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Ligand and DNA-dependent phosphorylation of human progesterone receptor *in vitro*

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ABSTRACT **The progesterone receptor (PR), like other members of the steroid receptor family, is a ligand-induced transcription factor. We have demonstrated previously that progesterone-induced binding of PR to a progesterone response element (PRE)-linked promoter stimulates RNA synthesis from that promoter in a cell-free transcription extract. It has been established that a hormone-mediated activation of PR beyond the removal of associated heat shock proteins is essential for efficient transactivation of the target gene. We now report that treatment with hormone leads rapidly to multiple phosphorylations of both the A and B forms of human PR in a HeLa nuclear extract. The putative kinase is present in the transcriptional extract but fails to phosphorylate the receptor significantly in the absence of specific hormone or DNA. Efficient phosphorylation of the PR occurs only in the presence of PREs, indicating that ligand-induced binding of PR to its cognate DNA response element makes it a preferred substrate for the kinase. The kinetics of the phosphorylation reaction overlap the kinetics of hormone-dependent RNA synthesis from a PRE-containing target promoter *in vitro*. We postulate that ligand and DNA-dependent phosphorylation of PR is an important functional event in the process leading to receptor-mediated transactivation of target genes.**

Steroid hormone receptors regulate the expression of steroid-responsive genes in a ligand-dependent manner (1–4). Unoccupied steroid receptor exists in a functionally inactive complex in association with a variety of heat shock proteins such as hsp90 (5–8), hsp70, (9) and hsp56 (10–12). Ligand binding triggers a poorly understood structural activation of the receptor. The hormone–receptor complex then binds to specific enhancer-like sequences referred to as steroid response elements (SREs) at the target gene and modulates gene transcription by RNA polymerase II. Although the overall pathway for transduction of the hormonal signal to the nucleus by steroid receptors has been known for several years, the precise molecular nature of ligand-induced activation of receptor, which converts it to a productive transcriptional regulator at the target promoter, has remained unsolved.

To dissect the functional role of steroidal ligand in receptor-mediated gene activation, we utilized a hormone-dependent cell-free gene expression system (13). In this system, addition of progesterone alone to a nuclear extract of human mammary carcinoma T47D cells, which are rich in progesterone receptor (PR), stimulated transcription from a progesterone response element (PRE)-linked target promoter. It has been proposed that ligand-induced activation of steroid receptors involves dissociation of receptor-associated heat shock proteins (5, 8, 14). To test if the hormonal ligand has a functional role beyond the removal of heat shock proteins from the receptor complex, we isolated a ligand-free PR essentially devoid of hsp90, hsp70, and hsp56 (15). We demonstrated that this receptor form still required progesterone for efficient binding to PREs and transactivation of a target promoter in a HeLa cell-free reconstituted system (15). Therefore, we established that the dissociation of heat shock proteins is not sufficient to generate a constitutively active receptor. An additional ligand-dependent activation step(s) appeared to be necessary to achieve a receptor conformation that is optimal for gene activation.

To investigate the molecular basis of this activation event, we considered the possibility that a ligand-induced covalent modification of the receptor such as phosphorylation may play a role in the activation process. PR, like other steroid receptors, is a phosphoprotein even in hormone-untreated cells or tissues (16–19). It is well documented that brief hormone treatment of PRs in cultured cells or tissue slices leads to an increased phosphorylation state of the receptor concomitant with its activation and nuclear accumulation (20–24). Phosphorylation has been shown to modulate the activities of other transcription factors in a number of different ways. For example, nuclear translocation (25–27), DNA binding (28–32), and transactivation properties (33–35) of various transcription factors have been reported to be influenced by phosphorylation. However, it is not clear how the ligand-induced phosphorylation modulates the biological activity of PR. Here we report that treatment with progesterone rapidly induces phosphorylation of human PR in our HeLa cell-free transcription extracts. The ligand-induced phosphorylation is dependent upon the binding of PR to its hormone response element. We explore the mechanism of this phosphorylation event and its potential functional role in receptor-mediated gene activation.

MATERIALS AND METHODS

Materials. Calf intestinal alkaline phosphatase (molecular biology grade) was purchased from Boehringer Mannheim. Ultrapure nucleotides and 3′-*O*-methyl-GTP was from Pharmacia LKB. [α-^{32}P]UTP and ^{35}S-labeled protein A were from Amersham. Rabbit anti-mouse IgG was from Zymed Laboratories. The anti-human PR (anti-hPR) monoclonal antibody (mAb) AB52 (36) was provided by Dean P. Edwards (University of Colorado, Denver). All SDS/PAGE reagents were from Bio-Rad.

DNA Templates. Methods of constructions of plasmids pLov, PRE$_2$pLov, and ERE$_2$pLov (in which ERE = estrogen-responsive element) have been described (37, 38).

Nuclear Extracts and hPR Preparations. HeLa nuclear extracts were prepared as described (38). hPR preparations purified by phosphocellulose (PC) column chromatography were obtained by published procedures (15) and were used in all experiments unless stated otherwise. The concentration of PR in this preparation was estimated by binding to [^{3}H]R5020 as described (13). PC-purified PR preparations consisted predominantly of PR$_A$ and small amounts of PR$_B$ as estimated by Western immunoblotting with mAb AB52.

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Abbreviations: PR, progesterone receptor; hPR, human PR; PRE, progesterone response element; mAb, monoclonal antibody; ERE, estrogen-responsive element; PC, phosphocellulose.

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Receptor Phosphorylation. The phosphorylation of PR was studied under conditions of *in vitro* transcription reaction. The protocol of a typical *in vitro* transcription assay has been described (38, 39). The partially purified PR fraction was combined with HeLa nuclear extract, treated with 0.1 μM progesterone (or RU 486 where mentioned) or left untreated, and incubated with template DNA [PRE_2pLov (200–300 ng)] and the nucleotides and other transcriptional components. Nonspecific herring sperm DNA was excluded from these reactions. The reaction mixtures were incubated at 30°C for 30 min. At the end of incubation, the reactions were immediately stopped by addition of a SDS/gel loading buffer [final concentration of 60 mM Tris·HCl, pH 6.8/3% SDS/5% mercaptoethanol/10% (vol/vol) glycerol], and the mixtures were heated to 90°C for 5 min and subjected to SDS/PAGE. The SDS/gels were run for a longer time than usual for better resolution of the phosphorylated PR bands.

Immunoblot. Immunoblotting was performed as described (15). Strips of membrane containing transferred proteins were incubated with mAb AB52, followed by rabbit anti-mouse IgG, and finally with ^{35}S-labeled protein A. The filters were then air-dried, and the signals were visualized by exposure to Kodak XAR-5 film at room temperature.

RESULTS

Ligand Induces Structural Changes in Both A and B Forms of Human PR in Cell-Free Transcription Extracts. hPR exists in two molecular forms, PR_A and PR_B. Upon SDS/PAGE, PR_A migrates as a single band with an apparent molecular mass of 94 kDa. PR_B, on the other hand, appears as a cluster of three bands with molecular masses between 114 and 120 kDa. Multiple PR_B species arise from an initial 114-kDa polypeptide by slow posttranslational phosphorylation events that do not require hormone (19). Previous studies showed that brief treatment of cultures of T47D cells with progesterone or antiprogesterone RU 486 resulted in the generation of progesterone receptor forms with decreased electrophoretic mobilities in SDS/polyacrylamide gels (19–21). The shift in mobility was shown to be caused by rapid secondary phosphorylation of both PR_A and PR_B.

The fact that ligand-induced phosphorylation of PR *in vivo* occurs concurrently with its activation and nuclear translocation opened up the possibility that this covalent modification of the receptor may play a role in the signal-transduction pathway of steroid hormones. We reasoned that if the ligand-dependent phosphorylation of PR as observed *in vivo* is indeed essential for the receptor to achieve competency in target-gene activation, then the same event must occur also in our hormone-regulated *in vitro* transcription system.

Partially purified PR (PC step; ref. 15) consisting predominantly of PR_A was incubated with HeLa nuclear extract in the presence or absence of hormone in an *in vitro* transcription reaction mixture that also included a PRE-linked test template and nucleotide triphosphates. When the incubation was carried out in the absence of hormone, PR_A appeared as a singlet (Fig. 1*A*, lanes 3 and 5) and PR_B appeared as a triplet (Fig. 1*B*, lane 1) upon analysis by SDS/PAGE. When the transcriptional incubation was performed in the presence of progesterone, however, a change in the electrophoretic mobilities of both PR_A (Fig. 1*A*, lane 4) and PR_B (Fig. 1*B*, lane 2) was observed. As a result of hormone treatment, a significant portion of PR_A migrated with reduced mobility. At least two slower migrating PR_A species were generated. A major portion of PR_B was shifted also to the slowest migrating form. When HeLa extract was omitted from the reaction mixture, incubation of PR with ligand alone did not result in a similar shift to slower receptor migration (Fig. 1*A*, lanes 1 and 2), indicating that the source of the modifying activity was in the HeLa extract. Addition of RU 486 to the transcription reaction led to similar shifts in the migration of PR_A and PR_B (Fig. 1 *A*, lane 6 and *B*, lane 4). However, treatment of PR_A with the antihormone generated a greater amount of the slowest migrating species compared with treatment with the agonist. Our results suggest that treatment of PR with hormone or antihormone in a cell-free transcription extract triggered structural modifications in both PR_A and PR_B that were reflected in their altered electrophoretic mobilities. Similar ligand-induced modification of PR was observed also when a more purified receptor preparation (ATP-agarose step; ref. 15) devoid of hsp90, hsp70, and hsp56 was used (data not shown).

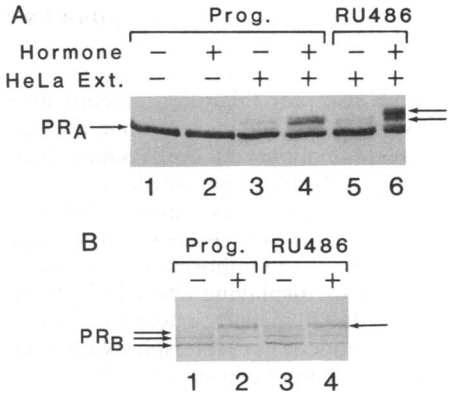

FIG. 1. Ligand treatment *in vitro* alters electrophoretic mobility of PR. Partially purified (PC-stage) PR consisting of 80–90% PR_A (*A*) and 10–20% PR_B (*B*) (estimated by densitometric scanning of the immunoblots) was used. PR (1.5 pmol) was preincubated with or without HeLa nuclear extract (Ext.; 50 μg) in the presence or in the absence of 0.1 μM progesterone (Prog.) or RU 486 and then incubated further in an *in vitro* transcription reaction (60 μl) containing 250 ng of PRE_2pLov DNA template as described (15, 39). The reactions were then analyzed by immunoblotting as described in text. The unmarked arrows on the right indicate the PR species of slower mobility generated by ligand treatment.

Ligand-Induced Modification of PR Is a Phosphorylation Event. We next confirmed that the ligand-induced modification of PR in HeLa extracts, leading to the electrophoretic mobility decrease, was due to increased phosphorylation of the receptor. Initially, we tested the requirement of ATP for this process. In the absence of ATP, no significant shift of PR_A to slower migrating forms was observed. Addition of ATP to the incubation mixture in the absence of hormone resulted only in very low levels of shift (Fig. 2*A*). Efficient shift of PR_A to slower migrating forms took place, however, when ATP was added to the transcription reaction in the

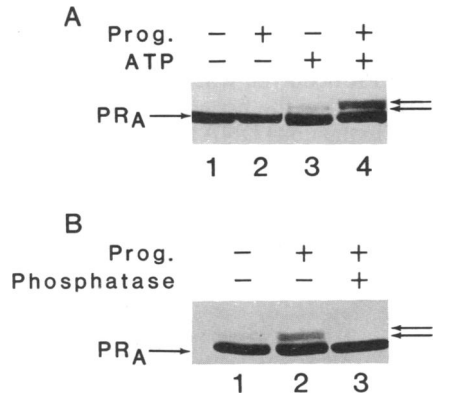

FIG. 2. Ligand-induced mobility shift of PR is due to phosphorylation. ATP (0.5 mM) (*A*) and calf intestinal alkaline phosphatase (10 units) (*B*) were included in the cell-free transcription reaction where indicated. Arrows indicate the PR species of slower mobility generated. Prog., progesterone.

presence of hormone. The shift of PR_B forms was similarly found to be ATP-dependent (data not shown).

We studied also the effects of treatment with alkaline phosphatase on receptor migration. Treatment of PR with alkaline phosphatase during incubation in the transcriptional extract completely abolished the ligand-induced mobility shifts of PR (Fig. 2*B*). Phosphatase also reversed the shift of PR to slower migrating forms when added to the reaction after incubation (data not shown). These results clearly indicate that hormone or antihormone-induced generation of new receptor forms displaying altered electrophoretic mobilities is indeed due to receptor phosphorylation. Therefore, the hormone-dependent hyperphosphorylation of PR seen in intact cells can be reproduced successfully *in vitro* under our cell-free transcription conditions. We estimated by densitometric scanning that under standard transcription reaction conditions, about 30–40% of PR_A or PR_B population undergoes hyperphosphorylation. This is consistent with the observation that in the *in vivo* situation also, hormone treatment resulted in only a partial conversion of the receptor to its fully phosphorylated form (refs. 19–21; also unpublished observation). The lack of quantitative phosphorylation of PR both *in vivo* and *in vitro* could be a reflection of the dynamic balance of phosphorylating and dephosphorylating activities that exist in this cell extract.

Multiple Ligand-Dependent Phosphorylations of PR Occur in a Sequential Manner. We next studied the kinetics of ligand-dependent phosphorylation of PR under cell-free transcription conditions. The results (Fig. 3) indicate that the generation of two PR_A bands of slower mobility proceeds in a sequential manner. Within the first 5 min of incubation, hormone rapidly induced phosphorylation of about 10% of the receptor. The phosphorylated species was almost entirely composed of the lower of the two shifted bands; no upper band was detected at this time. At 10 min, the lower band intensified, and the upper band composed of the slowest migrating species appeared. Maximum phosphorylation was attained within 20 min of incubation, generating equimolar amounts of both bands of reduced mobility. At time points of 30 min or longer, the predominant shifted species was the slowest migrating form. Therefore, the formation of the two hyperphosphorylated forms of PR_A followed distinct kinetics. We noted further that some phosphorylation of receptor also occurred in the reactions lacking hormone but at a much slower rate compared with the reaction containing hormone. Therefore, hormonal treatment clearly results in a faster kinetics of the phosphorylation reaction.

Ligand-Induced Phosphorylation of PR *in Vitro* Is PRE-Dependent. As our *in vitro* transcription system contains PRE-linked DNA templates, we next investigated whether the binding of PR to PREs is a prerequisite for efficient phosphorylation. To our surprise we noted that little phosphorylation of PR occurred when the transcriptional incubation was performed in the presence of hormone but without the addition of exogenous DNA template to the reaction [Fig. 4, bars 1 and 2 (first set of bars)]. Low concentrations (3–7 μg/ml) of a transcription template lacking PREs or a template containing EREs when added to our *in vitro* transcription reaction, led only to a marginal increase in phosphorylation

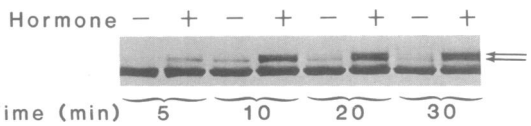

FIG. 3. Kinetics of ligand-induced phosphorylation of PR. Aliquots (60 μl) were taken at various time points from the cell-free transcription reaction mixture (300 μl) that was incubated in the presence (+) or absence (−) of progesterone and subjected to immunoblotting as described in text.

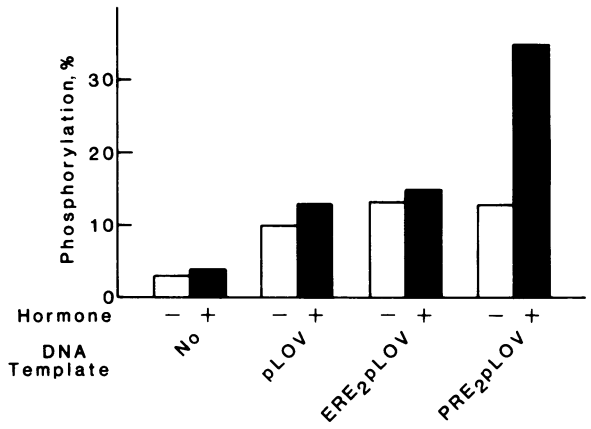

FIG. 4. DNA dependency of PR phosphorylation. No DNA template or various DNA templates (250 ng of each) were used as shown. The results represent an average of three experiments. The intensities of the signals were estimated by scanning autoradiograms with an LKB Ultrascan XL laser densitometer. Percent phosphorylation was calculated with respect to the total immunoreactive PR signal in the autoradiogram.

of PR in the absence of hormone (Fig. 4, bars 3 and 5). No significant enhancement of receptor phosphorylation was observed upon hormone addition (Fig. 4, bars 4 and 6). Similarly, addition of a template DNA containing PREs to the *in vitro* transcription reaction in the absence of ligand elicited only a marginal increase in phosphorylation of PR (Fig. 4, bar 7). However, addition of hormone and PRE-containing DNA template consistently triggered a significant stimulation in receptor phosphorylation (Fig. 4, bar 8). These results suggest that unliganded PR which fails to bind to DNA with high affinity is not a favored substrate of the putative DNA-dependent kinase present in the HeLa extract. Ligand-bound PR can bind to PREs efficiently, and the PR–PRE complex then becomes a preferred substrate for the kinase.

Kinetics of Ligand-Induced Phosphorylation of PR Correlate with the Kinetics of RNA Synthesis from a PRE-Linked Promoter. We compared the kinetics of ligand-induced phosphorylation of PR *in vitro* with the kinetics of progesterone-dependent RNA synthesis from a PRE-linked promoter in a cell-free gene expression system. For this purpose we performed a two-stage reaction in which PR was initially preincubated with HeLa nuclear extract in the presence of hormone; PRE-linked transcriptional template and NTPs were then added, and the kinetics of RNA synthesis was monitored. After an initial lag of about 5–7 min, the RNA synthesis increased linearly with time (Fig. 5). No significant ligand-induced phosphorylation of PR_A was detected during preincubation. However upon addition of DNA and NTP (zero time), phosphorylation of PR was initiated within the initial 5 min and reached a plateau by 20 min (Fig. 5). A significant population of the receptor was converted into the phosphorylated state during the time the functional transcription initiation complex was assembled, and RNA synthesis commenced. Our results show a direct correlation between the ligand-induced phosphorylation of PR and its gene regulatory activity and raise the interesting possibility that these two events are functionally coupled. Since the phosphorylated state is maintained over the course of a reaction (45–60 min) where PR-mediated transcription is maintained, our results appear to rule out a previous hypothesis that phosphorylation of nuclear receptor is related to inactivation of receptor.

DISCUSSION

In the present study, we demonstrate that progesterone induces multiple phosphorylations of its receptor in a cell-free transcription extract. The binding of PR to its cognate re-

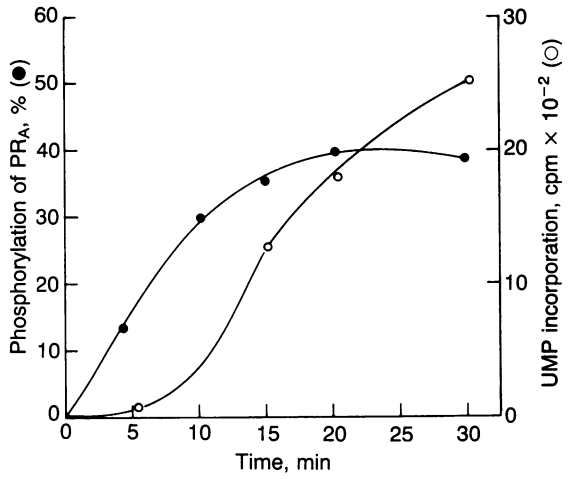

FIG. 5. Comparison of the kinetics of ligand-induced phosphorylation and RNA synthesis from a PRE-linked promoter *in vitro*. PC-purified PR (1.5 pmol) was mixed with HeLa extract (50 μg) and incubated with or without 0.1 μM progesterone for 10 min at room temperature. DNA template PRE$_2$pLov (600 ng) and the nucleotide mixtures were then added, and the reaction (90 μl) was continued at 30°C. The protocol of the transcription reaction was as described (15, 38, 39), but herring sperm DNA was excluded. Aliquots of 60 μl were taken at different times and analyzed by immunoblotting. Aliquots of 30 μl were taken at the same time and subjected to gel electrophoresis for estimation of RNA synthesis. The [^{32}P]UMP incorporation was measured by estimating the cpm present in individual bands in a dried gel in a Betagen analyzer (Betagen, Waltham, MA).

sponse element is a prerequisite for this phosphorylation reaction. The ligand and DNA-dependent phosphorylations of PR by a nuclear kinase suggest an important regulatory consequence for this phosphorylation event with regards to receptor function.

Previous studies have implied that phosphorylation is required to create hormone-binding states of glucocorticoid (40) and estrogen (41) receptors. Phosphorylation has also been shown to influence nuclear translocation (25–27) or DNA binding properties (28–32) of a number of transcription factors. We have observed previously that partially purified PR displayed high affinity binding to PREs when incubated with hormone or antihormone in the absence of HeLa extract or ATP (15) or in the presence of alkaline phosphatase. As shown in Fig. 2, no significant ligand-induced phosphorylation of PR was observed under these incubation conditions. Therefore, our studies in the cell-free system clearly indicate that ligand-induced phosphorylation of PR (*i*) is not an essential prerequisite for binding to PREs and (*ii*) occurs when the ligand–receptor complex is bound at specific nuclear target sites, thus rendering the above-mentioned possibilities rather unlikely. Furthermore, the kinetics of phosphorylation and receptor-mediated transcription in our cell-free system are totally inconsistent with the hypothesis that nuclear phosphorylation is a mechanism of deactivating functional PR. Our results are reminiscent of the results of Jackson *et al.* (42), who demonstrated that the infection of monkey CV-1 L cells with simian virus 40 induced multiple phosphorylations of the transcription factor Sp1. These phosphorylation events could be reproduced in an *in vitro* HeLa cell transcription extract and were shown to be dependent on the binding of Sp1 to the GC box sequences. These authors reported that the Sp1 kinase is a nuclear DNA-dependent protein kinase. Our results are also consistent with phosphorylation by a DNA-dependent kinase, since recent evidence has revealed DNA-bound chicken PR to be an excellent substrate for purified DNA-dependent kinase (48).

We have reported previously that PR regulates gene transcription by facilitating the formation of a stable preinitiation complex at the target promoter (38, 39). The receptor enhances RNA synthesis apparently by interacting directly or indirectly with the general transcription machinery. In addition, PR is known to interact cooperatively with other promoter-binding proteins, including NF-1 (43) and Oct-1 (44), to modulate target gene expression. Phosphorylation of PR could potentially influence any of these interactions. There is now ample evidence that phosphorylation modulates transactivation and transrepression functions of several gene regulatory factors, presumably by altering the interaction of these factors with other transcription regulatory components. Gonzales and Montminy (33) have demonstrated that phosphorylation of the cAMP response element-binding protein (CREB) at Ser-133 is essential for its transactivation function. The results of Sorger and Pelham (34) suggest that heat-induced phosphorylation of the DNA-bound heat shock factor (HSF) leads to a more efficient interaction of the factor with the transcription machinery. In contrast, Ofir *et al.* (35) have proposed that the net negative charge imparted by a phosphate moiety is crucial for the transrepression activity of c-fos but not for its transactivation function. Recent evidence also suggests that phosphorylation influences the transactivation function of several members of the steroid receptor superfamily. Glineur *et al.* (45) have reported that phosphorylation of v-erbA protein regulates its function as a transcriptional repressor of its target genes during erythroid differentiation. Studies by Denner *et al.* (46) indicate that phosphorylation of PR, by a pathway that apparently involves cAMP-dependent protein kinase, triggers its transcriptional activity.

Our kinetic data (Fig. 5) reveal a positive correlation between the progesterone-dependent phosphorylation of PR and the progesterone-induced enhancement of RNA synthesis from a PRE-driven promoter *in vitro*. The data are only correlative because the presence of a potent PR-kinase activity in the active transcription extract makes it virtually impossible to properly assess the transcriptional properties of PR, which has not undergone secondary phosphorylation. The precise location of the ligand-induced phosphorylation site(s) on human PR is not yet known. Future studies involving identification and subsequent mutation of those sites combined with an assessment of the effect of ligand on the functional activities of such mutants will be required to prove that the ligand-dependent phosphorylation of PR is obligatory for its gene regulatory activity.

It is interesting to note that the antihormone RU 486 also induces secondary phosphorylation of PR both *in vivo* (19) and *in vitro* (Fig. 1). In the cell and in cell-free extracts, RU 486-bound receptor displays lesser transcriptional activity compared with the agonist-bound receptor (13, 47). The phosphorylation pattern is slightly different but, most importantly, it is not known whether hormone and antihormone induce phosphorylation at the same sites on the PR. If the sites are distinct or the extent of phosphorylation differs, then differentially phosphorylated PR will be generated in response to hormone and antihormone and these, in turn, may regulate gene activity differently. Recent studies in our laboratory with limited proteolysis have revealed remarkable structural differences between unoccupied, progesterone-complexed, and RU 486-complexed PRs (G. F. Allan, personal communication). It has been demonstrated that PR complexed with progesterone displays a digestion pattern distinct from unoccupied PR or from PR complexed with RU 486, clearly indicating that ligand binding alone induces distinct allosteric conformational changes in PR. Taken together, these results suggest that although phosphorylation of PR may play an important functional role in influencing the nature of its interaction with the transcription machinery at the target promoter, it is the ligand-regulated conformation of the receptor that ultimately may determine whether the receptor will promote transcription efficiently.

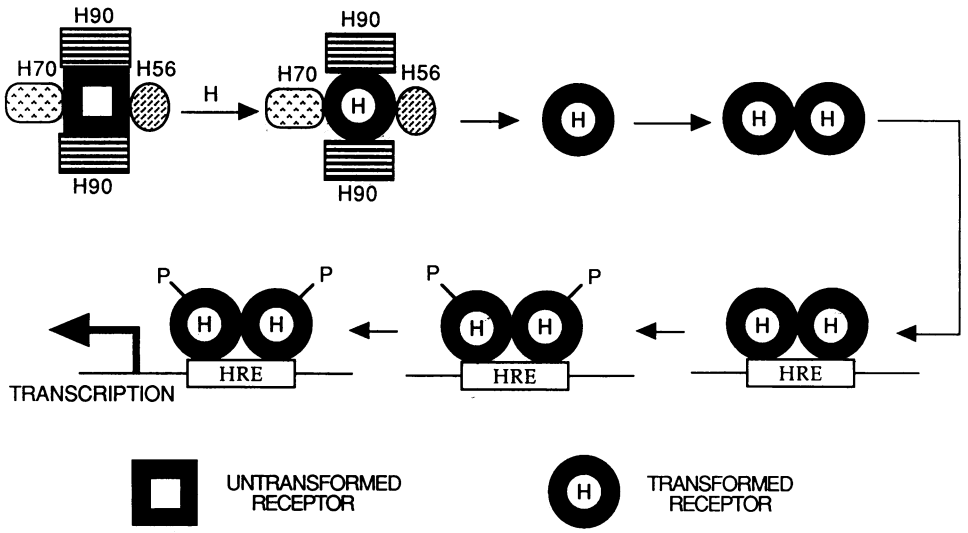

FIG. 6. A working model for ligand-induced gene activation by PR.

Based on these observations, we present a working model for ligand-induced gene activation by steroid receptors (Fig. 6). Following synthesis, cellular unoccupied receptor is complexed with nonreceptor proteins such as hsp90 and is functionally inert. Hormone binding provides an active conformation to the receptor protein and triggers the release of receptor-associated inhibitory proteins. The activated receptor then undergoes dimerization and binds to PREs at nuclear target loci. Binding to PREs facilitates the phosphorylation of PR by a nuclear kinase. According to this scenario, phosphorylated PR bound to an appropriate hormone agonist will possess the proper conformation to undergo productive interactions with the transcription apparatus at the target gene.

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