Chicken Progesterone Receptor Expressed in *Saccharomyces cerevisiae* Is Correctly Phosphorylated at All Four Ser-Pro Phosphorylation Sites†

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ABSTRACT: This study describes the phosphorylation of chicken progesterone receptor (cPR) produced in yeast, *Saccharomyces cerevisiae*, and examines the dependence of specific phosphorylations on hormone and DNA binding. The chicken progesterone receptor is expressed *in vivo* as two forms, cPR_B and a smaller form, cPR_A. Characterization of the phosphorylation sites in the cPR_B form expressed in yeast shows that progesterone receptor is phosphorylated on the three serines (Ser211, Ser260, and Ser530) reported previously in chicken oviduct. An additional site which was phosphorylated in response to hormone was also detected and was subsequently identified as Ser367. Although cPR_B and cPR_A are phosphorylated identically in chicken oviduct, cPR_A expressed in yeast is phosphorylated on Ser211, Ser260, and Ser367, but phosphorylation of Ser530 is almost undetectable. In contrast, cPR_B expressed in yeast is phosphorylated on all four sites. No phosphorylations were found in or near the region required for hormone binding, indicating that phosphorylation is not required for hormone binding. In order to determine whether any of the phosphorylations were DNA-dependent, phosphorylation was also studied using cPR_A containing a partial deletion of the DNA binding domain. Two of the sites, Ser211 and Ser367, showed reduced phosphorylation in this mutant, suggesting a possible requirement for DNA binding activity for the phosphorylation of these sites. To our knowledge, this is one of the first demonstrations that a eucaryotic protein expressed in yeast is correctly phosphorylated.

The chicken progesterone receptor is expressed as two forms, PR_B (M_r = 86K) and PR_A (M_r = 72K), in chicken oviduct. These forms arise by alternate initiation of translation from the same primary transcript (Conneely et al., 1987). The finding that steroid receptors are phosphoproteins (Dougherty et al., 1982; Housely & Pratt, 1983; Pike & Sleator, 1985; Horwitz et al., 1985; Migliaccio et al., 1986; Denner et al., 1987) and that some become hyperphosphorylated after binding their ligands suggests that phosphorylation plays a role in receptor function. Previous studies have suggested that phosphorylation is required for hormone binding of estrogen and glucocorticoid receptors [see Orti et al. (1992) for a review] and that phosphorylation can modify the affinity of progesterone receptor for DNA (Denner et al., 1989; Beck et al., 1992) and plays a role in transcriptional activation of the receptors (Denner et al., 1990a).

We have reported the identification of three phosphorylation sites in cPR using nonequilibrium labeling in chicken oviduct slices. Two of those, Ser211 and Ser260, are hormone-stimulated, and the third, Ser530, is phosphorylated only in response to hormone treatment (Denner et al., 1990b). We have recently identified a second hormone-dependent phosphorylation site, Ser367 (Poletti & Weigel, 1993), which corresponds to a phosphopeptide which was first detected in receptor expressed in yeast as is reported here. Interestingly, each hormone-dependent site lies in a separate transactivation domain flanking the DNA binding domain.

A previous study has shown that cPR is rapidly phosphorylated during *in vitro* transcription assays (Weigel et al., 1992) by a DNA-dependent protein kinase present in the HeLa nuclear extract used as a source of general transcription factors. Subsequent studies using human progesterone receptor showed that the DNA-dependent phosphorylation occurs prior to activation of target genes *in vitro* (Bagchi et al., 1992). Recent studies of human progesterone receptor suggest that the hormone-dependent phosphorylation occurs in two steps and that the final step is DNA-dependent (Beck et al., 1992; Takimoto et al., 1992).

We have developed a yeast expression system to produce wild-type and mutant receptors for structural and functional studies (Poletti et al., 1992). To our knowledge, few if any studies have been conducted to determine whether proteins from higher eucaryotes which have been expressed in yeast are correctly phosphorylated. The progesterone receptor expressed in yeast binds hormone with normal affinity and specificity, binds to DNA, and is transcriptionally active. However, since the known receptor phosphorylation sites (Denner et al., 1990b) are in regions which modulate receptor function rather than being in regions absolutely required for function, the detection of transcriptional activity does not answer the question of whether the receptor is correctly phosphorylated and has wild-type activity. Now, we have used this expression system to determine whether receptor expressed in yeast is correctly phosphorylated, to look for additional constitutive phosphorylations required for receptor activity, and to examine the hormone and DNA dependence of these modifications.

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EXPERIMENTAL PROCEDURES

Materials. Carrier-free $H_3{}^{32}PO_4$ (285 Ci/mg of phosphorus) and ^{125}I-labeled protein A were from ICN (Irvine, CA). [1,2-^{3}H]Progesterone (55 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL). Radioinert steroids were from Steraloids, Inc. (Wilton, NH). The monoclonal anti-cPR antibody (PR22) was kindly provided by Dr. David O. Toft (Mayo Clinic, Rochester, MN) (Sullivan et al., 1986). Rabbit anti-mouse IgG was from Zymed (South San Francisco, CA). Protein A–Sepharose was from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Acrylamide was from Serva Fine Biochemicals, Inc. (Paramus, NJ). All other gel electrophoresis supplies were from Bio-Rad (Richmond, CA). Oxalyticase was from Enzogenetics (Corvallis, OR). Trypsin (TPCK-treated) was from Worthington Biochemical Corp. (Freehold, NJ). Sequencing-grade endoproteinases AspN and GluC were from Boehringer Mannheim (Indianapolis, IN). HPLC reagents were from J. T. Baker Chemical Co. (Phillipsburg, NJ). Casamino acids, yeast nitrogen base without amino acids, and dextrose were obtained from Difco. Other reagents for yeast media, cell culture tested, were from Sigma (St. Louis, MO). Chemicals for manual [^{32}P]phosphate release were sequence-grade from Sigma or Aldrich (Milwaukee, WI). All other chemicals were reagent grade.

Yeast Strains and Plasmids. The *Saccharomyces cerevisiae* strain used was BJ3505 (*Mat α, pep4::HIS3 pr6-Δ* 1.6R *his3 lys2-208 trp1-delta101 ura3-52 gal2*can1) obtained from the Yeast Genetics Stock Center (Berkeley, CA).

Plasmids. Plasmid $YEpE_{21}$, which produces human estrogen receptor (hER) under the control of the copper-response CUP1 promoter (McDonnell et al., 1991), and plasmid $YEpA_2$, which produces full-length cPR_A under the control of two copies of the estrogen response element cloned upstream of the yeast CYC1 promoter, were described previously (Poletti et al., 1992). All receptor proteins in this study were produced as ubiquitin fusion proteins; the ubiquitin is rapidly cleaved *in vivo* by a host enzyme to produce authentic receptor (Butt et al., 1988). The $YEpB_3$ plasmid, which encodes cPR_B, was constructed as follows: plasmid $YEpP_2$ (Mak et al., 1989) was digested to completion with *Bgl*II and *Pfl*MI, and the resulting 1.3-kb fragment was cloned into the *Bgl*II/*Pfl*MI sites of $YEpA_2$. The $YEpB_3$ plasmid is equivalent to $YEpA_2$ in that the expression of PR_B is controlled by an upstream estrogen response element. The $YEpA_4$ plasmid was made by deleting the coding region from amino acids 455 to 483 of the cDNA of cPR_A using oligonucleotide-directed mutagenesis (Dobson et al., 1989). For insertion in the yeast vector, a polymerase chain reaction was utilized as previously described (Poletti et al., 1992) to create an *Nco*I site at the ATG start, and a *Kpn*I site 3′ of the TGA stop codon of the cPR_A cDNA. All PCR products were sequenced by the dideoxy method to confirm that no additional mutations were introduced during amplification (Sanger et al., 1977). $YRpP_2$ is a reporter plasmid in which two copies of a PRE/GRE were inserted upstream of the yeast CYC1 promoter fused to the *LacZ* gene. Details of this plasmid construction have been reported previously (Mak et al., 1989).

The yeast strain BJ3505 was transformed using a lithium acetate protocol (Ito et al., 1983), and transformants were selected by both tryptophan and uracil prototropy.

Yeast Growth and Labeling. As described previously (Poletti et al., 1992), the expression of cPR_A and cPR_B is regulated by upstream estrogen response elements in plasmids $YEpB_3$ and $YEpA_2$. Expression of progesterone receptor is induced by activation of the estrogen receptor which is expressed by the cotransformed plasmid $YEpE_2$. Yeast cells (50 000 cells/mL) were grown overnight at 30 °C in 600 mL of synthetic minimal medium (Sherman et al., 1982) in the presence of 100 μM $CuSO_4$ to induce synthesis of hER as described previously (Poletti et al., 1992). Twelve hours later, synthesis of cPR_A or cPR_B was initiated by adding 50 nM estradiol to the suspension (OD_{600} ~0.5–0.7) and incubating for 2 h at 30 °C.

$YEpA_4$ is regulated by the CUP1 promoter which responds to copper (Mak et al., 1989; Poletti et al., 1992). Production of the corresponding protein was initiated by adding 100 μM $CuSO_4$ and incubating for 2 h at 30 °C. Cells were collected by centrifugation at 3000 rpm for 10 min and resuspended in 50 mL of low-phosphate minimal media (in the presence of 2 mM mannose 6-phosphate barium salt and containing 0.1 of the initial amount of phosphate: 0.73 mM). Labeling experiments were done by adding 50 mCi of $H_3{}^{32}PO_4$ to the suspension and incubating 4 h at 30 °C in the presence or absence of 1 μM radioinert progesterone.

Receptor Purification and Identification of Phosphopeptides. All of the purification steps were done at 4 °C or on ice. Yeast cells were collected by centrifugation at 3000 rpm for 10 min and washed twice with homogenization buffer (50 mM potassium phosphate, 10 mM $NaMoO_4$, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, and 12 mM 1-thioglycerol, pH 7.0). Cells were resuspended in 15-mL tubes using 5 mL of homogenization buffer containing 0.5 M NaCl, in the presence of 5–6 g of acid-washed glass beads (0.5 mm; B. Braun Instruments, Baltimore, MD), and homogenized by vortexing 5 × 1 min at maximum speed. The suspension was then centrifuged at 2000 rpm for 10 min. The supernatant was saved, and the pellet was treated a second time as before with 0.5 M NaCl in homogenization buffer. After the second centrifugation, the two supernatants were combined and centrifuged at 100000*g* for 1 h to obtain yeast extract.

Receptor present in the high-speed extract was immunopurified by chromatography on a 1-mL PR22–protein A–Sepharose immunoaffinity column prepared as previously described (Schneider et al., 1982). A 2-mL protein A–Sepharose precolumn was utilized to remove nonspecific proteins which bind to the matrix. After extensive washing with immunopurification buffer (IPB = 0.5 M NaCl, 50 mM Tris, 0.02% NaN_3, and 0.2% Triton X-100, pH 7.5), followed by washing with H_2O, the column was eluted with 1 M CH_3-COOH. Samples were dried in a speed vacuum drier, resuspended in SDS–PAGE loading buffer, resolved by 7.5% SDS–PAGE (Laemmli, 1970), and stained with Coomassie Blue. Gels were exposed to X-AR film (Kodak) overnight. cPR bands were cut from gels, destained with 30% 2-propanol, washed twice with water, and then digested exhaustively with trypsin (typically each gel slice was placed in 0.5 mL of 50 mM NH_4HCO_3 at 37 °C, and six additions of 20 μg of trypsin were made at 4–6-h intervals). Tryptic peptides were analyzed by HPLC using a Vydac C_{18} reversed-phase column in 0.1% trifluoroacetic acid in water and eluted with an acetonitrile gradient (0.5%/min) (Denner et al., 1990b). Phosphotryptic peptides were identified using an on-line β-radioactive flow detector (Model IC Flo-One; Radiomatic Instruments, Inc., Tampa, FL).

AspN digestion was performed using 0.2 μg of AspN in 200 μL of 50 mM sodium phosphate buffer, pH 8.0, for 4 h at 37 °C. GluC digestion was performed using 1 μg of GluC in 200 μL of 25 mM ammonium bicarbonate, pH 7.8, for 8 h at 37 °C. At the end of the digestion, phosphopeptide cleavage was

analyzed by HPLC using the same conditions utilized for phosphotryptic peptide analysis.

Manual [^{32}P]phosphate release studies were done to localize the position of phosphoamino acids in the peptides using the method described by Sullivan and Wong (1991). Briefly, phosphotryptic peptides purified by HPLC or treated with a second endoproteinase were bound covalently to a disk of Arylamine-Sequelon (Milligen/Biosearch, Burlington, MA) using a solution of 10 mg/mL 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDAC) in 4-morpholineethanesulfonic acid (MES), pH 5.0. Disks were washed several times with trifluoroacetic acid (TFA) followed by methanol, counted to determine the initial amount of radioactivity, and subjected to manual Edman degradation. Disks were treated at 50 °C for 10 min with a coupling reagent containing methanol/water/triethylamine/phenyl isothiocyanate (7:1:1:1 v/v). After five washes with methanol, disks were then treated at 50 °C for 6 min with TFA to cleave the amino-terminal amino acid. The TFA solution was placed in a scintillation vial, and disks were washed with a mixture of TFA and 42.5% H_3PO_4 (9:1). The wash was combined with the TFA, and the released radioactivity was counted. Disks were washed 5 times with methanol prior to beginning another cycle.

Hormone Binding Studies. Scatchard plot analysis (Scatchard, 1949; Poletti et al., 1992) of [^{3}H]progesterone binding was done on cPR_B produced in yeast grown under the same conditions utilized for the labeling experiment, but the cells were disrupted by digestion of the yeast cell wall with oxalyticase, followed by spheroplast disruption by osmotic shock in TESH (10 mM Tris-HCl, 1 mM EDTA, and 12 mM monothioglycerol, pH 7.5) to minimize loss of binding activity due to mechanical shearing (Poletti et al., 1992).

Preparation of Nuclear Extracts and Western Analysis. Cytosolic extracts were prepared as described (Poletti et al., 1992) for Scatchard analysis. Nuclear extracts were prepared from the pellets recovered after osmotic shock by centrifugation at 1000*g* for 10 min. Pellets were washed with 0.02% Triton X-100 in TESH, followed by three washes with TESH. Extracts were obtained by incubating the nuclei with 0.5 M NaCl in TESH for 30 min on ice and then centrifuging at 100000*g* for 1 h.

Immunoblots were performed using an ECL chemiluminescent detection kit (Amersham, Arlington Heights, IL) using PR22 as the primary antibody and rabbit anti-mouse IgG (Weigel et al., 1992). Samples of cytosolic and nuclear extracts were prepared from cells treated for 4 h at 30 °C with 1 μM progesterone as described previously (Poletti et al., 1992).

RESULTS

Previous reports from this laboratory have shown that cPR_A and cPR_B produced in *S. cerevisiae* have biochemical properties similar to authentic receptor isolated from chicken oviducts (Mak et al., 1989; Poletti et al., 1992). The receptor forms used for these studies are shown in Figure 1. cPR_B (produced by $YEpB_3$) and cPR_A (produced by $YEpA_2$) are wild-type receptors; they share the same structure, but PR_A lacks the first 128 amino acids. In chicken oviducts, both forms are present because they appear to rise by alternate initiation of translation from the same mRNA (Conneely et al., 1989). $YEpA_4$ encodes cPR_A in which the second zinc finger has been deleted and does not bind either to specific or to nonspecific DNA.

In vivo ^{32}P incorporation into progesterone receptor was examined as shown in Figure 2. Receptors were immunopurified from control yeast cells bearing the plasmid $YEpA_2$

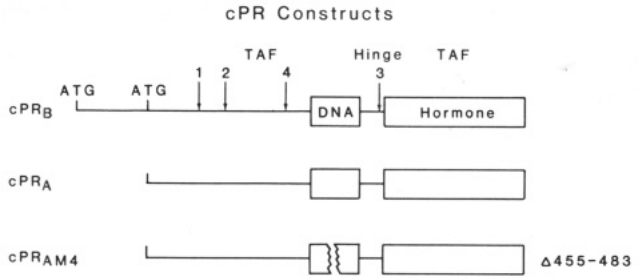

FIGURE 1: Structure of cPRs. cPR_B: chicken progesterone receptor form B. cPR_A: chicken progesterone receptor form A. cPR_{AM4}: cPR_A with a deletion of the second Zn finger. DNA and hormone indicate the DNA and hormone binding domains, respectively. TAFs indicate transactivation regions (Danielian et al., 1992), and Hinge is the hinge region connecting the DNA and hormone binding domains. 1, 2, 3, and 4 indicate the positions of the phosphorylation sites identified in authentic cPR; they are Ser211, Ser260, Ser530, and Ser367, respectively.

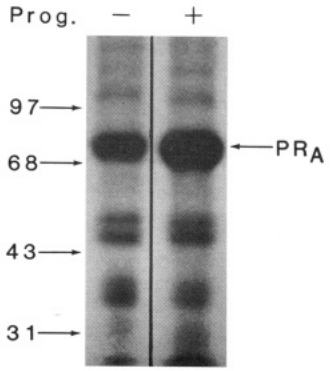

FIGURE 2: ^{32}P incorporation in cPR_A. PR_A indicates the location of the immunopurified cPR which was localized by Coomassie staining. Equal amounts of receptor were loaded in the two lanes, and phosphorylation was detected by autoradiography. Migration positions of molecular mass standards (in kilodaltons) are indicated. Prog: samples from control (–) or 1 μM progesterone-treated yeast.

encoding cPR_A or from cells which had been treated with progesterone; the receptor was analyzed by SDS–PAGE. Equal amounts of PR_A, determined by Coomassie staining, were present in the two lanes. The autoradiogram shows that hormone treatment increased receptor phosphorylation 4.5-fold (determined by counting the radioactivity present in the two bands after excision).

The bands of phosphorylated receptors were excised, exhaustively digested with trypsin, and analyzed by reversed-phase HPLC. Shown in Figure 3 are the phosphotryptic peptide maps of untreated cPR_A (panel A) and hormone-activated cPR_A (panel B) and cPR_B (panel C). Site 2 (Ser260) was present in the absence of hormone, but its ^{32}P incorporation was increased by hormone treatment (note change in scale). In PR_B, this site appeared to be a double peak, but further analysis of each peak (using digestion with endoproteinase and ^{32}P release) indicated that they both contained the same limit phosphopeptide (site 2). Whether the appearance of two peaks is due to partial digestion or to other causes is not known. Site 1 was present in both cases, but appeared to be extremely responsive to hormone. Site 3 (Ser530) was only detected in PR isolated from progesterone-treated yeast. Whereas PR_B exhibits a substantial amount of site 3 phosphorylation (Figure 3, panel C), PR_A contains almost no site 3 phosphopeptide (panel B). To confirm the identity of the site 2 and 3 peptides, the peptides were redigested with either AspN or GluC and reanalyzed. In each case, peptides with the elution positions reported for authentic cPR peptides (Denner et al., 1990b) were obtained (data not shown). In

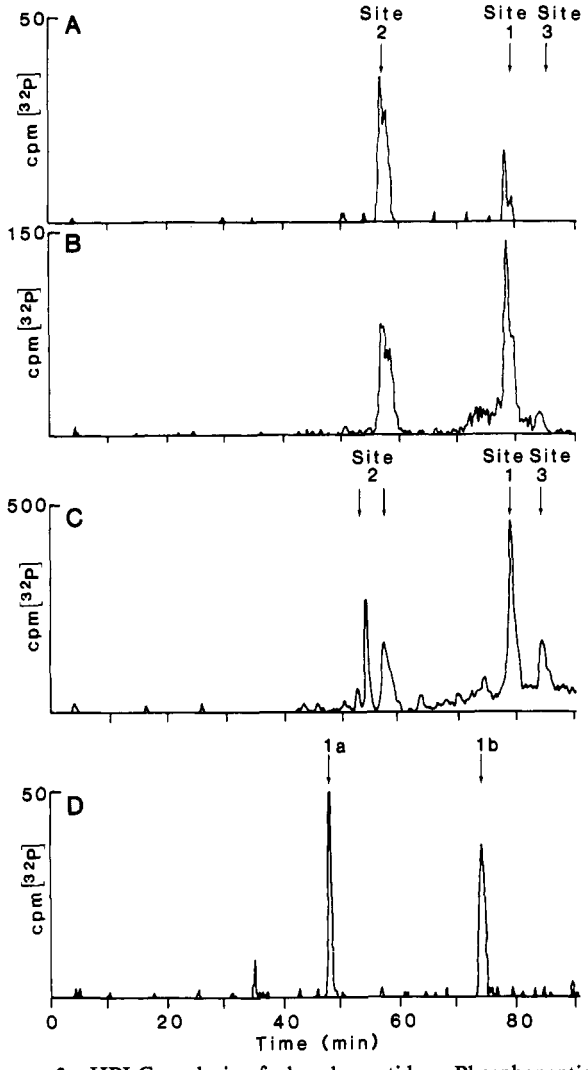

FIGURE 3: HPLC analysis of phosphopeptides. Phosphopeptides were prepared and analyzed on a C_{18} reversed-phase column as described under Experimental Procedures. Panel A, phosphotryptic peptides of cPR_A from control cells; panel B, phosphotryptic peptides of cPR_A from progesterone-treated cells; panel C, phosphotryptic peptides of cPR_B from progesterone-treated cells; panel D, redigestion of site 1 peak from PR_A with endoproteinase AspN. Site 1 indicates the elution position of Ser211- and Ser367-containing peptides; site 2 indicates the elution position of the Ser260-containing peptide; site 3 indicates the elution position of the Ser530-containing peptide. 1a and 1b represent the peptides containing Ser211 and Ser367, respectively, after digestion with AspN.

order to determine whether the large increase in site 1 was simply an increase in Ser211 phosphorylation or perhaps due to a new peptide, the site 1 phosphopeptide from progesterone-activated PR_A was treated with endoproteinase AspN, which cleaves on the N-terminal side of the aspartic acid. Peptides containing aspartic acid are cleaved and show different elution properties when rerun. HPLC analysis of this sample, shown in panel D, clearly shows the presence of two phosphopeptides; peak 1a corresponds to the peptide containing Ser211 (Denner et al., 1990b). Peak 1b is a novel peptide found in cPR from progesterone-treated yeast. On the basis of the absence of peak 1b in the AspN redigest of site 1 from receptor isolated from untreated yeast (data not shown), this new site is hormone-dependent. Recent studies (Poletti & Weigel, 1993) have confirmed that this site is also present in cPR isolated from oviducts and have identified the site as Ser367.

The peptide mapping data indicate that the same peptides are phosphorylated as in authentic cPR_A and cPR_B. However,

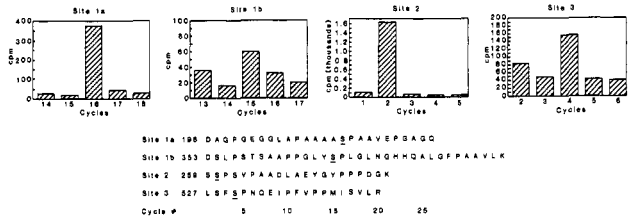

FIGURE 4: Manual ^{32}P release. Sites 1a, 1b, 2, and 3 indicate the ^{32}P release obtained from the corresponding peptides isolated from PR_B using the procedure described in Figure 3. The bottom of the figure shows the expected amino acid sequence of the peptides based on analyses of authentic chicken PR (Denner et al., 1990b). S indicates the phosphorylation sites in the peptides isolated from chicken receptor and thus the cycle in which the ^{32}P should be released if the yeast sites correspond to the chicken sites.

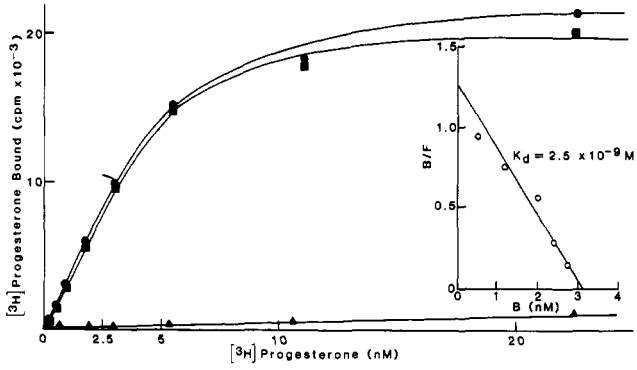

FIGURE 5: Hormone binding of cPR_B. Saturation analysis and Scatchard plot analysis of the cPR_B isolated from yeast cells bearing $YEpB_3$ plasmid, grown under the same conditions used in the phosphorylation studies (see Experimental Procedures for details). *B*, bound [^{3}H]progesterone; *F*, free [^{3}H]progesterone; K_d, dissociation constant. (●) Total hormone binding; (▲) nonspecific hormone binding; (■) specific hormone binding (total nonspecific).

these peptides contain multiple serines. To determine whether the same serines are phosphorylated in receptor isolated from yeast as are phosphorylated in authentic chicken progesterone receptor, manual Edman Degradations (see Experimental Procedures) were performed on the peptides to determine the cycles in which the ^{32}P is released. Since the only amino acid labeled in the receptor is phosphoserine (data not shown), the cycle containing the ^{32}P corresponds to the cycle containing the phosphoserine. Figure 4 shows the results of these analyses on peptides derived from PR_B. In each case, the ^{32}P was released in the expected cycle, confirming the identity of the sites. Thus, the receptor is correctly phosphorylated on all four Ser-Pro phosphorylation sites identified in authentic chicken progesterone receptor.

Previous studies suggested that phosphorylation might be required for hormone binding activity of steroid receptors such as glucocorticoid receptors (Nielsen et al., 1977) and estrogen receptors (Auricchio et al., 1981). The previous studies examining phosphorylation of chicken progesterone receptor in oviduct tissue slices (Denner et al., 1990b) were conducted under nonequilibrium labeling conditions such that constitutive sites which turn over slowly might not have been detected. We have previously shown that the progesterone receptor expressed in yeast has normal hormone binding affinity (Mak et al., 1989; Poletti et al., 1992). Figure 5 shows that under identical conditions used for induction of receptor for ^{32}P labeling studies (high concentrations of cells and low levels of inorganic phosphate), the receptor still retains normal hormone binding affinity. Only Ser211 and Ser260 are phosphorylated under these conditions. We have shown previously that receptor mutants lacking amino acids 1–400

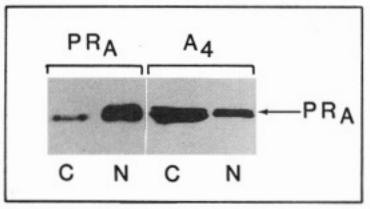

FIGURE 6: Distribution of cPR$_A$ and mutant expressed in yeast. Western blot analysis of samples obtained from cytosolic and nuclear extracts of yeast cells treated with 1 μM progesterone. C = cytosolic extracts; N = nuclear extracts.

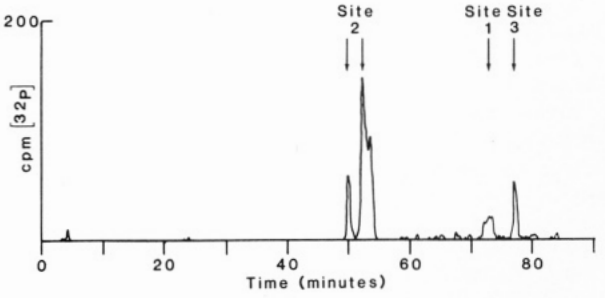

FIGURE 7: Phosphotryptic peptide mapping of receptor mutant. Samples were analyzed as in Figure 3, and the sites indicated correspond to those of Figure 3. The elution times differ somewhat from those in Figure 3 because a new column was used. However, the column was standardized with authentic chick PR peptides, and the elution positions for the known peptides are indicated.

retain normal hormone binding activity when expressed either in mammalian (Dobson et al., 1989) or in yeast (Mak et al., 1989) cells. Therefore, phosphorylation is not required to produce the hormone binding form of chicken progesterone receptor.

We have recently shown that the receptor is phosphorylated by a DNA-dependent kinase during *in vitro* transcription studies (Weigel et al., 1992). Takimoto et al. (1992) have reported that human progesterone receptor similarly undergoes DNA-dependent phosphorylation in T47D cells. To determine whether any of the progesterone receptor phosphorylations are DNA-dependent, we examined a mutant of cPR lacking a portion of the DNA binding domain (see Figure 1) to determine whether its phosphorylation was altered. This mutant still retains the region of the receptor which has been shown to be important for nuclear localization of the rabbit progesterone receptor (Guiochon-Mantel et al., 1989). As shown in Figure 6 a portion of this receptor is found in the nuclear fractions, but the nuclear/cytosol receptor ratio is decreased compared to wild type. The phosphorylation of the mutant in response to progesterone was determined as described for wild-type receptor. Figure 7 shows the map of phosphotryptic peptides obtained from cPR$_A$ with a deletion of the second zinc finger. Sites indicated correspond to those in Figure 3. Site 2 was phosphorylated in the mutant. The mutated receptor was also phosphorylated on site 3, Ser530, in response to hormone, indicating that DNA binding is not required for phosphorylation of this site. There is virtually no site 1 peak, indicating that neither the Ser211 nor the Ser367 peptide is phosphorylated to any significant extent (see Figure 3, panels A and B for comparison).

We have previously shown that receptor isolated from hormone-treated oviducts shows reduced mobility on SDS gels due to altered phosphorylation (Denner et al., 1990b). This change in phosphorylation correlates with the appearance of the two hormone-dependent phosphorylations. We have examined the mobility of the various PR$_A$ forms on SDS gels to determine which site is responsible for the change in mobility.

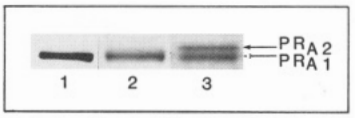

FIGURE 8: Analysis of immunopurified PR$_A$ by SDS gel electrophoresis. Shown is the PR$_A$ region of silver-stained gels. 1, untreated cPR$_A$; 2, progesterone-treated cPR$_A$; 3, progesterone-treated cPR$_A$ with deletion of second Zn finger. PR$_{A1}$ and PR$_{A2}$ indicate the two forms of cPR$_A$ detected after hormone treatment.

Figure 8 shows immunopurified wild-type and mutant PR$_A$ isolated from hormone-treated yeast and detected by silver staining of an SDS gel. The wild-type PR expressed in yeast appears as a single band, and hormone treatment produces essentially no receptor with reduced mobility. This receptor is essentially unphosphorylated on Ser530 (Figure 3B). The mutant shows a significant amount of receptor with slower mobility in response to progesterone treatment, indicating that DNA binding is not necessary for the phosphorylation responsible for altered mobility. Since the zinc finger mutant shows significant phosphorylation of Ser530 but essentially no phosphorylation of Ser367, yet its mobility is altered on SDS gels, it appears that phosphorylation of Ser530, not Ser367, is responsible for altered mobility.

DISCUSSION

The yeast expression system has become an important model to analyze the structure and function of numerous proteins. We previously have reported that progesterone receptor expressed in yeast has normal hormone binding properties and is transcriptionally active in yeast. Since there is good evidence that the activity of steroid receptors is regulated by phosphorylation, it was important to determine whether the progesterone receptor is correctly phosphorylated in yeast prior to undertaking functional studies of receptors containing mutated phosphorylation sites. The studies reported here show that the three sites (two hormone-stimulated and one hormone-dependent) previously identified in receptor isolated from cytosol prepared from chicken oviduct tissue slices (Denner et al., 1990b) are all phosphorylated in PR$_B$ expressed in yeast. In addition, we detected a second hormone-dependent phosphorylation site in receptor purified from whole cell extracts of progesterone-treated yeast. We have subsequently confirmed that this site is also phosphorylated in authentic chicken progesterone receptor isolated from whole cell extracts of oviduct tissue and have identified this site as Ser367 (Poletti & Weigel, 1993). The overall phosphorylation increases 3–5-fold in yeast in response to hormone. As in chicken, phosphorylation of Ser530 is observed only in the presence of hormone. Phosphorylation of Ser260 is somewhat enhanced in response to hormone, again as seen in authentic chicken receptor. Phosphorylation of the peptides in the site 1 peak is clearly very enhanced in response to hormone (note that site 1 is the major peak in the presence of hormone in Figure 3, but it is the smaller peak in the absence of hormone). Due to the low incorporation in the absence of hormone, it was impossible to obtain quantifiable double digests, but the Ser367 was clearly highly hormone-induced and there was also enhancement of Ser211, which is also consistent with authentic chicken receptor (Denner et al., 1990b; Poletti & Weigel, 1993). Thus, all four Ser-Pro motifs in cPR are phosphorylated both in chicken oviduct as well as in yeast. The kinase (or kinases) responsible for these phosphorylations has (have) not been identified.

We found that PR$_A$ expressed in yeast is not phosphorylated on Ser530 in response to hormone administration whereas it

shows phosphorylation indistinguishable from PR_B in chicken oviduct (Denner et al., 1990b). However, we have shown previously that activation of PR_A or a receptor deletion mutant containing only the DNA and hormone binding domains, but not PR_B, is harmful to yeast; the cells flocculate and appear to stop growing (Mak et al., 1989; Poletti et al., 1992). It is possible that the failure to phosphorylate this site is a result of this aberrant physiological situation or that the yeast cells rapidly degrade any PR_A which is phosphorylated on Ser530. The fact that the inactive PR_A mutant is phosphorylated on Ser530 suggests that there is no conformation intrinsic to PR_A expressed in yeast which prevents this phosphorylation.

One of the major goals of this study was to determine whether there are constitutive phosphorylations required for hormone binding which we could not detect in the oviduct slice system. These studies clearly show that phosphorylation is not required for hormone binding since the only constitutive phosphorylations are in regions which can be deleted without changing hormone binding (Mak et al., 1989).

Recent studies of human PR have shown that the phosphorylation which is responsible for the change in mobility on SDS gels is a DNA-dependent phosphorylation (Takimoto et al., 1992). Our studies indicate that this is not the case in cPR. The zinc finger deletion mutant cannot bind to DNA (O. Conneely, unpublished observation), yet it shows a band with altered mobility on SDS gels (Figure 8) presumably due to phosphorylation of Ser530 since the other hormone-dependent site, Ser367, is not phosphorylated. Surprisingly, Ser211 does not appear to be phosphorylated to any significant extent in the mutant. This site is normally partially phosphorylated even in the absence of hormone both in yeast (Figure 3A) and in chicken (Denner et al., 1990b), and it seems unlikely that at least the basal phosphorylation of this site would be DNA-dependent. However, Guiochon-Mantel et al. (1991) have reported that the rabbit progesterone receptor in the absence of hormone cycles between the cytosol and the nucleus, and DeFranco et al. (1991) have shown that the glucocorticoid receptor also shuttles beween the cytoplasm and the nucleus. It is possible, therefore, that the basal level of Ser211 phosphorylation occurs when the wild-type receptor is in the nucleus. Whether the changes in phosphorylation observed in these studies are related to a DNA-dependent phosphorylation, to altered receptor distribution, or to altered conformation remains to be determined. However, since the hormone-dependent Ser530 phosphorylation site is much closer to the second zinc finger than either of the affected sites, it seems unlikely that the altered phosphorylation is simply a result of a conformational change caused by the deletion.

Phosphorylation in proteins containing multiple phosphorylation sites sometimes occurs as a cascade with initial phosphorylation making the protein a substrate for subsequent phosphorylations (Pulverer et al., 1991). The fact that wild-type cPR_A shows Ser367 phosphorylation, but not Ser530 phosphorylation, whereas the Zn finger mutant shows phosphorylation of Ser530, but not Ser367, suggests that neither hormone-dependent phosphorylation is absolutely required for the second phosphorylation to occur. These sites are in separate transactivation domains whose activities vary depending upon the cell type and target gene (Bocquel et al., 1989). Thus, the relative importance of these two sites in receptor activation may be a function of the cell type and target gene.

This study is one of the first demonstrations that an exogenous eucaryotic protein expressed in yeast is correctly phosphorylated; these results suggest that the yeast expression system will be suitable for both *in vivo* and *in vitro* analyses of the role of phosphorylation in cPR_B function.

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