptor, the degree of transactivation induced by the andro-

gen receptor in the fibroblasts from our patient was still less than 13 percent of that in fibroblasts from the normal subjects. In contrast, the degree of transactivation induced by the glucocorticoid receptor was increased in the fibroblasts from our patient and the normal subjects.

Electrophoretic Analysis of the Molecules Interacting with the AF-1 Region of the Androgen Receptor

The glutathione *S*-transferase analysis revealed that ³⁵S-labeled proteins corresponding to an apparent molecular mass of 90 kd (Fig. 3) were interacting with the AF-1 region of the androgen receptor in the cellular extracts of the fibroblasts from a normal man (Fig. 3A and 3B, lane 3), LNCaP (Fig. 3A, lane 7), and CV-1 cells (Fig. 3A, lane 9). LNCaP and CV-1 cells are androgen-sensitive, as are normal fibroblasts.^{30,31} However, the 90-kd protein was not detected in the extracts of fibroblasts from our patient (Fig. 3A and 3B, lane 5). ³⁵S-labeled proteins corresponding to a molecular mass of 76 kd (Fig. 3) interacted with the AF-1 region of the glucocorticoid receptor in all the samples.

DISCUSSION

Our patient, who had the clinical and hormonal characteristics of complete androgen-insensitivity syndrome, had no abnormality in the androgen receptor gene. Detailed analyses of the action of androgen in the patient's fibroblasts indicated that the transactivation signal from the AF-2 region of the androgen receptor was transmitted normally to the basal tran-

scription machinery but that transmission of the activation signal from the AF-1 domain was disrupted. The finding that the deletion of 187 amino acid residues at the N-terminal of the androgen receptor, which includes the AF-1 region,^{11,12} caused complete androgen-insensitivity syndrome in another patient³ supports the conclusion that decreased transmission of a transactivation signal from the AF-1 region caused the syndrome in our patient. Since our patient's androgen receptor gene was normal, she must have had a deficiency in a coactivator necessary for the transfer of an activation signal from the AF-1 region of the androgen receptor to the transcription machinery.

Most of the known coactivators interact with the AF-2 region¹⁵ and are relatively nonspecific, in that they interact with multiple nuclear receptors. The protein type of coactivators, such as p300, androgen-receptor-associated protein₇₀, full-steroid-receptor coactivator-1, and transcriptional intermediary factor 2, all increase the transactivation of various nuclear receptors, including the androgen receptor, in the AF-2 region.^{13,24,25,28,29} However, induction of the expression of any of these coactivators did not correct the impairment in the transactivation function of the androgen receptor in the patient's fibroblasts.

Among the steroid hormone receptors, the structure of the N-terminal region varies, and this variation has been assumed to be responsible for the different effects elicited by the various receptors.³²⁻³⁴ Furthermore, the existence of an accessory coactivator that interacts specifically with the AF-1 region has been suggested. Recently, an RNA molecule, a steroid-

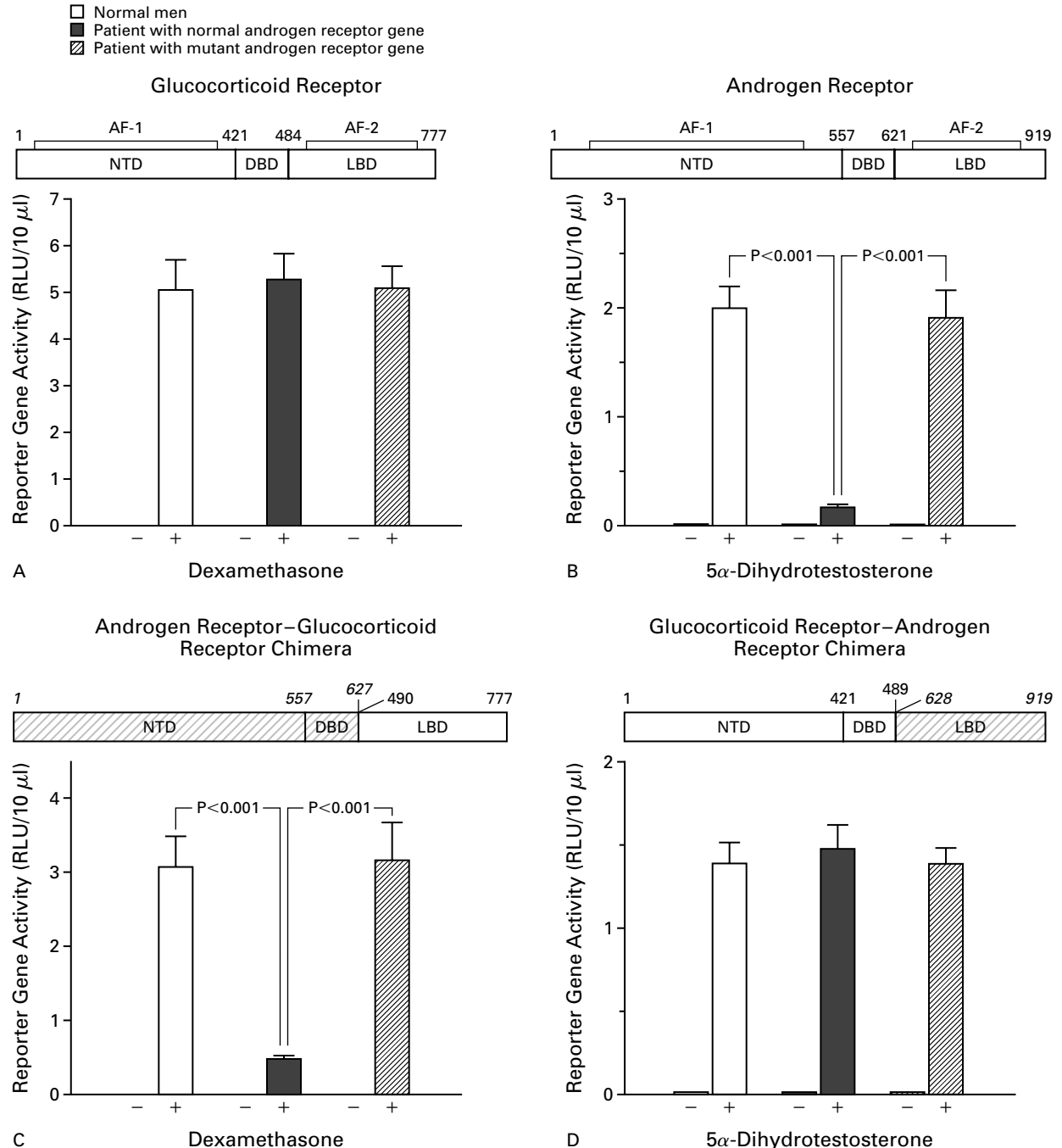
Figure 1 (facing page). Ligand-Dependent Transcriptional Activation by the Glucocorticoid Receptor (Panel A), the Androgen Receptor (Panel B), and Chimeras of the Glucocorticoid Receptor and the Androgen Receptor (Panels C and D) in Cultured Genital-Skin Fibroblasts from Five Normal Men, a Patient with Androgen-Insensitivity Syndrome Who Had a Normal Androgen Receptor Gene, and a Patient with Androgen-Insensitivity Syndrome Who Had a Mutation in the Androgen Receptor Gene.

In the fibroblasts from the patient with androgen-insensitivity syndrome who had a normal androgen receptor gene, the degree of transactivation by the transfected intact androgen receptor (Panel B) or the androgen receptor–glucocorticoid receptor chimera (Panel C) was low because of defects in a coactivator specific for the activation function 1 (AF-1) region in the N-terminal region of the androgen receptor. This coactivator is an essential part of the process of the transmission of the transactivating signal. In the fibroblasts of the patient with androgen-insensitivity syndrome who had a mutation in the androgen receptor gene,⁹ the endogenous androgen receptor is inactive, but the transfected androgen receptor (Panel B) and the androgen receptor–glucocorticoid receptor chimera (Panel C) are active, because a coactivator specific for the AF-1 region of the androgen receptor gene is present. The transfected glucocorticoid receptor (Panel A) and the glucocorticoid receptor–androgen receptor chimera (Panel D) are active in all the fibroblasts, because this coactivator is present. Each experiment was conducted in the presence (denoted by plus signs) or the absence (denoted by minus signs) of 10^{-7} M dexamethasone or 10^{-7} M 5α -dihydrotestosterone. Each bar represents the mean (\pm SD) of one experiment, which is representative of the results of five independent experiments. For each experiment, six dishes of fibroblasts from each subject were used. The mean activities of the reporter gene in the fibroblasts of the normal subjects were as follows: glucocorticoid receptor with 10^{-7} M dexamethasone, $202,414 \pm 18,653$ relative luciferase units (RLU) per $10 \mu\text{l}$ of sample; androgen receptor with 10^{-7} M 5α -dihydrotestosterone, $75,088 \pm 6832$ RLU per $10 \mu\text{l}$. From the five independent experiments, the reporter gene activities of the patient with the androgen-insensitivity syndrome who had a normal androgen receptor gene and the patient with androgen-insensitivity syndrome who had a mutation in the androgen receptor gene, relative to those in the normal subjects in the presence of ligand, were 108 ± 12 percent and 102 ± 13 percent, respectively, for the glucocorticoid receptor, and 9 ± 1 percent and 95 ± 10 percent, respectively, for the androgen receptor. The diagram above each graph shows the structure of the receptor. The italic numbers in the diagrams of the chimeric receptors represent the amino acid positions of the androgen receptor,¹⁰ and the roman numbers indicate the amino acid positions of the glucocorticoid receptor.¹⁹ The hatched regions and open regions in the chimeras represent the androgen receptor and glucocorticoid receptor origins, respectively. AF-2 denotes the activation function 2, NTD N-terminal domain, DBD DNA-binding domain, and LBD ligand-binding domain.

receptor coactivator,²⁶ has been shown to be an AF-1–specific coactivator of steroid receptors, including the androgen receptor, but supplementation with this molecule did not correct the impaired androgen-receptor–dependent transactivation in the fibroblasts of our patient. The absence of a 90-kd protein (which was present in fibroblasts from the normal subjects)

or the loss of the AF-1 region’s binding capability could be the cause of the androgen-insensitivity syndrome in our patient.

In conclusion, we have demonstrated that the transmission of a transactivating signal from the N-terminal region of the normal androgen receptor to the basal transcription machinery was disrupted in a pa-



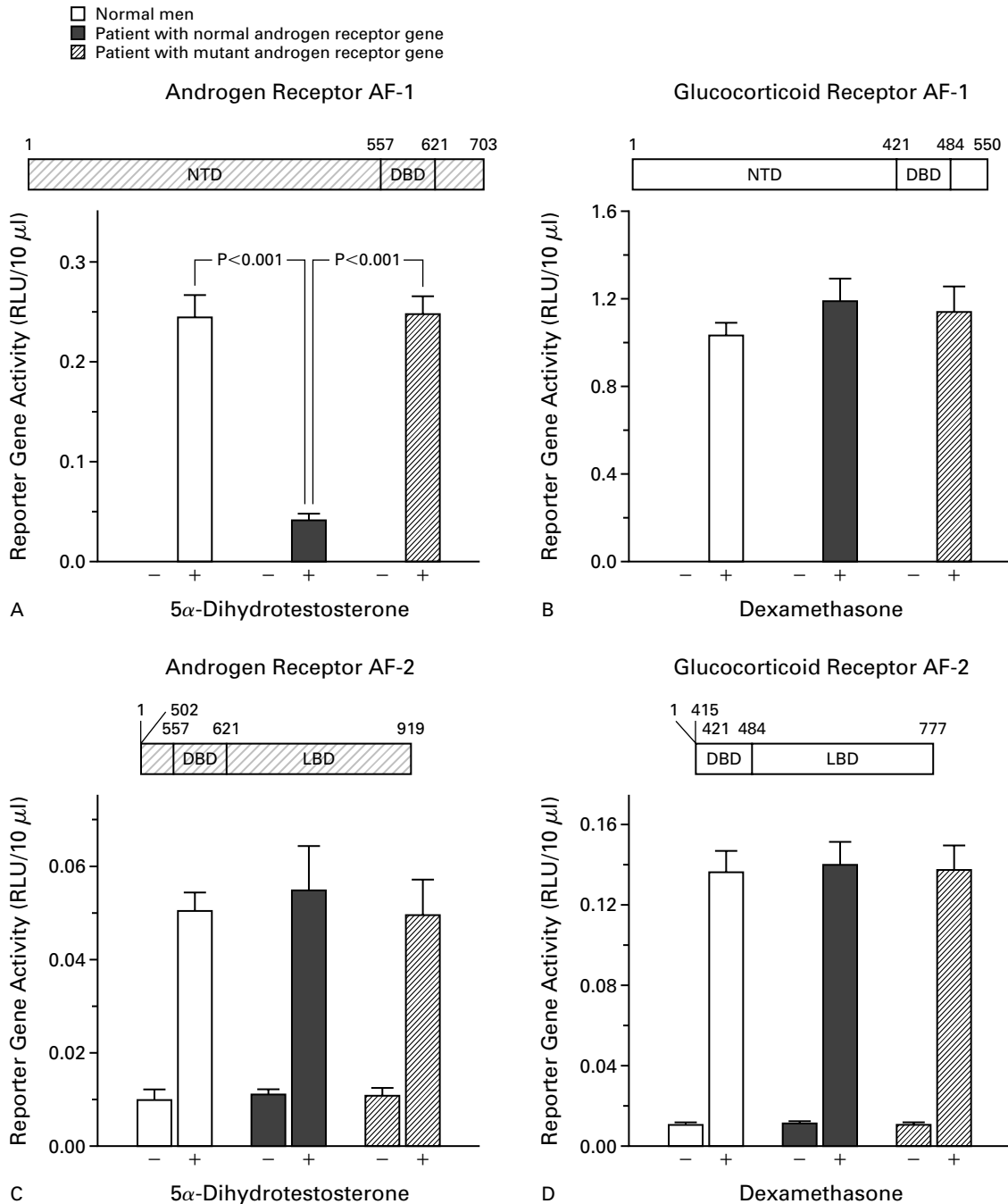


Figure 2. Transcriptional Activation by Truncated Androgen Receptors and Glucocorticoid Receptors in Cultured Genital-Skin Fibroblasts from Five Normal Men, a Patient with Androgen-Insensitivity Syndrome Who Had a Normal Androgen Receptor Gene, and a Patient with Androgen-Insensitivity Syndrome Who Had a Mutation in the Androgen Receptor Gene.

Panel A shows the degree of ligand-independent transactivation induced by the activation function 1 (AF-1) region of the androgen receptor. Panel B shows the degree of ligand-independent transactivation induced by the AF-1 region of the glucocorticoid receptor. Panel C shows the degree of 5α-dihydrotestosterone-dependent transactivation induced by the activation function 2 (AF-2) region of the androgen receptor. Panel D shows the degree of dexamethasone-dependent transactivation induced by the AF-2 region of the glucocorticoid receptor. Each experiment was conducted in the presence (denoted by plus signs) or the absence (denoted by minus signs) of 10⁻⁷ M dexamethasone or 10⁻⁷ M 5α-dihydrotestosterone. Each bar represents the mean (+SD) of one experiment, which is representative of the results of five independent experiments. For each experiment, six dishes of fibroblasts from each subject were used. The diagram above each graph shows the structure of the receptor. NTD denotes N-terminal domain, DBD DNA-binding domain, LBD ligand-binding domain, and RLU relative luciferase unit.

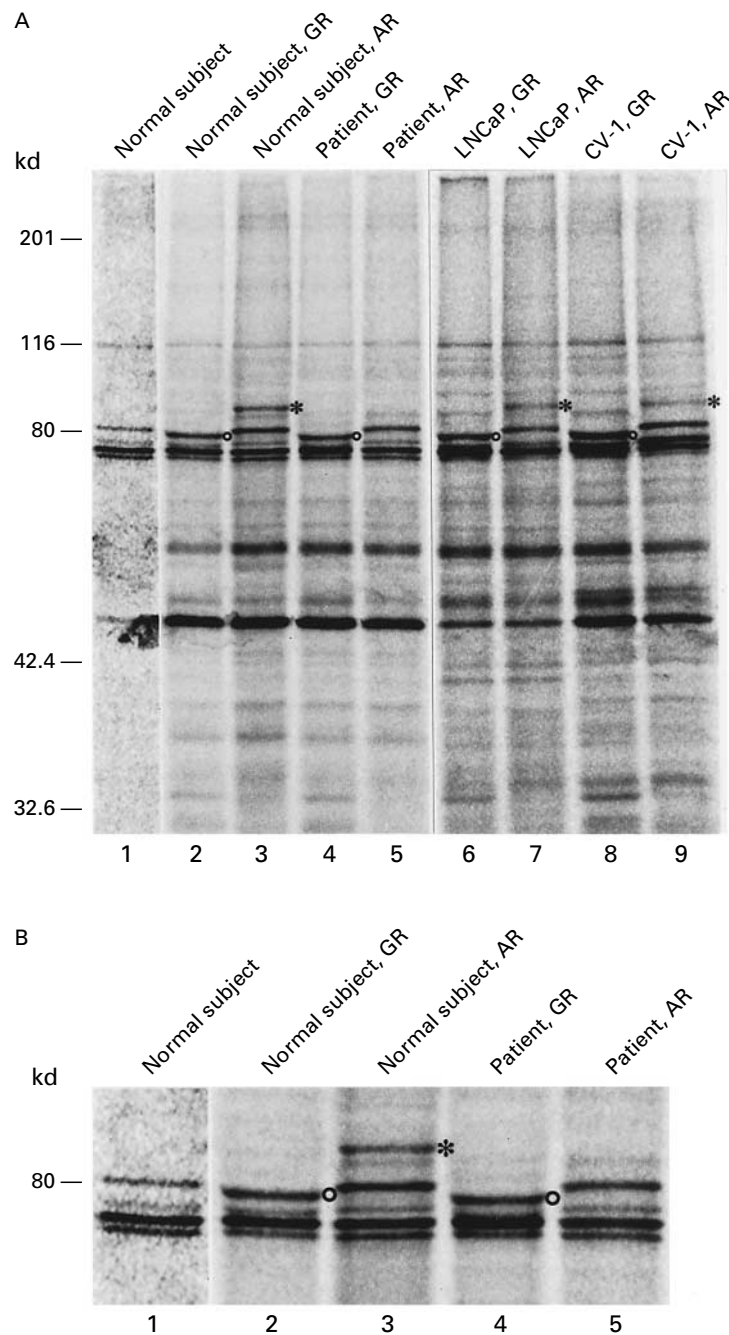


Figure 3. Electrophoretic Analysis of Proteins Bound to the Activation Function 1 (AF-1) Region of the Androgen Receptor (AR) and the Glucocorticoid Receptor (GR).

Glutathione *S*-transferase (lane 1 of Panels A and B), a glutathione *S*-transferase-fused AF-1 protein of the androgen receptor (lanes 3, 5, 7, and 9 of Panel A and lanes 3 and 5 of Panel B), or a glutathione *S*-transferase-fused AF-1 protein of the glucocorticoid receptor (lanes 2, 4, 6, and 8 of Panel A and lanes 2 and 4 of Panel B) was incubated with ³⁵S-labeled cellular extracts of genital-skin fibroblasts from four normal men and from our patient, an androgen-independent prostate-cancer cell line (LNCaP), and CV-1 cells. The bound proteins were subjected to sodium dodecyl sulfate–polyacrylamide-gel electrophoresis (7 percent separating gel). The asterisks indicate the 90-kd protein that bound specifically to the AF-1 region of the androgen receptor, and the circles indicate the 76-kd protein that bound specifically to the AF-1 region of the glucocorticoid receptor. The 90-kd protein was absent in the extracts of fibroblasts from our patient. Essentially the same 90-kd protein was detected in the extracts of fibroblasts from each of the four normal men.

tient with the androgen-insensitivity syndrome and that this disruption could not be corrected by supplementation with any known coactivators. We propose that there is a physiologically indispensable AF-1-specific coactivator crucial to the androgen receptor and that our patient had a newly identified form of steroid hormone insensitivity, a coactivator disease.

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CORRECTION

**Androgen-Insensitivity Syndrome as a Possible
Coactivator Disease**

Androgen-Insensitivity Syndrome as a Possible Coactivator Disease .
On page 860, in Figure 2, the labels under the x axis in Panels A and B
should be deleted, since 5 α -dihydrotestosterone and dexamethasone
were not added in these experiments. We regret the error.