Phosphorylation of serine-167 on the human oestrogen receptor is important for oestrogen response element binding and transcriptional activation

Enrique CASTAÑO[1], Daria P. VOROJEIKINA and Angelo C. NOTIDES[2]

Departments of Environmental Medicine, Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, U.S.A.

We have studied the role of phosphorylation of the human oestrogen receptor (hOR; otherwise known as hER) at serine-167, which has been identified previously as the major oestrogen-induced phosphorylation site. We have tested transactivation by the hOR in yeast and cell-free transcription assays, and shown that mutation of serine-167 results in a 70% decrease in hOR-dependent transcription. Furthermore we explored the functional significance of phosphorylation at this site by hormone binding and DNA binding. DNA binding affinity was 10-fold lower when serine-167 was changed to alanine in the hOR. Cell-free transcription experiments showed that casein kinase II is the enzyme responsible for oestradiol-dependent phosphorylation of the hOR at serine-167. This suggests that a conformational change of the hOR must occur upon hormone binding that exposes serine-167 to casein kinase II, resulting in transactivation of oestrogen-responsive genes.

INTRODUCTION

The human oestrogen receptor (hOR; otherwise known as hER), a member of the superfamily of ligand-activated transcription factors, mediates the effects of oestrogens on sexual development, reproduction and growth [1]. The binding of an oestrogen to the hOR induces a cascade of events, including the release of accessory proteins (e.g. the heat-shock proteins), increased nuclear retention, DNA binding and the transcription of oestrogen-responsive genes [2]. Steroid receptors are also regulated by phosphorylation at functionally significant sites by specific protein kinases. In general, phosphorylation regulates many functions of transcription factors, including protein–protein interactions, nuclear translocation [3], DNA binding [4] and transcriptional activation [5]. The activity of protein kinases towards steroid receptors is regulated, in turn, by a number of biochemical pathways that make the phosphorylation of steroid receptors cell-specific. The involvement of multiple biochemical pathways exerts a higher order of control on the function of the steroid receptors in different cell types.

The hOR is a phosphoprotein that is hyperphosphorylated in response to hormone binding [6]. Phosphorylation of the hOR has also been shown to correlate with nuclear retention [6], *in vitro* DNA binding [6] and transactivation *in vivo* [7]. Following oestradiol binding, residues serine-154 and serine-167 of the hOR become phosphorylated in MCF-7 cells [8]. Other amino acids are phosphorylated in MCF-7 cells independently of oestradiol binding, including serine-118 and tyrosine-537 [8]. When the hOR is expressed in transfected COS-1 cells, it undergoes hormone-induced phosphorylation at serine-104, -106 and -118 [9]. It has been shown that serine-118 is phosphorylated by mitogen-activated protein kinase, and that this phosphorylation is involved in receptor activation by growth factors [10,11]. This site has been studied extensively, and it has been shown that mutation of serine-118 to alanine results in a decrease in transactivation in COS-1 and HeLa cells [9–12].

The mutation of tyrosine-537 to serine in the hOR causes constitutive transactivation in transient transfection assays. The result from constitutive transactivation was postulated to be due to the binding of SRC-1, a co-activator that only binds to the oestradiol–hOR complex, but can bind to the mutant Y537S hOR in the presence or absence of oestradiol [13].

The hOR can be phosphorylated *in vitro* at serine-167 by casein kinase II (CK II) [14]. CK II also phosphorylates the calf oestrogen receptor, resulting in an increased DNA binding affinity for an oestrogen response element (ORE; otherwise known as ERE) [15]. Phosphorylation by CK II increases the level of transcription with the progesterone [16] and vitamin D [17] receptors. Additionally, CK II phosphorylates other transcription factors, resulting in a change in either their DNA binding or transactivation properties [17–20]. From these results it has been shown that phosphorylation of the hOR has multiple effects on transactivation. However, the effect of phosphorylation of serine-167 on transcription has not been reported previously.

In the present study, we demonstrate that phosphorylation of serine-167 of the hOR is required for full hormonal transactivation of the hOR in yeast. This was accomplished using two yeast expression systems for the hOR. In the first system, the wild-type hOR and the S167A mutant receptor were expressed in a yeast strain containing a β-galactosidase gene downstream of a promoter driven by two perfect OREs. In the second system, the hOR and the S167A mutant hOR fused to the LexA DNA binding domain were expressed in a strain containing the β-galactosidase gene downstream of the LexA promoter. We have also developed a cell-free transcription system that differentiates between the unliganded hOR and the oestradiol-bound hOR. This system has allowed us to demonstrate that phosphorylation of the hOR by CK II enhances the receptor's affinity for an ORE and increases the transcription of oestrogen-responsive genes.

MATERIALS AND METHODS

Plasmids

The expression plasmids phER and pS167AhER were constructed by subcloning the cDNA of either the wild-type hOR or

Abbreviations used: (h)OR, (human) oestrogen receptor; CK II, casein kinase II; ORE, oestrogen response element.

[1] To whom correspondence should be addressed.

[2] Deceased 10 November 1996. This paper is dedicated to our beloved mentor and friend.

the S167A hOR mutant from the puc19-hER plasmid [21] into the *Eco*RI site of pSCW231. The plasmids pLexAhER and pLexAS167AhER were described previously [21], and contain the whole sequence of the hOR or the S167A hOR fused to the DNA sequence of the DNA binding domain of the LexA protein, such that the LexA DNA binding domain is N-terminal to the entire hOR. The plasmid pEC7 was constructed by removing the fragment between the *Eco*RI and *Bgl*II sites of pLovTATA, and cloning in the fragment of the vitellogenin II sequence from positions −744 to −313, which includes a perfect ORE. The plasmids pLovTATA and pML or MLP-C2AT(190) contain a guanidine-free sequence. This sequence is not digested by RNase T1 and serves as a reporter for the *in vitro* transcription assays [22]. YRPE2 and pSCW231 have been described previously [23,24], and were generously provided by Bert O'Malley (Baylor College of Medicine, Houston, TX, U.S.A.). The plasmid pVitII [25] was provided by John Burch (Institute for Cancer Research, Philadelphia, PA, U.S.A.).

Site-directed mutagenesis of the hOR

Oligonucleotide site-directed mutagenesis of the hOR was performed by the method of Kunkel et al. [26]. The mutated cDNA of the hOR was cloned into the *Eco*RI site of the pVL1393 baculovirus transfer vector. The orientation of the cloned fragment was confirmed by a *Bgl*II digestion, and the sequence of the mutant S167A hOR was verified by DNA sequencing.

Transactivation of the hOR in a yeast expression/reporter system

The system was constructed by co-transforming the yeast strain 939 [(MATa prb1-1122 prc1-407 pep4-3 leu2 trp1 ura3-52)/(MATα prb1-1122 prc1-407 pep4-3 leu2 trp1 ura3-52)] [27] with two different vectors, an effector plasmid (either phER or pS167AhER) and a reporter plasmid YRPE2, using the one-step transformation method [27]. Yeast cells were grown overnight at 30 °C to an A_{600} of 0.5. A 2 ml portion of the yeast strain culture was distributed into test tubes without or with 10 nM oestradiol and grown on synthetic dextrose minimal medium supplemented with 100 mg/l leucine for 4 h. Plasmids LexAhER and LexAS167AhER were transformed in the strain CTY10-5d (MATa ade2 trp1-901 leu2-3,112 his3-200 gal4 gal80 URA3::lexA op-lacZ) using the one-step transformation method. For transactivation assays, the yeast cells were grown up for 18 h in a synthetic dextrose minimal medium containing 0.67 % Bacto-Yeast nitrogen base and 2 % glucose. In addition, the media for each of the yeast strains were supplemented with 100 mg/l leucine and 20 mg/l each of adenine, histidine and methionine. The activity of the reporter gene product, β-galactosidase, was assayed at 420 nm as described previously [27]. Miller units were calculated as follows, as described by Miller [28]:

Miller units = $1000 \times A_{420}$ (absorbance of the product)/A_{600} of the assayed culture × volume (ml) × time (min).

Preparation of recombinant hORs and human progesterone receptors

Spodptera frugiperda Sf9 cells were infected with the baculovirus containing the hOR cDNA, the S167A hOR cDNA or human progesterone receptor cDNA for 4 days at 27 °C, and then lysed by freezing and thawing in a hypotonic buffer (20 mM Tris, 1 mM EDTA, 1 mM NaF, 0.5 mM PMSF and 1 mM EGTA, pH 7.5). KCl was added to a final concentration of 400 mM, then the lysate was centrifuged at 28000 ***g*** for 10 min. Ammonium sulphate was added slowly to the supernatant to 40 % satn., and the mixture was incubated for 30 min on ice. The precipitate was pelleted by centrifugation at 28000 ***g***, then resuspended in a buffer (1 mM EDTA, 1 mM EGTA, 150 mM KCl, 10 % glycerol, 0.5 mM PMSF and 40 mM Tris, pH 7.4). This extract was used as the source of non-pure hOR. Purification of the hOR and S167A hOR was carried out by ORE-affinity chromatography, conducted as described previously [29]. The hOR obtained from this procedure was estimated to be 85 % pure, based on silver staining of a PAGE separation.

DNA probes and gel mobility shift assays

For gel mobility shift assays, the following probes, containing a perfect ORE, were used: 5′-GATCTCGTAGGTCACTGTGACCTCTA and 5′-GATCTAGAGGTCACAGTGACCTACGA. The oligonucleotides were labelled and annealed as described previously [30]. Both the wild-type and mutant receptors were incubated with 20 nM CK II and 0.5 mM ATP for 15 min at 30 °C.

The ORE binding reaction contained between 2 nM and 20 nM baculovirus-expressed hOR or S167A hOR, 1 μg of poly(dI-dC), 5 ng of probe (5000 c.p.m./ng), 25 mM Hepes (pH 7.4), 1 mM Na_2VO_4, 80 mM NaCl, 10 % glycerol, 0.5 mM PMSF and 1 mM leupeptin in a final volume of 20 μl. The ORE binding reaction was incubated on ice for 50 min before electrophoresis. Relative band intensities were quantified by volume integration, using Image Quant software and a PhosphorImager SF from Molecular Dynamics. The reported K_d values are means ± S.E.M. of values determined in three independent experiments.

Western blot analysis

Lysates of Sf9 cells infected with baculovirus expressing the hOR or S167A hOR were mixed with SDS sample buffer, boiled for 5 min and loaded on to an SDS/10 %-polyacrylamide gel. Yeast extracts were made utilizing the glass beads method as described previously [27]. After electrophoresis, the proteins were electrotransferred on to a PVDF membrane. The hOR was visualized on Western blots using previously described methods [29].

Cell-free transcription assay

Where noted, the hORs or S167A hORs were first incubated without or with 100 nM oestradiol in the presence of 20 nM CK II or 1 μM CK II substrate peptide (Promega) for 15 min at 30 °C with 0.5 mM ATP in transcription buffer. CK II was generously donated by Dr. D. W. Litchfield and Dr. E. G. Krebs (University of Washington, Seattle, WA, U.S.A.). In all cases, the hORs or S167A hORs were incubated without or with 100 nM oestradiol for 1 h on ice in a transcription buffer (10 % glycerol, 0.5 mg salmon sperm DNA, 1 unit of RNasin, 1 mM PMSF, 1 mM leupeptin, 200 ng of plasmid pEC7, 80 mM NaCl and 20 mM Hepes, pH 7.9). HeLa nuclear extract (8 units; Promega) was added to the 20 μl reaction along with 6 mM $MgCl_2$, 1 mM 3′-*O*-methyl-GTP, 5 mM phosphocreatine, 10 units of RNase T1 and 50 ng of plasmid pML. The reaction was incubated for 15 min at 21 °C, followed by the addition of 0.5 mM rCTP, 0.5 mM rATP and 10 μCi of [α-^{32}P]UTP for 45 min at 30 °C. The reaction was terminated as described previously [22]. Relative band intensities were quantified by volume integration, using Image Quant software and a PhosphorImager SF.

Immunoprecipitation of the hOR from the cell-free transcription assay

The assay was carried out by substituting 20 μCi of [γ-^{32}P]ATP for the [α-^{32}P]UTP. After 45 min, a buffer containing 50 mM Tris (pH 7.5), 1 % Triton X-100 and 1 % sodium azide was added to the mixture containing the hOR, and the proteins were immunoprecipitated with 100 ng of anti-hOR antibody 6 for 3 h on ice. Protein A–Sepharose CL-4B (10 μg) was incubated in the transcription reaction for 3 h; the reaction mixture was then washed three times with 150 mM NaCl, then with 500 mM NaCl and 1 M NaCl. The reaction mixture was centrifuged and the pellet was resuspended in SDS sample buffer and denatured at 95 °C for 5 min before SDS/PAGE. Relative band intensities were quantified by volume integration, using Image Quant software and a PhosphorImager SF. The anti-hOR antibody 6 is a polyclonal antibody made by immunizing rabbits with a peptide corresponding to amino acids 259–278 in the hinge region of the hOR.

RESULTS

Transcriptional activation of the hOR in yeast

Yeast systems have been useful for the assay of transcriptional properties of steroid receptors [27]. We utilized the yeast assay to check the effect of mutating serine-167 of the hOR into alanine on transactivation. The hOR expressed in the yeast expression reporter system was transcriptionally active and responsive to oestrogens, but not to anti-oestrogens or other steroid hormones (Table 1). Incubation of the yeast cells with 5 nM oestradiol increased β-galactosidase activity by an average of 345 Miller units. Oestradiol was approx. 6-fold more effective than oestriol in activating the hOR for transcription. This result is expected, since oestradiol has a 10-fold higher affinity for the hOR than does oestriol [30]. Tamoxifen or ICI 164,384 (1 μM) had little or no effect in activating transcription in this system. The transactivation of the hOR by oestradiol was reduced by 40 % in the presence of 1 μM tamoxifen and by 30 % in the presence of 1 μM ICI 164,384. Non-oestrogenic steroids, such as progesterone and testosterone, were inactive at concentrations up to 1 μM. Thus the yeast expression reporter system is a useful method with which to study the transactivation properties of the hOR.

The effect of phosphorylation of serine-167 of the hOR was studied by mutating this residue to alanine. The mutant S167A hOR showed a 75 % decrease in transactivation in comparison with the wild-type hOR (Figure 1A). Western blots with an anti-hOR antibody [29] showed similar expression levels of wild-type hOR and mutant S167A hOR in equivalent numbers of yeast cells (Figure 1B). Thus the decrease in transactivation of the mutant S167A hOR was not due to a lower level of hOR in the yeast cells.

Table 1 Steroid specificity of the hOR in a yeast expression/reporter system

Ligand	Concentration (nM)	β-Galactoside activity (Miller units)	Activity (%)
Oestradiol	5	345 ± 9	100
Oestriol	5	60.9 ± 8.0	17
Dexamethasone	1000	0.9 ± 0.2	< 1
Testosterone	1000	3.8 ± 1.5	1
Progesterone	1000	3.6 ± 1.7	1
Tamoxifen	1000	2.6 ± 0.6	< 1
ICI 164,384	1000	4.6 ± 2.2	1
Oestradiol + tamoxifen	5 + 1000	208 ± 10	58
Oestradiol + ICI 164,384	5 + 1000	231 ± 5	67

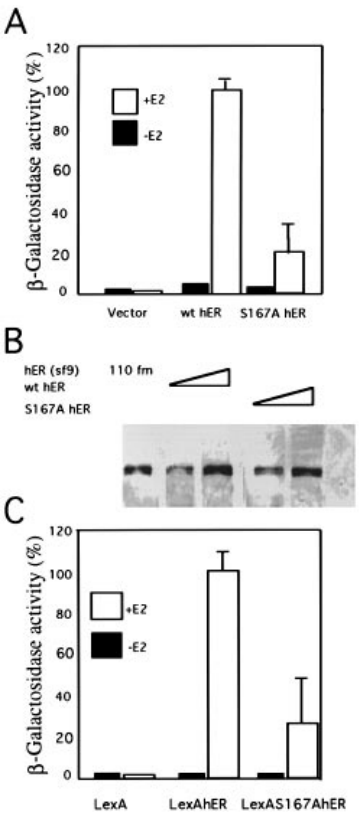

Figure 1 Transactivation by wild-type and S167A hORs in yeast

(**A**) The expression vectors phER and phERS167A were transformed into yeast strain 939 with the reporter plasmid YRPE2 containing two OREs upstream of the *lacZ* gene. The yeast were incubated without or with 10 nM oestradiol for 18 h at 30 °C and then assayed for β-galactosidase activity. The 100% value was equal to 345 Miller units. (**B**) Western blot with 110 femtomol of SF9 hOR previously quantified by [^{3}H]oestradiol is shown in lane 1, and was used to indicate the expression levels of the wild-type hOR and S167A hOR in yeast strain 939; 50 and 100 μl of yeast extract was used for hOR and S167A hOR respectively. (**C**) Yeast transformed with pLexA, pLexAhER or pLexAS167AhER were incubated without or with 10 nM oestradiol for 18 h at 30 °C, and then assayed for β-galactosidase activity. The 100% value was equal to 15 Miller units. Values are means ± S.E.M. of six independent experiments. Abbreviations: E2, oestradiol; wt, wild type; ER ≡ OR.

In order to investigate only the transcriptional properties of the hOR as well as those of the S167A hOR, we took advantage of the well known characteristics of the LexA system in yeast [31] by cloning the cDNA of the hOR to the DNA sequence of the DNA binding domain of the LexA protein, resulting in the expression of a LexA–hOR fusion protein. Furthermore the yeast strain CTY contains a β-galactosidase gene under the control of four LexA binding sequences, and has been used previously to test transactivation by LexA [21,31]. In this assay, β-galactosidase activity depends on the transactivation properties of the fused protein, and is independent of the DNA binding properties of the hOR or the S167A hOR.

The results from the yeast assay showed a 60 % difference in β-galactosidase activity between cells transformed with pLexAhER and pLexAS167AhER when 10 nM oestradiol was added to the cells (Figure 1C). There was almost no β-galactosidase activity in the absence of hormone or in the case of LexA

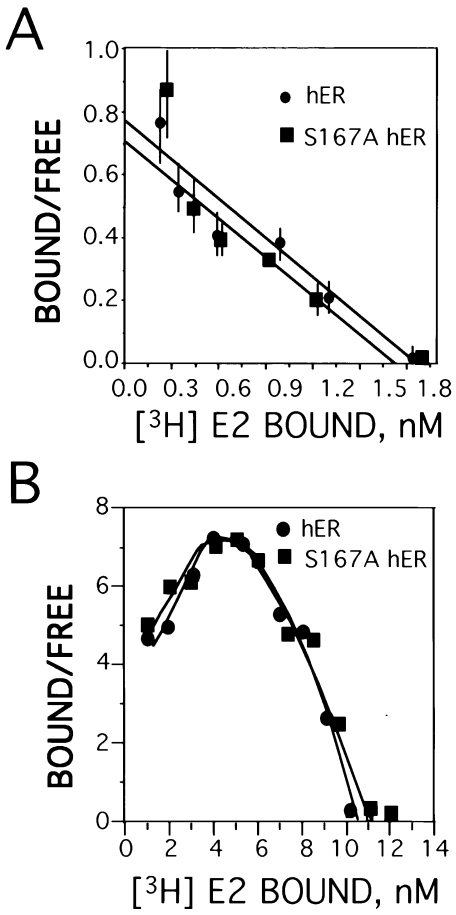

Figure 2 Binding affinities of the wild-type hOR and the S167A hOR for oestradiol

Scatchard analysis of the specific binding of [^{3}H]oestradiol to: (**A**) 2 nM wild-type hOR or S167A hOR; (**B**) 10 nM hOR or S167A hOR. Abbreviations: E2, oestradiol; ER ≡ OR.

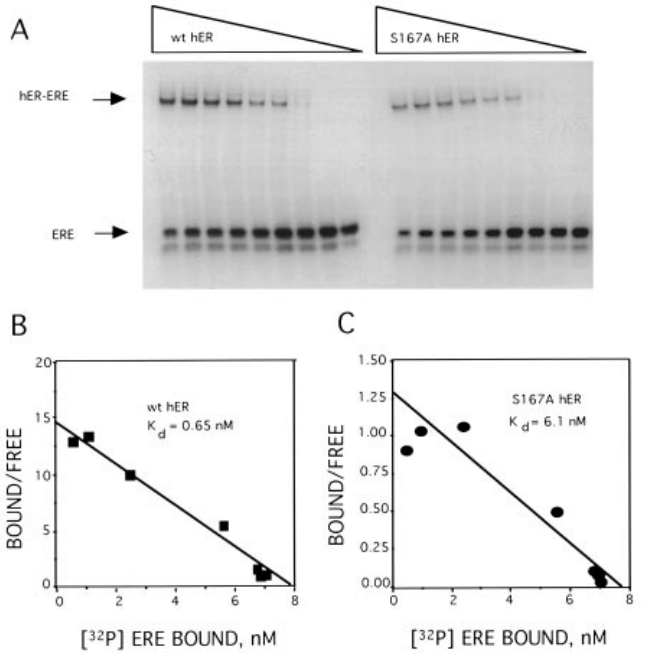

Figure 3 Binding affinities of the wild-type hOR and the S167A hOR for an ORE

(**A**) Increasing concentrations of hOR or S167A hOR, from 1 to 20 nM, were incubated with 5 ng of ^{32}P-labelled ORE for 45 min on ice. The hOR–ORE complexes were separated on a 5% non-denaturing polyacrylamide gel. (**B**) Scatchard analysis of the specific binding of ^{32}P-labelled ORE to the hOR. (**C**) Scatchard analysis of the specific binding of ^{32}P-labelled ORE to the S167A hOR. The dissociation constants shown are the means from three separate experiments. Abbreviations: wt, wild type; ER ≡ OR; ERE ≡ ORE.

expressed by itself. This experiment shows that, at least in yeast, a single mutation alters the transcriptional response, suggesting that this amino acid is crucial for transactivation. Serine-167 may need to be phosphorylated for the hOR to have full transcriptional activity.

Mutation of the hOR at serine-167 does not alter hormone binding

The yeast system was useful for measuring differences in transcriptional responses between wild-type and S167A hORs. However, we were unable to characterize the binding of receptors from yeast because our yeast extracts contained a native yeast oestrogen binding protein, which has been observed previously [32], and because receptors were partially denatured during extraction from yeast. For these reasons, we instead utilized the Sf9 expression system to obtain purified receptor. The Sf9 system was chosen for its ability to produce high levels of hOR with a known phosphorylation pattern [8]. Protein extracts from baculovirus-infected Sf9 cells expressing either wild-type or S167A hOR were obtained and the hOR purified. CK II has been shown previously to phosphorylate the purified hOR at serine-167, preferentially in the presence of oestradiol [14].

Pure hOR or S167A hOR was phosphorylated in a CK II reaction, which was followed by an oestradiol binding analysis, and showed no significant differences in oestradiol binding capacity or affinity. The hormone binding properties were investigated at two different concentrations of receptor, in order to evaluate the binding affinity and co-operativity of the hOR and the S167A hOR. The positive co-operativity of the hOR is related to its dimerization properties, and is observed when the receptor concentration is higher than 4 nM [33]. The Scatchard plots were linear, and the dissociation constants were 0.82 nM and 0.80 nM for the wild-type hOR and the S167A hOR mutant respectively (Figures 2A and 2B) at 2 nM hOR. Furthermore, at a concentration of 10 nM, both the hOR and the S167A hOR showed positive co-operativity and Hill coefficients of ~ 1.35.

DNA binding affinity is decreased in the mutant S167A hOR

To investigate whether phosphorylation of serine-167 of the hOR is involved in DNA binding, gel mobility shift assays were carried out using the wild-type or S167A hOR purified from Sf9 cells. The receptors were first incubated with CK II to phosphorylate serine-167 prior to the interaction with the ORE; 20 nM CK II and 10 nM oestradiol were also present in the CK II reaction. The ORE binding affinities of the hOR and the S167A hOR were compared on a gel mobility shift assay. Increasing concentrations of CK II-treated hOR or S167A hOR were added to 5 ng of ^{32}P-labelled ORE (Figure 3). Scatchard plots were obtained from these data and the dissociation constants of the hOR and the S167A hOR were calculated. The DNA binding affinity of the mutant S167A hOR for the ORE was approx. 10-fold lower (K_d 6.10 ± 0.39 nM) than that of the wild-type hOR (K_d 0.65 ± 0.27 nM) (Figure 3). The data suggest

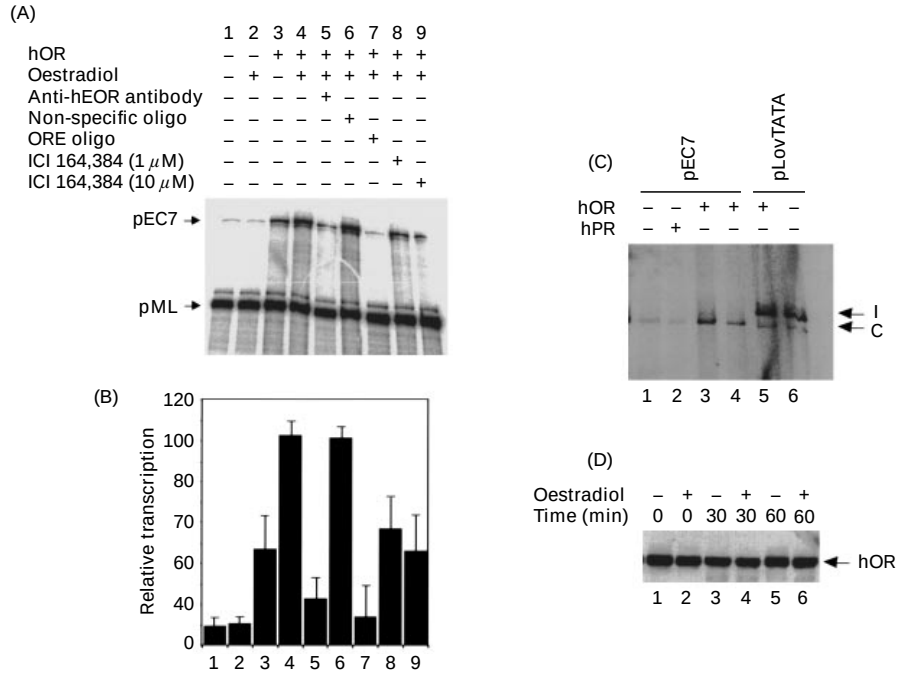

Figure 4 Cell-free transcriptional activation by the hOR

(**A**) *In vitro* transcription from the pEC7 template in the absence of hOR (lanes 1 and 2) or in the presence of 9 nM hOR (lanes 3–9), with (lanes 2, 4–9) or without (lanes 1 and 3) 100 nM oestradiol. The hOR was preincubated with anti-hOR antibody 6 (lane 5), 10 ng of a non-specific oligonucleotide (oligo; lane 6), 10 ng of an ORE (lane 7), 1 μM ICI 164,384 (lane 8) or 10 μM ICI 164,384 (lane 9). The transcripts from pML were a control for the *in vitro* transcription assay, and are driven by a adenovirus major late promoter independent of the hOR. (**B**) Relative amounts of pEC7 transcripts normalized to the intensity of the pML transcripts. The radioactivity of the transcripts was in the linear range of the PhosphorImager SI. The values are means $\pm$ range of two independent experiments and are expressed as a percentage of the maximal value (lane 4), set at 100%. (**C**) The transcription reactions were preincubated with 100 nM oestradiol, with the exception of the transcription reaction of lane 4. Reactions included 9 nM hOR (lanes 3 and 4) or 10 nM human progesterone receptor (hPR; lane 2); 600 ng of pLovTATA was used in lanes 4 and 5 instead of 200 ng of pEC7 (lanes 1–3). C stands for correct transcription initiation and I for incorrect transcription initiation. (**D**) Western blot analysis with anti-hOR antibody 6 showing the stability of the receptor for up to 1 h when the hOR was incubated at 30 °C in HeLa nuclear extract.

that the hormone-dependent phosphorylation of serine-167 by CK II enhances the affinity of the hOR for its ORE. We also used a second procedure to measure the binding affinity of the hOR and the S167A hOR for the ORE, based on changing the concentration of DNA rather than receptor. In this case, the K_d of the wild-type receptor was 1.1 ± 0.2 nM and that of the S167A mutant hOR was 4.3 ± 0.2 nM, confirming the large influence of serine-167 on the affinity of the receptor for the ORE.

When the hOR was assayed by gel mobility shift assay without CK II added to phosphorylate the hOR, the K_d for DNA binding was 1.2 ± 0.32 nM, while the mutant showed no significant difference in DNA binding in the presence or the absence of CK II (K_d 6.1 ± 0.39 nM with, compared with 6.21 ± 0.42 nM without, CK II treatment). The difference in the wild-type hOR without and with CK II suggests that a fraction of the receptor is already phosphorylated at serine-167 and a fraction is phosphorylated by CK II.

Cell-free transcriptional activation of the hOR

Cell-free transcription systems have been useful in dissecting specific aspects of transactivation of the hOR [34] as well as other steroid receptors [22]. Figure 4 shows the stimulation of accurate RNA transcripts by purified hOR. The purified hOR induced the production of transcripts from the expression vector pEC7 *in vitro* (Figure 4A). Furthermore, hOR–oestradiol complexes enhanced the transcription of specific G-free transcripts by 2-fold, indicating that the hOR was transcriptionally more efficient when complexed with oestradiol. It should be noted that transcription from the control plasmid (pML), which contains the adenovirus major late promoter in front of a 200 bp G-free cassette, was not increased by the oestrogen–hOR complex, and therefore it was used as a standard to compare levels of transcription from pEC7 in different experiments and to normalize the data for the relative transcription from pEC7. Moreover, incubation of the hOR with 100 nM oestradiol and 1 μM ICI 164,384, before the transcription reaction, resulted in a 50% decrease in the production of transcripts as compared with oestradiol alone (Figure 4A, lane 8 and 9). Thus, as expected, ICI 164,384 has antagonist activity at the level of transcription.

To test further whether the cell-free transcription assay was hOR-mediated, we incubated the hOR with an anti-hOR antibody before the cell-free transcription assay. The anti-hOR antibody 6 caused an 80% decrease in the specific transcripts produced when compared with the control (Figure 4B, bars 5 and 4 respectively). Prior incubation of the hOR with rabbit sera did not decrease the level of transcription (results not shown). The addition of a 100-fold molar excess of ORE oligonucleotide compared with the ORE from pEC7 reduced the transcription level by 90%, while the same amount of an unrelated oligonucleotide did not affect transcription (Figure 4B, bars 7 and 6 respectively). Thus transcription from the expression vector is dependent on the added hOR, specific for an ORE, and is enhanced by oestradiol binding. Transcription from the expression plasmid pLovTATA, which lacks OREs, was not activated by the addition of hOR or oestradiol (Figure 4C, lanes 4 and 5). These control reactions included pLovTATA at a high concentration, which resulted in a high proportion of incorrect

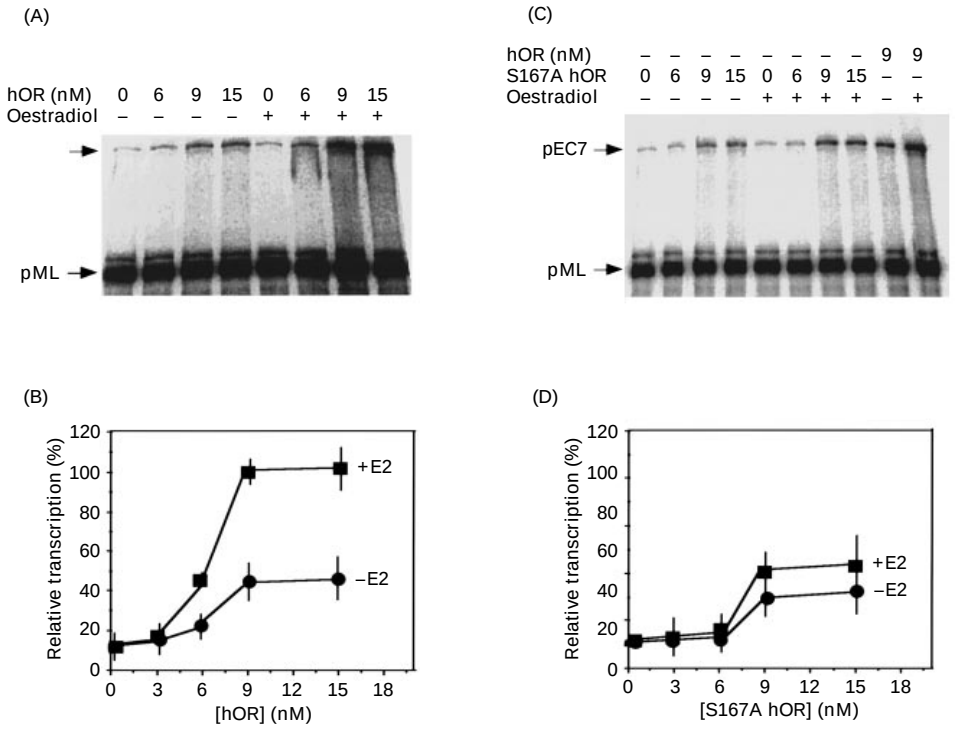

Figure 5 Cell-free transactivation by wild-type hOR and S167A hOR is receptor-concentration-dependent in the presence and the absence of oestradiol

(**A**) The transactivation activity of 0, 6, 9 and 15 nM hOR was assayed *in vitro* with the pEC7 template. The hOR was incubated without (lanes 1–4) or with (lanes 5–8) 100 nM oestradiol. (**B**) Relative transcription from the pEC7 template. (**C**) The transactivation activity of 0, 3, 9 and 15 nM S167A hOR was assayed *in vitro* with the pEC7 template. The S167A hOR was incubated without (lanes 1–4) or with (lanes 5–8) 100 nM oestradiol. Lane 9 shows the hOR without oestradiol and lane 10 the hOR with 100 nM oestradiol. (**D**) Relative transcription from pEC7. Transcription from pEC7 was normalized as described in the legend to Figure 4. The values are means ± range of two independent experiments. Abbreviation: E2, oestradiol.

transcripts; if pLovTATA was used at the same concentration as the template containing OREs, transcripts were barely detectable, although correct. The cell-free transcription reaction showed specificity for the OR, since the progesterone receptor did not stimulate transcription (Figure 4C, lane 2). The progesterone receptor in this experiment was obtained from the baculovirus system expressing the human progesterone receptor. Addition of 10 nM progesterone receptor and 100 nM oestradiol to the cell-free transcription reaction did not result in any stimulation of transcription of the expression plasmid pEC7. The hOR was not degraded during the cell-free transcription reaction, as shown in Figure 4(D). The amount of intact receptor recovered at the end of a cell-free transcription reaction was the same whether oestradiol was included or not; therefore the effects of oestradiol on transcription are not due to effects on protease sensitivities.

Phosphorylation of serine-167 of the hOR is required for full hormonal transactivation *in vitro*

We investigated the effect of serine-167 phosphorylation on the hOR in the cell-free transcription system. Unliganded hOR or S167A hOR induced maximal transactivation in the cell-free transcription assay at 9 nM (Figure 5). The mutant S167A hOR was approx. 20 % less active than the hOR in the cell-free transcription system (Figure 5D). Cell-free transactivation reached a maximum at 9 nM liganded hOR or S167A hOR. However, the amount of G-free transcripts produced by the hOR was twice that produced in the absence of hormone. These data suggest that the oestradiol-bound hOR is more efficient than the unliganded receptor at increasing the level of transcription.

The addition of 100 nM oestradiol to the S167A hOR in the cell-free transcription assay produced an increase in transactivation of less than 40 % of that produced by the hOR–oestradiol complex, indicating the significance of phosphorylation of the hOR at this site.

Phosphorylation of the hOR and the S167A hOR in a cell-free transcription assay

We further investigated whether the hOR was phosphorylated in the cell-free transcription assay, as it has been shown previously that the progesterone receptor is phosphorylated during a similar cell-free transcription assay [35]. We used the wild-type hOR as well as the S167A hOR in the absence or the presence of 100 nM oestradiol. The hOR was immunoprecipitated with anti-hOR antibody from the cell-free transcription assay that included [γ-^{32}P]ATP, but not from that including [α-^{32}P]UTP. The addition of oestradiol to the assay increased by about 2-fold the labelling of the hOR with ^{32}P (Figures 6A and 6B). However, no similar increase in incorporation of ^{32}P was observed with the S167A hOR, suggesting that, after oestradiol binds to the hOR, only serine-167 is significantly phosphorylated in the cell-free transcription system. Probing the membrane with an anti-hOR antibody verified that equivalent amounts of wild-type and S167A hOR were present in the assays (Figure 6C).

CK II phosphorylates and activates the hOR

We have shown previously that CK II phosphorylates the hOR on serine-167 and that this phosphorylation is enhanced by

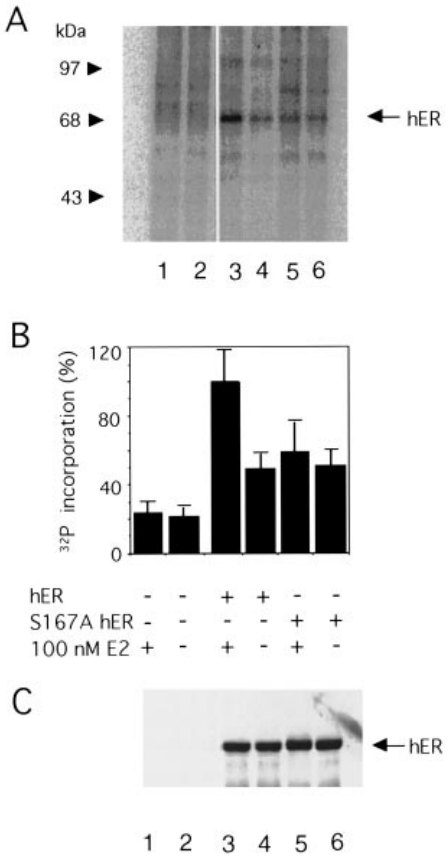

Figure 6 ^{32}P incorporation into the wild-type hOR and S167A hOR during the cell-free transcription assay

The wild-type hOR and S167A hOR were incubated with 10 μCi of [γ-^{32}P]ATP in the cell-free transcription assay. The hOR was immunoprecipitated with anti-hOR antibody 6 and separated by SDS/PAGE. The proteins were transferred to a PVDF membrane, and the radioactivity localized between 66 and 67 kDa was quantified in a PhosphorImager. (**A**) Representative experiment showing the ^{32}P incorporated during the cell-free transcription assay in the presence of hOR (lanes 3 and 4) or S167A hOR (lanes 5 and 6). Oestradiol (100 nM) was included in the cell-free transcription assay (lanes 1, 3 and 5). (**B**) The results are shown as a percentage of the ^{32}P incorporated into the hOR in the presence of 100 nM oestradiol, and the values are means $\pm$ range of two independent experiments. (**C**) Western blot analysis with anti-hOR antibody 6 demonstrating equivalent amounts of the wild-type hOR and the S167A hOR. Abbreviations: E2, oestradiol; ER $\equiv$ OR.

oestradiol [14]. To test further whether CK II phosphorylates the hOR in the cell-free transcription assay, the hOR was incubated with CK II for 30 min and then added to the transcription reaction mixture. No significant increase in transactivation by the hOR was noted in the absence of oestradiol, whereas a consistent 20 % increase in transcription was observed when 100 nM oestradiol was added (Figure 7A, lanes 5–7).

It was shown previously that inclusion of CK II substrate peptide in a cell-free transcription assay resulted in a decreased level of general transcription by RNA polymerase II [36]. We decided to utilize this procedure in our cell-free transcription assay. The transactivation level of the hOR after adding 1 μg of a CK II substrate peptide was the same as that of the hOR without oestradiol. However, a 60 % reduction in transactivation of the hOR–oestradiol complex resulted when the CK II substrate peptide was added to the transcription assay containing CK II and oestradiol (Figure 7A, lanes 7 and 9). The use of a non-specific peptide resulted in no significant decrease in transactivation by the hOR (Table 2).

Incubation of CK II with the mutant S167A hOR had no effect on the receptor's ability to stimulate transcription *in vitro* in the absence or presence of 100 nM oestradiol (Table 2). Addition of the CK II peptide did not reduce the level of transactivation of the mutant S167A hOR in the absence or the presence of 100 nM oestradiol, implying that CK II is not involved in the phosphorylation of any other sites on the S167A hOR or in the phosphorylation of another transcription factor crucial to the transactivation of the hOR.

DISCUSSION

The hOR produced in the yeast expression/reporter system is transcriptionally active and specifically responsive to oestrogenic compounds such as oestradiol and estriol. Other steroids, such as progesterone and testosterone, failed to induce transcription. However, the pure anti-oestrogen ICI 164,384 at 1 μM was only able to inhibit the effect of 5 nM β-oestradiol by 40 % in our yeast system. This may be because anti-oestrogens were unable to penetrate the cell wall of our yeast strain, as seen in other yeast strains [37,38], but not in the yeast assay described by McDonnell and co-workers [39]. Our yeast system showed a 100-fold increase in the expression of β-galactosidase over the YES system, another yeast expression/reporter system described recently [40,41]. This may be due to the higher level of expression of hOR in our system, as seen by Western blot analysis (results not shown).

The mechanism by which oestradiol induces activation of the OR is not fully understood. Previously we showed that serine-167 is the major site of oestradiol-dependent phosphorylation in the hOR in MCF-7 cells [14]. In the present study we have shown that serine-167 is important for transactivation, since its replacement by alanine reduced oestrogen-dependent transcription in both a yeast expression/reporter system and cell-free systems. The reduced transcriptional activity of the S167A hOR may result from the lack of phosphorylation on serine-167. The finding that CK II enhances oestrogen-dependent transcription from the wild-type receptor also supports the importance of phosphorylation of serine-167.

The mutation of serine-167 to alanine had no effect on oestradiol binding affinities and Hill coefficients, indicating that the mutant receptor was stable and folded properly. The S167A hOR showed a 4–10-fold reduction in affinity for the ORE. This finding is consistent with a report by Tzeng and Klinge [15] showing that the calf OR shows increased DNA affinity when CK II is added to the reaction. Since serine-167 of the hOR is a conserved amino acid in all the known sequences of ORs, phosphorylation at this site may represent a conserved feature of activation by oestrogen. The data imply that the receptor is phosphorylated at serine-167 prior to DNA binding. Moreover, oestradiol did not significantly increase the phosphorylation of S167A hOR, even though other amino acids of the mutant receptor were phosphorylated by the nuclear extract, suggesting that serine-167 of the hOR is the main site of phosphorylation after oestradiol is bound.

To verify the phosphorylation of serine-167 in the hOR by CK II, we developed a cell-free assay that is dependent on the hOR and demonstrates a 2-fold increase in transcription in the presence of oestradiol. Similar results showing *in vitro* transactivation by the hOR were obtained by Beekman et al. [34]. Our procedure differed from the previously reported assay in several respects. We found that it takes 1 h at 4 °C for 100 nM oestradiol to bind the 9 nM hOR used in the transcription assay. We followed this preincubation period with a 15 min incubation after the addition

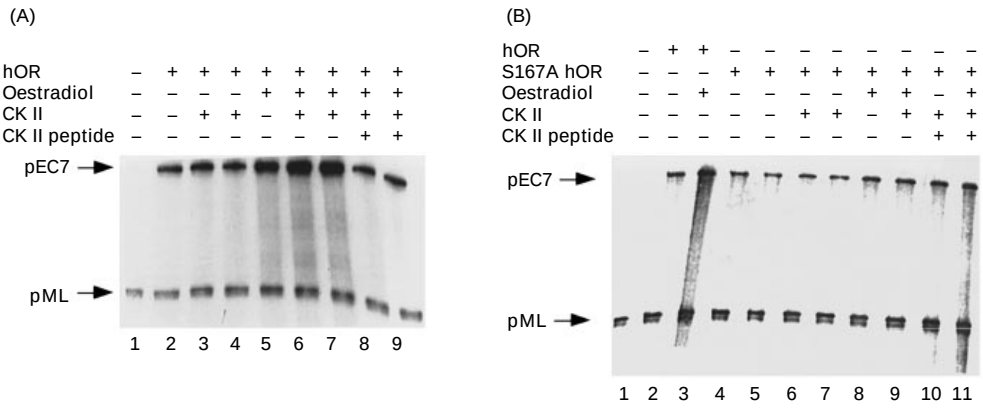

Figure 7 Transactivation by the hOR is enhanced by CK II

(**A**) Wild-type hOR (9 nM) was incubated with 20 nM CK II at 25 °C for 30 min before the transcription assay (lanes 3, 4 and 6–9). Some reactions also included 1 μM CK II substrate peptide (lanes 8 and 9) and 100 nM oestradiol (lanes 5–9). (**B**) S167A hOR (9 nM) was incubated with 20 nM CK II (lanes 6, 7 and 9–11). Some reactions also included 1 μM CK II substrate peptide (lanes 10 and 11) and 100 nM oestradiol (lanes 3, 8, 9 and 11). In order to allow comparison of the efficiency of transactivation, wild-type hOR was included in the experiment with S167A hOR (lanes 2 and 3).

Table 2 Transactivation of the hOR in a cell-free transcription system

The reactions were carried out as described in the Materials and methods section. Transcription from plasmid pEC7 was normalized as described in the legend to Figure 4. The values are means ± range of two independent experiments.

Receptor (9 nM)	Oestradiol (nM)	CK II (nM)	CK II peptide (μM)	Control peptide (μM)	Activity (%)
Control					10 ± 4
Control	100				11 ± 4
Control	100	20			14 ± 7
hOR					48 ± 9
hOR			1		46 ± 11
hOR		20			51 ± 8
hOR		20	1		52 ± 17
hOR	100				100
hOR	100		1		65 ± 13
hOR	100			1	97 ± 7
hOR	100	20			127 ± 10
hOR	100	20	1		55 ± 14
S167A hOR					29 ± 8
S167A hOR		20			33 ± 6
S167A hOR			1		34 ± 8
S167A hOR	100				50 ± 15
S167A hOR	100	20			52 ± 14
S167A hOR	100	20	1		54 ± 16

of the nuclear extract, to allow assembly of the transcription factors. A final 45 min incubation at 30 °C was performed after the ribonucleotides were added, to allow the production of transcripts.

The optimum concentration in this assay was 9 nM hOR hormone binding sites or 4.5 nM hOR dimers. The amount of specific transcripts formed depended on the hOR concentration, and reached a maximum at 9 nM for both the wild-type and S167A hORs (Figure 5B). This was unexpected, since the mutant S167A hOR showed significantly decreased DNA binding affinity and might have been expected to activate transcription only at higher concentrations than the wild-type receptor. The results imply that DNA binding is not rate limiting in the cell-free transcription assay. The S167A hOR supported lower transcriptional activity in the presence of oestradiol at any concentration, suggesting that there are differences in the interaction of the two hORs with other proteins such as receptor-interacting proteins (RIPs), SPT6 or SRC-1, which are known to interact with the hOR [42–44], or differences in interactions with the basal transcription factors [45]. This hypothesis is further supported by the results obtained with the LexA–hOR fusion protein. In the LexA assay, the receptor is no longer responsible for binding to DNA, and the differences between the wild-type and S167A hORs must result from other steps in transcription. With the LexA–hOR, the conversion of serine-167 into an alanine still led to a decrease in oestrogen-dependent transcriptional activation by the LexA–S167A hOR.

The cell-free transcription assay shows some transactivation upon the addition of hOR without hormone. Similar results were obtained by Nigro et al. [46]. It has been shown that the OR can bend the DNA by 56 ° upon binding, and that such bending occurs regardless of whether the receptor has hormone bound [47]. It was also shown that such DNA bends promote transcription in an cell-free assay [48]. These bends in the DNA may explain why, in cell-free systems, the steroid receptors still induce transcription in the absence of hormone. Two possible mechanisms of activating transcription can be suggested: one in which the binding of the receptor to the ORE bends the DNA and stimulates transcription [44], and a second in which protein–protein interaction between the receptor and another transcription factor has an effect on the transcriptional machinery. It is also possible that an accessory protein interacts with the dephosphorylated receptor and prevents the receptor from interacting with transcription factors, or that phosphorylation of the receptor affects the binding of a co-repressor.

We also investigated the role of CK II in phosphorylating the hOR in the cell-free transcription system. The addition of this kinase did not affect activation by the hOR in the absence of oestradiol. However, CK II treatment caused a 20% increase in transcription measured after oestradiol was added. This may suggest that, even though the nuclear extract contains CK II, it does not support maximal phosphorylation of the hOR. The substrate peptide for CK II prevented the effects of added CK II (Figure 7) and also reduced oestrogen-dependent transcription when CK II had not been added (results not shown).

CK II has been shown to phosphorylate vitamin D, progesterone and thyroid receptors [16,17,49]. Glucocorticoid receptors also have a potential casein kinase consensus sequence. Jurutka et al. [17] speculated that phosphorylation by this kinase may be a conserved regulatory feature of the steroid receptor superfamily. CK II may be associated with steroid receptors, since the enzyme is associated with heat-shock protein 90 [50]. Upon hormone binding, this heat-shock protein and other accessory proteins are released, and the CK II is probably in close proximity to the steroid receptors.

In summary, serine-167 is the major site of oestrogen-dependent phosphorylation of the hOR. Our results indicate that phosphorylation of serine-167 is important for hormone-dependent transcriptional activation by the hOR, and suggest that CK II may be the enzyme responsible for phosphorylation at this site. Phosphorylation at serine-167 increases the transactivation of the hOR both by increasing the affinity of the hormone–receptor complex for OREs and by activating transcription initiation at steps after DNA binding.

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REFERENCES

1 Jensen, E. V. (1996) Ann. N. Y. Acad. Sci. **784**, 1–17
2 Parker, M. G. (1995) Vitam. Horm. **51**, 267–287
3 Boulikas, T. (1995) Crit. Rev. Eukaryotic Gene Expression **1**, 1–77
4 Denton, R. R., Koszewski, N. J. and Notides, A. C. (1992) J. Biol. Chem. **267**, 7263–7268
5 Sassone-Corsi, P. (1994) EMBO J. **13**, 4717–4728
6 Koszewski, N. J. and Notides, A. C. (1991) Mol. Endocrinol. **5**, 1129–1136
7 Ali, S., Metzger, D., Bonert, J. M. and Chambon, P. (1993) EMBO J. **12**, 1153–1160
8 Arnold, S., Obourn, J., Yudt, M., Carter, T. and Notides, A. C. (1995) J. Steroid Biochem. Mol. Biol. **52**, 159–171
9 Le Goff, P., Montano, M. M., Schodin, D. J. and Katzenellenbogen, B. S. (1994) J. Biol. Chem. **269**, 4458–4466
10 Castoria, G., Migliaccio, A., Green, S., DiDomenico, M., Chambon, P. and Aurricchio, F. (1993) Biochemistry **32**, 1740–1750
11 Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D. and Chambon, P. (1995) Science **270**, 1491–1494
12 Bunone, G., Briand, P., Miksicek, R. J. and Picard, D. (1996) EMBO J. **15**, 2174–2183
13 Weis, K. E., Ekena, K., Thomas, J. A., Lazennec, G. and Katzenellenbogen, B. S. (1996) Mol. Endocrinol. **10**, 1388–1398
14 Arnold, S., Obourn, J., Jaffe, H. and Notides, A. (1994) Mol. Endocrinol. **8**, 1208–1214
15 Tzeng, D. Z. and Klinge, C. M. (1996) Biochem. Biophys. Res. Commun. **223**, 554–560
16 Denner, L. A., Shrader, W. T., O'Malley, B. W. and Weigel, N. L. (1990) J. Biol. Chem. **265**, 16548–16555
17 Jurutka, P. W., Hsieh, J. C., MacKonald, P. N., Terpening, C. M., Haussler, C. A., Haussler, M. R. and Whitfield, G. K. (1993) J. Biol. Chem. **268**, 6791–6799
18 Jurutka, P. W., Hsieh, J. C., Nakajima, S., Haussler, C. A., Whitfield, G. K. and Haussler, M. R. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 3519–3524
19 Osada, S., Yamamoto, H., Nishihara, T. and Imagawa, M. (1996) J. Biol. Chem. **271**, 3891–3896
20 Sailen, B. and Amiya, K. B. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 6570–6574
21 Chen, W. C., Hurd, C., Vorojeikina, D. V., Arnold, S. F. and Notides, A. C. (1997) Biochem. Pharmacol., **53**, 1161–1172
22 Elliston, J. F., Beekman, J. M., Tsai, S. Y., O'Malley, B. W. and Tsai, M. J. (1992) J. Biol. Chem. **267**, 5193–5198
23 McDonnel, D. P., Nawaz, Z., Dnesmore, C., Weigel, N. L., Pham, T. A., Clark, J. H. and O'Malley, B. W. (1991) J. Steroid Biochem. Mol. Biol. **39**, 291–297
24 Schaufele, F., West, B. L. and Baxter, J. D. (1992) Mol. Endocrinol. **6**, 656–665
25 Burch, J. B. and Fischer, A. H. (1992) Nucleic Acids Res. **18**, 4157–4165
26 Kunkel, T. A., Bebenek, K. and McClary, J. (1991) Methods Enzymol. **204**, 125–139
27 Kaiser, C., Michaelis, S. and Mitchel, A. (1994) Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
28 Miller, J. H. (1972). Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
29 Obourn, J. D., Koszewski, N. J. and Notides, A. C. (1993) Biochemistry **32**, 6229–6236
30 Sasson, S. and Notides, A. C. (1983) J. Biol. Chem. **258**, 8113–8117
31 Burshell, A., Stathis, P. A., Yung, D., Miller, S. C. and Feldman, D. (1984) J. Biol. Chem. **259**, 3450–3456
32 Candau, R. and Berger, S. L. (1996) J. Biol. Chem. **271**, 5237–5245
33 Arnold, S. F., Melamed, M., Vorojeikina, D. P., Notides, A. C. and Sasson, S. (1997) Mol. Endocrinol. **11**, 48–53
34 Beekman, J. M., Allan, G. F., Tsai, S. Y., Tsai, M. J. and O'Malley, B. W. (1993) Mol. Endocrinol. **7**, 1266–1274
35 Weigel, N. L., Carter, T. H., Schrader, W. T. and O'Malley, B. W. (1992) Mol. Endocrinol. **6**, 8–14
36 Angiolillo, A., Desgro, A., Marsili, V., Panara, F. and Giangranceshi, G. L. (1993) Experimentia **49**, 902–905
37 Zysk, J. R., Johnson, B., Ozenberger, B. A., Bingham, B. and Gorski, J. (1995) Endocrinology **136**, 1323–1326
38 Lyttle, C. R., Damian-Matsumura, P., Juul, H. and Butt, T. R. (1992) J. Steroid Biochem. Mol. Biol. **42**, 677–685
39 Pham, T. A., Hwung, Y. P., Santiso-Mere, P., McDonnell, D. P. and O'Malley, B. W. (1992) Mol. Endocrinol. **6**, 1043–1050
40 Arnold, S. F., Klotz, D. M., Collins, B. M., Vonier, P. M., Guillette, L. J. and McLachlan, J. A. (1996) Science **272**, 1489–1492
41 Arnold, S. F., Robinson, M. K., Notides, A. C., Guillette, L. J. and McLachlan, J. A. (1996) Environ. Health Perspect. **104**, 544–548
42 Cavilles, V., Dauvois, S., Danielian, P. and Parker, M. G. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 10009–10013
43 Voegel, J. J., Heine, M. J., Zechel, C., Chambon, P. and Gronemeyer, H. (1996) EMBO J. **15**, 3667–3675
44 Onate, S. A., Tsai, S. Y., Tsai, M. J. and O'Malley, B. W. (1995) Science **270**, 1354–1357
45 Jacq, X., Brou, C., Lutz, Y., Davidson, I., Chambon, P. and Tora, L. (1994) Cell **79**, 107–117
46 Nigro, V., Molinari, A. M., Armetta, I., de Falco, A., Abbodanza, C., Medici, N. and Puca, G. (1992) Biochem. Biophys. Res. Commun. **186**, 803–810
47 Nardulli, A. M. and Shapiro, D. J. (1992) Mol. Cell. Biol. **12**, 2037–2042
48 Nardulli, A. M., Grobner, C. and Cotter, D. (1995) Mol. Endocrinol. **9**, 1064–1076
49 Kat, D., Reginato, M. J. and Lazar, M. A. (1995) Mol. Cell. Biol. **15**, 2341–2348
50 Csermely, P. and Kahn, C. R. (1991) J. Biol. Chem. **266**, 4943–4950

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