Changes in the Abundance of Androgen Receptor Isotypes: Effects of Ligand Treatment, Glutamine-Stretch Variation, and Mutation of Putative Phosphorylation Sites†

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ABSTRACT: The SDS−polyacrylamide gel electrophoresis (SDS−PAGE) migration pattern of wild-type and mutated human androgen receptors (ARs) expressed in COS-1 cells was analyzed. In the absence of hormone, the wild-type AR migrated as a closely spaced 110-112 kDa doublet. Alkaline phosphatase treatment resulted in a single 110 kDa band showing that the 112 kDa upshift reflects receptor phosphorylation. Deletion of the N-terminal amino acids 46−101 or 100−142 resulted in mutant ARs migrating as single protein bands. Three consensus phosphorylation sites in this region were substituted, and the resulting mutated proteins were analyzed. Two Ser-Pro-directed kinase consensus sites at positions Ser-80 and Ser-93 were both necessary for the AR 112 kDa upshift. Substitution of the putative casein kinase II Ser-118 site had no effect on the AR migration pattern. Surprisingly, deletion of the glutamine repeat, located directly N-terminal of the Ser-Pro sites, resulted also in an AR single form. Lengthening of the glutamine repeat caused an increase in the spacing between the two isotypes of the doublet, showing that the number of glutamine residues determines the extent of the upshift. Hormone treatment induced an extra isotype with an apparent molecular mass of 114 kDa, resulting in a 110−112−114 kDa AR triplet. The hormone-induced upshift was dependent on the Ser-80 consensus phosphorylation site. Mutations in the DNA binding domain caused a different distribution of receptor protein over the three AR isotypes. In the presence of ligand, the amount of the hormone-induced 114 kDa isotype was reduced compared to the 110 and 112 kDa isotypes. A reduction in the amount of hormone-induced isotype was also observed for an AR mutant with an intact DNA binding domain, but it was incapable of activating transcription due to the deletion of a 244−528 fragment in the N-terminal domain. This suggests that part of the hormone-induced phosphorylation occurs following DNA binding and during or following transcription regulation. These findings also apply to AR mutants that lack the steroid binding domain. The constitutively active mutant AR13 migrated as three separable isotypes. Mutations in the DNA binding domain or reduction in the transactivating capacity due to a deletion in the N-terminal domain resulted in a reduced amount of the slowest migrating isotype. Although the loss of transactivating capacity seems to impair phosphorylation, substitution of the Ser-80, Ser-93, or Ser-118 by either alanine or glycine residues had no significant effect on the capacity of the respective mutants to activate the transcription of a $(GRE)_2$tkCAT reporter in HeLa cells.

The androgen receptor (AR) is a member of the superfamily of ligand-dependent transcription factors, which interacts through a ligand-dependent mechanism with specific DNA sequences, thereby regulating the transcription of target genes (Evans, 1988; Green & Chambon, 1988; Truss & Beato, 1993). These nuclear receptors show a high level of molecular identity, and the arrangement of the different, separate functional/structural domains is essentially the same: an N-terminal modulating domain, a central DNA binding domain, a hinge region, and a C-terminal ligand binding domain. The N-terminal regions of the different members of the nuclear receptor family are highly variable in size and amino acid composition, but the DNA and ligand binding domains share a high degree of sequence homology (Laudet et al., 1992).

Many transcription factors, including steroid hormone receptors, are phosphoproteins [reviewed in Moudgil (1990); Hunter & Karin, 1992; Meek & Street, 1992; Ortí et al., 1992; Takimoto & Horwitz, 1993; Kuiper & Brinkmann, 1994]. Mutational analysis, phosphoamino acid analysis, tryptic phosphopeptide mapping, and microsequencing have identified phosphorylation sites in the glucocorticoid (GR) (Bodwell et al., 1991), progesterone (PR) (Denner et al., 1990; Poletti & Weigel, 1993), estrogen (ER) (Ali et al., 1993; Le Goff et al., 1994), and vitamin D_3 receptors (VDR) (Hsieh et al., 1991). Phosphorylation mainly occurs in the N-terminal domain of these receptors, although sites in the hinge region (chicken PR; Denner et al., 1990), hinge region and ligand binding domain (VDR; Brown & DeLuca, 1991; Hsieh et al., 1991), and DNA binding domain (VDR; Hsieh et al., 1991) have also been reported. Almost all of the characterized phosphorylation sites are on serine residues

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(Dalman et al., 1988; Sheridan et al., 1988; Denner et al., 1990; Bodwell et al., 1991; Hsieh et al., 1991; Washburn et al., 1991; Denton et al., 1992; Ali et al., 1993; Poletti & Weigel, 1993; Le Goff et al., 1994). In some cases, minor phosphorylation on threonine residues (Hoeck & Groner, 1990; Bodwell et al., 1991) has been observed, but rarely on tyrosine residues (Migliaccio et al., 1986; Auricchio, 1989). With respect to the human AR, no specific phosphorylation sites have been identified as yet. From limited proteolysis studies of ^{32}P-labeled AR, it is evident that phosphorylation occurs in the first 300 amino acid residues of the N-terminal domain (Kuiper et al., 1993). Phosphoamino acid analysis revealed that AR phosphorylation takes place on serine residues (G. G. J. M. Kuiper, personal communications).

Several different protein kinases (protein kinase A, protein kinase C, casein kinase II, Ser-Pro-directed kinases) have been described to phosphorylate steroid receptors [reviewed in Moudgil (1990); Ortí et al., 1992]. In the region in which the AR is shown to be phosphorylated, several consensus sites for these kinases can be found.

Upon incubation of cells in culture with the respective ligands, steroid receptors become hyperphosphorylated (Ortí et al., 1989, 1992; Brown & DeLuca, 1990; Moudgil, 1990; Chauchereau et al., 1991; van Laar et al., 1991; Washburn et al., 1991; Beck et al., 1992; Denton et al., 1992; Kuiper et al., 1993). Since this rapid additional phosphorylation has been associated with hormone binding, nuclear import, DNA binding, and transcription activation, the importance of receptor phosphorylation has often been emphasized [reviewed in Moudgil (1990); Ortí et al., 1992; Takimoto & Horwitz, 1993]. Mutational analysis of phosphorylation sites in the human ER and human VDR indeed showed that particular sites are important for the receptor's capacity to activate transcription (Hsieh et al., 1991; Ali et al., 1993; Le Goff et al., 1994). However, site-directed mutagenesis of the phosphorylation sites in the mouse GR had no, or only a minor, effect on transcription activity (Mason & Housley, 1993).

For several nuclear receptors [PR (Sheridan et al., 1988, 1989; Beck et al., 1992), ER (Washburn et al., 1991), AR (Krongrad et al., 1991; Kuiper et al., 1991), and VDR (Jurutka et al., 1993)], phosphorylation is accompanied by a characteristic decrease in electrophoretic mobility (upshift) during SDS−PAGE analysis, resulting in the separation of different isotypes. Immediately following synthesis, the nascent human AR has an apparent molecular mass of 110 kDa and matures into two bands (110−112 kDa) over the ensuing 30 min. Alkaline phosphatase treatment restored the 110 kDa single protein band, showing that the upshift to 112 kDa reflects phosphorylation (Kuiper et al., 1991).

For the human PR, it has been shown that hormone-stimulated hyperphosphorylation induced additional receptor isotypes. Both hyperphosphorylation and coincident PR upshifts during SDS−PAGE were partially inhibited in PR mutants unable to bind DNA or by progesterone antagonists that prevented PR−DNA interaction (Bagchi et al., 1992; Takimoto et al., 1992).

In our quest to locate and analyze functional domains of the human AR, many deletion mustants have been generated and tested for their correct length and expression level by SDS−PAGE (Jenster et al., 1991, 1993). In these studies, divergence from the wild-type AR doublet migration pattern was observed. In order to elucidate the nature of the doublet migration pattern and use it as a marker for the identification of phosphorylation sites, deletion and substitution mutants were constructed and analyzed by SDS−PAGE and immunoblotting.

EXPERIMETNAL PROCEDURES

Cell Culture, Transfection, and Western Blotting. COS-1 cells were maintained and transfected in Eagle's minimum essential medium (MEM) supplemented with 5% fetal calf serum, nonessential amino acids, and antibiotics (medium). The cells were cultured in 150 cm^2 T-flasks (3×10^6 cells/flask) and transfected using the DEAE-dextran method. The COS-1 cells were incubated with 15 mL of MEM containing 20 μg of AR expression plasmid and 0.2 mg of DEAE-dextran. After 6 h, cells were treated with 15 mL of MEM and 25% (v/v) glycerol for 1 min, washed twice with MEM, and reincubated with medium for 2 days. Sixteen hours prior to cell lysis, medium was replaced and 1 nM R1881 (17α-methyl-17β-hydroxyestra-4,9,11-trien-3-one, NEN, Boston, MA) was added. Cells transfected in 150 cm^2 T-flasks were lysed in 1 mL of lysis buffer (40 mM Tris·HCl (pH 7.0), 1 mM EDTA, 4% (v/v) glycerol, 10 mM dithiothreitol, 2% (w/v) SDS, 0.6 mM PMSF, and 0.5 mM bacitracin). In most cases, part of the cell lysis mix (approximately 20 μL) was mixed with 5× sample buffer (200 mM Tris·HCl (pH 6.8), 25% (v/v) glycerol, 10% (w/v) SDS, 50 mM dithiothreitol, and 1% (w/v) bromophenol blue), heated, and loaded on a 7% SDS−PAGE gel as described previously (Kuiper et al., 1991). Sometimes AR protein was first immunoprecipitated using monoclonal antibody F39.4.1 coupled to goat anti-mouse agarose beads (Zegers et al., 1991). Western blotting and immunostaining were performed as described previously (Kuiper et al., 1991). The Sp061 (Zegers et al., 1991) and Sp197 (Kuiper et al., 1993) polyclonal anti-AR antibodies were used to visualize the different AR proteins.

Alkaline Phosphatase Treatment. Cells transfected in 150 cm^2 T-flasks were suspended in 1 mL of TGD buffer (40 mM Tris·HCl (pH 8.5), 10% (v/v) glycerol, 1 mM dithiothreitol, 0.6 mM PMSF, 0.5 mM bacitracin, and 0.2 mg/mL leupeptin). Cytosols (400 μL) were prepared and incubated for 60 min at 37 °C with 200 units of calf alkaline phosphatase (Boehringer Mannheim), as described previously (Kuiper et al., 1991).

Chloramphenicol Acetyl Transferase (CAT) Assays and Subcellular Location. AR functional activity was assessed in HeLa or COS-1 cells by transient transfection using the calcium phosphate precipitation method (Chen & Okayama, 1987). Transfections of AR mutants were performed four or five times in duplo, using at least two independent plasmid extracts (Jenster et al., 1991). In each experiment, CAT activities were corrected for the reporter background, and the percentage relative to that of the wild-type AR (set to 100%) was determined. The means (±SEM) were calculated. CAT assays were performed as described previously (Jenster et al., 1991). For AR subcellular localization, COS-1 cells were cultured directly on microscope glass slides and transfected using the calcium phosphate precipitation method. Fixation, permeabilization, and immunocytochemistry of the transfected cells were performed essentially as described previously (Jenster et al., 1991, 1993). The anti-AR monoclonal F39.4.1 and polyclonal Sp197 antibodies were used to visualize the different AR proteins.

AR Expression Vectors. A human cDNA expression vector (pAR0) was constructed using the SV40 early

promoter and the rabbit β-globin polyadenylation signal (Brinkmann et al., 1989). The AR cDNA encodes 910 amino acids of which the polymorphic glutamine stretch contains 20 Gln residues and the glycine stretch consists of 16 Gly residues. The construction of pAR7, pAR13, pAR22, and pAR64 has been described previously (Jenster et al., 1991, 1993). pAR30.3 contains the same residue substitutions as pAR28.3 and was constructed in the same way (Jenster et al., 1993). The mutants pAR9 (deletion codons 46-101), pAR10 (deletion codons 100−142), pAR11 (deletion codons 139−188), pAR19 (deletion codons 186−244 and an additional mutation in codon 249 resulting in Glu to Val substitution), pAR34 (deletion codons 626−654), pAR62 (deletion codon 485−528), pARS80G (=pAR79), pARS93A (=pAR78), pARS118A (=pAR96), and pAR70 were constructed by site-directed mutagenesis, using PCR DNA amplification techniques (Higuchi et al., 1988). The following primers, introducing the deletion or substitution, were used:

AR9: 5′-AGGCACCCAGAGGCCGCATCGAT-CACAGGCTACCTGGTC-3′

AR10: 5′-CCAAGCCCATCGTAGATCGATG-CCGCAGCAGCTGCCA-3′

AR11: 5′-GAGCCGCCGTGGCCGCATCGAT-GCAACTCCTTCAGC-3′

AR19: 5′-ACATCCTGAGCGAGGCATCGAT-GGGCCTGGGTGTGG-3′

AR34: 5′-GCTGAAGAAACTTGGATCGATT-GAAGGCTATGAATG-3′

AR62: 5′-GCTGGCGGGCCAGGAATCGAT-GCGTTTGGAGACTG-3′

AR70: 5′-GCCAGTTTGCTGCTGCTCGAGA-CTAGCCCCAGGCAG-3′

AR78: 5′-GAGGATGGTGCTCCCCAAGCC-3′

AR79: 5′-CAACAGACTGGCCCCAGGCAG-3′

AR96: 5′-CACAGCCGCAGGGCGCCCTGGAGTG-3′

pARS118A (pAR96) lacked one CAG codon at position 83, resulting in the deletion of one glutamine residue in a short glutamine stretch of normally six residues (Figure 2) (Faber et al., 1989).

In the AR mutants pAR9, pAR10, pAR11, pAR19, pAR34, and pAR62, a unique Cl*a*I site was introduced in the same matching frame in all constructs [identical to the *Cla*I site present in pAR22 (Jenster et al., 1993)]. In this way, parts of the AR cDNA can easily be swapped between these mutants, resulting in all kinds of in frame deletion or duplication. pAR60 was constructed by removing the 1.74 kb *Cla*I/*Bam*HI fragment, containing most of the AR cDNA, from pAR22 (deletion of codons 244−360) and inserting the 1.24 kb *Cla*I/*Bam*HI fragment from pAR62 into pAR22. pAR 5 (deletion codons 627−910) was constructed by ligation of an *Xba*I linker (Promega, Madison, WI) that contains an in-frame stop codon into the blunt-ended *Cla*I site of pAR34. pAR113 (deletion codons 370−528) was constructed by removal of the internal fragment of pAR62

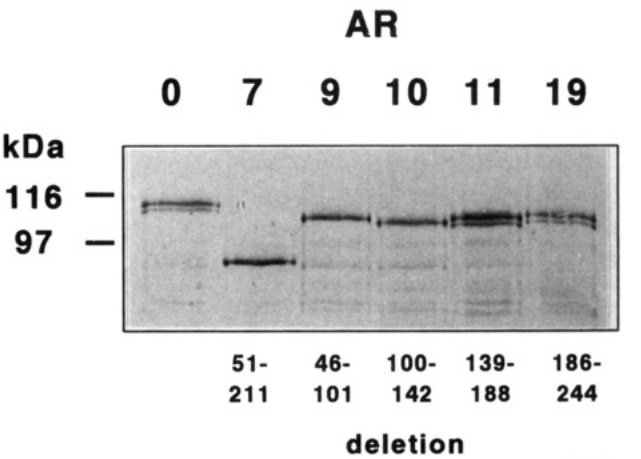

FIGURE 1: Immunoblot of the wild-type AR and AR deletion mutants. AR proteins expressed in COS-1 cells in the absence of hormone were immunoprecipitated with the monoclonal antibody F39.4.1, separated by 7% SDS−PAGE, blotted, and immunostained with the polyclonal antibody Sp061. Molecular weight markers (kDa) were run in a parallel lane and their positions are indicated on the left. The AR mutant number and respective residues deleted are indicated.

digested with *Rsr*II and *Cla*I. Ligation of the blunt-ended plasmid resulted in an in-frame deletion. From pAR113, pAR126 was constructed by introducing the 370−528 deletion present in pAR113 into the pAR5 plasmid.

pAR75 was constructed by PCR amplification of part of the first exon containing the $(CAG)_{47}CAA$ stretch on genomic DNA isolated from a patient with Kennedy's disease and exchanged with the pAR0 sequences using the *Sma*I and *Afl*II restriction sites. pAR73, pAR76, pAR77, and pAR74 were constructed in the same way using genomic DNA, with the respective $(CAG)_n$ repeat lengths from androgen insensitivity patients or cell line materials. All AR mutants were sequenced to verify the correct reading frame.

RESULTS

AR Migration Pattern in the Absence of Hormone. As shown previously, the wild-type AR (length: 910 amino acids) expressed in LNCaP cells (lymph node carcinoma of the prostate) or in transfected COS-1 cells migrated as two protein bands (110−112 kDa) when analyzed by Western blotting (van Laar et al., 1990; Jenster et al., 1991; Kuiper et al., 1991). Deletion of the N-terminal residues 51−211 (AR7) resulted in a protein that migrated as a single (84 kDa) band, suggesting the loss of phosphorylation sites (Figure 1) (Jenster et al., 1991). To locate the putative phosphorylation sites responsible for the partial protein upshift in the wild-type AR (AR0), four AR mutants lacking smaller parts of the same region were generated and analyzed by SDS−PAGE (AR9[Δ46−101], AR10[Δ100−142], AR11[Δ139−188], and AR19[Δ186−244]) (Figures 1 and 2). Figure 1 shows that mutants AR9 and AR10 both migrated as a single protein band, whereas AR11 and AR19, like the wild-type AR, were doublets. This indicated that the region responsible for the AR upshift could be narrowed to the region 46−139. This part of the N-terminal domain contains six serine residues, three of which are consensus sites for known protein kinases: two Ser-Pro-directed kinase sites (Ser-80 and Ser-93) and one casein kinase II site (Ser-118) (Kemp & Pearson, 1990; Kennelly & Krebs, 1991). To study in more detail the presumed role of the potential phosphorylation sites in AR migration, the serine residues at positions 80, 93, and

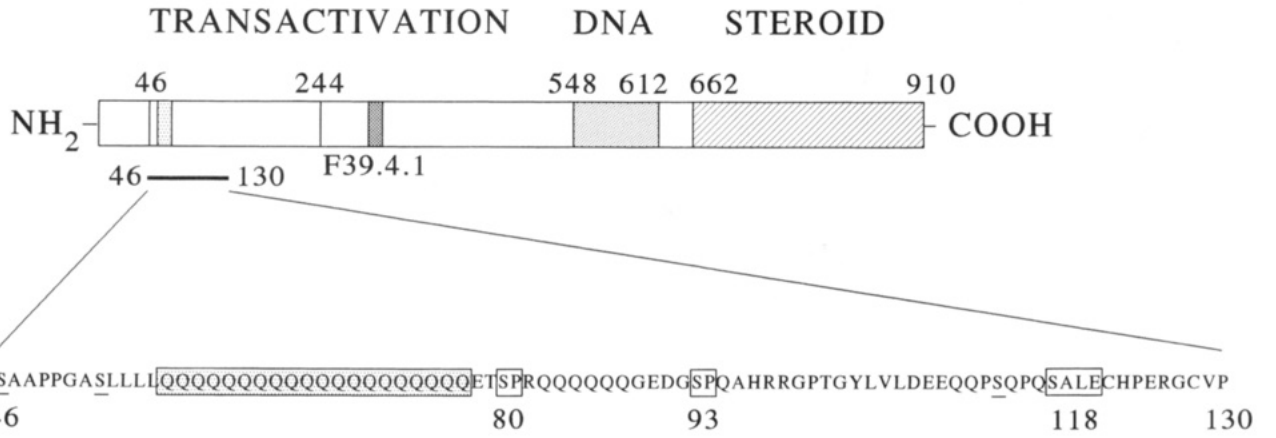

FIGURE 2: Location of the AR functional domains and putative phosphorylation sites in part of the N-terminal domain. The wild-type human AR consists of four major domains: (1) an N-terminal domain involved in transactivation (residues 1−548); (2) the DNA binding domain (residues 548−612); (3) a hinge region (residues 612−662); and (4) the steroid binding domain (residues 662−910). Part of the N-terminal domain is enlarged showing the region containing amino acid residues 46−130, including the polymorphic glutamine stretch (stippled box; residues 58−77) and six serine residues (underlined), three of which are kinase consensus sites (boxed). Serine residues 80 and 93 are potential phosphorylation sites for the Ser-Pro-directed kinase. Serine-118 is a potential phosphorylation site for casein kinase II.

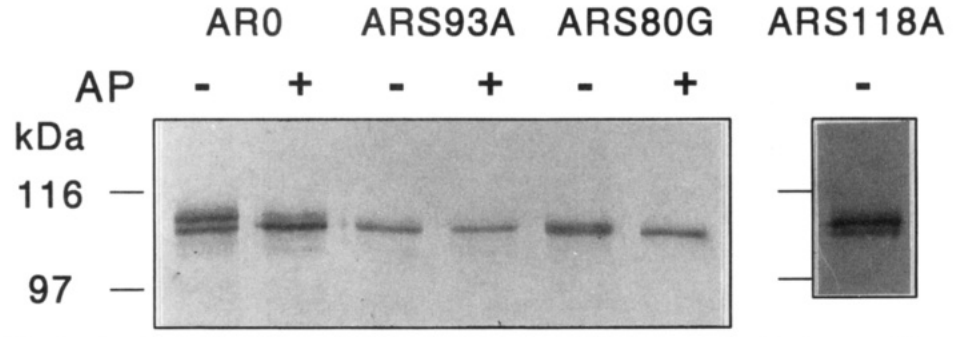

FIGURE 3: Immunoblot showing dephosphorylation of wild-type and mutated ARs expressed in the absence of hormone. Cytosols were prepared from COS-1 cells expressing AR proteins in the absence of hormone and incubated with (+) or without (−) alkaline phosphatase (AP). AR proteins were immunoprecipitated with the monoclonal antibody F39.4.1, separated by 7% SDS−PAGE, immunoblotted, and visualized using the Sp061 antibody. Molecular mass markers (kDa) are indicated on the left.

118 were substituted by a glycine or an alanine residue in the mutants ARS80G, ARS93A, and ARS118A, respectively. The SDS−PAGE migration pattern of these three AR mutants showed that serine residues on positions 80 and 93 were essential for the AR 110 kDa to 112 kDa upshift, whereas the serine-118 substitution had no effect on the migration pattern of the two isotypes (Figure 3). To demonstrate that phosphorylation causes the AR mobility upshift, alkaline phosphatase (AP) treatment of cytosolic extracts was performed. As shown in Figure 3, the upper 112 kDa band of the wild-type AR0 reverted to the 110 kDa isotype upon alkaline phosphatase treatment, while the 110 kDa ARS80G and ARS93A single protein bands were practically unaffected.

In addition to the serine mutations, the influence of the length of the flanking glutamine stretch (Figure 2; residues 58−77) on the SDS−PAGE migration pattern of the AR was investigated. Surprisingly, deletion of the glutamine repeat resulted in an AR, migrating as a single 105 kDa band (Figure 4). Lengthening the stretch to 7, 12, 16, 20, 29, or 48 glutamine residues revealed not only an expected increase in the size of the proteins but also a gradual appearance of two bands with increasing spacing between the two AR isotypes (Figure 4).

Hormone-Induced AR Migration Pattern. Hormone (1 nM R1881) added over 16 h to the COS-1 cells expressing the wild-type AR induced an additional protein form, resulting in three separable receptor isotypes (Figure 5). The same

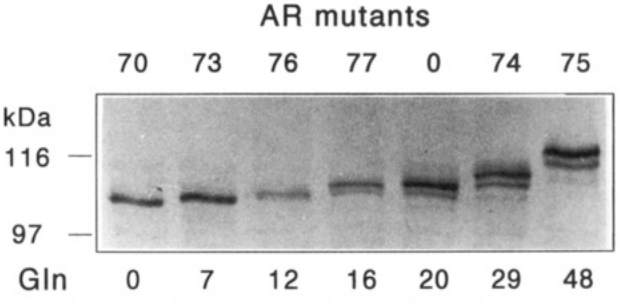

FIGURE 4: Immunoblot of AR proteins containing glutamine repeats with different lengths. AR proteins expressed in COS-1 cells in the absence of hormone were immunoprecipitated with the monoclonal antibody F39.4.1, separated by 7% SDS−PAGE, blotted, and immunostained with the polyclonal antibody Sp061. Molecular weight markers (kDa) were run in a parallel lane and their positions are indicated on the left. Mutant number and length of the glutamine stretch (Gln) are indicated.

hormone-induced triplet was observed for the ARS118A mutant, showing that Ser-118 is not essential for the different AR protein migration upshifts in the absence or presence of ligand (Figure 5). In contrast, the ARS80G migration pattern revealed no additional significant upshift upon hormone exposure. The single protein bands of ARS93A and AR70 (ΔGln stretch) both converted into doublets upon R1881 treatment, although the hormone-induced upshift of ARS93A was faint (Figure 5).

AR Isotypes and Transcription Activity. To pinpoint the step in the AR activation cascade during which the hormone-

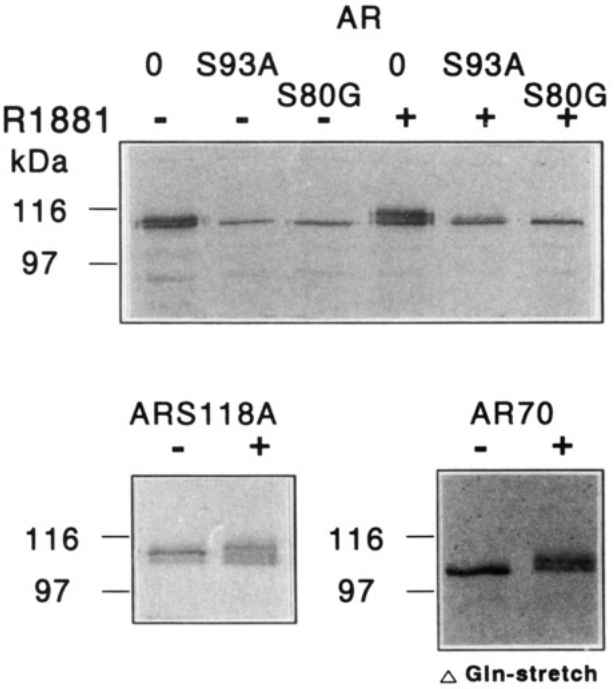

FIGURE 5: Hormone-induced protein upshifts of wild-type AR and AR mutants. AR proteins expressed in COS-1 cells and incubated in the absence (−) or presence (+) of 1 nM R1881 for approximately 16 h were separated by 7% SDS−PAGE, blotted, and immunostained with the polyclonal antibody Sp061. Molecular weight markers (kDa) were run in a parallel lane and their positions are indicated on the left of each blot.

stimulated phosphorylation takes place, a selected set of receptor mutants was analyzed in more detail. Figure 6 summarizes the subcellular localizations and the transactivation capacities of the wild-type and mutant ARs studied. All of these AR mutants were exclusively or predominantly nuclear in the presence of hormone [see also Jenster et al. (1993)]. As has been described previously, part of the AR64 protein population formed large clusters in the nucleus upon ligand treatment (Jenster et al., 1993). AR30.3 was partially cytoplasmic because of the substitution of the two basic arginine and lysine residues, which are part of the nuclear localization signal (Jenster et al., 1993) (Figure 6).

AR64, which is unable to bind DNA due to substitutions of two essential cysteine residues in the first zinc finger, migrated as a doublet in the absence of R1881 (Figure 7). Hormone treatment of AR64 generated less of the slowest migrating, 114 kDa form compared to its 110 and 112 kDa isotypes, suggesting that DNA binding is necessary for part of the ligand-stimulated phosphorylation (Figure 7). In order to be able to investigate further whether DNA binding or transcription activation is needed for phosphorylation, an AR mutant was required that is still able to bind DNA, but incapable of activating transcription. Because a deletion should be far from the proposed phosphorylation sites, AR60, which lacks amino acids 244−528, was used for these experiments (Figure 6). The AR60 migration patterns showed the appearance of a doublet when expressed in the absence of ligand. Hormone exposure resulted in an additional faint third isotype (partial triplet), indicating that not DNA binding but transcription activation would be the step during which part of the hormone-stimulated phosphorylation takes place (Figure 7). Additional evidence was provided by experiments with the constitutively active mutant AR13, which lacks the ligand binding domain. AR13 migrated as three separable isotypes (Figure 7). Substitution of the residues Arg-Lys by Gly-Ala at positions 608−609 (AR30.3) inactivated the constitutive receptor, most likely due to the inability to bind DNA (Figure 6). Analogous to the AR0 versus AR64 SDS−PAGE protein pattern, the distribution of receptor protein over the three isotypes was different for AR13 compared to AR30.3. An equal distribution over the isotypes is observed in the AR13 migration pattern. In contrast, there is considerably more of the second upshift and less of the third, slowest migrating isotype in the AR30.3 sample (Figure 7). A similar pattern is observed for AR126. This mutant contains an intact DNA binding domain, but is barely able to activate transcription due to a deletion in the N-terminal region (Figure 6). This again suggests that part of the hormone-induced receptor phosphorylation occurs during or following transcription activation.

To test whether substitution of the proposed phosphorylation sites would affect transactivation, the capacity of ARS80G, ARS93A, and ARS118A to induce transcription was investigated. Cotransfection experiments of these AR mutants with the $(GRE)_2$tkCAT reporter [pG29GtkCAT (Schüle et al., 1988)] in HeLa cells showed that their transcription activation capacity was comparable to the wild-type AR activity (Figure 6). Since Gln stretches and glutamine-rich domains have been implicated in transcription activation, and since the length of the polyglutamine stretch considerably influences the isotype pattern on SDS−PAGE, AR70 (lacking the Gln stretch) and AR75 (containing 48 glutamines) were tested for their capacity to activate transcription. The wild-type AR (20 Gln), AR70, and AR75 were cotransfected with either the MMTV-CAT or the pPA2-CAT reporter construct in COS-1 cells. Both reporters can be induced by androgens when cotransfected with AR0. The MMTV-CAT contains the MMTV-LTR promoter, which harbors four glucocorticoid response elements, while the pPA2-CAT reporter accommodates 630 base pairs of the promoter region of the prostate specific antigen (PA) gene containing one androgen response element (Riegman et al., 1991). Deletion of the glutamine stretch resulted in an increased activity of AR70, but only when tested on the pPA2-CAT reporter (Figure 8). Lengthening of the repeat to 48 residues decreased the transactivation capacity of AR75 on both promoters, showing that the (extended) Gln stretch inhibits transcription.

DISCUSSION

Several reports have shown that there is a causal link between phosphorylation and electrophoretic mobility during SDS−PAGE of the PR (Sheridan et al., 1988, 1989; Beck et al., 1992), ER (Washburn et al., 1991), AR (Krongrad et al., 1991; Kuiper et al., 1991), and VDR (Jurutka et al., 1993). Therefore, analysis of protein isotypes could indirectly provide information on receptor phosphorylation sites and function. In the present study, the SDS−PAGE protein patterns of wild-type and mutated human ARs and their ability to activate transcription were investigated.

Narrowing down the region responsible for AR doublet appearance to residues 46−139 reduced the more than 10 possible N-terminal serine phosphorylation motifs to only three obvious consensus serine phosphorylation sites. Substitution analysis revealed that at least the two Ser-Pro-directed kinase sites (Ser-80 and Ser-93) are essential for the 112 kDa AR upshift. The putative casein kinase II

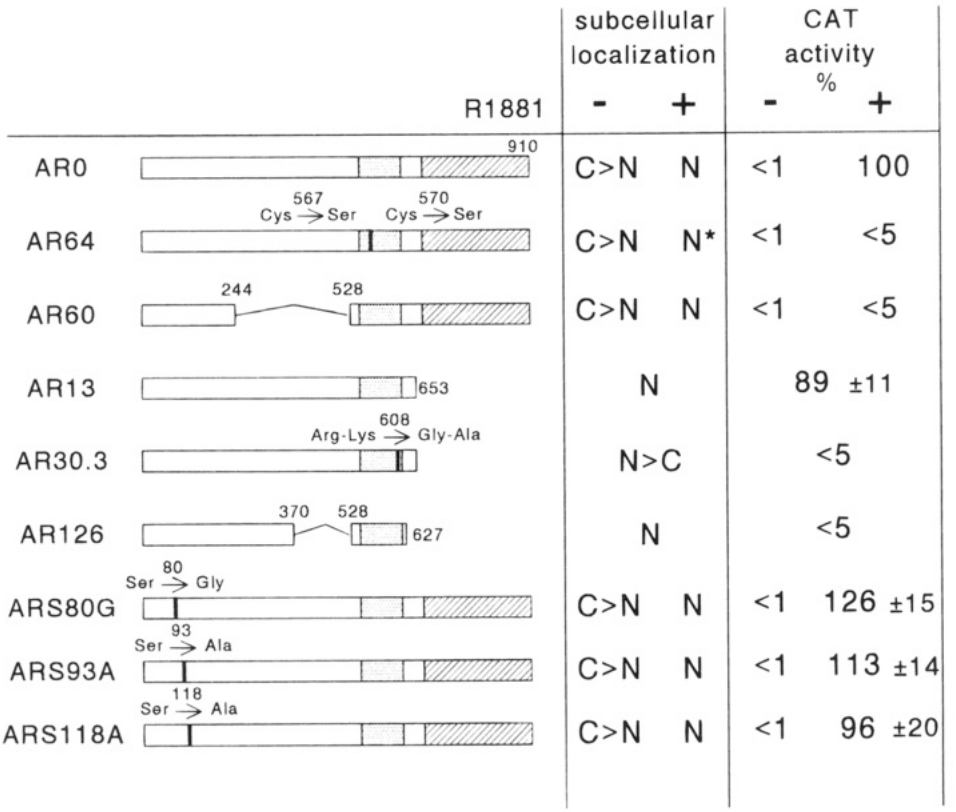

FIGURE 6: Subcellular localization and transcription activation of the wild-type AR and AR mutants. Nuclear (N) and cytoplasmic (C) localization of AR proteins expressed in COS-1 cells in the absence (−) or presence (+) of 1 nM R1881. The receptor mutants were visualized using the F39.4.1. antibody except for AR60, for which the Sp197 antibody was used. In the presence of hormone, large AR64 clusters were formed in the nucleus (indicated by N•) (Jenster et al., 1993). CAT activity was determined in cell lysates of transfected HeLa cells cultured in the absence (−) or presence (+) of 1 nM R1881. Activities were corrected for the $(GRE)_2$tkCAT background, and the percentages (±SEM) relative to that of the wild type AR0 of four independent assays are shown.

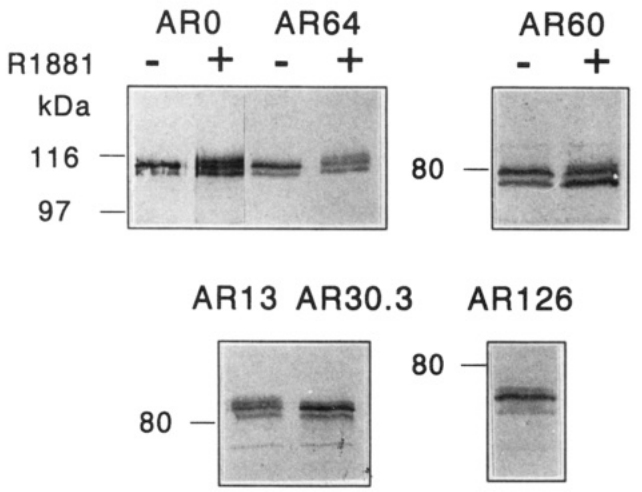

FIGURE 7: Hormone-induced protein upshifts of the wild-type AR and AR mutants. AR proteins expressed in COS-1 cells and incubated in the absence (−) or presence (+) of 1 nM R1881 for approximately 16 h were separated by 7% SDS−PAGE, blotted, and immunostained with the polyclonal antibody Sp061. Molecular weight markers (kDa) were run in a parallel lane and their positions are indicated on the left for each blot.

phosphorylation site at position 118 is not important for the AR migration pattern. Besides the potential Ser-80 and Ser-93 sites, it cannot be excluded that other phosphorylation sites are also essential for the 112 and 114 kDa upshifts. The observation that the unliganded AR10 (lacking amino acids 100−142) migrated as a single protein band suggests that, except for Ser-118, other serine residues in this region contribute to an upshift when phosphorylated.

Interestingly, deletion of the glutamine stretch (a repeat of 20 glutamine residues in the AR0 protein) also resulted in a single receptor band. It is unlikely that deletion of the glutamine residues directly affected phosphorylation of the neighboring phosphorylation sites and therefore resulted in a single band, since lengthening of the stretch to different sizes increased the spacing between the two AR isotypes. If the phosphorylation of surrounding sites was reduced, one would rather expect the amount of the upshifted band to vary and the spacing to be constant. Two possible explanations for phosphorylation-induced AR doublet appearance in the absence of hormone are proposed. Firstly, phosphorylation on sites Ser-80 and Ser-93 results in a conformational change in the neighboring glutamine repeat, causing the migration upshift visible as a 112 kDa isotype. Deletion of the glutamine stretch and elimination of the phosphorylation sites would both result in a single AR protein band. Shortening of the glutamine stretch would reduce the extent of the conformational change and, consequently, the spacing between the receptor isotypes. Secondly, Ser-80 and Ser-93 phosphorylation might retard AR protein migration by decreasing SDS binding to the AR. The negatively charged detergent SDS provides the negative charge to the SDS−protein complex and therefore largely determines the migration pattern of proteins in the SDS−PAGE system. Possibly, SDS molecules that bind to the glutamine stretch will be supplanted by the negative phosphate of the phosphorylated Ser-80 and Ser-93. Phosphorylation of Ser-80 and Ser-93 in AR mutants containing a reduced glutamine repeat length might supplant fewer SDS molecules, resulting in a decreased difference in negative charge between the two isotypes,

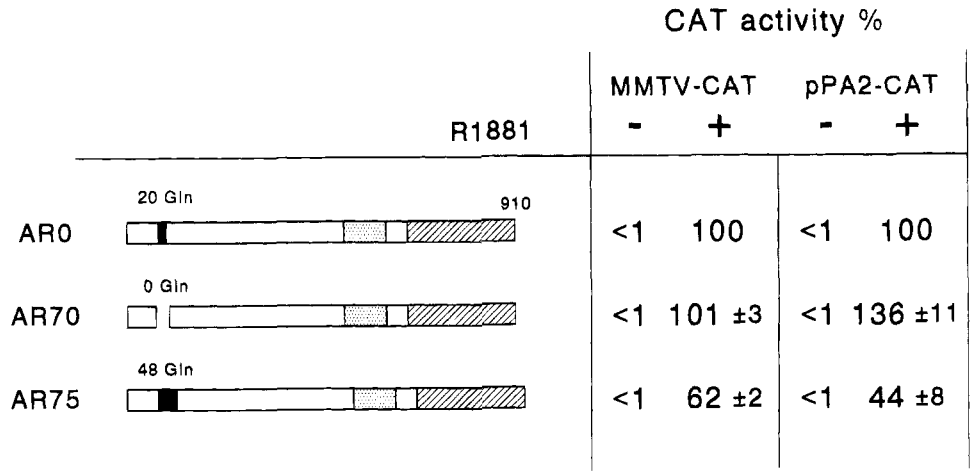

FIGURE 8: Functional analysis of AR mutants with different lengths of the glutamine stretch. Transcriptional activity was examined by cotransfection of AR0 (20 Gln), AR70 (0 Gln), and AR75 (48 Gln) expression plasmids with the MMTV-CAT or pPA2-CAT reporter. CAT activity was determined from cell lysates of transfected COS-1 cells cultured in the absence or presence of 1 nM R1881. Activities were corrected for the reporter background, and the means (±SEM) of the R1881-treated samples of five independent assays are presented as a percentage relative to that of wild-type AR0.

which subsequently results in decreased spacing between the AR isotypes.

Deletion of the Gln repeate (AR70) resulted in increased activity when tested in COS-1 cells on a reporter containing part of the prostate specific antigen (PA) promoter (pPA2CAT). The same AR70 mutant, however, had wild-type activity when tested on the MMTV-LTR reporter (MMTV-CAT). Extension of the Gln repeat from 20 to 48 residues resulted in decreased AR activity on both promoters to approximately 50% compared to the wild-type AR, which confirms the observations of Mhatre and co-workers (Mhatre et al., 1993). This indicates that the glutamine stretch inhibits AR transcription activation. Lengthening of the Gln repeat to more than 40 residues is associated with Kennedy's disease, an X-linked neuro-degenerative disorder characterized by a slowly progressing muscle weakness (La Spada et al., 1991).

The androgen R1881 induced a second upshift, resulting in three AR isotypes (110, 112, and 114 kDa). These ligand-induced changes could be due to additional phosphorylation and/or relocation of phosphorylated sites. The R1881-induced upshift is dependent on the Ser-80 consensus phosphorylation site. Substitution of the Ser-93 consensus phosphorylation site resulted in a reduction in the amount of the ligand-induced isotype.

Interestingly, the migration pattern of AR13 consisted of three major isotypes, which might represent the triplet found for the wild-type AR0 when expressed in the presence of ligand. Thus, in addition to being constitutively active with respect to transcription, AR13 also seems to be constitutively phosphorylated.

Analogous to the human PR (Bagchi et al., 1992; Takimoto et al., 1992) and mouse ER (Lahooti et al., 1994), AR DNA binding is important for the hormone-induced 114 kDa upshift. The immunoblot pattern of AR64 (unable to bind DNA because of the substitution of two essential cysteines in the first zinc finger) revealed a different distribution of receptor protein over the three isotypes compared to the AR0 triplet. There was less of the slowest 114 kDa isotype present in the AR64 sample. A comparable observation was made when a mutation was introduced in the DNA binding domain of the constitutively active AR13 (AR30.3). The slowest migrating isotype of AR30.3 became less intense compared to the other isotypes. Both AR64 and AR30.3 contain mutations in the DNA binding domain and subsequently are unable to activate transcription. It could still be possible that the hormone-induced upshift is not only DNA dependent but occurs predominantly during or after transcription activation. The R1881-induced migration pattern of AR60, which contains an intact DNA binding domain but is unable to transactivate due to the deletion of amino acids 244−528, was clearly aberrant. A comparable observation was made for AR126, which was only weakly constitutively active due to an N-terminal deletion (Δ370−528). As was found for AR60, its slowest migrating isotype was less intense. These observations suggest that part of the hormone-induced phosphorylation is not solely dependent on DNA binding, but rather occurs during or following transcription activation. However, it cannot be excluded that due to the large N-terminal deletion, conformational changes were introduced or phosphorylation sites erased.

Interestingly, although elimination of the transactivating capacity seemed to affect hormone-induced phosphorylation, substitution of the proposed phosphorylation sites did not affect the transactivating capacity of the AR. Similarly, Mason and Housley (1993) have shown that substitution of phosphorylation sites in the mouse GR had little effect on its capacity to activate transcription. On the other hand, phosphorylation sites in the human ER and human VDR have been shown to be important for the transactivating capacity of these receptors (Hsieh et al., 1991; Ali et al., 1993; Le Goff et al., 1994).

As has been observed for several other steroid receptors, a significant discrepancy exists between the predicted molecular mass based on the AR sequence (98.4 kDa) and the AR protein size determined by SDS−PAGE (110 kDa) (Table 1). The region responsible for this mobility retardation of approximately 12 kDa is largely deleted in AR7 (Δ51−211) (Table 1). The calculated molecular mass (81.2 kDa) and its SDS−PAGE size determination (~84 kDa) are comparable. Recently, it has been shown that the mobility retardation of the mouse GR can be attributed to an N-terminal region of approximately 200 amino acids containing all identified phosphorylation sites within the receptor (Hutchison et al., 1993). With the SDS−PAGE analysis of many different AR deletion mutants (Jenster et al., 1991,

Table 1: Summary of AR Mutants Showing the Effect of the Mutation on SDS−PAGE Size and Migration Pattern[a]

AR mutant	mutation	calculated mass (kDa)	SDS−PAGE mass (kDa)	migration pattern −R1881	migration pattern +R1881
AR0 (20 Gln)	wild-type	98.4	110	D	T
AR7	Δ51−211	81.2	84	S	
AR9	Δ46−101	92.3	104	S	S
AR10	Δ100−142	94.3	103	S	D
AR11	Δ139−188	93.6	103	D	
AR19	Δ186−244	92.6	104	D	
ARS80G	S80G	98.4	110	S	S
ARS93A	S93A	98.4	110	S	pD
ARS118A	S118A	98.4	110	D	T
AR64	C567S; C570F	98.4	110	D	pT
AR60	Δ244−528	70.2	76	D	pT
AR13	Δ653−910	68.5	84	T	T
AR30.3	Δ653−910 RK608GA	68.5	84	pT	pT
AR126	Δ370−528 Δ627−910	50.4	60	pT	pT
AR70 (0 Gln)	Δ58−77	95.9	105	S	T
AR73 (7 Gln)		96.8	107	"D"	
AR76 (12 Gln)		97.4	108	"D"	
AR77 (16 Gln)		98.0	109	"D"	
AR74 (29 Gln)		99.6	112	D	
AR75 (48 Gln)		102.0	114	D	

[a] Calculated molecular mass is based on the amino acid residue constitution, and SDS−PAGE mass is an estimation of the size determined after gel electrophoresis. Migration patterns are indicated as S (single form), D (doublet), "D" (less spaced doublet), pD (partial doublet), T (triplet), and pT (partial triplet) in the absence (−) or presence (+) of 1 nM R1881.

1993), it became clear that the first part of the AR N-terminal domain (amino acids 46−244) is responsible for the discrepancy between the predicted and SDS−PAGE-determined molecular masses. Comparable to the mouse GR, this region contains most, if not all, AR phosphorylation sites (Kuiper et al., 1993). Deletion of this region in the human AR and mouse GR resulted in the loss of the capacity to activate transcription (Danielsen et al., 1987; Jenster et al., 1991). The basis for the molecular mass discrepancy is unknown, although it is unlikely that it is caused by phosphorylation, since alkaline phosphatase treatment had no effect on the apparent 110 kDa molecular mass of the wild-type AR determined by SDS−PAGE. Possibly, the region between amino acids 46 and 244 binds relatively few SDS molecules, resulting in a less negatively charged protein and the observed mobility retardation during SDS−PAGE. So far, it has not been possible to attribute the mobility retardation to a smaller domain, since the SDS−PAGE molecular masses of AR9, AR10, AR11, and AR19 were all 10−12 kDa above their calculated sizes (Table 1).

It becomes evident that the N-terminal region between residues 46 and 244 in the hAR is of high interest since it (1) is essential for transcription activation (Jenster et al., 1991), (2) contains a glutamine stretch that is involved in the capacity of the AR to activate transcription, (3) harbors most of the AR phosphorylation sites (Kuiper et al., 1993), and (4) is responsible for the mobility retardation.

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REFERENCES

Ali, S., Metzger, D., Bornert, J.-M., & Chambon, P. (1993) Modulation of transcriptional activation by ligand-dependent phosphorylation of the human oestrogen receptor A/B region. *EMBO J. 12*, 1153−1160.

Auricchio, F. (1989) Phosphorylation of steroid receptors. *J. Steroid Biochem. 32*, 613−622.

Bagchi, M. K., Tsai, S. Y., Tsai, M.-J., & O'Malley, B. W. (1992) Ligand and DNA-dependent phosphorylation of human progesterone receptor in vitro. *Proc. Natl. Acad. Sci. U.S.A. 89*, 2664−2668.

Beck, C. A., Weigel, N. L., & Edwards, D. P. (1992) Effects of hormone and cellular modulators of protein phosphorylation on transcription activity, DNA binding, and phosphorylation of human progesterone receptors. *Mol. Endocrinol. 6*, 607−620.

Bodwell, J. E., Ortí, E., Coull, J. M., Pappin, D. J. C., Smith, L. I., & Swift, F. (1991) Identification of phosphorylated sites in the mouse glucocorticoid receptor. *J. Biol. Chem. 266*, 7549−7555.

Brinkmann, A. O., Faber, P. W., van Rooij, H. C. J., Kuiper, G. G. J. M., Ris, C., Klaassen, P., van der Korput, J. A. G. M., Voorhorst, M. M., van Laar, J. H., Mulder, E., & Trapman, J. (1989) The human androgen receptor: domain structure, genomic organization and regulation of expression. *J. Steroid Biochem. 34*, 307−310.

Brown, T. A., & DeLuca, H. F. (1990) Phosphorylation of the 1,25-dihydroxyvitamin D_3 receptor. *J. Biol. Chem. 265*, 10025−10029.

Brown, T. A., & DeLuca, H. F. (1991) Sites of phosphorylation and photoaffinity labeling of the 1,25-dihydroxyvitamin D_3 receptor. *Arch. Biochem. Biophys. 286*, 466−472.

Chauchereau, A., Loosfelt, H., & Milgrom, E. (1991) Phosphorylation of transfected wild type and mutated progesterone receptors. *J. Biol. Chem. 266*, 18280−18286.

Chen, C., & Okayama, H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol. 7*, 2745−2752.

Dalman, F. C., Sanchez, E. R., Perini, F., & Pratt, W. B. (1988) Localization of phosphorylation sites with respect to the functional domains of the mouse L cell glucocorticoid receptor. *J. Biol. Chem. 263*, 12259−12267.

Danielsen, M., Northrop, J. P., Jonklaas, J., & Ringold, G. M. (1987) Domains of the glucocorticoid receptor involved in specific and nonspecific deoxyribonucleic acid binding, hormone activation, and transcriptional enhancement. *Mol. Endocrinol. 1*, 816−822.

Denner, L. A., Schrader, W. T., O'Malley, B. W., & Weigel, N. L. (1990) Hormonal regulation and identification of chicken progesterone receptor phosphorylation. *J. Biol. Chem. 265*, 16548−16555.

Denton, R. R., Koszewski, N. J., & Notides, A. C. (1992) Estrogen receptor phosphorylation. Hormonal dependence and consequence on specific DNA binding. *J. Biol. Chem. 267*, 7263−7268.

Evans, R. M. (1988) The steroid and thyroid hormone receptor superfamily. *Science 240*, 889−895.

Faber, P. W., Kuiper, G. G. J. M., van Rooij, H. C. J., van der Korput, J. A. G. M., Brinkmann, A. O., & Trapman, J. (1989) The N-terminal domain of the human androgen receptor is encoded by one large exon. *Mol. Cell. Endocrinol. 61*, 257−262.

Green, S., & Chambon, P. (1988) Nuclear receptors enhance our understanding of transcription regulation. *Trends Genet. 4*, 309−314.

Higuchi, R., Krummel, B., & Saiki, R. K. (1988) A general method of in vitro preparation and specific mutagenesis of DNA fragment: study of protein and DNA interactions. *Nucleic Acids Res. 16*, 7351−7367.

Hoeck, W., & Groner, B. (1990) Hormone-dependent phosphorylation of the glucocorticoid receptor occurs mainly in the amino-terminal transactivation domain. *J. Biol. Chem. 265*, 5403−5408.

Hsieh, J. C., Jurutka, P. W., Galligan, M. A., Terpening, C. M., Haussler, C. A., Samuels, D. S., Shimizu, Y., Shimizu, N., & Haussler, M. R. (1991) Human vitamin D receptor is selectively phosphorylated by protein kinase C serine 51, a residue crucial to its trans-activation function. *Proc. Natl. Acad. Sci. U.S.A. 88*, 9315−9319.

Hunter, T., & Karin, M. (1992) The regulation of transcription by phosphorylation. *Cell 70*, 375−387.

Hutchison, K. A., Dalman, F. C., Hoeck, W., Groner, B., & Pratt, W. B. (1993) Localization of the ~12 kDa Mr discrepancy in gel migration of the mouse glucocorticoid receptor to the major phosphorylated cyanogen bromide fragment in the transactivating domain. *J. Steroid Biochem. Mol. Biol. 46*, 681−686.

Jenster, G., van der Korput, J. A. G. M., van Vroonhoven, C., van der Kwast, Th. H., Trapman, J., & Brinkmann, A. O. (1991) Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. *Mol. Endocrinol. 5*, 1396−1404.

Jenster, G., Trapman, J., & Brinkmann, A. O. (1993) Nuclear import of the human androgen receptor. *Biochem. J. 293*, 761−768.

Jurutka, P. W., Terpening, C. M., & Haussler, M. R. (1993) The 1,25-dihydroxy-vitamin D_3 receptor is phosphorylated in response to 1,25-dihydroxy-vitamin D_3 and 22-oxacalcitriol in rat osteoblasts, and by casein kinase II in vitro. *Biochemistry 32*, 8184−8192.

Kemp, B. E., & Pearson, R. B. (1990) Protein kinase recognition sequence motifs. *Trends Biochem. Sci. 15*, 342−346.

Kennelly, P. J., & Krebs, E. G. (1991) Consensus sequences as substrates specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem. 266*, 15555−15558.

Krongrad, A., Wilson, C. M., Wilson, J. D., Allman, D. R., & McPhaul, M. J. (1991) Androgen increases androgen receptor protein while decreasing receptor mRNA in LNCaP cells. *Mol. Cell. Endocrinol. 76*, 79−88.

Kuiper, G. G. J. M., & Brinkmann, A. O. (1994) Steroid hormone receptor phosphorylation: is there a physiological role? *Mol. Cell. Endocrinol. 100*, 103−107.

Kuiper, G. G. J. M., de Ruiter, P. E., Grootegoed, J. A., & Brinkmann, A. O. (1991) Synthesis and post-translational modification of the androgen receptor in LNCaP cells. *Mol. Cell. Endocrinol. 80*, 65−73.

Kuiper, G. G. J. M., de Ruiter, P. E., Trapman, J., Boersma, W. J. A., Grootegoed, J. A., & Brinkmann, A. O. (1993) Localization and hormonal stimulation of phosphorylation sites in the LNCaP-cell androgen receptor. *Biochem. J. 291*, 95−101.

Lahooti, H., White, R., Danielian, P. S., & Parker, M. G. (1994) Characterization of ligand dependent phosphorylation of the estrogen receptor. *Mol. Endocrinol. 8*, 182−188.

La Spada, A. R., Wilson, E. M., Lubahn, D. B., Harding, A. E., & Fischbeck, K. H. (1991) Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature 352*, 77−79.

Laudet, V., Hänni, C., Coll, J., Catzeflis, F., & Stéhelin, D. (1992) Evolution of the nuclear receptor gene superfamily. *EMBO J. 11*, 1003−1013.

Le Goff, P., Montano, M. M., Schodin, D. J., & Katzenellenbogen, B. S. (1994) Phosphorylation of the human estrogen receptor. *J. Biol. Chem. 269*, 4458−4466.

Mason, S. A., & Housley, P. R. (1993) Site-directed mutagenesis of the phosphorylation sites in the mouse glucocorticoid receptor. *J. Biol. Chem. 268*, 21501−21504.

Meek, D. W., & Street, A. J. (1992) Nuclear protein phosphorylation and growth control. *Biochem. J. 287*, 1−15.

Mhatre, A. N., Trifiro, M. A., Kaufman, M., Kazemi-Esfarjani, P., Figlewicz, D., Rouleau, G., & Pinsky, L. (1993) Reduced transcriptional regulatory competence of the androgen receptor in X-linked spinal and bulbar muscular atrophy. *Nature Genet. 5*, 184−188.

Migliaccio, A., Rotondi, A., & Auricchio, F. (1986) Estradiol receptor: phosphorylation on tyrosine in uterus and interaction with antiphosphotyrosine antibody. *EMBO J. 5*, 2867−2872.

Moudgil, V. K. (1990) Phosphorylation of steroid hormone receptors. *Biochim. Biophys. Acta 1055*, 243−258.

Ortí, E., Mendel, D. B., Smith, L. I., & Munck, A. (1989) Agonist-dependent phosphorylation and nuclear dephosphorylation of glucocorticoid receptors in intact cells. *J. Biol. Chem. 264*, 9728−9731.

Ortí, E., Bodwell, J. E., & Munck, A. (1992) Phosphorylation of steroid hormone receptors. *Endocrine Rev. 13*, 105−128.

Poletti, A., & Weigel, N. L. (1993) Identification of a hormone-dependent phosphorylation site adjacent to the DNA-binding domain of the chicken progesterone receptor. *Mol. Endocrinol. 7*, 241−246.

Riegman, P. H. J., Vlietstra, R. J., van der Korput, J. A. G. M., Brinkmann, A. O., & Trapman, J. (1991) The promoter of the prostate-specific antigen gene contains a functional androgen response element. *Mol. Endocrinol. 5*, 1921−1930.

Schüle, R., Muller, M., Kaltschmidt, C., & Renkawitz, R. (1988) Many transcription factors interact synergistically with steroid receptors. *Science 242*, 1418−1420.

Sheridan, P. L., Krett, N. L., Gordon, J. A., & Horwitz, K. B. (1988) Human progesterone receptor transformation and nuclear down-regulation are independent of phosphorylation. *Mol. Endocrinol. 2*, 1329−1342.

Sheridan, P. L., Francis, M. D., & Horwitz, K. B. (1989) Synthesis of human progesterone receptors in T47D cells. *J. Biol. Chem. 264*, 7054−7058.

Takimoto, G. S., & Horwitz, K. B. (1993) Progesterone receptor phosphorylation. Complexities in defining a functional role. *Trends Endocrinol. Metab. 4*, 1−7.

Takimoto, G. S., Tasset, D. M., Eppert, A. C., & Horwitz, K. B. (1992) Hormone-induced progesterone receptor phosphorylation consists of sequential DNA-independent and DNA-dependent stages: analysis with zinc finger mutants and the progesterone antagonist ZK98299. *Proc. Natl. Acad. Sci. U.S.A. 89*, 3050−3054.

Truss, M., & Beato, M. (1993) Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. *Endocrinol. Rev. 14*, 459−479.

van Laar, J. H., Bolt-de Vries, J., Zegers, N. D., Trapman, J., & Brinkmann, A. O. (1990) Androgen receptor heterogeneity and phosphorylation in human LNCaP cells. *Biochem. Biophys. Res. Commun. 166*, 193−200.

van Laar, J. H., Berrevoets, C. A., Trapman, J., Zegers, N. D., & Brinkmann, A. O. (1991) Hormone-dependent androgen receptor phosphorylation is accompanied by receptor transformation in human lymph node carcinoma of the prostate cells. *J. Biol. Chem. 266*, 3734−3738.

Washburn, T., Hocutt, A., Brautigan, D. L., & Korach, K. S. (1991) Uterine estrogen receptor in vivo: phosphorylation of nuclear specific forms on serine residues. *Mol. Endocrinol. 5*, 235−242.

Zegers, N. D., Claassen, E., Neelen, C., Mulder, E., van Laar, J. H., Voorhorst, M. M., Berrevoets, C. A., Brinkmann, A. O., van der Kwast, Th. H., de Winter, J. A. R., Trapman, J., & Boersma, W. J. A. (1991) Epitope prediction and confirmation for the human androgen receptor: generation of monoclonal antibodies for multi-assay performance following the synthetic peptide strategy. *Biochim. Biophys. Acta 1073*, 23−32.