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Agonistic and antagonistic activities of RU486 on the functions of the human progesterone receptor

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RU486 induced the binding to a palindromic progestin responsive element (PRE) *in vitro* of homo- and heterodimers of the human progesterone receptor (hPR) isoforms A and B, present in T47D breast cancer cells or in HeLa cells transiently expressing the recombinant proteins. The resulting complexes were indistinguishable from those induced with the agonist R5020 with respect to specificity, affinity and stability. Ligand exposure was a necessary prerequisite to observe PR/PRE complexes. Antagonist-induced complexes migrated more rapidly during electrophoresis than agonist-induced ones, and no 'mixed' PR/RU486-PR/R5020 complexes were observed, suggesting that the dimerization interfaces of agonist- and antagonist-bound molecules are non-compatible. The analysis of a series of deletion mutants and chimeric receptors revealed the presence of two transcription activation functions (TAFs), located in the N-terminal region A/B (TAF-1) and the hormone binding domain (TAF-2). In the presence of agonists, both TAFs were active in HeLa cells. In the presence of RU486 TAF-2 was inactive, while TAF-1 within the hPR form B/RU486 complex activated transcription from a reporter gene containing a single palindromic PRE. We consider this to be the most convincing evidence that the receptor/RU486-complex does in fact bind to PREs *in vivo*. No transcriptional activation was observed in the presence of RU486 from a reporter gene containing the complex MMTV-LTR PRE. In contrast to hPR form B, form A was not able to activate transcription from PRE/GRE-tk-CAT in the presence of RU486. *In vivo* competition between hPR/RU486 and either cPR/R5020 or the human glucocorticoid receptor/dexamethasone (hGR/Dex) complex further supported that hPR/RU486 bound *in vivo* to its cognate responsive element. Indeed, the observed inhibition of transcription was shown to be due to competition for the MMTV PRE, since no transcriptional interference by the hPR/RU486 was observed, and since no heterodimers were formed between hPR/RU486 and cPR/R5020 or hGR/Dex. That the ligand-free hPR, however, was unable to compete, demonstrated that ligand binding is the prerequisite for DNA binding of hPR *in vivo*.

Key words: agonist-antagonist/progesterone receptor/RU486/target gene specificity/transcription activation

Introduction

As a member of the nuclear receptor superfamily, the progesterone receptor (PR) exhibits the characteristic modular structure with conserved DNA (region C, DBD) and hormone binding (region E, HBD) domains flanked by the less well conserved regions A/B and D (Figure 3A and B; Beato, 1989; Evans, 1988; Gronemeyer *et al.*, 1987; Green and Chambon, 1988; Kastner *et al.*, 1990a and refs therein). However, the PR is unique in the steroid receptor subset of this family because two isoforms (form A and form B) encoded in the same gene have been observed for the chicken and human homologues (Schrader and O'Malley, 1972; Gronemeyer *et al.*, 1985, 1988; Horwitz and Alexander, 1983; Horwitz and Francis, 1988; note that only one form has been described for the rabbit PR, Loosfelt *et al.*, 1984; Logeat *et al.*, 1985) which originate from translational initiation at two in-phase ATG codons (Conneely *et al.*, 1987; Gronemeyer *et al.*, 1987; Kastner *et al.*, 1990b).

RU486 is an antagonist of glucocorticoid and progestin action in man and binds with high affinity to the corresponding receptors (review Baulieu, 1989). Interestingly, RU486 does not bind to the PRs of all species; it is, for example, unable to interact with the chicken or hamster homologues (Baulieu, 1985). Baulieu has suggested that RU486 acts by stabilizing the so-called '8S non-transformed' heteromeric receptor complex, thus precluding interaction with the cognate HRE (Baulieu, 1989 and refs 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 (Becker *et al.*, 1986). Evidence has been presented, however, which may indicate that the PR-RU486 complex is able to bind to PREs both *in vitro* and *in vivo* (Bailly *et al.*, 1986; Guiochon-Mantel *et al.*, 1988; El-Ashry *et al.*, 1989; Turcotte *et al.*, 1990). Unfortunately, the *in vivo* data presented for the PR were based on competition experiments which did not distinguish between competition for DNA binding or for a factor mediating the activity of the transcription activation functions of the receptor. We have recently presented evidence for the existence of the latter phenomenon which we termed 'transcriptional interference' (Meyer *et al.*, 1989). An apparently related phenomenon, based on the over-expression of a transcriptional activator, has been called 'squelching' (Gill and Ptashne, 1988; Bocquel *et al.*, 1989; Tasset *et al.*, 1990).

In the present study we have used a transient expression/gel retardation system that faithfully mimics the *in vivo* hormonal requirement for PR-PRE interaction and found no differences with respect to specificity, affinity or stability *in vitro* in the presence of R5020 or RU486. We furthermore demonstrate that RU486 promotes DNA binding of hPR *in vivo* and, in analysing two transcription activation functions (TAFs) of hPR, we show that only one TAF

(TAF-2), located within the region containing the hormone binding domain, is inactive in the presence of RU486. Consequently, due to the presence of a second TAF (TAF-1) which is located in the N-terminal region A/B, RU486 has the potential to behave as an agonist for hPR dependent transcriptional activation. We show that this is indeed the case for certain target genes.

Results

The hormone or the anti-hormone induces binding in vitro of homo or heterodimers of the human progesterone receptor isoforms A and B to the cognate responsive element

Breast cancer T47D cells, which contain high levels of endogenous PR (Horwitz and Francis, 1988), were exposed to either the progestin R5020 or to the anti-progestin RU486 (Baulieu, 1989 and refs therein). Whole-cell extracts (WCE; Kumar and Chambon, 1988; Eul *et al.*, 1989) prepared from these cells formed specific complexes *in vitro* with a palindromic PRE, as demonstrated by gel shift assays (Figure 1A, lanes 2 and 3, respectively). Three 'retarded' complexes (arrows) were observed and corresponded to specific PR–PRE complexes, since they were absent in extracts from untreated cells [lane 1; note the presence of a non-specific band (open triangle), see Eul *et al.*, 1989]. Moreover, mutations in the PRE at positions which are essential for receptor binding *in vitro* (Eul *et al.*, 1989), and which abrogate progestin inducibility *in vivo* (Meyer *et al.*, 1989), resulted in the disappearance of these complexes (data not shown). T47D cells contain two PR isoforms (see Introduction) of different molecular weights. To assess the contribution of each form to the formation of these complexes expression vectors generating each isoform [hPR1 (form B) and hPR2 (form A); Kastner *et al.*, 1990a, b; see Figure 3] were transiently transfected into HeLa cells and WCEs were prepared for gel retardation. Major retarded bands (Figure 1B, lanes 1, 5, 11 and 18) corresponding to specific hormone (lanes 1 and 5) or anti-hormone (lanes 11 and 18) induced PR–PRE complexes, were observed following incubation *in vivo* with the respective ligand. They were not seen in absence of ligand (Figure 1D, lanes 1 and 4) nor with a mutated PRE (data not shown). No differences were observed between PR–PRE complexes formed with extracts prepared from cells incubated with hormone *in vivo* and extracts from cells incubated with hormone *in vitro* (data not shown). In agreement with the molecular weight difference between the hPR isoforms of ~30 kd, the corresponding DNA complexes migrated with different mobilities [for an example, see Figure 1C, compare lanes 1 (form A) and 2 (form B)]. When run on the same gel and exposed to the same ligand (not shown), their mobilities were identical to those of the lower and upper bands (arrowheads) seen on retardation gels performed with T47D cell extracts (Figure 1A, lanes 2 and 3).

It has been shown that the estrogen and glucocorticoid receptors bind to their cognate responsive elements as dimers (Kumar and Chambon, 1988; Tsai *et al.*, 1988; Wrange *et al.*, 1989). This raised the possibility that the intermediary band observed with T47D cell extracts could correspond to a complex containing a heterodimer of forms A and B [note that neither we nor others have rigorously demonstrated the existence of protein–protein interaction resulting in PR dimer formation; it is, however, highly likely that such an interaction stabilizes PR–PRE complexes, since the PR does not bind to 'half-palindromic' PREs *in vitro* (our unpublished results)]. When extracts prepared from HeLa cells transiently expressing either form A or B and treated with hormone (Figure 1B, lanes 1–5) or anti-hormone (lanes 11–18), were mixed at varying ratios, three bands (denoted A_2, AB and B_2 in Figure 1C) were observed, as with T47D cell extracts. The presence of the intermediary band, which is indicative of AB heterodimers and which was observed also with RU486-treated extracts, is particularly obvious on long-run retardation gels (Figure 1C, compare lane 4 with lanes 1 and 2). Similar results were obtained when extracts, prepared from HeLa cells co-expressing both PR isoforms, were exposed to R5020 or RU486 (*in vitro* or *in vivo*) and used for gel retardation (data not shown). Thus, we concluded that the two hPR isoforms bind as homo and heterodimers to the PRE *in vitro*, and that this binding is strictly hormone or anti-hormone dependent, irrespective of whether cells, *in vivo*, or extracts, *in vitro*, were exposed to the ligands.

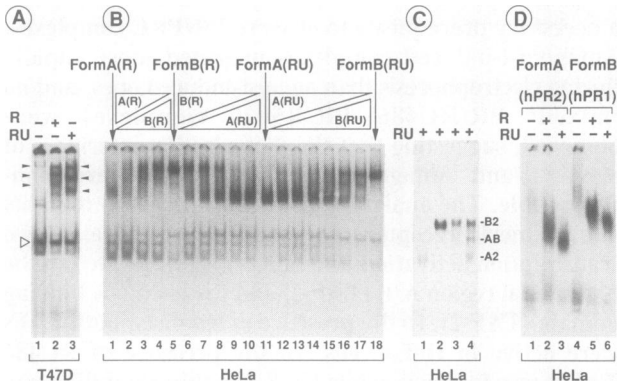

Fig. 1. hPR isoforms A and B generate agonist and antagonist-induced homo and heterodimeric complexes with a palindromic PRE. **A.** Gel retardation assays using extracts of T47D breast cancer cells and a palindromic synthetic PRE. Incubation was *in vivo* without (lane 1) or with 20 nM R5020 (lane 2) or RU486 (lane 3). **B.** Extracts of HeLa cells transiently expressing hPR form A (lanes 1 and 11) or form B (lanes 5 and 18) from hPR2 and hPR1 (Kastner *et al.*, 1990b), respectively, incubated *in vivo* with R5020 (lanes 1 and 5) or RU486 (lanes 11 and 18), were used for gel retardation assays with PRE as described in Materials and methods. Identical data were obtained when extracts were incubated with ligands *in vitro* (not shown). Mixtures at varying ratios {as illustrated by triangles at the top; for example, the ratio of isoform B bound to R5020 [B(R)] over isoform A bound to RU486 [A(RU)] decreases from lane 6 to 10} of the respectively treated isoforms were incubated for 30 min on ice and 10 min at 25°C before running on a 7% native 0.5 × TBE polyacrylamide gel at 25°C. Heterodimers were also formed between mutant cPR25 (lacking amino acids 1–289; Turcotte *et al.*, 1990) and hPR form B when both were incubated *in vivo* or *in vitro* with R5020, but no RU486/hPR–R5020/cPR complexes were seen (data not shown). **C.** RU486 induces homo and heterodimeric hPR–PRE complexes ('long-run' retardation gel). Gel retardation performed with extracts of hPR2 (lane 1) or hPR1 (lane 2) transfected HeLa cells, incubated *in vivo* in the presence of RU486. Form A- and form B-containing extracts were mixed at 1:1 (lane 3) and 2:1 (lane 4) ratios. **D.** Long-run retardation gel with extracts of HeLa cells transfected with hPR2 (lanes 1–3) or hPR1 (lanes 4–6), incubated *in vivo* either without (lanes 1 and 4) or with R5020 ('R', lane 2 and 5) or RU486 ('RU', lane 3 and 6) as indicated at the top.

PR−PRE complexes induced by RU486 and R5020 have similar stability, but their electrophoretic migrations are different

Complexes formed between the PRE and the PR isoform homo or heterodimers migrated faster when the receptor was exposed to RU486 rather than to R5020. This effect was seen for extracts of hormone-treated T47D cells (Figure 1A, lanes 2 and 3) and could be reproduced by incubating transfected HeLa cells with ligands *in vivo* (Figure 1D, compare lanes 2 and 3 for form A and lanes 5 and 6 for form B). Identical results were obtained when extracts of HeLa cells transiently expressing PR isoforms were incubated with the agonist or the antagonist *in vitro* (not shown).

To investigate the stability of progestin and antiprogestin-bound PR−PRE complexes, we analysed their dissociation kinetics by gel retardation 'off-curves' (Figure 2; Eul *et al.*, 1989). No significant differences were observed between complexes formed in the presence of agonist or antagonist (compare lanes 4−7 with 11−14, and data not shown). In addition, similar amounts of PR−PRE complexes were formed when identical aliquots of WCE of HeLa cells transiently expressing hPR were incubated *in vitro* with RU486 or R5020 (not shown; identical results were obtained when exposure to hormone was done *in vivo*, see Figure 2, lanes 1−3 and 8−10). Thus we concluded that the hPR−RU486 complex binds with similar affinity to the PRE to the hPR−R5020 complex.

RU486-liganded PR does not form heterodimers with R5020-liganded PR

The above results suggested to us that the differences in migration observed on retardation gels between the agonist- and antagonist-induced PR−PRE complexes did not reflect differences in the interaction with the DNA, but rather conformational differences, most likely, of the hormone binding domain. In the case of the estrogen receptor it has been shown that the region containing the hormone binding domain is involved in dimerization (Kumar and Chambon, 1988; Sabbah *et al.*, 1989; Fawell *et al.*, 1990). In keeping with this result, our preliminary data indicate that at least the N-terminal 289 amino acids of the chicken PR are not required for homodimerization (M.-E. Meyer, unpublished results). Therefore, we tested whether the conformations of the hormone binding domain generated by the hormone and the anti-hormone would be compatible with dimerization. Extracts of HeLa cells, expressing hPR form B and incubated *in vivo* with R5020, were mixed with extracts of HeLa cells, expressing hPR form A and incubated *in vivo* with RU486. No intermediary complex was observed {Figure 1B, lanes 6−10; the corresponding homodimer complexes [Form B(R) and Form A(RU)] are shown in lanes 5 and 11, respectively}. Heterodimer formation was also observed between the human and chicken PR [e.g. hPR form B and cPR25 which lacks the N-terminal 289 amino acids (Turcotte *et al.*, 1990)] when exposed to R5020. However, in keeping with the above described results, no hPR form B−cPR25 heterodimers were observed when the corresponding expression vectors were co-transfected and cells were exposed to R5020 and an excess of RU486 *in vivo* (remember that cPR does not bind to RU486) (data not shown). Thus we conclude that R5020- and RU486-liganded receptor molecules are unable to bind to a PRE as heterodimers.

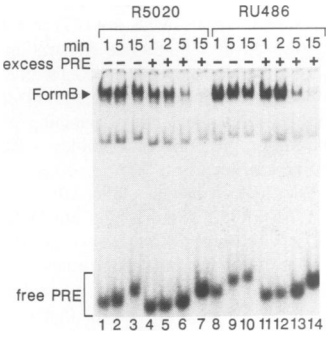

Fig. 2. hPR−RU486 and hPR−R5020 complexes with a palindromic PRE have similar stability. Gel retardation 'off-curves' (Eul *et al.*, 1989) were performed by using R5020- or RU486-treated extracts of HeLa cells expressing hPR form B to generate complexes with end-labelled PRE and incubating the complexes for the indicated times (1−15 min as shown on the top) without (lanes 1−3 and 8−10) or with (lanes 4−7 and 11−14) an excess of non-radioactive PRE. Samples were directly loaded onto running gels, accounting for differences in migration (as is most obvious for the free PRE). Similar data were observed when cells expressing hPR form A were analysed (not shown).

Human and chicken PR each contain two autonomous transcriptional activating functions

Our previous analysis of cPR mutants revealed the presence of two transcription activation functions, located in the N-terminal region A/B (TAF-1, see Figure 3B) and in the E region containing the hormone binding domain (TAF-2, Figure 3B) (Gronemeyer *et al.*, 1987; Bocquel *et al.*, 1989). In fact, chimeric receptors containing the DNA binding domain of the yeast transcription factor GAL4 (amino acids 1−147) and either the hormone binding domain of hPR [GAL−hPR(E), Figure 3A] or cPR [GAL−cPR(E), Figure 3B] or the regions A/B [GAL−hPR(AB1), Figure 3A; GAL−cPR(AB1), Figure 3B] all activated transcription (Figure 3D, lanes 3, 6, 8 and 12) from the cognate reporter gene 17M-tk-CAT (Figure 3C; Webster *et al.*, 1988a). As expected, the hormone was required for transcriptional activation by GAL−hPRE(E) and GAL−cPR(E) (Figure 3D, compare lanes 7 and 11 with 8 and 12, respectively). That GAL−cPR(AB1) and GAL−cPR(AB2) were active, but not GAL−cPR(AB8), indicates that the core of TAF-1 is located between amino acids 128 and 373 (Figure 3D, compare lanes 3−5 with lane 1; Figure 3B for illustration of the chimerae). Deletion mapping of hPR supported the data obtained with the GAL4−hPR chimerae: hPR3 (containing TAF-2, Figure 3A) in the presence of hormone and hPR5 (containing TAF-1, Figure 3A) activated transcription of PRE/GRE-tk-CAT (Figure 3E, lanes 9, 10 and 13, 14, respectively), though less efficiently than hPR1 (lanes 1 and 2). Deletion of either the N-terminal region A/B or of the hormone binding domain of cPR (giving cPR3 and cPR5, respectively) similarly created mutants which activated transcription, although rather weakly (Figure 3E, lanes 21−24). It is unclear why cPR3 was less active than hPR3, particularly in view of the similar transcriptional stimulation observed with Gal−cPR(E) and Gal−hPR(E). Since the two deletion mutants were similarly expressed when analysed with the same monoclonal antibody (data not

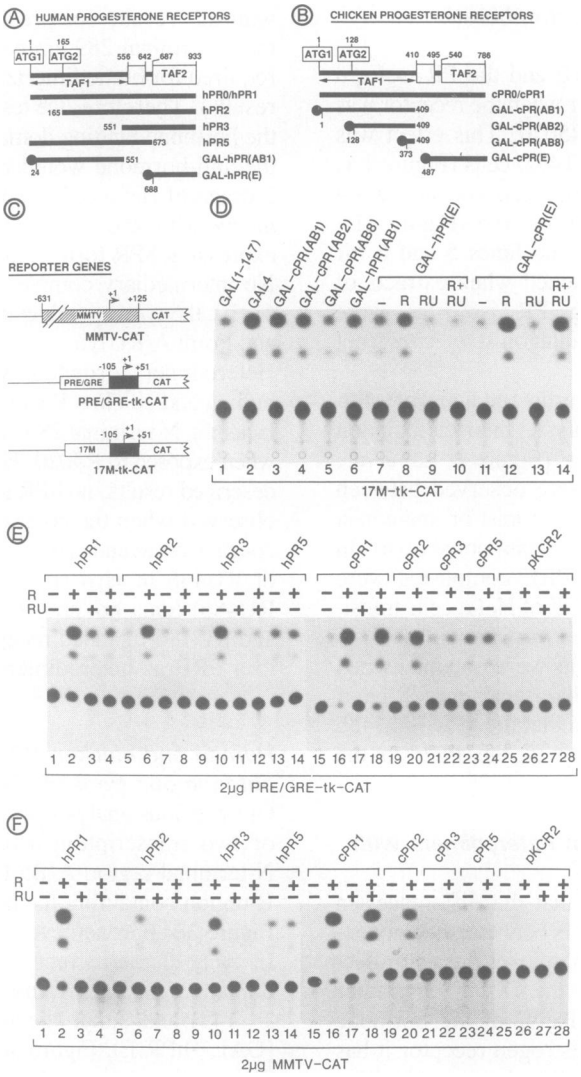

Fig. 3. RU486 binding does not generate an active TAF-2 while promoter specific activation of transcription by TAF-1 is not affected by the antagonist. **A**. Schematic illustration of hPR mutants and chimerae. The modular structure of hPR is depicted at the top with the DNA (amino acids 556–642) and hormone binding domain (amino acids 687–933) indicated as boxes. The two in-frame ATG codons, giving rise to form A (amino acids 165–933) and form B (amino acids 1–933) are shown. The regions containing TAF-1 and TAF-2 are indicated. Below, black bars correspond to expressed regions for individual hPR mutants, black circles represent the Gal4 DNA binding domain (amino acids 1–147) in Gal4–hPR chimerae. First and last amino acids are numbered when different from the N- and C-terminus, respectively. **B**. Similar illustration of cPR mutants and chimerae. For further details see Gronemeyer *et al.* (1987). **C**. The reporter recombinants used in this study are schematically depicted. MMTV-CAT contains the indicated sequences of the MMTV-LTR in front of the promoterless chloramphenicol acetyl transferase (CAT) gene (Cato *et al.*, 1986). In PRE/GRE-tk-CAT (Green *et al.*, 1988) and 17M-tk-CAT (Webster *et al.*, 1988a) synthetic oligonucleotides containing palindromic binding sites for the PR/GR family and Gal4, respectively, were linked to the Herpes simplex thymidine kinase promoter (nucleotides −105 to +51) placed in front of the promoterless CAT gene. **D**. Chicken and human PR contain two transcription activating functions. 500 ng of Gal 1–147 (lane 1) or 200 ng of either Gal4 (lane 2), Gal–cPR(AB1) (lane 3), Gal–cPR(AB2) (lane 4), Gal–cPR(AB8) (lane 5) or Gal–hPR(AB1) (lane 6) or 500 ng of Gal–hPR(E) (lanes 7–10) or Gal–cPR(E) (lanes 11–14) were transfected together with 1 µg 17M-tk-CAT (Figure 3C) into HeLa cells and incubated in absence (lanes 1–7 and 11) or presence of 20 nM R5020 (lanes 8 and 12), 20 nM RU486 (lanes 9 and 13) or 20 nM R5020 + 2 µM RU486 (lanes 10 and 14). CAT assays were performed as described in Materials and methods. The following conversion rates (in % of total chloramphenicol) were measured: 6, 36, 30, 18, 6, 3, 6, 17, 3, 3, 3, 36, 4, 34 for lanes 1–14, respectively. **E**. Transcriptional activation of 2 µg PRE/GRE-tk-CAT by receptors expressed by transfection into HeLa cells of hPR1 (lanes 1–4), hPR2 (lanes 5–8), hPR3 (lanes 9–12), hPR5 (lanes 13 and 14), cPR1 (lanes 15–18), cPR2 (lanes 19 and 20), cPR3 (lanes 21 and 22). cPR5 (lanes 23 and 24) or of the parental vector pKCR2 (lanes 25–28) in absence of ligand (lanes 1, 5, 9, 13, 15, 19, 23 and 25), or in presence of 10 nM R5020 ('R'; lanes 2, 6, 10, 14, 16, 20, 22, 24 and 26), 10 nM RU486 ('RU'; lanes 3, 7, 11, 17 and 27) or 10 nM R5020 + 1 µM RU486 (lanes 4, 8, 12, 18 and 28). In all cases 100 ng of PR expression vectors and 1 µg of pKCR2 was used. The following conversion rates (in % of total chloramphenicol, for 100 ng receptor expression vectors, data are given for 5 units of galactosidase) were measured: 3.9, 69.7, 15.1, 16.4, 3.2, 45.8, 4.8, 5.0, 2.5, 45.6, 5.3, 4.9, 14.4, 15.5, 6.6, 96.9, 4.5, 92.3, 5.6, 68.8, 4.7, 6.8, 15.8, 18.1, 3.5, 3.8, 4.6, 7.5 for lanes 1–28, respectively. **F**. Identical transfection scheme as in Figure 3E, but replacing the reporter gene with MMTV-CAT. The following conversion rates (given exactly as in **E**) correspond to lanes 1–28, respectively: 0.4, 83.6, 0.6, 0.7, 0.5, 6.6, 0.3, 0.2, 0.4, 30.8, 0.3, 0.3, 7.0, 8.4, 0.5, 95.6, 0.5, 96.1, 0.5, 32.5, 0.4, 0.5, 0.9, 0.4, 2.1, 2.4, 0.4, 0.4, 0.4, 0.4.

shown), it is possible that the small differences in the constructions may affect the DNA binding affinity.

When transcriptional activation of a reporter gene containing a 'simple' progestin responsive element (PRE/GRE-tk-CAT, Figure 3C) was compared with that one seen using a reporter gene harbouring a complex PRE

[MMTV-CAT, Figure 3C; see Beato, 1989], important differences were observed. All of the N- or C-terminally truncated mutants were less active with the MMTV-PRE than with the monomeric palindromic PRE in PRE/GRE-tk-CAT, when compared with the vectors expressing the chicken and human PR forms B (compare cPR3 and cPR5, and hPR3 and hPR5, with cPR1 and hPR1, respectively, in Figure 3E and F). In addition the two PR isoforms A and B exhibited striking differences: transcriptional activation of PRE/GRE-tk-CAT by hPR form A [expressed from hPR2, Kastner *et al.* (1990b)] was ~60% of the activity induced by hPR form B [expressed from hPR1, Kastner *et al.* (1990b)], whereas it was <10% when the reporter gene was MMTV-CAT (compare Figure 3E and F, lanes 1–8). Similar results were obtained with the two chicken PR isoforms (compare Figure 3E and F, lanes 15–20). Thus, two TAFs are present in the chicken and human PRs and their activity appears to be influenced by the promoter context of the target genes.

RU486 does not generate an active TAF-2 and acts as a promoter specific agonist/antagonist

No transcriptional activation was exerted by GAL–hPR(E) in presence of RU486 (Figure 3D, lane 9) and a 100-fold excess of RU486 over R5020 completely abolished the activity induced by R5020 alone (compare lanes 8 and 10). This was not due to the inability of the Gal–hPR(E)–RU486 complex to bind to its cognate responsive element, since (i) it could compete out the transcriptional activation of a 17M-tk-CAT reporter gene by Gal–cPR(E)–R5020 or Gal-hPR(AB1) (data not shown) and (ii) we have excluded the possibility that this competition could be due to transcriptional interference (Meyer *et al.*, 1989 and see below). Thus, RU486 can induce DNA binding of Gal–hPR(E) *in vivo*, leading to competition between the two Gal–PR chimerae for the common RE. In keeping with the above results, hPR3–RU486 did not activate transcription (Figure 3E, lanes 11, 12 and Figure 3F lanes 11, 12), although it could bind to DNA; indeed hPR3 could compete out the activity of cPR–R5020 or hGR–dexamethasone *in vivo*, but did not interfere transcriptionally in the presence of RU486 (not shown). As expected, GAL–cPR(E) was insensitive to RU486 (Figure 3D, lanes 13 and 14), since the cPR hormone binding domain does not bind the anti-progestin (our unpublished results).

Using MMTV-CAT as the reporter recombinant, RU486 was a pure antagonist of the stimulation of transcription by the intact hPR (Figure 4A, compare lanes 2 and 4). However, using a reporter gene (PRE/GRE-tk-CAT, Figure 3C) containing only a single palindromic PRE as responsive element, RU486 did not fully antagonize the transcriptional activation induced by R5020 (Figure 3E, lane 4). In fact, RU486 was ~20% as effective as the agonist R5020 at stimulating transcription from PRE/GRE-tk-CAT when used alone (Figure 4E, lane 3) or in excess over R5020 (lane 4), a value which was very similar to that one obtained with hPR5 which lacks the hormone binding domain (Figure 4E, lanes 13 and 14). As expected, no stimulation by RU486 was seen with the same reporter gene when the chicken homologue was tested (Figure 3E, lane 17). Interestingly, hPR isoform A, which lacks the N-terminal 164 amino acids of form B (Kastner *et al.*, 1990a, b), did not activate PRE/GRE-tk-CAT transcription in presence of RU486 (Figure 3E, lanes 7 and 8), thus giving results identical to

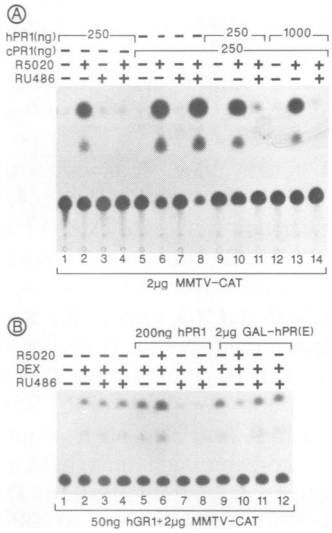

Fig. 4. RU486-bound hPR antagonizes transcriptional activation of MMTV-CAT by cPR–R5020 and hGR–dexamethasone, but the ligand-free hPR does not. **A.** 250 ng of hPR1 (lanes 1–4), 250 ng cPR1 (lanes 5–8) or hPR1 plus cPR1 at ratios of 1:1 (lanes 9–11) or 4:1 (lanes 12–14) were transfected into HeLa cells together with 2 µg MMTV-CAT and cells were incubated with hormone or anti-hormone (10 nM R5020; 10 nM RU486 in lanes 3 and 7; 1 µM RU486 in lanes 4, 8, 11 and 14) as indicated at the top. **B.** Transfection into HeLa cells of 50 ng hGR1 and 2 µg MMTV-CAT alone (lanes 1–4), or together with 200 ng hPR1 (lanes 5–8) or 2 µg GAL–hPR(E) (lanes 9–12). Cells were treated (5 nM R5020; 1 µM Dex, dexamethasone; 1 nM RU486 in lanes 3, 7 and 11; 5 nM RU486 in lanes 4, 8 and 12) as indicated. The following amounts (in % of total chloramphenicol) of acetylated chloramphenicol were measured for 5 units of the internal standard β-galactosidase: 0.3, 9.1, 8.8, 8.4, 14.4, 29.1 2.7, 1.9, 15.7, 6.3, 14.4, 16.3 for lanes 1–12, respectively.

those obtained with hPR3 (lanes 11 and 12) or with hPR form A using MMTV-CAT as reporter gene (Figure 3F, lanes 7 and 8). Note, however, that in the presence of excess RU486, both hPR form A and B bound efficiently to their responsive element, since they inhibited cPR–R5020 induced MMTV-CAT transcription (see below and data not shown).

In conclusion, the transcriptional activation seen with hPR form B–RU486 suggests very strongly that RU486 can promote the binding of the hPR to its cognate HRE *in vivo* (see below). Moreover, it appears that hPR TAF-2 is inactive in the presence of RU486, whereas TAF-1 is able to *trans*-activate the tk promoter. However, the MMTV promoter is not at all activated by TAF-1 when hPR form B is bound to RU486 (Figure 3F, lanes 3 and 4), and hPR5, which lacks the hormone binding domain and has TAF-1 as the only transcriptional activation function, is ~10% as active as hPR–R5020 (Figure 3F, lanes 13 and 14). This residual transcriptional activation by hPR5 indicates that TAF-1 can stimulate the MMTV promoter to some extent. The complete failure of hPR form B–RU486 to activate MMTV-CAT may be related to a possible negative effect of the RU486-bound hPR hormone binding domain on TAF-1 action due to some steric hindrance problem.

Competition experiments demonstrate that hPR binds in vivo to its cognate responsive element in the presence, but not in the absence of R5020 or RU486

The following can be predicted from the above results: by virtue of its DNA binding ability *in vivo*, and provided that

TAF-1 is inactive, the hPR−RU486 complex should be able to inhibit the transcriptional activation brought about by the cPR−R5020 complex (cPR does not bind RU486) or by the human glucocorticoid receptor (hGR)−dexamethasone (Dex) complex (remember that PR and GR recognize common responsive elements).

RU486−hPR could indeed antagonize transcriptional activation exerted by cPR−R5020 (Figure 4A). hPR form B activated transcription from MMTV-CAT in presence of R5020 (Figure 4A, lane 2), but not in the presence of RU486 alone (lane 3) or in excess over R5020 (lane 4). As expected, an excess of RU486 did not affect cPR form B−R5020 induced transcription (lanes 5−8). However, co-expression of hPR and cPR in presence of both, R5020 and an excess of RU486, severely impaired cPR−R5020 induced transcription in a hPR dose dependent manner (see lanes 9−14). Similarly, co-expression of the hGR and hPR in the presence of micromolar concentrations of Dex and 1 nM (Figure 4B, lane 7) or 5 nM (lane 8) RU486 inhibited the transcriptional activation of the MMTV-CAT reporter gene induced by the hGR−Dex complex (lanes 2 and 5). Note that such concentrations of RU486 are too low to compete with Dex for binding to the hGR (lanes 3 and 4) and that the activation seen in lane 6 results from the combined agonistic effects of the two receptors. Thus hPR form B−RU486 (as well as hPR form A−RU486; data not shown) inhibits cPR−R5020- and hGR−Dex-induced MMTV-CAT transcription.

Three mechanisms could account for the inhibitory effects: (i) the anti-hormone promotes binding of hPR to the PRE, but does not allow efficient transcriptional activation, (ii) binding of RU486 enables the hPR to interfere with the transcriptional activation exerted by agonist-bound cPR or hGR and (iii) cPR−R5020 and hPR−RU486, and hGR−Dex and hPR−RU486, form transcriptionally inactive heterodimers. In gel retardation experiments, we observed that heterodimers were formed in cells co-expressing hPR and cPR in presence of R5020. In presence of R5020 and an excess of RU486, however, no cPR(R5020)−hPR(RU486) heterodimers were seen (see above). Furthermore, we found no evidence indicating that heterodimers might be formed between hGR and hPR, and therefore we concluded that only two mechanisms could explain the above results: either RU486 induces DNA binding of hPR *in vivo*, and/or it enables at least one of the hPR activation functions to interfere with transcriptional activation by cPR or hGR.

In order to investigate whether hPR−RU486 competes with cPR−R5020 for binding to the MMTV-HRE or rather competes for (common) factor(s) mediating TAF action [a phenomenon referred to as 'transcriptional interference' (Meyer *et al.*, 1989) or 'squelching' (Ptashne, 1988; Bocquel *et al.*, 1989; Tasset *et al.*, 1990) in the case of overexpressed activators], we tested whether expression of either hPR TAF-1 or TAF-2 could result in an inhibition of cPR−R5020-induced transcription. cPR1, as the transcriptional activator of MMTV-CAT, was co-transfected with the chimeric receptors Gal−hPR(AB1) or Gal−hPR(E), which contain hPR TAF-1 and TAF-2, respectively. Note that in our previous studies, we did not obtain any evidence that Gal chimerae could bind to progestin responsive elements (Meyer *et al.*, 1989; see also below). As a positive control for transcriptional interference we used Gal−ER(EF) (Webster *et al.*, 1988b; Tora *et al.*, 1989) which clearly interfered with cPR form B−R5020-induced transcription (Figure 5A lanes 2 and 3) in a estrogen (E2) dependent fashion (lanes 12 and 13). While co-expression of hPR form B inhibited cPR form B-induced transcription from MMTV-CAT in presence of an excess of RU486 over R5020 (lanes 4 and 5), no significant effect was seen by co-transfecting Gal−hPR(E) (compare lane 3 with lanes 7, 9 and 11). In contrast, in the presence of R5020 as the only steroid, Gal−hPR(E) significantly interfered with cPR-induced transcription in a dose dependent manner (compare lane 3 with lanes 6, 8 and 10). Similarly, no interference of hGR−Dex-induced transcription from MMTV-CAT was observed when Gal−hPR(E) was co-transfected, and cells were incubated with RU486 (Figure 4B, compare lane 9 with lanes 11 and 12). As expected, however, the same chimera interfered efficiently with Dex-induced transcription in presence of the agonist R5020 (compare lanes 9 and 10). On the other hand, very little, if any, interference was exerted by Gal−hPR(AB1) on cPR *trans*-activation (Figure 5B, lanes 4−7), even though it was efficient at activating transcription (Figure 3D, lane 6). Similarly the Gal4 DNA binding domain [GAL(1−147); Figure 5B, lane 3] and Gal4 itself (lanes 1 and 2) did not interfere. That Gal4 did not inhibit transcriptional activation by cPR form B provides further evidence that it does not recognize the MMTV-PRE, since we have previously shown that it is efficiently produced in HeLa cells (Webster *et al.*, 1988b).

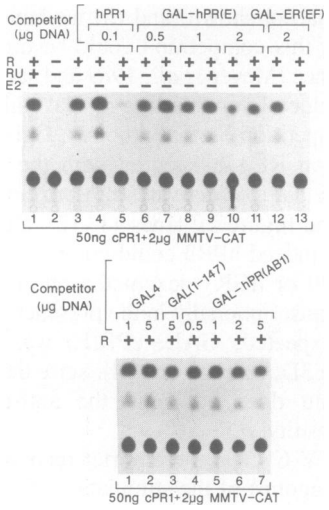

Fig. 5. In the presence of progestins—but not in presence of the antagonist RU486—hPR TAF-2 is able to interfere with the transcriptional activation of MMTV-CAT by cPR form B (or hGR, see Figure 4 lanes 9−12). **A.** Transient transfection into HeLa cells of 50 ng cPR1 and 2 μg MMTV-CAT alone (lanes 1−3), or together with the indicated amounts of hPR1 (lanes 4 and 5), GAL-hPR(E) (lanes 6−11) or GAL−ER(EF) (lanes 12 and 13). Hormonal treatment of the transfected cells is indicated at the top (R, R5020; RU, RU486; E2, estradiol). The following conversion rates (in %) were obtained for 5 units β-galactosidase: 28, 0.4, 25, 39, 1, 15, 23, 13, 18, 6, 12, 16, 0.7, in lanes 1−13, respectively. **B**, Gal4 and GAL−hPR(AB1), the latter containing hPR TAF-1 (see Figure 3D, lane 6), do not significantly interfere with transcriptional activation of MMTV-CAT by cPR form B. 50 ng cPR1 and 2 μg MMTV-CAT were transfected together with indicated amounts of GAL4 (lanes 1 and 2), GAL(1−147) (lane 3) or GAL−hPR(AB1) (lanes 4−7) and HeLa cells incubated with 10 nM R5020. Quantitation revealed an inhibition in lane 7 of ~20%, no inhibition was found for lane 2.

In conclusion, it is clear that hPR is unable to transcriptionally interfere/'squelch' in presence of RU486 with the transcriptional activation exerted by cPR or hGR. Thus, the only remaining interpretation of the inhibitory effect of hPR−RU486 on transcriptional activation exerted by cPR−R5020 or hGR−dexamethasone is a competition *in vivo* for the common responsive element (MMTV-PRE). Most importantly, this competition was seen in the presence (Figure 4A, lanes 11 and 14; Figure 4B, lane 7), but not in the absence of ligand (Figure 4B, lane 5). We take this as convincing evidence that either the hormone or the antihormone is required for hPR to bind to the PRE of target genes *in vivo*.

Discussion

The anti-progestin RU486 induces the binding of hPR to its cognate responsive element in vivo

RU486, the first available active antiprogesterone (Baulieu, 1989) has attracted considerable attention not only for the novel aspects of its use for abortion or 'contragestion' (Baulieu, 1989), but also because different models have been proposed for its mode of action. Conceptually, anti-steroids can act at least at two distinct levels. They either fail to enable the receptor to interact with its cognate responsive element, leaving it in its non-DNA binding state which may involve hsp90 binding (Baulieu, 1989), or they may interfere with subsequent processes linked to the activation of transcription. While Baulieu and colleagues reported that RU486 may stabilize heterooligomeric receptor−hsp 90 complexes and, thus, prevent receptor−HRE interaction (Baulieu, 1989 and refs therein), other observations do not support such a model. We demonstrated for example, that a chimera containing the GAL4 DNA binding domain linked to the hormone binding domain of the human glucocorticoid receptor was able to compete in the presence of RU486 with GAL4 for a GAL4-responsive element *in vivo* (Webster *et al.*, 1988b). Similar results were subsequently reported by Guiochon-Mantel *et al.* (1988) who expressed a rabbit PR lacking the hormone binding domain, but still activating transcription; co-transfected wild-type rabbit PR was shown to inhibit this activation in the presence of RU486. In these experiments, however, mutant receptors were used which exhibit only some properties of the wild-type homologue (see Bocquel *et al.*, 1989). In particular, we observed that the DNA binding affinity of cPR mutants lacking region A/B or the hormone binding domain, are severely decreased when compared with the wild-type receptor (Turcotte *et al.*, 1990). It is thus possible that a very weak DNA binding activity of the PR−RU486 complex will score positive in an assay using receptor mutants. The major criticism to this type of competition experiments is, however, that the observed inhibition of transcription by the receptor−RU486 complex was taken as evidence demonstrating competition for DNA binding, without taking into consideration that this inhibition may be due to transcription interference/'squelching'.

Using two different approaches, we demonstrate here that RU486 does in fact promote binding of the human progesterone receptor to its cognate responsive element. First, in presence of RU486, hPR form B activates transcription from PRE/GRE-tk-CAT 20% as efficiently as in the presence of the agonist R5020; this clearly requires binding of the hPR to DNA. Second, we demonstrate that the hPR−RU486 complex is able to compete out either the cPR−5020 or the hGR−Dex complex on the common responsive element *in vivo*. Note that we have indeed excluded RU486-mediated transcriptional interference and heterodimer formation to account for the observed competition (see results section for details).

The unliganded hPR does not bind to PRE both in vivo and in vitro

Our *in vivo* competition assays demonstrated clearly that the unliganded hPR was unable to compete with the glucocorticoid receptor−dexamethasone complex for the common responsive element, while it did so in the presence of R5020 or RU486. Thus, only exposure to hormone or to the antihormone promotes DNA binding of hPR *in vivo*. Similarly *in vitro* DNA binding of transiently expressed hPR and cPR, as visualized by the gel retardation assays with a palindromic PRE, was completely dependent on prior ligand binding (Figure 1A and D). This observation is in contrast with previous reports on the *in vitro* binding of (partially) purified ligand-free progesterone or glucocorticoid receptors (Bailly *et al.*, 1986; Willmann and Beato, 1986). It is likely that the manipulations *in vitro* during extraction and purification lead to the 'activation' of the receptor, a process which is induced by the hormone *in vivo* and which may be linked to the dissociation of a heterooligomeric receptor−hsp90 complex. However, Schauer *et al.* (1989) reported that both hormone-free and hormone-occupied rabbit PR, present in unfractionated uterine cytosol, possess similar total DNA binding activity for PREs at equilibrium conditions but differ with respect to their kinetics of interaction with DNA. These results are at variance with the data reported here and by El-Ashry *et al.* (1989), since very little, if any, binding of unfractionated steroid-free cytosolic PR to responsive DNA elements was observed. In contrast to the reports by Schauer *et al.* (1989) no significant differences in the kinetics of PR DNA binding *in vitro* have been observed for receptors exposed to R5020 or RU486 (this study, compare also El-Ashry *et al.*, 1989). While the reason for the discrepancies with the report of Schauer *et al.* (1989) is unclear to us, we note that the *in vitro* DNA binding system, as reported here, apparently reflects the basic characteristics of receptor−DNA binding *in vivo*.

When bound to RU486, hPR TAF-2 does not activate transcription nor interfere with transcriptional activation

We show here that hPR and cPR—similar to the human estrogen and glucocorticoid receptors (Godowski *et al.*, 1988; Hollenberg and Evans, 1988; Webster *et al.*, 1988b; Bocquel *et al.*, 1989; Tora *et al.*, 1989; Tasset *et al.*, 1990)—contain two transcriptional activation functions, a constitutively active TAF-1 located in the N-terminal regions A/B, and a hormone-dependent TAF-2 located in the hormone binding domain. We note, that in contrast to TAF-1 of human estrogen receptor which is active in chicken embryo fibroblasts, but not in HeLa cells (Tora *et al.*, 1989), TAF-1 of hPR and cPR activate also transcription in HeLa cells, thus indicating that these two TAFs may activate transcription differently. hPR TAF-2 in the chimera Gal−hPR(E) was completely inactive in the presence of RU486, although *in vivo* competition experiments demonstrated that this chimera−RU486 complex bound effi-

ciently to the Gal4 responsive element. Furthermore, Gal−hPR(E)−RU486 was unable to interfere/'squelch' with the transcriptional activation exerted by cPR−R5020 or hGR−Dex. In the presence of R5020, however, Gal−hPR(E) efficiently 'squelched' hGR−Dex-induced transcription. Thus these experiments indicate that TAF-2, in presence of RU486, is unable to interact with its putative cognate transcriptional intermediary factor(s). However, it is unclear whether TAF-2 is constitutively active, or whether it requires a progestin to be activated. Therefore, we do not know whether RU486 binding results in a 'distortion' of a preexisting 'active' TAF-2 conformation, or whether RU486 is simply unable to generate an 'active' TAF-2 in the same way as the agonist would do it.

Altered conformation of hPR bound to RU486

Two types of experiments support the hypothesis that RU486 and R5020 binding results in different conformations of the hPR. Firstly, no 'mixed' heterodimer complexes, containing one isoform bound to R5020 and the other bound to RU486, were observed (Figure 1B, lanes 5−11). Secondly, agonist and antagonist PR−PRE complexes migrated with different mobilities on native polyacrylamide gels. Since RU486 as well as R5020 binding is mediated exclusively via the hormone binding domain of hPR (our unpublished results), these experiments suggest that R5020 and RU486 binding results in structurally distinct and non-compatible (with respect to dimerization) conformations of the hormone binding domain, which may also differentially affect the activity of TAF-2.

The agonistic activity of RU486 is both TAF-1 and promoter context-dependent (Table I).

The present *in vitro* DNA binding results and those of others (El-Ashry *et al.*, 1989) indicated that RU486 and R5020−PR−PRE complexes have similar binding characteristics. Thus the differences in the agonistic activities observed between hPR−RU486 and hPR−R5020 are unlikely to be due to differences in the interaction with PRE, but rather reveal differential TAF activities.

Apparently TAF-1 and TAF-2 of hPR can act autonomously, since hPR3, hPR5, as well as the Gal4-chimerae containing hPR TAF-1 or TAF-2, all activate transcription. Consequently, given that the hPR−RU486 complex binds to cognate PREs *in vivo*, we can predict that it should activate transcription by virtue of its TAF-1, even though TAF-2 is inactive. In fact, when using 'simple' reporter genes with a single PRE (e.g. PRE/GRE-tk-CAT, Figure 3C), we observed an efficient transcriptional activation by hPR form B in presence of RU486. This activation was similar to that one observed for the same reporter gene when using hPR5, which contains TAF-1 as the only transcription activation function. Note that the mechanism of action of RU486 bears similarities to that of hydroxy-tamoxifen in the case of the human estrogen receptor, since in the presence of this anti-estrogen transcriptional activation was observed also to be due to TAF-1 (Berry *et al.*, 1990). While the two hPR isoforms were similarly efficient at activating transcription from PRE/GRE-tk-CAT in the presence of R5020, no activation was observed when hPR form A was exposed to RU486 (Table I). This suggested that the A/B region of hPR form A does not contain a functional TAF-1 on its own. It is possible, for example, that the hPR form B region A/B contains two TAFs, one of them located in the N-terminal 164 amino acids and that the latter is necessary for TAF-1 activity in presence of RU486.

Table I. RU486 agonistic activity is isoform specific and dependent on the target gene promoter context

	Form B		Form A	
	R5020	RU486	R5020	RU486
PRE−GRE-tk	++	+	++	−
MMTV	++	−	+	−

In contrast to the tk promoter, other promoters, such as the MMTV and the ovalbumin one are differentially activated by the two PR isoforms in the presence of R5020 (Figure 3E and F, compare lanes 2 and 6, and lanes 16 and 19; Tora *et al.*, 1988; Kastner *et al.*, 1990a). Since the isoforms differ only by their N-terminal regions, it is likely that TAF-1 is involved in this promoter specific effect. Indeed, in the presence of RU486, hPR form B (and also form A) were unable to activate the MMTV promoter (Table I). Remember that hPR form B−RU486 did activate the tk promoter. Since TAF-1 is the only potentially active transcription activation function in the presence of RU486, we conclude that it is in fact TAF-1 of a given isoform which activates transcription in a promoter context-dependent manner. It is not completely understood why hPR5 but not hPR form B−RU486 activates transcription from the MMTV promoter, but we cannot exclude that the RU486 liganded hormone binding domain interferes with TAF-1 activity in the intact hPR.

Our data clearly suggest the possible existence of agonistic activities of RU486 in humans. We note in this respect that progestomimetic activities of RU486 have indeed been described in a pharmacological study with post-menopausal women (Gravanis *et al.*, 1985).

What could be the biological significance of hPR isoform heterodimers?

Apparently, the endogenous (T47D) and recombinant hPR isoforms A and B form heterodimeric complexes with palindromic and 'imperfect' PREs (this report and El-Ashry *et al.*, 1989). Although cPR isoforms A and B may be differentially expressed during development (P. Tuohimaa, personal communication), co-expression of the two isoforms was observed in human and chicken target tissues. In view of the differential promoter specificity of these isoforms, the formation of heterodimers may reveal a novel regulatory aspect of control of gene expression by progestins, as the PR AB heterodimer may have transcriptional activation properties different from those of the PR A and B homodimers. Thus activation of transcription of a specific gene, in a specific target cell, at a specific time, might result from combinatorial mechanisms which act in addition to the basic receptor−HRE recognition. These mechanisms include cell specific activity of the transcriptional activation functions (TAFs) (Bocquel *et al.*, 1989), promoter specificity of the two isoforms (this study; Tora *et al.*, 1988; Kastner *et al.*, 1990a), heterodimer formation between the two isoforms

(this study) and possibly differential control of expression of the two PR isoforms.

Materials and methods

Receptor expression vectors

cPR0, cPR1, cPR2, cPR3, cPR5, cPR25, hPR0, hPR1 and hPR2 have been described (Gronemeyer *et al.*, 1987; Kastner *et al.*, 1990b, Turcotte *et al.*, 1990). hPR3 and hPR5 were constructed by site-directed mutagenesis and express amino acids 551–933 and 1–673, respectively (see Figure 3A). Due to the method of construction the hPR5 open reading frame is stopped in pSG5 downstream of the unique *Bam*HI site such that C-terminal of hPR amino acid 673 an additional sequence (GSRSY, followed by a stop codon) is expressed. All chimerae with the Gal4 DNA binding domain originated from pG4MpolyI or pG4MpolyII (Webster *et al.*, 1989; sequences of the polylinkers are available on request). Gal–hPR(AB1) was constructed by inserting into pG4MpolyII a hPR *Bam*HI fragment spanning from a natural *Bam*HI site to a *de novo* introduced one such that this chimera expresses amino acids 24–551 (inclusive) C-terminal of the Gal4 DNA binding domain (see Figure 3A). Gal-hPR(E) originates from pG4MpolyI into whose *Bam*HI site the *BclI–BglII* fragment of hPR1 was introduced (thus expressing the hPR amino acids 688–933). Gal–cPR(AB1), Gal–cPR(AB2) and Gal–cPR(AB8) are derived from pG4MpolyII by introducing into the corresponding sites the *Xho*I–*Sac*I [Gal–cPR(AB1)], *Bam*HI–*Sac*I [Gal–cPR(AB2) or Gal–cPR(AB8)] fragments of cPR1, cPR2 and cPR8 (Turcotte *et al.*, 1990), respectively (see Figure 3B). Gal–ER(EF), Gal4, and Gal(1–147) have been described (Webster *et al.*, 1988a, b). All novel recombinants were verified to express similar amounts of mutant/chimeric receptors with the expected molecular weights by immunoblotting with suitable antibodies (data not shown).

Reporter recombinants

MMTV-CAT, PRE/GRE-tk-CAT and 17M-tk-CAT are schematically illustrated in Figure 3C and have been described previously (Cato *et al.*, 1986; Gronemeyer *et al.*, 1987; Green *et al.*, 1988; Webster *et al.*, 1988a; Meyer *et al.*, 1989).

Cell culture, transient transfection, CAT assays

All procedures were done as described previously, using as transfection internal standard the amount of β-galactosidase expressed from pCH110 (Gronemeyer *et al.*, 1987; Bocquel *et al.*, 1989; Meyer *et al.*, 1989).

Gel retardation

For a description of the palindromic PRE and the mutated PRE used in this study, see Eul *et al.* (1989). Cells expressing transiently a particular human or chicken PR isoform were treated with 20 nM of the respective hormones 1 h prior to scraping (*in vivo* incubation), and extracts were prepared as described below. For *in vitro* incubation extracts of hormone deprived transfected cells were first prepared as follows: cells were washed with PBS, scraped and resuspended [250 μl/3 Petri dishes (9 cm diameter)] in homogenization buffer (Eul *et al.*, 1989), followed by two freezing (−80°C)/thawing(ice)/cycles. 15 min centrifugation (10 000 *g*) yielded the extract which was frozen in liquid nitrogen and stored aliquotted at −80°C. Subsequently hormones were added [1 μl/20 μl extract of 100 μM R5020 or RU486 (in 1% ETOH)] for 40 min at room temperature. For generation of heterodimers *in vitro* or *in vivo* hormonally treated extracts were mixed *in vitro* (no difference in the results described were observed when isoforms were co-expressed *in vitro*), the PRE was added and the mixture was kept for 30 min on ice and then for 10 min at room temperature. Electrophoresis was performed with a 7% polyacryamide gel (30/0.8%) in 0.5 × TBE at 100 V for 5 h at 20°C. T47D cell extract was prepared identically, except that cells from three plates were lysed in 150 μl homogenization buffer. All other procedures, in particular gel retardation 'off-curves', were done as described (Eul *et al.*, 1989).

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