Activation of Transcription by Progesterone Receptor Involves Derepression of Activation Functions by a Cofactor

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Hormone-induced progesterone receptors (PR) bound to response elements stimulate transcription initiation at target promoters through a mechanism that presumably involves cofactors or coactivators. To allow identification of such cofactors of transcriptional activation in a functional assay, we have established a reconstituted transcription system that is characterized by a specific loss of responsiveness to purified baculovirus-expressed wild type PR. In contrast to wild type PR, a C-terminally truncated PR mutant displayed strong activation potential in this system. As the purified recombinant full-length PR is capable of DNA binding, our results suggest that C-terminal sequences of PR mediate a cis-repression of N-terminal activation functions. Moreover, using this PR-nonresponsive transcription system, we identified and partially purified an activity from rat liver, termed COPRA (cofactor of PR activation), that restores transactivation by full-length PR. Characterization of COPRA revealed that this cofactor exhibits activator specificity and is not involved in basal transcription. We postulate that COPRA acts by relieving the repression of activation functions mediated by C-terminal sequences. (Molecular Endocrinology 11: 768–778, 1997)

INTRODUCTION

Regulation of mRNA synthesis is achieved partly through the coordinate action of general transcription factors (GTFs), RNA-polymerase II (RNAPII), and activators. GTFs (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIF) mediate binding of RNAPII to the promoters (1), but also have important functions at later steps of the initiation process. In cell-free transcription systems GTFs and RNAPII are sufficient to initiate basal levels of transcription from TATA-only promoters (2). Activators bind to promoters or enhancers in a sequence-specific manner and thereby stimulate the rate of transcription initiation at the target promoter above basal levels. The underlying mechanisms of transcriptional stimulation are not well understood, but many activators have been shown to interact with various components of the initiation complex (1). These interactions are thought to help to recruit RNAPII, GTFs, or the RNAPII holoenzyme complex, to stabilize initiation complex intermediates, or to induce conformational changes within components of the initiation complex that may activate subsequent steps (for review, see Refs. 1 and 3).

There is increasing evidence that some activators, in order to function, require another class of factors, so-called coactivators (also termed positive cofactors, intermediary factors, adaptors, or mediators; Refs. 1 and 4). By definition, coactivators do not bind sequence-specifically to DNA and are not involved in basal transcription. These factors may exert their effects on transcriptional activation through various mechanisms, including stimulation of DNA-binding of activators (5, 6), by affecting chromatin structure (7–9) or by mediating activator-initiation complex interactions (10–12). Although the TATA box-binding protein (TBP)-associated factors (TAFs) are stably associated subunits of the TFIID complex, they can also be regarded as coactivators because they are dispensable for basal transcription but absolutely essential for activated transcription in vitro (13, 14).

The progesterone receptor (PR) is a transcriptional activator of the nuclear receptor superfamily whose function is strictly dependent on the presence of progesterone (15–17). PR exhibits the modular structure common to all steroid receptors (Fig. 1A). The DNA-binding domain (domain C) is located in the center of the molecule and consists of two zinc-fingers. Initially, two independent activation functions, AF1 and AF2, have been localized to the N- and C-terminal domains of the receptor, respectively (18, 19). A more detailed deletion analysis has shown that AF1 consists of a core region, which displays activation potential when
linked to a heterologous DNA-binding domain, and a
modulatory region that cannot act on its own but mod-
ulates the activity of the core region (20). As we have
recently shown in cell-free transcription and cotrans-
fecions (21), the DNA-binding domain of PR appears
to contain another independent activation function,
AF3. The C-terminal part of PR also harbors the
hormone-binding domain, a dimerization motif (22),
and sequences involved in the interaction with chap-
erones that maintain the unliganded receptor in an
inactive state (23, 24).

In vivo, binding of progesterone induces high-affinity binding of the receptor to pro-
gesterone response elements (PREs) through a com-
plex activation cascade that includes conformational
changes, release of chaperones, and multiple phos-
phorylation events (25, 26). Presumably, due to the
separation of chaperones, PR purified from chicken
oviduct and recombinant human PR purified from bac-
ulovirus-infected Spodoptera cells bind to PREs in a
hormone-independent manner and thus display an ac-
tivated state (27, 28). Consistently, in cell-free tran-
scription systems based on crude nuclear extracts,
such purified PR preparations efficiently stimulate
transcription initiation of PRE-containing reporter
genes (27, 28). In vitro assays revealed binding of PR
to TFIIIB and the TFIIID subunit dTAF, 110 (29, 21),
which possibly contribute to transcriptional stimu-
lization by PR. However, there is evidence from
squeezing experiments in cell culture and cell-free
transcription systems that transactivation by PR
might involve additional cofactors (20, 30–32). Re-
cently, several candidate genes, steroid receptor
coactivator-1 (SRC-1), hRPF1, transcriptional inter-
mediary factor 2 (TIF2), and binding protein of CRE-
binding protein (CBP) have been identified that,
when overexpressed, stimulate PR-dependent tran-
scription (33–36). In the case of SRC-1 and TIF2,
interactions with the ligand-binding domain of PR
have been demonstrated, suggesting that they may
act as mediators between PR and the transcription
machinery. However, the mechanisms by which
these coactivators effect transactivation by PR
remains largely uncharacterized.

To identify cofactors of transcription of mammalian
origin, two basically different approaches have been
mainly used. With the help of the yeast two-hybrid
system (37), cDNA libraries can be screened for pro-
teins that interact with the activator of choice and that
may ultimately turn out to act as cofactors of transcrip-
tion. The immediate access to the cloned genes for
these proteins is, of course, the major advantage of
this approach. However, a large number of false pos-
purposes are frequently obtained and, more importantly, cofactors that act through mechanisms that do not involve stable interactions with the activator may not be recovered by this genetic assay. Moreover, final proof for a functional role of the cloned gene in transcription activation and the analysis of its mechanism of action requires additional assays. Such efforts are, however, often hampered by the lack of appropriate cell lines or in vitro transcription systems containing limiting amounts of the candidate factor (38).

Most of these limitations are circumvented by the second type of approach, which uses cell-free transcription systems to identify cofactors of transcriptional activation. This biochemical approach is based on the observation that in vitro transcription systems reconstituted from highly purified and/or recombinant GTFs and RNAPII often display reduced effects to activators of transcription (39, 40). This loss of activator responsiveness can be attributed to the removal of important cofactors during purification and offers the opportunity to identify and characterize complementing activities in a functional test system. We have chosen this biochemical approach to study a possible role of cofactors of PR-mediated transcriptional activation. Using partially purified GTFs and RNAPII from rat liver as well as recombinant GTFs, we have established an efficient basal transcription system that is characterized by a strongly reduced response to highly purified recombinant full-length PR (form B). By screening various chromatographic fractions from rat liver nuclear extract, we identified and partially purified an activity that restored transactivation by PR in vitro but did not influence DNA binding of the activator. Because it stimulated PR activity but not two other activators tested, we termed this activity cofactor of PR activation (COPRA). Comparison of full-length PR and deletion mutants in the COPRA-deficient system revealed that C-terminal sequences of PR impose an inhibitory effect on the activation functions. We postulate a model in which COPRA acts by relieving this repression.

RESULTS

Activation of Cell-Free Transcription by Recombinant Human PR

To obtain sufficient amounts of PR for in vitro transcription studies, we overexpressed isoform B of human (h) PR as a His-tagged fusion protein (MH₆₇-hPR0) in Spodoptera frugiperda cells with the help of a recombinant baculovirus. To investigate the role of the two major activation functions, AF1 and AF2, we also expressed two deletion mutants, MH₆₇-hPR0Ácore and MH₆₇-ABC (Fig. 1A; Ref. 21). Mutant MH₆₇-hPR0Ácore was generated by deletion of the core domain of AF1, while MH₆₇-ABC was generated by deleting C-terminal sequences containing AF2. Purification of the recombinant proteins by nickel-nitrilotriacetic acid (NTA)-chromatography resulted in highly purified protein preparations (21). As analyzed by electrophoretic mobility shift assay (EMSA) and in accordance with published data (27, 28), the purified wild type PR, as well as deletion mutants, bound to DNA in a constitutive fashion.

The transcriptional activation potential of these activators was first compared in a cell-free transcription system that is based on crude nuclear extract from rat liver and does not contain significant amounts of PR. To detect basal transcription, a TATA-only promoter linked to a G-free cassette of 400 bp was used (TATA, Fig. 1B). Human PR-mediated activation was monitored using a construct that contains two PREs upstream of the TATA-box and a G-free cassette of 300 bp (P₂-TATA, Fig. 1B). In the absence of activators, basal transcription could be observed from both templates (Fig. 1C, lane 1). Addition of increasing amounts of MH₆₇-hPR0 stimulated transcription from the P₂-TATA-promoter up to 23-fold, whereas transcription from the TATA-promoter remained unchanged (Fig. 1C, lanes 2–5), indicating that transactivation by the recombinant PR is strictly PRE-dependent. Specific stimulation of the P₂-TATA-promoter was also seen with the mutants MH₆₇-hPR0Ácore (Fig. 1C, lanes 7–10) and MH₆₇-ABC (Fig. 1C, lanes 12–15). However, compared with full-length PR, the deletion mutants lacking the core of AF1 (MH₆₇-hPR0Ácore) or AF2 (MH₆₇-ABC) showed clearly decreased transcriptional stimulation, indicating that both the core of AF1 and AF2 contribute to transactivation by full-length receptor. To achieve nearly saturated transcription activation, about 5-fold higher molar amounts of MH₆₇-ABC were required (Fig. 1C, right panel). This correlates well with the reduced DNA-binding activity of this mutant (data not shown), which most likely results from the deletion of a dimerization motif located in the C-terminal domain of steroid receptors (22).

Reconstitution of a Cell-Free Transcription System with Reduced PR Response

As a convenient source for the purification of GTFs and RNAPII, we used rat liver nuclear extracts, which are highly active and obviously contain all factors required for PR-mediated transcription (Fig. 1). As indicated in Fig. 2A, in a first purification step nuclear extracts were fractionated by phosphocellulose chromatography. Subsequently, fractions A, C, and D were subjected to diethylaminoethyl (DEAE)-Sepharose chromatography, yielding a total of nine chromatographic fractions (Fig. 2A). At this stage of purification, three fractions (AB, CC, and DA) were required to reconstitute efficient basal transcription (data not shown). As summarized in Fig. 2A, using various assays we determined that DA contained TFIIA, TFIIF, and TFIID. CC contained TFIIE, TFIIF, RNAPII, and another TFIIID complex. Based on chromatographic behavior we assume that fraction AB contains TFIIA.
The reconstituted transcription system containing AB, CC, and DA showed still a strong response to added PR (data not shown). To obtain a more defined system, we thus replaced CC by recombinant TFIIE and TFIIF as well as a RNAPII fraction that had been obtained by separation of CC on MonoQ (Fig. 2A). As documented in Fig. 2B (lane 1), by combining AB, DA, and RNAPII from rat liver with recombinant human TFIIE and TFIIF, efficient basal transcription could be observed. Transcription proved to be highly dependent on each of the five components (lanes 2–5). Importantly, addition of saturating amounts of MH6-hPR0 did not result in significant stimulation of the PRE-containing template in this system (Fig. 3A, compare lanes 1 and 2). Because increasing the GTFs to promoter ratio by lowering the template concentration or elevating the concentrations of the various GTFs in the reconstituted system did not improve the response to added MH6-hPR0 (data not shown), we can eliminate the possibility that the poor responsiveness is due to a limiting concentration of a GTF. Therefore, these results suggest that a cofactor essential for transactivation by hPR became limiting in the reconstituted system.

Identification of COPRA

The dramatic loss of PR response observed by replacing CC with more purified RNAPII and recombinant TFIIE and TFIIF immediately suggested that CC might contain a cofactor important for PR activation. Indeed, addition of low amounts of CC to the nonresponsive system significantly increased fold activation by MH6-hPR0 from 1.4- to 9.3-fold while little effect on basal transcription was observed (Fig. 3A, lanes 2 and 3). We next screened the fractions obtained from separation of CC on MonoQ for the activity-enhancing PR activation. The activity eluted between 325 and 400 mM COPRA, a Cofactor of PR Activation
KCl (Fig. 2A, data not shown) and was termed COPRA. To characterize COPRA in more detail a titration experiment was performed. As shown in Fig. 3B, addition of increasing amounts of COPRA to reactions containing MH<sub>6</sub>-hPR<sub>0</sub> increased fold activation by PR from 2.1-fold to 14.3-fold in a dose-dependent manner. Thus, at the maximum dose of COPRA about 7-fold potentiation of PR activity was obtained. Importantly, basal transcription of the TATA-only template remained unchanged (Fig. 3B, lanes 1–5). Moreover, in the absence of the activator, COPRA did not influence transcription from the P<sub>2</sub>-TATA-promoter (Fig. 3B, lane 6), excluding the possibility that COPRA might represent an activator that acts through binding to PREs. Together, these experiments show that COPRA does not affect basal transcription, but greatly stimulates transactivation by hPR. Therefore, COPRA might represent a bona fide coactivator involved in enhancement of transcription by hPR in vitro. Further searches for additional fractions containing activities with similar function or able to cooperate with COPRA in transactivation of PR proved to be unsuccessful (data not shown).

Different Activators Exhibit Distinct COPRA Requirements

To determine whether transactivation by other activators might also be dependent on COPRA in our reconstituted transcription system, we investigated transactivation by hepatic nuclear factor-4 (HNF4), an orphan receptor of the nuclear receptor superfamily, which is expressed in the liver (41). The template we used consisted of three HNF4-binding sites in front of a TATA-box (H<sub>4</sub><sub>3</sub>-TATA). To assay activation in vitro we used purified recombinant HNF4 expressed as a His-tagged fusion protein in the baculovirus system. By EMSA we could show that rat liver fraction AB, which is required for efficient transcription in the reconstituted system, formed some protein-DNA complexes with the HNF4-binding site (data not shown). As determined by competition with oligonucleotides containing HNF4-binding sites (H4), these endogenous DNA-binding proteins proved to be responsible for a minor (1.6-fold) activation of the reporter gene H<sub>4</sub><sub>3</sub>-TATA (Fig. 4A, compare lanes 1 and 2). In the absence of COPRA, reactions containing MH<sub>6</sub>-HNF4 supported 6.7-fold activation of H<sub>4</sub><sub>3</sub>-TATA (lane 3), whereas reactions containing COPRA supported 8-fold activation (lane 4). Thus, COPRA potentiates transactivation by HNF4 by a factor of only 1.2.

Next, we investigated the COPRA requirement of HNF1, a member of the POU-homeobox family, which is also highly expressed in liver (42). By EMSA, endogenous HNF1 from rat liver could be detected in fraction AB and is, thus, present in our reconstituted transcription system (data not shown). To analyze whether these endogenous amounts of HNF1 support activation of the reporter gene H1-TATA, which contains a single HNF1-binding site in front of a TATA-box, we performed oligonucleotide competition. Clearly, addition of an excess of HNF1 competitor oligonucleotides (H1) specifically decreased transcription of H1-TATA by a factor of 5 (Fig. 4B, compare lanes 1 and 3), indicating that endogenous HNF1 is able to mediate about 5-fold activation of the promoter in the absence of COPRA. Reactions supplemented with COPRA supported about 7-fold activation (lane 2). Thus, addition of COPRA to the system resulted only in a 1.4-fold potentiation of HNF1-mediated transactivation.

Together, these data demonstrate that COPRA does not play a major role in activation of transcription by HNF4 and HNF1. Fig. 4. Transactivation by HNF4 and HNF1 Is Not Dependent on COPRA A, Cell-free transcription from the TATA template (TATA) and a template containing three HNF4-binding sites in front of the TATA-box (H<sub>4</sub><sub>3</sub>-TATA) was analyzed in the reconstituted system. Reactions received either no activator (lanes 1, 2, and 5) or a saturating amount of purified baculovirus-expressed HNF4 (MH<sub>6</sub>-HNF4, 5 pmol) in the absence (lanes 1–3) or presence of 1 μl COPRA (lanes 4 and 5). Lane 2 contained 20 ng H4 competitor oligonucleotides (H4-comp.). B, Transcription from reporter plasmid syn0-TG1 containing one HNF1-binding site (H1-TATA) was analyzed in the same system as in panel A except that plasmid PL-TG was used to measure basal transcription (TATA). As HNF1 is present in rat liver fraction AB, all reactions contained the activator. Lane 2 received 1 μl COPRA, whereas lane 3 received 70 ng oligonucleotides containing HNF1-binding sites (H1-comp.). Fold activation values, as normalized to the reaction containing H1 competitor, are given in the diagram on the right.
HNF1 and HNF4 while PR activity is strongly dependent on COPRA. Hence, COPRA is not a general co-factor of transcriptional activation but displays activator specificity.

A PR Deletion Mutant Lacking C-Terminal Sequences Is Active in the Absence of COPRA

To explore the role of the N- and C-terminal activation functions of hPR in mediating COPRA action, we compared the transactivation potential and COPRA requirement of full-length MH6-hPR0 with the two deletion mutants, MH6-hPR0Δcore and MH6-ABC (Fig. 1), in the reconstituted transcription system. In accordance with the results presented in Fig. 3, MH6-hPR0 displayed little activity in the basal system, but transactivation could be clearly potentiated up to 4.5-fold by addition of COPRA (Fig. 5, lanes 3–8). Potentiation of MH6-hPR0 activity was already evident at a limiting dose of activator (lanes 3 and 4), excluding the possibility that COPRA might relieve self-squelching caused by excess of activator. MH6-hPR0Δcore also showed little activity in the absence of COPRA, and addition of COPRA reproducibly resulted in about 2-fold potentiation (lanes 13–16). The apparently lower potentiation factor observed with the mutant might indicate that the core of AF1 is necessary for full COPRA action. Alternatively, it seems possible that the lower potentiation factor observed with MH6-hPR0Δcore is a consequence of the lower intrinsic activation potential of this mutant. Unexpectedly, in contrast to MH6-hPR0, the deletion mutant lacking the C-terminal part of hPR, MH6-ABC, supported activation of transcription in the absence of COPRA, and no significant potentiation was observed upon addition of COPRA (lanes 9–12). Importantly, COPRA-independent transactivation and the lack of potentiation by COPRA was observed both at a limiting (lanes 9 and 10) as well as a nearly saturating dose of MH6-ABC (lanes 11 and 12). First of all, these results indicate that the basal system is obviously capable of mediating transactivation by the N-terminal activation functions (AF1/AF3). Furthermore, the fact that in the absence of COPRA transactivation by AF1/AF3 is not evident in the context of full-length PR, although the recombinant full-length PR binds efficiently to PPREs, suggests that N-terminal activation functions are repressed through the presence of C-terminal sequences. Consequently, we postulate that COPRA might play a role in relieving this repression.

**DISCUSSION**

A Basal Transcription System Exhibiting a Specific Loss of PR Response

To establish a reconstituted transcription system allowing identification of cofactors of PR-mediated transactivation on a functional basis, we have used recombinant GTFs (TFIIE, TFIIF) as well as partially purified GTFs and RNAPII from rat liver. Rat liver GTFs and RNAPII had to be purified through two and three columns, respectively, to observe a dramatic loss of PR response. As two other activators tested, HNF1 and HNF4, were still able to mediate transactivation in this system (Fig. 4), it is clear that our system lacks a component that plays a rather specific role in transcription activation by PR. Because chromatographic purification of GTFs to homogeneity requires far more than two or three chromatographic steps, our basal transcription system most likely contains still a number of cofactors or coactivators that have been described by other groups using HeLa cell-derived transcription systems (11, 39, 40, 43–45). Assuming similar chromatographic behavior of human and rat coactivators, e.g. upstream stimulatory activity (USA)-derived cofactors PC1 to PC4 (39) should be contained in DEAE-Sepharose fraction DA, which supplies TFIIB, TFIID, and TFIIH to our system. Therefore, our data do not exclude the possibility that PCs or other factors with

| pmol hPR0 | COPRA | ABC | Δcore |
|----------|-------|-----|-------|
| 0        | –     | 12  | 12    |
| 2        | +     | 24  | 24    |
| 12       | –     | 2   | 12    |

**Fig. 5.** Deletion of the C Terminus of PR Reveals a cis-Represenation of Activation Functions That Can Be Relieved by Addition of COPRA

*In vitro* transcription was analyzed with the reconstituted system as described in the legend of Fig. 3. Reactions containing either no activator (lanes 1 and 2) or the indicated amounts of MH6-hPR0 (PR, lanes 3–8), MH6-hPR0Δcore (Δcore, lanes 13–16), or MH6-ABC (ABC, lanes 9–12) in the absence (−) or presence (+) of 1 μl COPRA are shown. Normalized fold activation values are indicated in the diagram below the autoradiogram. Open and filled bars represent reactions without or with COPRA, respectively. The difference between full-length PR and the ABC mutant was consistently observed in four independent experiments using two different receptor and COPRA preparations.
coactivator properties might play essential roles in PR-mediated transcription, most likely at steps after COPRA-mediated activation of PR.

**COPRA, an Activator-Specific Cofactor of Transactivation**

Using this system, which is characterized by a specific loss of PR response, we have identified an activity from rat liver, termed COPRA, which restores transcriptional activation by full-length PR in vitro. COPRA alone is not capable of mediating a transcriptional response through PREs (Fig. 3) and does not form specific protein-PRE complexes in EMSA (data not shown), suggesting that it does not represent a protein that acts through binding to PREs. As COPRA has no influence on PR-PRE complex formation in EMSAs (data not shown), we determined that it cannot influence activation by increasing the DNA-binding affinity of PR, e.g. by removing chaperones or by stabilizing PR dimers. COPRA has no effect on basal transcription and thus increases activation solely by a positive effect on PR-mediated transcription. This property distinguishes COPRA from cofactors such as DR2/PC3/Topoisomerase I or NC2/Dr1, which increase relative stimulation by activators by specifically repressing basal promoter activity (45, 46). In summary, COPRA displays several hallmarks of a coactivator of transcriptional activation. However, although COPRA clearly potentiated PR activity, it had little effect on transactivation by HNF1, a member of the POU-homeodomain transcription factor family. Moreover, COPRA also did not function in conjunction with HNF4, which is an orphan member of the nuclear receptor family and, thus, distantly related to PR. These observations indicate that COPRA is a rather specific cofactor that functions with a restricted set of activators only. However, we cannot rule out the possibility that HNF1 and HNF4 possess higher affinity for COPRA than PR and thus are saturated by small amounts of COPRA that might contaminate components of the basal transcription system.

Western blot analysis indicated that COPRA co-purifies with a TFID complex through three columns (Fig. 2, data not shown). However, as heat treatment for 10 min at 47 C known to destroy TFID activity (47) did not inactivate COPRA (data not shown), we consider it unlikely that COPRA corresponds to a specific TFID subtype (48) that, due to a specific TAF or TAF combination, might be particularly responsive to PR. We have not been able to demonstrate a stable association of COPRA and PR in EMSAs (data not shown). Moreover, using antibodies against CBP and SRC-1 (kindly provided by T.-P. Yao and D. Livingston, Boston, MA) in Western blotting experiments we found no indication that CBP or the major 160-kDa variant of SRC-1 is particularly enriched in the COPRA fraction (data not shown), suggesting that COPRA might not correspond to CBP or SRC-1 (33, 36). However, a relationship of COPRA with the PR cofactor hRPF1 (34), which has been shown to act as a positive cofactor of PR, TIF-2 (35), and other less characterized interacting factors (49, 50) cannot be excluded at present and requires further analysis.

**Analysis of PR Mutants in the COPRA-Deficient System Reveals a cis-Repression of N-Terminal Activation Functions**

In contrast to MHt-hPR0 and MHt-hPR0Δcore, the C-terminally truncated mutant MHt-ABC efficiently activated transcription, presumably through AF1 and AF3 (AF1/AF3), in the absence of COPRA, and no potentiation of MHt-ABC activity was observed in the presence of COPRA (Fig. 5). From these results we conclude that all factors required for AF1/AF3 activity are present in the basal system and that COPRA does not act as a mediator of transactivation for these activation functions. As noted above, EMSAs showed that MHt-hPR0 and MHt-hPR0Δcore, as well as MHt-ABC, are able to bind to DNA in a constitutive fashion, when tested alone or after mixing with GTFs and RNA-Pil fractions (data not shown). Thus, the two PR constructs containing C-terminal sequences, MHt-hPR0 and MHt-hPR0Δcore, do not reveal full AF1/AF3 activity, although the activators are in a DNA-binding state and transactivation by AF1/AF3 is basically possible under these conditions. This clearly suggests that in these constructs AF1/AF3 is inactivated due to a repressive effect imposed or mediated by C-terminal sequences. As depicted in Fig. 6A, this repression might be due to an intramolecular masking of AF1/AF3 by the C-terminal sequences containing AF2, which possibly also eliminates AF2 activity. Alternatively, repression of PR activation functions might be mediated by a repressor whose binding depends on the presence of C-terminal PR sequences (Fig. 6B). Consistent with the performance of MHt-ABC, in both models deletion of C-terminal PR sequences would allow transactivation through AF1/AF3 either by relieving the intramolecular inhibition or by eliminating inhibitor binding (Fig. 6C). Presumably, due to the presence of COPRA, the repressed state of activation functions is not apparent in crude nuclear extracts, inasmuch as MHt-hPR0 and MHt-hPR0Δcore display transactivation potential (Fig. 1). Consistently, addition of COPRA did not further potentiate PR-mediated activation in crude nuclear extracts (data not shown), indicating that they contain nearly saturating amounts of COPRA. It is noteworthy that, based on the analysis of a PR deletion mutant lacking the C-terminal 42 amino acids that could be activated by the antagonist RU486, while full-length PR remained inactive, Vegeto et al. (51) proposed a very similar model in which sequences at the very C-terminal end of PR play a role in silencing transactivation domains.
Possible Mechanisms of COPRA Action

Based on our findings that PR activation functions are repressed in the absence of COPRA and that COPRA restores transactivation by full-length PR as well as MH6-hPR0, we postulate that COPRA functions by relieving the intramolecular or repressor-mediated inhibition of activation functions (Fig. 6, A and B). Consistent with the result that MH6-ABC cannot be stimulated by COPRA (Fig. 5), this may occur through a conformational change induced by binding of COPRA to the C-terminal part of PR. In this scenario, the associated cofactor might not only cause derepression of activation functions but also act as a mediator of transactivation, which links AF2 to targets of the general transcription apparatus. Due to the lack of evidence for a stable complex of PR and COPRA, a more likely explanation might be that derepression occurs through mechanisms that involve a transient interaction of COPRA and PR or modifications of PR or repressor mediated by COPRA. It is attractive to speculate that derepression of activation functions might be the step that triggers stable association of additional molecules (e.g. SRC-1 or TIF2) contributing to the transactivation potential of AF2. In any case, we believe that our report is the first one to suggest that transactivation by PR might involve a cofactor-mediated derepression of activation functions.

Because DNA-binding is constitutive and hormone does not affect transactivation by purified PRs (27, 28), the role of the hormone cannot be directly assessed in our system. However, Allan et al. (52) have presented evidence that DNA binding of PR induced by antibody-mediated dimerization is not sufficient for transactivation, but rather hormone, in addition, is required. Because purified baculovirus-expressed PR activates transcription in a hormone-independent manner, we therefore speculate that its conformation might resemble that of hormonally activated receptor. This assumption is supported by the comparison of the activation potentials of full-length PR and PR deletion constructs (Fig. 1), which indicated that AF2, which is thought to require hormone-induced conformational changes for its activity, contributes to transactivation of full-length PR. Another indication results from a recent study of Beck et al. (1996), who demonstrated that the phosphorylation state of unliganded baculovirus-expressed PR resembles that of hormonally activated native PR (53). If baculovirus-expressed PR indeed corresponds to a hormonally activated PR, then our results imply that neither DNA binding nor hormone-induced conformational changes suffice to render PR active, but that derepression of activation functions through COPRA is another obligatory and separable step of the activation cascade. Finally, although the physiological relevance is not proven to date, the mechanism by which COPRA regulates transactivation by PR may represent another level of regulatory complexity for PR at the level of target genes, as variations in COPRA concentrations and/or the ratio of coactivator/repressor might alter the magnitude of the progesterone response in different PR-positive target tissues.

MATERIALS AND METHODS

Baculovirus Expression

Construction of baculovirus transfer vectors encoding the histidine-tagged fusion proteins MH6-hPR0, MH6-hPR0, and MH6-ABC was described in detail elsewhere (21). To generate a HNF4-encoding transfer vector the complete open reading frame of the Xenopus laevis HNF4α cDNA (54) was amplified by PCR with primers generating BglI site at both ends. After BglII digestion, products were ligated into pBlueBacHisB (Invitrogen, San Diego, CA). Construction of baculovirus transfer vectors encoding histidine-tagged 56-kDa and untagged 34-kDa subunits of human TFIIE will be described elsewhere (M. Klotzbücher and L. Klein-Hitpass, manuscript in preparation). Generation of recombinant baculoviruses, infection of Spodoptera frugiperda (Sf21) cells,
preparation of SF21 cell extracts, and affinity purification of overexpressed proteins on Ni-NTA-agarose (Qiagen, Hilden, Germany) were performed as described (55) with minor modifications. Cells were swollen in five packed cell volumes of buffer I, Ni-NTA-agarose bound proteins were washed three times with 20 volumes of buffer III (20 mM HEPES, pH 7.9, 10% glycerol, 100 mM KCl, 1.5 mM MgCl\textsubscript{2}, 0.5 mM dithiothreitol, 5 mM NaF, 0.5 mg/ml aprotinin, 0.1 mM benzamidine, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride) including 5 mM imidazole, transferred to a column, eluted in buffer III containing 120 mM imidazole, and dialyzed against buffer III containing 0.2 mM EDTA. In the case of TFIIH, SF21 cells were coinfected with viruses encoding both subunits, and \textit{in vivo} assembled TFIIH complexes (rTFIIH) were isolated from SF21-cell extracts by Ni-NTA affinity chromatography.

\textbf{Overexpression in \textit{Escherichia coli}}

TFIIH subunits RP74 and RP30 were overexpressed in \textit{E. coli} BL21(DE3) (Novagen, Madison, WI) using pET23/d/RAP74\textsubscript{H}4 and pET11d/RAP30 (56, 57). Expression, purification, and assembly of recombinant TFIIH complexes (rTFIIH) by renaturation were performed essentially as described by Wang et al. (56).

\textbf{Purification of GTFs, RNAPII and COPRA}

Nuclear extracts were prepared from livers of 3- to 6-month-old rats according to the procedure of Gorski \textit{et al.} (58) with modifications described by Döbbeling \textit{et al.} (59). For preparation of partially purified transcription factors, nuclear extracts were adjusted to buffer D (25 mM HEPES, 10% glycerol, 2 mM dithiothreitol, 0.2 mM EDTA, and protease inhibitors as indicated above) containing 0.1 M KCl and loaded on a phosphocellulose column P11 (Whatman) column. The 0.1 M KCl flowthrough (fraction A) and proteins eluted between 0.3–0.475 M KCl (fraction C) and 0.475–0.85 M KCl (fraction D) were required for transcription and further purification. Fraction A was eluted on a DEAE-Sepharose column (CL-6B, Pharmacia Biotech, Uppsala, Sweden), and bound proteins were eluted with 0.3 M KCl (fraction AB) and 0.5 M KCl (fraction AC). P11 fraction C was dialyzed against buffer D/100 mM KCl before DEAE-Sepharose chromatography. Bound proteins were eluted with 0.25 M KCl (fraction CB) and 0.85 M KCl (fraction CC). P11 fraction D was dialyzed against 0.1 M KCl before DEAE-Sepharose chromatography. The 180 mM flowthrough fraction (DA) as well as 0.32 M KCl (fraction DB) and 0.85 M KCl (fraction DC) eluates were collected. For further purification of RNAPII and COPRA, DEAE-Sepharose fraction CC was dialyzed against buffer D/100 mM KCl, loaded on a MonoQ column (Pharmacia), and eluted with a 0.1 to 0.6 M KCl gradient. COPRA activity was found in the range of 325 to 400 mM KCl, while RNAPII activity eluted in the range of 400–500 mM KCl. Pooled fractions were dialyzed against buffer D/100 mM KCl/20% glycerol, frozen in liquid nitrogen, and stored at −80 °C.

\textbf{Western Blot Analysis}

Fractions were subjected to SDS-PAGE in 0.1% SDS-10% polyacrylamide gel. Proteins were electrotransferred to nitrocellulose filters by semidry blotting. Blots were blocked with 2.5% blocking reagent (Boehringer, Mannheim, Germany), followed by incubation with primary antibodies and appropriate horseradish peroxidase-conjugated anti-IgG in blocking solution. Bound antibodies were detected using the ECL (enhanced chemiluminescence) system (Amerham, Braunschweig, Germany) for detection of TFIIH, TFIIA, TFIIF, and TBP, commercially available (UBI, Santa Cruz, CA) cross-reactive antibodies were used. TFIIH was detected with the help of monoclonal antibody 3c9 directed against p62 (60).

\textbf{RNAPII Assay}

RNAPII activity was measured as described by Hodo and Blatti (61).

\textbf{Transcription Templates}

Reporter plasmids P\textsubscript{2}-TATA (PRE\textsubscript{2}-TATA-G300) and TATA (TATA-G300 and TATA-G400) containing the TATA-box of the ovalbumin promoter were as described (31, 62). Transcription templates PL-TG, containing the TATA-box of the \textit{Xenopus} albumin gene in front of a 380 nucleotides (nt) G-free cassette and Syn0-TG1 carrying the HNF1-binding site of the albumin promoter in front of a 300 nt long G-free cassette, were as described by Schorpp \textit{et al.} (63). H\textsubscript{4}/TATA was generated by inserting three copies of the HNF4-binding site of the human α1-antitrypsin promoter (64) into the BglII-site of TATA-G300.

\textbf{Cell-Free Transcription}

Transcription reactions with 4.5 µl of rat liver nuclear extract and the indicated amounts of recombinant activators were performed in a total volume of 20 µl as described previously (31). Reconstituted transcription reactions contained 0.8 µl AB, 2.3 µl DA, 1.2 µl RNAPII 40 ng rTFII, and 60 ng rTFIIH. Sixty nanograms of the TATA constructs and 90 ng of reporter constructs containing activator-binding sites were used. Reactions with crude nuclear extract additionally contained 200 ng sonicated salmon sperm DNA. The transcription reactions were incubated, processed, and analyzed as described (27). For quantitative analysis, autoradiograms were scanned using a laser densitometer (Pharmacia Biotech). Fold activation values (activated/basal) were normalized to the ratio obtained in reactions without activator, which was set to be 1.

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