Transcriptional Activation and Nuclear Targeting Signals of the Human Androgen Receptor*

(Received for publication, August 1, 1990)

Jorge A. Simental‡, Madhabananda Sar§, Malcolm V. Lane‡, Frank S. French‡, and Elizabeth M. Wilson‡†

From the Laboratories for Reproductive Biology and the Departments of §Pediatrics, §Cell Biology and Anatomy, and ‡Biochemistry, University of North Carolina, Chapel Hill, North Carolina 27599

The androgen receptor (AR) is a signal-transducing protein required for sexual differentiation, development, and expression of the male phenotype. A series of human AR deletion mutants were created either by site-directed mutagenesis using restriction enzyme digestion, the polymerase chain reaction, or, for a series of unidirectional NH2-terminal deletions, exonuclease III digestion. Receptor mutants were expressed in monkey kidney COS cells as truncated AR proteins between 20 and 107 kDa as revealed on immunoblots, where wild type AR was a doublet of 114 and 108 kDa. Subcellular localization by immunocytochemical staining demonstrated androgen-dependent nuclear uptake of AR from a perinuclear region of the cytoplasm. A nuclear targeting signal similar in sequence and position to the glucocorticoid receptor and homologous to the SV40 large T antigen was required for androgen-induced nuclear uptake of wild type AR. AR mutants lacking the NH2-terminal and/or steroid binding domains were constitutively nuclear with reduced transcriptional activity. Transcriptional activation by wild type AR was androgen-dependent in cotransfection studies of CV1 cells using the chloramphenicol acetyltransferase reporter gene linked to the mouse mammary tumor virus promoter. Deletion mutagenesis revealed within the NH2-terminal region a domain required for androgen-induced nuclear uptake of wild type AR. AR mutants lacking the NH2-terminal and/or steroid binding domains were constitutively nuclear with reduced transcriptional activity. Transcriptional activation by wild type AR was androgen-dependent in cotransfection studies of CV1 cells using the chloramphenicol acetyltransferase reporter gene linked to the mouse mammary tumor virus promoter. Deletion mutagenesis revealed within the NH2-terminal region a domain required for full transcriptional activity and within the steroid binding domain, an inhibitory function, deletion of which yielded a constitutively active receptor. Inhibition of wild type AR by coexpression with an inactive NH2-terminal fragment suggested competition for nuclear factors required for transcriptional regulation. These studies demonstrate a concerted interplay among the domains of the AR protein in regulating gene transcription.

The androgen receptor (AR) is a transcription enhancer

* This work was supported by Grants HD10690 and P30-HD18968 (recombinant DNA and histochemistry cores) from the National Institute of Child Health and Human Development Center for Population Research and National Institutes of Health Grant NS17479. 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.

† To whom correspondence should be sent: Laboratories for Reproductive Biology, CB 7500 MacNider Bldg., University of North Carolina, Chapel Hill, NC 27599. Tel.: 919-966-5159; Fax: 919-966-2423.

‡ The abbreviations used are: AR, androgen receptor; R1881, methyltrienolone; DS, sodium dodecyl sulfate; PCR, polymerase chain reaction; MMTV-CAT, mouse mammary tumor virus promoter linked to the chloramphenicol acetyltransferase gene; PBS, phosphate-buffered saline; NTS, nuclear targeting signal.

factor that belongs to a superfamily of nuclear receptors whose function is dependent upon the binding of cognate steroid hormones. Steroid receptors act as transcriptional activators by binding hormone regulatory elements of specific genes to trigger a cascade of transcriptional events (1-4). The functional domains of these ligand-activated transcription factors are undergoing intense investigation and have been clarified to varying degrees (5-12). Sequence comparison and deletion mutagenesis have revealed three major domains: a NH2-terminal region involved in transcriptional activation; a highly conserved, cysteine-rich DNA binding domain; and a carboxy-terminal steroid binding domain (1, 4). Subsequently, signal sequences for nuclear translocation were recognized in the hinge region between the DNA and steroid binding domains for the glucocorticoid and progesterone receptors (13, 14). Sites for receptor dimerization occur within the DNA and steroid binding domains of the mouse estrogen receptor (15, 16). The DNA binding region contains highly conserved amino acids critical for receptor function (17), the importance of which is exemplified by natural mutations in the AR gene in a patient with androgen insensitivity (18, 19) and in the vitamin D receptor gene in the syndrome of hypocalcemic vitamin D-resistant rickets (20). The variable amino acids within the DNA binding domain determine specificity for hormone response elements, particularly two amino acids in the P box toward the carboxyl side of the first zinc finger (21-23).

While the DNA binding domain is absolutely required for transcriptional activation (24, 25), dimerization (15, 26), and nuclear transport (14), sequences outside this region are involved in functional activity (27-29). Short acidic regions in the NH2- and COOH-terminal domains of the glucocorticoid receptor have roles in transcriptional activation which are position-independent and cooperative (28, 30). Sequences toward the carboxyl- and NH2-terminal ends of the estrogen and progesterone receptors determine receptor specificity for target genes in a cell-specific manner (27, 31, 32). Although the precise mechanism of transcriptional activation by steroid hormones is not well understood, it is likely that cooperative interactions occur between liganded activated steroid receptors and upstream transcription factors to form active transcription initiation complexes (33, 34). In this model, the transcriptional activator signals are reversibly inhibited by the ligand-free steroid binding domain (25, 28).

Studies on the estrogen receptor by King and Greene (35) and Welschon et al. (36) challenged the concept of steroid receptor transformation in association with receptor translocation to the nucleus. Their results suggested that nuclear localization of steroid receptors is independent of hormone binding, a concept later supported by studies on the progesterone receptor (14, 37). However, ligand-dependent nuclear
localization of the glucocorticoid receptor (13, 38) raised the possibility that not all steroid receptors follow the same pathway to the nucleus.

In the present report, truncation and deletion mutants of the human AR cDNA were created to characterize sequences involved in nuclear translocation and transcriptional activation. To functionally map potential nuclear targeting signals, mutant receptors were expressed in COS cells and assayed by immunocytochemistry using two polyclonal antibodies directed against epitopes in the NH\(_2\)-terminal region of AR (39). Ligand-dependent activation of gene transcription was assayed by cotransfection of wild type mutant receptors with a reporter gene in monkey kidney CV1 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following reagents were purchased: D-threo-[1,2-\(^{14}C\)]chloromethylpheno|lon (40-60 mCi/mmol) from ICN Biomedicals; 131I-protein A and [\(^3H\)]methyltrienolone ([17a-methyl-\(^3H\)]R1881, 80 Ci/\(\mu\)mol) from Du Pont-New England Nuclear; unlabeled deoxynucleotide triphosphates, acetyl coenzyme A, and DEAE-dextran from Pharmacia LKB Biotechnology Inc.; Taq polymerase from Perkin-Elmer Cetus; T4 polynucleotide kinase, T4 DNA ligase, and M13 bacteriophage from New England Biolabs; exonuclease III, T4 DNA ligase, and S1 nuclease from Promega-Biotec; restriction endonucleases from Promega-Biotec; restriction endonucleases from New England Biolabs; Dulbecco's modified essential medium with high glucose and fetal bovine serum from GIBCO; X-Omat-AR diagnostic x-ray film from Kodak Chemical Co.; buffers and chemicals from Fisher, Bethesda Research Laboratories, and Sigma. Stock cultures of CV1 and COS-7 cells were obtained from the American Type Culture Collection.

**Cell Culture and Biochemical Analysis**—Monkey kidney CV1 and COS-7 cells were maintained in 10% fetal calf serum in Dulbecco's minimal essential medium containing high glucose and antibiotics. COS cells were transfected using the DEAE-dextran method, and CV1 cells were transfected using calcium phosphate DNA precipitation as described previously (40). Immunoblot analysis of AR mutants expressed in COS cells was performed as described by Yarbrough et al. (40). Binding of [\(^3H\)]R1881 was measured after culturing transfected COS cells in serum-free medium 1 day before harvest. Cells were incubated with 5 \(\mu\)M [\(^3H\)]R1881 for 2 h at 37 \(^\circ\)C in the presence or absence of 100-fold excess unlabelled R1881. Free steroid was removed, and the cells were washed in phosphate-buffered saline and harvested in SDS sample buffer. Aliquots were counted for radioactivity with specific binding representing the difference between counts in the presence and absence of excess unlabelled hormone.

**Materials and PCR-mediated Mutagenesis**—The full-length human AR coding sequence was constructed in pCMV by ligating three fragments of 5' overlapping genomic clones containing the first exon (41) and a partial 3' cDNA clone (42). AR1 (p6HBhAR-A) was made by subcloning the full-length human AR cDNA BglII-BglII blunt-ended fragment into BglII-HindIII blunt-ended restriction sites of the eukaryotic expression vector pCMV5 (43). AR mutants in pCMV5 are driven by the human cytomegalovirus (Towne strain) early gene promoter and contain the human growth hormone transcription termination and polyadenylation signals. Mutants AR2 (amino acids 72-919), AR3 (amino acids 1-411), and AR4 (amino acids 338-919) are NH\(_2\)-terminal deletions of AR1 prepared by unidirectional exonuclease III digestion as recommended by Promega Biotec (44). A translation initiation Kozak sequence was inserted into the BglII site of AR1 prior to the exonuclease III digestions. AR5 (amino acids 507-919) lacks most of the NH\(_2\)-terminal domain and was created by inserting the KpnI-BamHI fragment of AR1 into the KpnI-BamHI sites of pCMV5. Human AR contains an endogenous translation initiation sequence at methionine 507 which acts as an initiation site for AR. AR6 (amino acids 566-919) is a partial clone used in the initial characterization of AR cDNA (42). Mutants AR7, -8, -9, and -10 were created by site-directed mutagenesis as described below. AR11 (amino acids 1-660) was made by digesting AR1 with Thnl1, blunt-ended, and digested with Smal to produce a receptor lacking the steroid binding domain. AR12 (amino acids 1-507) was created by digesting AR1 with KpnI and BamHI to delete the NH\(_2\)-terminal binding domain. The fragment was blunt-ended and religated to produce a vector containing only the NH\(_2\)-terminal region. AR13 (amino acids 507-660) contains the DNA binding domain/hinge region and was made by digestion of AR5 with Thnl1 and blunt-ended and digested with Smal. Truncated mutants were sequenced to verify the correct reading frame. Vector DNA was amplified in DH5\(\alpha\) Escherichia coli cells.

A rapid method for site-specific mutagenesis using the polymerase chain reaction (PCR) (-47) generated AR7, -8, -9, and -10 and the following mutants: AR12 (amino acids 1-660) was used in the initial characterization of AR cDNA (42). Mutants AR7, -8, -9, and -10 were created by site-directed mutagenesis as described below. AR11 (amino acids 1-660) was made by digesting AR1 with Thnl1, blunt-ended, and digested with Smal to produce a receptor lacking the steroid binding domain. AR12 (amino acids 1-507) was created by digesting AR1 with KpnI and BamHI to delete the NH\(_2\)-terminal binding domain. The fragment was blunt-ended and religated to produce a vector containing only the NH\(_2\)-terminal region. AR13 (amino acids 507-660) contains the DNA binding domain/hinge region and was made by digestion of AR5 with Thnl1 and blunt-ended and digested with Smal. Truncated mutants were sequenced to verify the correct reading frame. Vector DNA was amplified in DH5\(\alpha\) Escherichia coli cells.

**RESULTS**

**Mutagenesis of AR**—The full-length human AR expression vector (Fig. 1) was used to generate a variety of deletion mutants of the receptor in order to identify and further...
Mutagenesis and Functional Analysis of the Androgen Receptor

512 Mutagenesis and Functional Analysis

of entire AR expression vector, designated AR1, is 7.3 kilobases (kb) in length and was used as parent vector for site-directed mutagenesis.

analyze domains required for receptor function. The restriction enzyme sites unique to the vector and coding sequence are indicated. The vector contains the cytomegalovirus promoter (CMV), the human growth hormone transcription termination and polyadenylation signal (hGH), the SV40 origin of replication (SV 40), the fl origin of replication, and the ampicillin resistance gene (amp)

Due to blunt end ligation. The insert was digested with BglII, made blunt ended, and digested with BglII prior to insertion. The ligated vector was used to transform DHE E. coli and was sequenced directly using single-stranded sequencing from the fl origin. Several restriction enzyme sites indicated in Fig. 1 facilitated the construction of site-directed mutagenesis.

FIG. 1. Schematic diagram of the full-length wild type human AR1 expression vector. The 3.1-kilobase full coding sequence of human AR was inserted into the pCMV5 expression vector (4.2 kilobases) at the BglII and HindIII sites. The HindIII site was lost due to blunt end ligation. The insert was digested with BglII, made blunt ended, and digested with BglII prior to insertion. The ligated vector was used to transform DHE E. coli and was sequenced directly using single-stranded sequencing from the fl origin. Several restriction enzyme sites indicated in Fig. 1 facilitated the construction of site-directed mutagenesis.

Transient Expression of Mutant AR Proteins in COS Cells— Mutant AR proteins expressed in COS cells were analyzed on immunoblots to determine their approximate molecular weights. Two antipeptide antibodies facilitated AR detection: one was raised against a 15-amino acid sequence (amino acids 544–559) adjacent to and 5' of the DNA binding domain (51) positioned in exon B (52); a second antibody was to a 21-amino acid peptide near the NH2-terminal (amino acids 9–29) (39). The full-length wild type receptor migrated as a doublet of 114 and 108 kDa (Fig. 2, lane 2), possibly reflecting different degrees of receptor phosphorylation. Expression of AR mutants containing major domain truncations or serial deletions of the NH2-terminal domain resulted in AR proteins reduced in size as shown in Fig. 2 for AR2, -3, -4, and -5 (lanes 3–6). The smallest AR mutant with deletions of both the NH2- and COOH-terminal domains was approximately 20 kDa (Table I). An internal deletion of a putative nuclear translocation signal (AR8, see below) resulted also in a doublet of 107 and 102 kDa (Fig. 2, lane 7), again likely resulting from receptor phosphorylation. Controls for nonspecific interactions of antibody included preadsorption of antibody with peptide (data not shown) and analysis of cell extracts transfected with the parent vector lacking an AR cDNA insert (Fig. 2, lane 1). Neither control resulted in staining of prominent bands as also shown previously (39). AR mutant constructs AR6 and AR7 (Table II) lacked both antibody epitopes, and therefore these two expressed proteins could not be assessed for molecular size using the immunoblot technique. A summary of receptor sizes determined by immunoblot analysis is provided in Tables I and II.

Androgen Binding—All truncated receptors displayed high affinity androgen binding characteristic of the wild type receptor if the carboxyl-terminal region was intact as summarized in Tables I and II. No binding of the synthetic androgen, [3H]R1881, was detected with mutants AR11, AR12, or AR13 lacking the carboxyl-terminal region (Table I). Deletion of a 23-amino acid region in the hormone binding domain diminished, but did not abolish, androgen binding. Between 8 and 25% of wild type binding activity was retained. This 23-amino acid deleted region shares sequence similarity with other steroid receptors (41) and has been reported to be a site of interaction with the 90-kDa heat shock protein (48).

Domain Requirements for Transcriptional Activation— Transcriptional activation domains of the AR were identified by coexpression of wild type or deletion mutants with a reporter plasmid. Induction of chloramphenicol acetyltransferase (CAT) activity under the control of the MMTV promoter was androgen-dependent using wild type AR (Fig. 3). R1881 stimulated a 70-fold increase in induction of CAT activity. Deletion of the steroid binding domain (AR11) resulted in constitutive CAT activity at approximately 10% of wild type, indicating that the steroid binding domain represses transcriptional activity of wild type AR in the absence of androgen. This constraint is lost with deletion of the steroid binding domain. Furthermore, the low level of constitutive activity achieved with the AR11 deletion mutant is consistent with the presence of a transcriptional activation function within the steroid binding domain itself. Deletions of the entire NH2-terminal domain either with (AR7) or without (AR5) deletion of the DNA binding domain were totally lacking in transcriptional activation activity (Fig. 3, Table II), suggesting that the NH2-terminal domain has a role in modulating promoter activity. These results are summarized in Table I. Essentially identical results were obtained using the reporter vector, pMTV29VTM (33, 53), which contains two glucocorticoid response elements from the MMTV long terminal repeat spaced 29 residues apart and linked to the thymidine kinase promoter.

To further map transcriptional activator signals within the NH2-terminal domain, each of a series of NH2-terminal deletion mutants was coexpressed with the MMTV-CAT reporter plasmid. As shown in Figs. 4, full inductive activity was retained with deletion of 72 (AR2) and 141 (AR3) amino acids.

3J. A. Kempsainen, J. A. Simental, and E. M. Wilson, unpublished observations.
**Mutagenesis and Functional Analysis of the Androgen Receptor**

**TABLE I**

**Functional analysis of mutant human androgen receptors**

AR mutant DNAs were constructed and tested as described under "Experimental Procedures." The inclusive amino acid numbers (AA#) are indicated for each followed by the approximate molecular weights (MW (kD)) determined by migration on immunoblots of extracts from COS cells expressing mutant receptors. Binding of [3H]R1881 was determined by whole cell labeling and indicated by the presence (+) or absence (−) of high affinity binding activity. In cells expressing AR10, a mutant with partial deletion of the steroid binding domain, binding of [3H]R1881 was reduced to 8−25% of control (+).

Subcellular localization was determined by transient expression of wild type and mutant AR in transfected COS cells in the presence (+A) or absence (−A) of 50 nM R1881 as described under "Experimental Procedures." Immunostaining of COS cells is indicated as either nuclear (N) or perinuclear and cytoplasmic (C), with some AR mutants localized in both, e.g., C>N or N>C. Functional activity was determined by transient cotransfection of mutant AR vector DNA and a reporter plasmid containing the MMTV-CAT fusion gene. CAT activity was determined from transfected CV1 cells incubated in the presence (+) or absence (−) of 50 nM R1881. Activity is expressed as a percentage relative to wild type AR1.

| AR mutant DNA | MW (kD) | Binding | Localization | MMTV-CAT |
|---------------|---------|---------|--------------|-----------|
| AR1           | 919     | +       | C N          | 100       |
| AR8           | 507-919 | 33      | N N          | <1        |
| AR10          | 507-919 | 37      | (−)          | N N <1    |
| AR11          | 1-880   |         | N N          | 13        |
| AR12          | 1-503   |         | N N          | 1         |
| AR13          | 507-660 |         | N N          | 2         |

**TABLE II**

**Functional analysis of NH2-terminal deletion mutants of the human androgen receptor**

A series of NH2-terminal deletions was created using unidirectional exonuclease III digestion of wild type AR1 as described under "Experimental Procedures." Functional activities of the mutant receptors were analyzed as described in the Table I legend and under "Experimental Procedures." Mutants AR6 and AR7 lacked the antibody epitope for the polyclonal antibody AR52 which lies NH2-terminal of the DNA binding domain; molecular weights of these two expressed protein mutants could therefore not be determined by immunoblot analysis. All NH2-terminal deletions retained high affinity binding of [3H]R1881.

| AR mutant DNA | MW (kD) | Binding | Localization | MMTV-CAT |
|---------------|---------|---------|--------------|-----------|
| AR1           | 1-915   | +       | C N          | 100       |
| AR2           | 75-919  | +       | C N          | 103       |
| AR3           | 141-919 | +       | NC N         | <1        |
| AR4           | 536-919 | +       | NC N         | <1        |
| AR5           | 557-919 | +       | N N          | <1        |
| AR6           | 586-919 |         |              | <1        |
| AR7           | 628-919 |         |              | <1        |

from the NH2-terminus. However, deletion of 338 amino acids (AR4) or further deletions (AR5, -6, and -7, see also Table II) caused complete loss of transcriptional activity. Thus, a region of the AR between amino acids 141 and 338 appears to contain a signal sequence essential for transcriptional activation. This region contains sequences rich in acidic residues similar to the glucocorticoid receptor (30) and yeast transcription factors GCN4 and GAL4 (54, 55).

Since the NH2-terminal transcriptional activator signal may interact with nuclear proteins involved in transcriptional initiation, we tested whether the transcriptionally inactive mutant AR12 expressing only the NH2-terminal domain could inhibit transcriptional activation by wild type AR1. AR12 and AR1 vector DNA were cotransfected together with the MMTV-CAT reporter plasmid into CV1 cells, and CAT activity was determined by eluting the major radioactive spots from thin layer chromatography plates. Inhibition of wild type AR1 transcriptional activity by AR12 was 17% when
Mutagenesis and Functional Analysis of the Androgen Receptor

Fig. 3. Functional analysis of wild type AR and receptor mutants lacking major domains. CV1 cells were transfected with parent or AR vector DNA using the calcium phosphate precipitation method. Immediately after transfection, cells were placed in medium containing 0.2% serum with (+) or without (−) 50 nM R1881 and incubated for 24 h. Medium was replaced, and after an additional 24 h of incubation, the cells were harvested and assayed for CAT activity as described under “Experimental Procedures.” Quantitative evaluation of CAT activity is provided in Table I.

Fig. 4. Functional analysis of NH2-terminal deletion mutants of the AR. CV1 cells were transfected and incubated in the presence (+) or absence (−) of 50 nM R1881 as described in Fig. 3 and under “Experimental Procedures.” Equal molar amounts of these vector DNAs were cotransfected. Inhibition increased to 55% when a 10- or 20-fold higher level of AR12 DNA was cotransfected relative to AR1. Both AR5 and AR13, which contain the DNA binding domain, could fully inhibit CAT activity of wild type AR1 (data not shown).

Immunocytochemistry of Expressed AR Mutants—The subcellular distribution of AR and its mutant forms was investigated by transient expression in COS cells in the presence or absence of androgen. In a control study in which cells were transfected with the parent pCMV5 vector lacking AR sequence, minimal background staining was observed with or without androgen (Fig. 5, A and B). Wild type AR displayed strong nuclear immunostaining in the presence of R1881 (Fig. 5D), but in the absence of androgen, nuclear staining was greatly diminished and specific AR staining was localized in the perinuclear region of the cytoplasm (Fig. 5C). Subsequent addition of androgen 2 h prior to fixation resulted in a shift from the cytoplasmic/perinuclear region to the nucleus (data not shown). These results suggest that AR undergoes hormone-dependent translocation from the cytoplasmic/perinuclear region to the nucleus. In control transfection studies using the same staining conditions for the progesterone and estrogen receptors in the absence of hormone, very little cytoplasmic/perinuclear staining was observed like that seen for the AR. The estrogen and progesterone receptors were
located in the nucleus both in the absence and presence of hormone as previously reported (14, 35, 36, 38). The glucocorticoid receptor was both cytoplasmic and nuclear in the absence of hormone with a major portion of receptor in the cytoplasm; staining shifted to the nucleus with hormone addition. Previous reports have indicated a predominantly cytoplasmic distribution of the glucocorticoid receptor in the absence of hormone (13, 38, 56).

Progressive deletion of the NH2-terminus resulted in increased nuclear immunostaining in the absence of androgen, beginning with deletion of 211 amino acids (AR3, Table II). Mutant AR5 which lacks almost the entire NH2-terminal domain localized in the nucleus both in the absence and presence of androgen (Fig. 5, G and H). Deletion of the COOH-terminal domain (AR11 and -12) or the COOH- and NH2-terminal domains (AR13) also resulted in constitutively nuclear AR (Table I). Thus, the NH2-terminal and carboxy-terminal regions of AR act to inhibit nuclear transport of the unliganded receptor. This inhibition is released by androgen binding in the wild type receptor or by deletion of the major domains. This inhibition could result from specific protein interactions or a size constraint. It was somewhat surprising that the NH2-terminal domain alone (AR12) was nuclear (Table I). In this instance, the truncated AR protein, with an estimated molecular mass of 70 kDa (Table I), must be small enough to pass unimpeded through nuclear pores. In the absence of a DNA binding sequence, it may nevertheless remain associated with the nucleus due to protein/protein interactions.

A Nuclear Targeting Signal in AR—Sequence requirements for nuclear translocation of AR were further investigated using site-directed mutagenesis and immunocytochemistry. A sequence located between amino acids 628 and 657 within the hinge region of AR contains short stretches of basic amino acids that resemble nuclear targeting signals (NTS) of the glucocorticoid receptor (13) and the SV40 large T antigen (55). A comparison of this sequence with other published NTS is shown in Table III. PCR mutagenesis was used to prepare two mutant AR vectors: a full-length AR lacking 32 amino acids in the hinge region of the D exon (AR8), shown diagrammatically in Fig. 6, and a mutant lacking this same sequence and most of the NH2-terminal domain (AR9, Table I). Deletion of the putative NTS from the full-length receptor (AR8) increased cytoplasmic/perinuclear staining in the presence of androgen and markedly reduced, but did not abolish, nuclear staining (Fig. 5F). The striking perinuclear staining of AR8 in the presence of androgen was also observed by immunofluorescence (Fig. 7B) in marked contrast to androgen-induced nuclear localization of the full-length wild type receptor (Fig. 7A). These results indicate that the NTS potentiates androgen-dependent nuclear uptake of the full-length wild type AR and that absence of the NTS reduces the androgen stimulus for nuclear translocation of AR. Deletion of NTS from AR5 did not alter its constitutive nuclear location (see AR9, Table I).

An experiment was designed to determine if two mutants, AR8 and AR5, might interact such that the NTS of AR5 would assist in nuclear translocation of AR8 which lacks the putative NTS. The AR antibody raised against a NH2-terminal epitope was used so that only AR8 would be detected immunocytochemically. Staining in the presence of androgen remained predominantly perinuclear. Thus, it can be concluded that either the two mutant ARs did not interact or the NTS of AR5 could not accommodate nuclear transport of AR8. AR5 alone is constitutively nuclear and lacking the NH2-terminal domain.

The functional activity of mutant receptors lacking the NTS was assessed in cotransfection assays. Deletion of NTS from wild type AR resulted in androgen-dependent CAT activity similar to wild type (Fig. 8). This result was surprising since transfection studies in COS cells revealed predominantly cytoplasmic/perinuclear staining even in the presence of androgen. However, since there was no total loss of nuclear transport with deletion of the NTS, a low level of receptor entering the nucleus may have been sufficient for full induction of CAT activity. A similar retention of activity by the large T antigen after deletion of a homologous NTS has been reported (57, 58). In the mutant lacking a NTS and the NH2-terminal domain, complete loss of CAT activity was observed (Fig. 8).

**DISCUSSION**

Deletion mutagenesis and expression of human AR cDNA has facilitated functional analysis of the AR domains, including regions for hormone binding, nuclear transport, and transcriptional activation. As predicted from previous studies on a partial AR cDNA (42) and from homology considerations within the family of steroid receptors (1, 4), high affinity hormone binding was restricted to the carboxy one-third of the protein. Hormone binding in this region was required for transcriptional activity of the full-length, wild type human AR. This hormone requirement was lost, however, with deletion of the carboxyl-terminal region, resulting in a constitutively active regulatory protein with 10% of the transcriptional enhancement activity of wild type AR. Thus, the steroid binding domain represses transcriptional activation in the

**TABLE III**

| Protein                        | Amino acid no. | Sequence                                                                 |
|--------------------------------|----------------|--------------------------------------------------------------------------|
| SV40 large T antigen           | 126            | YKKRRKVK                                                                |
| Human androgen receptor        | 627            | GARKLKLGNLKLQEGEEGASSSTSPTETTTQK                                       |
| Rat glucocorticoid receptor    | 497            | YRKCLRQAGNNLLEARKTXXKG11QQAT                                            |
| Rabbit progesterone receptor   | 638            | RKRKKKKKwen                                                             |
| Human c-myc                    | 320            | PAARKVRLD                                                              |
| Yeast histone H2B              | 28             | DGKKR8R                                                               |
| Xenopus nucleoplasmin          | 160            | QQARKKRLD                                                             |

**Comperative alignment of nuclear localization signals**

Amino acid numbers are indicated for the first residue of each sequence containing the putative nuclear localization signal. The SV40 large T antigen sequence has served as a model for nuclear targeting signals (55). The amino acid sequence shown for human AR immediately follows the DNA binding domain and begins the hinge region. The rat glucocorticoid receptor sequence (13) extends 11 amino acids into the DNA binding domain and continues into the hinge region preceding the steroid binding domain. Additional homologous sequences are shown for the rabbit progesterone receptor (14), the human c-myc (79), yeast histone H2B (80), and Xenopus nucleoplasm (81). All sequences have stretches of basic amino acids that characterize nuclear translocation signals.
with the glucocorticoid receptor and the SV40 large T antigen nuclear targeting signals. This region was deleted from the full-length AR to create AR8.

**Fig. 6. A putative nuclear targeting signal for AR.** A 32-amino acid sequence was deleted based on homology comparisons with the glucocorticoid receptor and the SV40 large T antigen nuclear targeting signals. This region was deleted from the full-length AR to create AR8.

**Fig. 7. Immunofluorescent localization of AR in COS cells.** COS cells were transfected with either the full-length, wild type AR1 (A) or the deletion mutant AR8 (B) which lacks the putative nuclear targeting signal shown in Fig. 6. After transfection, the cells were maintained in medium containing 0.2% serum and 50 nM R1881. Immunofluorescent staining was performed as described under “Experimental Procedures.”

**Fig. 8. Functional activity of wild type and mutant receptors lacking the putative nuclear targeting signal.** CV1 cells were transfected as described under “Experimental Procedures” and incubated in the presence or absence of R1881 as indicated. Shown is CAT activity for the parent pCMV plasmid, wild type (AR1), full-length AR lacking the NTS (AR8), and the NH2-terminal and NTS deletion (AR9). The upper spots represent acetylated forms of [3H] chloramphenicol.

Absence of androgen and thereby regulates the activator capability of AR. The overall reduction in transcriptional activity upon deletion of the steroid binding domain suggests that this region itself contains one or more transcriptional activator signals. Alternatively, this domain may influence overall receptor conformation necessary for full transcriptional activation. Similar inhibition by the steroid binding domain has been reported for the glucocorticoid (59, 60), estrogen (61, 62), progesterone (63), and vitamin D receptors (9) and, most recently, for the AR (64).

A concerted interplay among other domains of AR was observed in addition to the steroid binding domain. The DNA binding domain presumably anchors the receptor to its hormone response element, which in these studies was contained within the MMTV long terminal repeat. The DNA binding domain is also likely involved in receptor dimerization as demonstrated with other steroid receptors (15, 26, 65). This domain is essential for transcriptional activation by steroid receptors (1, 7, 25, 61, 66, 67) and may contain an intrinsic activator function (25). The low but detectable CAT activity (1% of wild type AR) when the AR DNA binding domain was expressed without the COOH- or NH2-terminal domains supports this hypothesis. Moreover, single base mutations in the second zinc finger region of the glucocorticoid receptor were reported to result in loss of transcriptional enhancer activity without affecting specific DNA binding (68). A three-dimensional model depicting interaction of the glucocorticoid receptor DNA binding domain with its response element indicates several amino acids in the second zinc fingers of the homodimer are potentially available for contact with proteins of the transcriptional complex (69).

The NH2-terminal domain of AR was essential for gene activation, shown here by loss of transcriptional activity with deletion of the first 338 amino acids. Similar transcriptional activator functions are reported in NH2-terminal regions of the glucocorticoid and progesterone receptors that resemble AR in being relatively hydrophilic and negatively charged. A 40-kDa mutant glucocorticoid receptor, nt', which lacks the NH2-terminal domain, is biologically inactive even though it contains both hormone and DNA binding domains (5, 70, 71). Acidic regions between amino acids 77 and 293 of the human and mouse glucocorticoid receptor (6, 25, 30, 66) and within this region, amino acids 106–318, enhanced transcription (28, 29). A 50% reduction in transcriptional enhancement was observed following deletion of 128 amino acids from the NH2-terminus of the chicken progesterone receptor, which also includes a highly acidic region, while cleavage of the entire NH2-terminal region abolished transcriptional activity (7). A smaller deletion (amino acids 262–287) in a conserved area near a potential leucine zipper motif also reduced transcriptional activation by the progesterone receptor (67). Although these regions may be directly involved in transcriptional activation, it cannot be ruled out that such deletions change the conformation of the remaining receptor to make it transcriptionally inactive.

The mechanisms involving NH2-terminal sequences of steroid receptors in gene activation are unknown; however, protein/protein interactions likely occur with other transcription factors (33, 34). Both promoter and host cell specificity appear to influence the requirement for the NH2-terminal domains in transcriptional activation (27, 31, 32, 61, 62), suggesting that these regions interact with cell-specific transcription factors. The ability of the AR NH2-terminal domain to inhibit transcription when coexpressed in excess of wild type AR suggests that it competes for binding to one or more nuclear regulatory proteins required for activation of transcription initiation. NH2-terminal domain sequences are the most divergent among the family of steroid receptors (1, 4). Even the rat and human AR differ significantly in this region, primarily by the presence and length of repeated single amino acid motifs (41, 52). Sequence specificity among steroid receptors in this domain contrasts with the striking sequence similarity in other regions, particularly the DNA binding domain. It is therefore the NH2-terminal domain that may contribute to specificity of gene activation by steroid hormone receptors through interaction with specific transcription factors.

A surprising finding of these studies was the subcellular localization of AR expressed in COS cells. In the absence of hormone, AR was predominantly cytoplasmic, with staining largely in the perinuclear region. Immunofluorescent studies...
further revealed the ligand-free AR in cytoplasmic organelles surrounding the nucleus, possibly vesicles of the endoplasmic reticulum or Golgi, while androgen caused rapid and complete nuclear translocation. The glucocorticoid receptor also undergoes hormone-induced nuclear uptake (13, 38); however, in the absence of hormone, it was distributed more uniformly through the cytoplasm. In control studies not shown in this report, the estrogen, progesterone, and glucocorticoid receptors were expressed in COS cells to assess whether the fixation and staining procedures contributed to the perinuclear distribution of AR. As reported previously by others, estrogen and progesterone receptor immunostaining was nuclear in the absence of hormone (14, 35, 38, 72), and using the same conditions, the glucocorticoid receptor was cytoplasmic (13, 38, 56), with some nuclear and minimal peripheral staining. Thus the striking perinuclear staining of AR does not appear to result from fixation or staining procedures and indicates that the subcellular localization of the ligand-free AR differs from that of the other major steroid receptors.

The transfection system described in this report was useful in establishing a sequence required for nuclear transport within the hinge region 3' of the DNA binding domain. In the absence of this putative nuclear targeting signal, the AR remained predominantly cytoplasmic. The inability of the ligand-free AR or the androgen-bound AR lacking the NTS to enter the nucleus suggests the presence of a nuclear barrier. Size constraints are thought to limit protein access to the nucleus: 60-kDa or smaller proteins diffuse freely into the nucleus while larger proteins require nuclear targeting mechanisms (58, 73). In agreement with this hypothesis, large deletions of AR rendered it constitutively nuclear. The putative targeting signal shown here shares homology with those of other steroid receptors. Targeting signals of the glucocorticoid receptor are located within two domains: one in close association with the DNA binding domain and homologous in sequence to the SV40 large T antigen (13), and the other in the carboxyl-terminal steroid binding domain. While the receptors for progesterone, glucocorticoid, and mineralocorticoid share homologous sequences with the large T antigen, the estrogen, vitamin D, thyroid, and retinoic acid receptors have less homologous sequences (4), suggesting a different mechanism for nuclear uptake. It may be significant that this latter group of receptors is smaller in size due to shorter NH2-terminal sequences.

High-level transient expression of AR in COS cells contrasts with the low concentration of AR in androgen-responsive cells. Light microscopy immunocytochemical analyses of reproductive tract tissue sections from intact male rats have demonstrated AR in nuclei (41, 51). In tissue sections from rats 1 day after castration to remove the source of endogenous androgen, nuclear staining remained detectable but somewhat diminished (74). Three days after castration, AR became immunocytochemically undetectable. Strong nuclear immunostaining restored within 15 min of androgen administration to castrated rats indicated that AR remains in tissues following castration as supported by ligand binding studies of cytosol fractions (75–78). Persistence of nuclear staining 24 h after castration (74) may reflect delayed clearance of nuclear AR after androgen depletion by castration. The presence of other androgen binding proteins (55) may contribute to more prolonged local elevation of androgen after castration in certain tissues, particularly rat ventral prostate. Thus, the subcellular localization of endogenous androgen-free AR remains uncertain due to what appears to be low reactivity with antibody when assayed immunocytochemically.

Acknowledgment—We thank Jon Kemppainen for excellent technical assistance and D. O. Toft, G. L. Greene, J. A. Gustafsson, and M. V. Govindan for other steroid receptor antibodies and cDNA used in control studies.

REFERENCES

1. Evans, R. M. (1988) Science 240, 889–895
2. Yamamoto, K. R. (1985) Annu. Rev. Genet. 19, 209–252
3. Green, S., and Champon, P. (1986) Nature 324, 615–617
4. Carston-Jurios, M. A., Schrader, W. T., and O'Malley, B. W. (1990) Mol. Endocrinol. 4, 991–1002
5. Danielsen, M., Northrop, J. P., and Ringold, G. M. (1986) EMBO J. 5, 2513–2522
6. Giguere, V., Hollenberg, S. M., Rosenfeld, M. G., and Evans, R. M. (1986) Cell 46, 645–652
7. Gronemeyer, H., Turcotte, B., Quirin-Sticker, C., Bocquel, M. T., Meyer, M. E., Kroczowski, Z., Jeltsch, J. M., Lerouge, T., Garnier, J. M., and Champon, P. (1987) EMBO J. 6, 3985–3994
8. Carston, M. A., Tsai, M. J., Conney, O. M., Maxwell, B. L., Carston-Jurios, M. A., Dubson, A. D. W., Elbrecht, A., Toft, D. O., Schrader, W. T., and O'Malley, B. W. (1987) Mol. Endocrinol. 1, 791–801
9. McDonnell, D. P., Scott, R. A., Kerner, S. A., O'Malley, B. W., and Pike, J. W. (1988) Mol. Endocrinol. 3, 635–644
10. Weinberger, C., Thompson, C. C., Ong, E. S., Lebo, R., Grulich, D. M. V., and Evans, R. M. (1986) Nature 324, 641–646
11. Green, S., Kumar, V., Knust, A., Walter, P., and Champon, P. (1986) Cold Spring Harbor Symp. Quant. Biol. 51, 751–758
12. Ariza, J. L., Weinberger, C., Cerelli, G., Glaser, T. M., Handelin, B. L., Housman, D. E., and Evans, R. M. (1987) Science 237, 265–275
13. Picard, D., and Yamamoto, K. R. (1987) EMBO J. 6, 3339–3340
14. Gaiachon-Mantel, A., Loosfelt, H., Lescop, P., Sar, S., Atger, M., Ferrot-Applanat, M., and Milgrom, E. (1989) Cell 57, 1147–1154
15. Kumar, V., and Champon, P. (1988) Cell 55, 145–156
16. Fawell, S. E., Lees, J. A., White, R., and Parker, M. G. (1990) Cell 60, 953–962
17. Schena, M., Friedman, L. P., and Yamamoto, K. R. (1989) Genes & Dev. 3, 1590–1601
18. Quigley, C. A., Sinental, J. A., Evans, B. A., Lumbah, D. B., Hughes, I. A., and French, F. S. (1990) 72nd Annual Meeting of the Endocrine Society, Abstracts, p. 223, The Endocrine Society, Bethesda, MD
19. French, F. S., Lumbah, D. B., Brown, T. R., Sinental, J. A., Quigley, C. A., Yarborough, W. G., Tan, J. A., Sar, M., Joseph, D. L., Evans, B. A., Hughes, I. A., Migeon, C.-J., and Wilson, E. M. (1990) Recent Prog. Horm. Res., in press
20. Hughes, M. R., Malloy, P. J., Kiebach, D. G., Kesterson, R. A., Pike, J. W., Feldman, D. and O'Malley, B. W. (1988) Science 242, 1702–1705
21. Green, S., Kumar, V., Theules, L., Wahli, W., and Champon, P. (1988) EMBO J. 7, 3097–3104
22. Danielsen, M., Hinck, L., and Ringold, G. M. (1989) Cell 57, 1311–1318
23. Umerson, K., and Evans, R. M. (1989) Cell 57, 1139–1146
24. Miesfeld, R., Godowski, P. J., Maler, B. A., and Yamamoto, K. R. (1987) Science 236, 421–427
25. Hollenberg, S. M., Giguere, V., Segui, P., and Evans, R. M. (1987) Cell 49, 39–46
26. Tsai, S. A., Carlstedt-Duke, J., Weigel, N. L., Dahlman, K., Gustafsson, J. A., Tsai, M. J., and O'Malley, B. W. (1988) Cell 55, 361–369
27. Tora, L., Gronemeyer, H., Turcotte, B., Gaub, M. P., and Champon, P. (1988) Nature 333, 185–188
28. Godowski, P. J., Picard, D., and Yamamoto, K. R. (1988) Science 241, 812–816
29. Freedman, L. P., Yoshinaga, S. K., Vanderbilt, J. N., and Yamamoto, K. R. (1988) Science 245, 298–300
30. Hollenberg, S. M., and Evans, R. M. (1988) Cell 55, 899–906
31. Lees, J. A., Fawell, S. E., and Parker, M. G. (1989) Nucleic Acids Res. 17, 5477–5488
32. Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., and Champon, P. (1989) Cell 59, 477–487

*3 Sar, M., Lumbah, D. B., French, F. S., and Wilson, E. M. (1990) Endocrinology, in press.\n
\n
Mutagenesis and Functional Analysis of the Androgen Receptor
