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PROGESTIN RECEPTORS: ISOFORMS AND ANTIHORMONE ACTION

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Summary—We present evidence that the two isoforms of A and B of the chicken (cPR) and human progesterone receptor (hPR) originate from two different mRNA populations. One of these encodes the isoforms A which originate by initiation of translation at an in-frame AUG found 127 (cPR) and 165 (hPR) codons downstream of the AUG which gives rise to the isoforms B. Two estrogen-inducible hPR promoters were identified which are responsible for the generation of these two classes of transcripts. Characterization of the cPR promoter suggested the possible existence of cell-type and isoform-specific auto-regulation of cPR transcription and provided evidence that estrogen-induction of cPR expression occurs at a post-transcriptional level. Finally, we demonstrate promoter-specific transcriptional activation by the hPR isoforms A and B, and we discuss the mechanism of action of the anti-progestin RU486.

INTRODUCTION

The progesterone receptor (PR) belongs to the superfamily of nuclear receptors (including those for steroid and thyroid hormones and for retinoids) whose members coordinate homeostasis and morphogenesis. These receptors act as transcriptional factors that regulate gene expression positively or negatively by interacting with cognate DNA sequences [ligand responsive elements (REs), enhancers in case of positive regulation]. Extensive structure–function analyses have localized and characterized multiple domains which are contained in differently conserved segments (termed A to F) of steroid receptor primary structure; for reviews, see [1–4]. The DNA binding domain (DBD, region C) is thought to fold into so-called "zinc-fingers" which provide the appropriate 3D-structure for DNA interaction, while only a very limited number of amino acids is decisive for specific recognition of the cognate REs [5–9]. In the absence of hormone the receptor does not bind to its cognate RE *in vivo* [10, 11], possibly due to an inhibitory potential of the ligand-free hormone binding domain (HBD) [12, 13]. A constitutive transcription activation function (TAF-1) is present in the N-terminal region A/B of steroid hormone receptors. Ligand-binding initiates steroid receptor–DNA interaction and transcriptional activation of target genes takes place due to the action of TAF-1 and a second transcription activation function, TAF-2, which is located in the HBD and is active only in the presence of hormone [11, 14–18]. Evidence has been presented that the activity of steroid receptor TAFs is mediated to the basic transcriptional machinery by intermediary factors which are apparently limiting in amounts [19, 20].

RU486 is an antagonist of glucocorticoid and progestin action in man and binds with high affinity to the corresponding receptors [21]. Interestingly, RU486 does not bind to the PRs of all species; it is, for example, unable to interact with the chicken or hamster homologues [22]. Baulieu has suggested that RU486 acts by stabilizing the so-called "8S non-transformed" heteromeric receptor complex, thus precluding interaction with the cognate HRE ([21] and references therein). In support of this interpretation the glucocorticoid receptor in the presence of RU486 did not induce footprints *in vivo* on the HRE of the tyrosine aminotransferase promoter [10]. Evidence has been presented, however, which may indicate that the PR–RU486 complex is able to bind to DNA [23–26].

The human (hPR) and chicken (cPR) PRs are unique in the superfamily of nuclear receptors (for a review, see Ref. [3]) in that two isoforms (designated form A and form B) of different molecular weights have been observed in the cytosol of human breast cancer (e.g. T47D cells) and chicken oviduct tubular gland cells, respectively, at approximately equimolar

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ratios [27–29]. Cloning of the corresponding cDNAs and genes revealed a single-copy PR gene, producing multiple transcripts. Two in-frame ATG codons were found in the cDNA sequence corresponding to region A/B of the human (codons 1 and 165, designated ATG1 and ATG2, respectively) and chicken (codons 1 and 128, designated ATG1 and ATG2, respectively) homologues. hPR or cPR expression vectors in which the region upstream of ATG2 was deleted generated a protein which was indistinguishable from the natural hPR or cPR form A with respect to its apparent molecular weight, immunoreactivity and hormone-binding capability. Vectors containing cDNA sequences upstream of, and including ATG1, expressed the PR isoforms B [30–35]. However, it was unclear from these studies by which mechanism forms A were generated.

Here we will review results obtained mainly in our laboratory. First we will describe experiments demonstrating that the PR isoforms are functionally different and that they arise most likely from two different populations of PR mRNAs which, in the case of hPR, are expressed from two different promoters. In the second part we will review our experiments addressing the question of how the anti-progestin RU486 generates its antagonistic action.

EVIDENCE FOR cPR ISOFORM A-SPECIFIC TRANSCRIPTS IN THE CHICK OVIDUCT

Given that cPR is a single-copy gene three mechanisms have to be considered which may be involved in the generation of the cPR isoforms A and B: form A is produced (i) by proteolysis of form B, (ii) by alternative initiation of translation at ATG2 or (iii) from a variant cPR mRNA generated by either 5′-trimming, alternative splicing or due to alternative promoters yielding form A- and form B-specific transcripts.

Since it is nearly impossible to experimentally exclude option (i), and since we had obtained no evidence in our transient transfection studies which supported option (ii) [31, 35], we cloned the cPR gene and determined its exon–intron organization in order to define the various cPR transcripts (Fig. 1; [34]). In addition to the previously isolated 4.5 kb cPR–cDNA (boxed in Fig. 2; [33]), using a variety of cDNA- and gene-derived probes for Northern blot analysis of chick oviduct mRNA, we characterized five further PR mRNA variants (Fig. 2, [34]). A 3.4 kb cDNA was sequenced in its entirety and corresponded to a RNA which resulted from aberrant splicing and alternative polyadenylation. A 8.2 and 3.3 kb mRNA were also found to be the result of alternative polyadenylation. Most importantly, however, we detected the presence of an abundant 4.1 kb cPR mRNA species (and of a corresponding 3.0 kb variant) which apparently lacked the 5′ region of the first exon (it did hybridize to probe **b** but not to probe **a**; see Fig. 2). This mRNA should be unable to give rise to cPR isoform B, while it could still be translated into form A. Our attempts to determine the 5′ boundary of this cPR form A mRNA failed, since S1-mapping analysis did not reveal any significant protected fragment [34]. It is possible that this mRNA species possesses heterogenous 5′-ends (see also below for hPR form A), thus precluding its identification in this assay.

We concluded from these data that chick oviduct cells express two cPR mRNA popu-

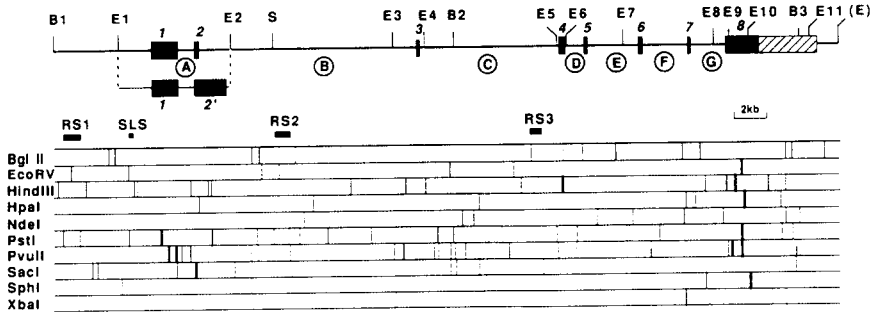


Fig. 1. Organization and physical map of the cPR. The structural organization of the cPR gene is schematically illustrated with ■ corresponding to exons 1–8. The encircled letters denote introns A–G. The positions of EcoRI (E1–E11), BamHI (B1–B3) and a single SalI (S) site are indicated. A variant cPR mRNA assembled from exons 1 and 2′ is shown separately. The hatched box corresponds to additional exonic sequences present in the longest detected 8.2 kb cPR mRNA. Highly repeated sequences (RS1–RS3) and the satellite-like sequence (SLS) are indicated by **bold** lines below the map. In the lower part of the figure, a restriction map for 10 additional enzymes is given (exonic sites are in **bold**). From Ref. [34].

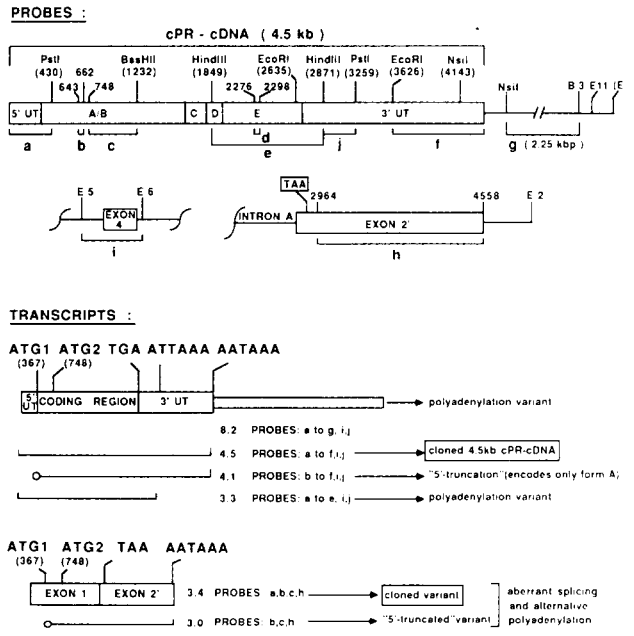


Fig. 2. Definition of six chick oviduct cPR transcripts. PROBES: schematic illustration of the probes used for Northern blot analysis. The locations of probes **a–f** are depicted relative to the cloned 4.5 kb cPR cDNA encoding regions A–E of the receptor. Untranslated regions (5′ and 3′ UT) of this cPR mRNA are indicated as well as a downstream region harbouring probe **g** (revealing cPR mRNAs extending beyond the 3′ border of the 4.5 kb RNA). Below, two portions of the cPR gene (see Fig. 1) are shown to illustrate the location of probes **i** and **h**, the latter of which reveals cPR mRNAs containing exon 2′. This exon results from a lack of splicing at the exon 2 splice-donor site. A cDNA composed of exon 1 and exon 2′ has been cloned and sequenced in its entirety [34]. TRANSCRIPTS: schematic representation of 6 transcripts originating from the cPR transcription unit having (from top to bottom) sizes of 8.2, 4.5, 4.1, 3.3, 3.4 and 3.0 kb. Probes detecting individual mRNAs are denoted and the mechanism of their generation as inferred from the Northern blot analysis, is stated. Note that the 4.1 and 3.0 kb RNAs are 5′ truncated (illustrated by a ○ at their 5′ ends) and do not contain ATG1 but ATG2. Therefore, these transcripts can only encode cPR form A which originated from initiation of translation at ATG2.

lations, one of which is specific for isoform A. While these data provide a mechanistic explanation for the existence of the two cPR isoforms, they do not, however, rule out that additional mechanisms (see above) may also be operative *in vivo*.

CHARACTERIZATION OF cPR PROMOTER

Sequence analysis of the 5′-flanking region of the cPR gene demonstrated the absence of any TATA or CCAAT box upstream of the transcription initiation start-site. Instead this region is very GC-rich and has a putative Sp1 binding site at −110 [note that several additional Sp1-sites are found in the first exon (Fig. 3; [34, 36])]. While no consensus palindromic or "halfpalindromic" estrogen responsive elements (ERE) could be detected, there is a "halfpalindromic" progestin responsive element (PRE) at −251 and a cluster of four additional "halfpalindromic" PREs between −890 and −1120

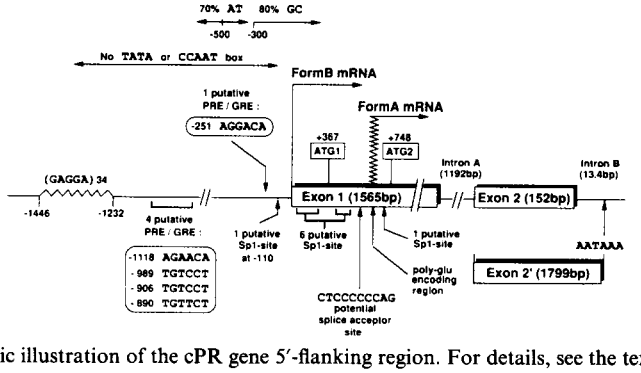


Fig. 3. Schematic illustration of the cPR gene 5′-flanking region. For details, see the text and Ref. [34, 36].

(Fig. 3). In fact, using DNase I footprinting, we showed that some of these sites, in particular the one at −251, can bind a bacterially overexpressed cPR DBD fusion protein [37] *in vitro*. We demonstrated by transient transfection and S1 nuclease mapping that the sequence encompassing 867 bp upstream of the cPR cap-site contains a promoter which gives rise to transcripts properly initiated at +1 and which is functional in chicken embryo fibroblasts (CEF), but not in HeLa cells. Moreover, transient co-transfection into CEF of cPR isoform expression vectors resulted in a progestin-dependent stimulation of transcription from cPR promoter-CAT constructs by cPR form A but not by form B([36]; see also below). In contrast, co-transfection of the human or chicken estrogen receptor in the presence of estrogen did not stimulate cPR promoter-CAT constructs containing up to 1683 nucleotides of the cPR gene 5′-flanking region. Moreover, run-on experiments with oviduct nuclei of chicken treated with various hormonal regimes confirmed that the cPR gene transcription is not under the control of estrogen. Since we observed on Northern blots of chick oviduct mRNA that withdrawal from estrogen-treatment resulted in a clear decrease of cPR mRNA levels, we concluded that estrogen-regulation of cPR gene expression occurs at a post-transcriptional step. Unexpectedly, and despite the cPR from A/R5020-dependent stimulation of cPR promoter-CAT constructs in CEF, we did not observe any stimulation of cPR gene transcription by progesterone, glucocorticoid or androgen in nuclear run-on assays using chick oviduct nuclei [36]. We believe that the transient transfection experiments indicate a potentially existing cell type—specific autoregulation of cPR gene transcription *in vivo*. It will be important in this respect to investigate whether the two cPR isoforms may be differentially expressed, for example, in a cell- or development-specific fashion.

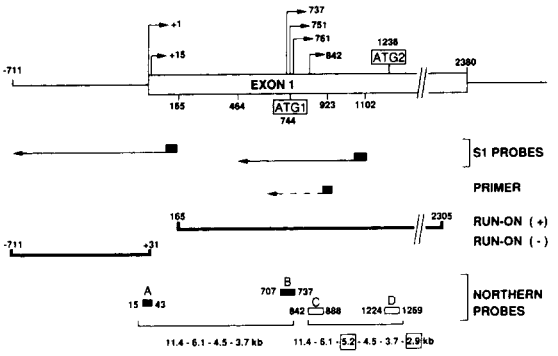


Fig. 4. Illustration of the experimental approaches used to define the existence of two classes of hPR mRNAs, one of which can encode only hPR isoform A. See the text and Ref. [38].

TWO DISTINCT ESTROGEN-REGULATED PROMOTERS GENERATE TRANSCRIPTS ENCODING THE hPR ISOFORMS A AND B

To investigate whether, similarly as for cPR, distinct transcripts encode the two forms of hPR, we performed S1 nuclease mapping and primer extension analyses to identify potentially existing hPR mRNAs initiated between ATG1 and ATG2, the only two in-phase ATGs found in the sequence encoding the N-terminal region A/B (see Fig. 6; [38, 39]). In fact using the two "S1 probes" illustrated in Fig. 4, two clusters of transcriptional start sites were detected in T47D mRNA. While two cap-sites were identified at +1 and +15, a second cluster was found downstream (+751, +761 and +842) of ATG1 [38]. A further cap-site at +737 is 7 nucleotides upstream of ATG1, a distance too short to allow translational initiation at ATG1. Thus, two classes of hPR mRNA are expressed

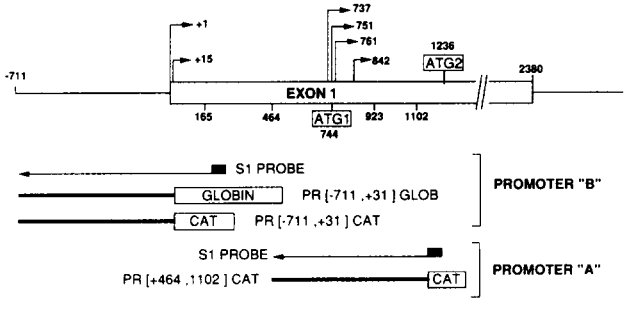


Fig. 5. Illustration of the experimental approach to define two estrogen-inducible hPR gene promoters. See the text and Ref. [38].

in T47D cells, one of which can code for form A but not for form B. Nuclear run-on, and primer extension experiments confirmed that the downstream cluster of sites in fact corresponded to transcriptional start-sites and not to splice-acceptor sites of a potentially existing upstream exon (see Fig. 4). To correlate the multiple transcriptional start-sites with the multiple hPR mRNA species seen on Northern blots, oligonucleotide probes (A–D in Fig. 4) were used. Probes A and B, located upstream of ATG1, detected four mRNAs of 11.4, 6.1, 4.5 and 3.7 kb size. The same species showed up with probes C and D (located downstream of ATG1) which, interestingly, revealed a 5.2 and a 2.9 kb species in addition [38]. We note that the difference between these two RNAs and the 6.1 and 3.7 kb, respectively, is about 800 bp which corresponds to the distance between the two clusters of transcriptional start-sites (see Fig. 4). Thus, we concluded that the hPR gene is transcribed to give rise to two different RNA families, one of which has cap-sites around ATG1 and, consequently, encodes only hPR isoform A.

What is the origin of the two classes of hPR mRNAs? One hypothesis is that the hPR gene is transcribed from two different promoters. We tested this possibility by constructing promoter-chimeric reporter genes [containing either the bacterial chloramphenicol acetyl transferase (CAT) gene for CAT-assays or the rabbit β-globin gene for S1 nuclease mapping; see Fig. 5] with 711 bp hPR upstream sequences, or with an internal region of the first exon (+464 to +1102) which contained the second cluster of start-sites and about 300 bp upstream. In fact, both of these regions showed promoter activity when transfected into HeLa cells as determined by CAT assay and S1 nuclease mapping analysis [38]. Moreover, promoter B (giving rise to isoform B) and promoter A (giving rise to isoform A) were estrogen-inducible when the human estrogen receptor was co-expressed. Estrogen-inducibility of hPR transcription was confirmed by nuclear run-on experiments using MCF7 cell nuclei.

We therefore concluded that in man and chickens, the PR isoforms A and B originate

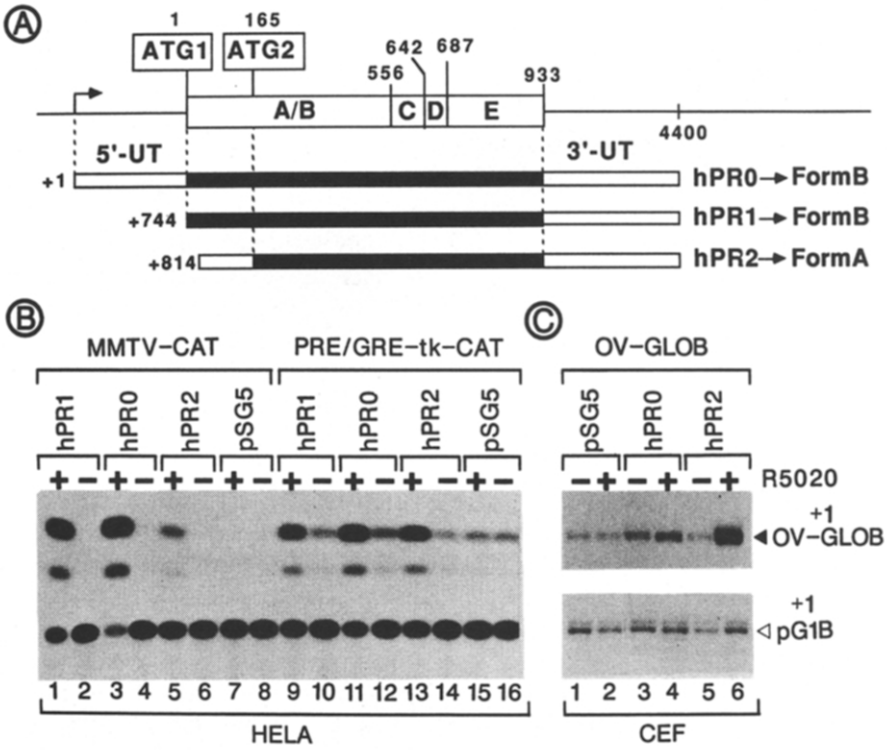


Fig. 6. Promoter-specific activation of transcription by the hPR forms A and B. (A) Schematic illustration of the expression vectors hPR0 and hPR1, generating hPR form B, and hPR2, generating hPR form A as described previously [35]. ■ indicates coding sequences, sequences the 5′ and 3′ non-coding regions. At the top the modular structure of the hPR is depicted. (B) Progestin-dependent activation of transcription from the MMTV-CAT (lanes 1–8) and PRE/GRE-tk-CAT (lanes 9–16) reporter recombinants by hPR forms A and B in HeLa cells. (C) Transcription from the OV-GLOB reporter gene in CEF is stimulated by form A but not form B. From Ref. [38].

from two different PR mRNA species, and that in the case of hPR these mRNAs are produced from two different promoters. Interestingly, the hormonal control of cPR and PR is exerted at different levels: while the two hPR promoters are estrogen-inducible, the estrogen-regulation of cPR expression is not a transcriptional phenomenon but occurs at a post-transcriptional step.

THE PR ISOFORMS A AND B ARE FUNCTIONALLY DIFFERENT

One of the most important questions concerning the existence of PR isoforms is whether they might exert different functions. Indeed, we have demonstrated that the PR isoforms A and B have a differential target gene specificity, most likely due to the fact that they differentially interact with a given promoter-specific environment [38, 40]. This isoform-specificity can be characterized as follows: while the reporter gene PRF/GRE-tk-CAT is equally activated by the two isoforms, the MMTV-CAT [containing the complex PRE of the mouse mammary tumour virus (MMTV) long terminal repeat (LTR)] is preferentially activated by isoforms B, and the ovalbumin promoter is only activated by isoform A (Fig. 6). Interestingly, the cPR and hPR acted synonymously on the various target genes. This is somewhat surprising, since there is little sequence conservation in the N-terminal 164

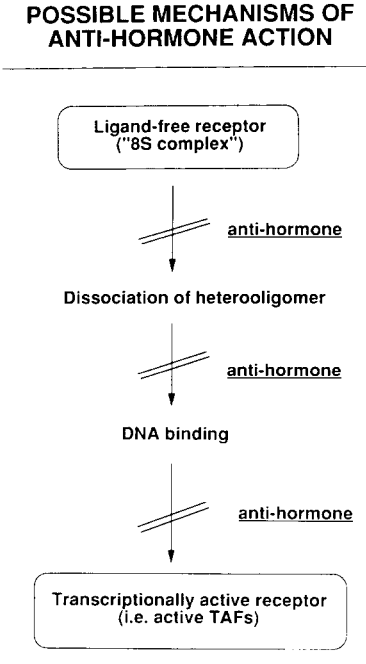
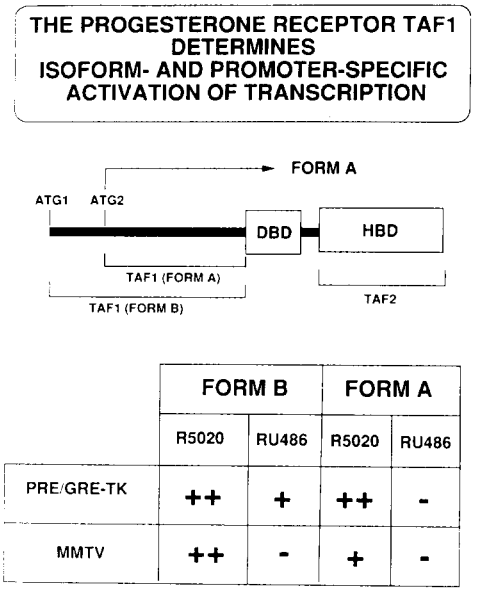


Fig. 7



	FORM B		FORM A	
	R5020	RU486	R5020	RU486
PRE/GRE-TK	++	+	++	-
MMTV	++	-	+	-

Fig. 8. Transcriptional activation of PRE/GRE-tk-CAT or MMTV-CAT by the two hPR isoforms A (translated from ATG2) and B (translated from ATG1) in presence of the agonist R5020 or the antagonist RU486 ("+ +" strong, "+" moderate, "−" no activation of transcription.) See the text and Ref. [11].

and 127 amino acids of the hPR and cPR, respectively, which corresponds to the sequence differing between forms A and B [38].

It will be challenging to define the mechanism(s) responsible for this isoform-specific target gene activation, not only with respect to the PR system but also in view of the multiple isoforms now identified in various mammalian and invertebrate members of the superfamily of nuclear receptors [41–45]. It is tempting to speculate that differential isoform expression in a development- and tissue/cell-specific fashion may be a mechanism to diversify the response to an individual signal in order to establish multiple signal-regulated gene networks.

MECHANISM OF ACTION OF RU486

Conceptually antihormones may act at three distinct levels (Fig. 7). They may interfere with the conversion of the ligand-free "heterooligomeric 8S-complex" to the 4S DNA binding form, they may interfere with the ability of the 4S form to bind to cognate responsive elements, or they might interfere with the processes subsequent to DNA binding which lead to transcriptional activation. Although we and others have shown that the hPR–RU486-complex can bind to DNA *in vitro* [23, 24, 11, 46], it was unclear whether binding would occur also

in vivo. In fact, genomic footprinting revealed that the glucocorticoid receptor bound to RU486 was unable to bind to the tyrosine aminotransferase promoter, while it did so in the presence of agonist, and it has been proposed that RU486 may act by stabilizing the 8S-complex [10, 21].

In order to test whether hPR–RU486 could potentially bind to its responsive element *in vivo*, we designed an *in vivo* competition assay that took advantage of the fact that RU486 is an antagonist in man but not in chicken, since it binds to hPR but not to cPR. We demonstrated that transcriptional activation of the MMTV promoter exerted by cPR–R5020 could be inhibited by co-expressed hPR when the transfected cells were exposed to both R5020 and RU486 (note that the experimental conditions were chosen such that only cPR–R5020 and hPR–RU486 complexes were generated). We excluded that this inhibition could be due to the formation of transcriptionally incompetent cPR–R5020–hPR–RU486 complexes, or that hPR–RU486 could "transcriptionally interfere/squelch" cPR–R5020-induced transcription [11, 19]. Thus the observed inhibition was apparently a result of a competition between cPR–R5020 and hPR–RU486 for the common responsive element and we concluded that RU486-liganded hPR can in fact bind to target genes *in vivo*. Therefore, the antihormonal effect is likely to occur at a step subsequent to DNA binding.

Similarly as for the estrogen receptor [17, 18] we established also for the cPR and hPR, that transcriptional activation is based on the action of two TAFs, located in the N-terminal region A/B (TAF-1) and in the HBD (TAF-2; Fig. 8). We also showed that TAF-2 is inactive in the presence of RU486 and that RU486 binding occurs exclusively in the HBD ([11], our unpublished results). Consequently, we expected that the hPR–RU486-complex should activate transcription by virtue of TAF-1. Indeed this was the case, since we demonstrated that RU486 is an agonist for PRE/GRE-tk-CAT induction. However, this activation was target gene-specific, since we could observe it for the PRE/GRE-tk-CAT but not for the MMTV-CAT (Fig. 8; [11]). We consider the partial agonist activity as a most convincing argument for the *in vivo*-DNA binding ability of the hPR–RU486-complex. Interestingly, only isoform B activated PRE/GRE-tk promoter, while form A was completely inactive in presence of RU486 (Fig. 8).

Thus it appears that TAF-1 is involved in both, the promoter-specific transcriptional activation by PR isoforms (since the isoforms differ in their N-terminal region which harbours TAF-1) and in the isoform- and promoter-specific transcriptional activation in the presence of RU486. However, in order to understand the molecular mechanisms which are responsible for these specificities, detailed studies are necessary, defining the TAF(s)-1 and the role of the additional N-terminal region present in isoform B, as well as the potential interaction of the hormone- and antihormone-bound HBD with these regions. These studies will have to take into consideration the 3D structure of the (anti)hormone receptor complex.

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