Multiple Human Progesterone Receptor Messenger Ribonucleic Acids and Their Autoregulation by Progestin Agonists and Antagonists in Breast Cancer Cells

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We have used AB-52, a monoclonal antibody which recognizes both the A (94,000 daltons) and B (120,000 daltons) proteins of human progesterone receptors (hPR), and hPR-50, a PR complementary DNA probe isolated from a T47D-pcD library, to study the structure and hormonal regulation of the hPR mRNAs and proteins in human breast cancer cells. RNA blot hybridization analysis of poly(A^+) RNA shows that $T47D_{co}$, an estrogen resistant human breast tumor cell line in which PR are constitutively expressed, contain at least six PR mRNAs ranging in size from 2.5 to 11.4 kilobases. All six are mature cytoplasmic messages that are also present in normal human endometrium and in PR-positive MCF-7 breast cancer cells, but not in PR-negative cells. Using hPR-50 RNA synthesized *in vitro* as a 1.3 kilobase standard, we calculate that MCF-7 cells contain approximately 16 message molecules per cell which are increased to approximately 45 by estradiol treatment; $T47D_{co}$ cells contain approximately 90 message molecules per cell constitutively expressed. Treatment of $T47D_{co}$ cells with progesterone leads to down-regulation of immunoreactive A- and B-receptors in the first 8–12 h, followed by their replenishment during the next 48 h. In parallel, hPR message levels initially decrease and then return to pretreatment levels. The synthetic progestin R5020 chronically down-regulates A- and B-receptors; the proteins are profoundly suppressed for at least 48 h, while PR mRNAs fall to less than 15% of control. However, with both hormones, parallel changes in protein and message levels are observed, suggesting that progestational agonists autoregulate the levels of their own receptors by inhibiting transcription of the PR gene. Antagonists appear to have different effects. With the antiprogestin RU 486 there is discordance between hPR protein and message levels which may be due to an ineffective inhibitory interaction between the antagonist-occupied receptors and PR genes, thereby disrupting the negative feedback loop. (Molecular Endocrinology 2: 62–72, 1988)

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INTRODUCTION

Progesterone has profound developmental and physiological effects in the reproductive tract and central nervous system; its synthetic derivatives are widely used to treat hormone dependent cancers and in contraception. The actions of this steroid hormone are mediated by low abundance intracellular progesterone receptor proteins (PR) whose levels are generally up-regulated by estradiol, a property that makes PR useful markers of hormone dependence in breast cancer. PR are down-regulated by progesterone and its analogs (for review see Ref. 1). The molecular mechanisms underlying these changes in PR levels are not known. Spurred perhaps by the clinical importance of these proteins, a number of technological advances have recently been made which will undoubtedly lead to increased understanding of PR structure and mechanisms of action. These advances include the successful purification of the receptors (2–5), the production of antireceptor monoclonal antibodies (MAbs) (6–8), and the isolation of cDNAs synthesized from PR messages (9, 10). Both polyclonal and MAbs have now been produced against purified PR. Logeat *et al.* (6) generated five MAbs using rabbit uterine receptors as immunogens, and Sullivan *et al.* (8) produced five monoclonals to chicken oviduct PR. One of these, PR-6, was

used by us to immunoaffinity purify human PR (hPR) from $T47D_{co}$ human breast cancer cells and to generate three MAbs (11). The antichick and antirabbit PR antibodies have been used to screen expression libraries, resulting in the successful isolation of cDNA clones encoding chicken and rabbit PR (9, 10) and most recently hPR (12). Additionally, we and others have used these immunological reagents to characterize hPR structure, particularly the phosphorylation and structural relationships between the A- and B-forms of hPR (13).

We have previously described the autoregulation of hPR levels by progestins. This is characterized by a down-regulation of receptors in nuclei that occurs several hours after hormone administration (14). We have also shown by immunological and *in situ* photoaffinity labeling methods that native hPRs consist of two nonassociated 8S complexes: one 8S complex contains B-proteins [120,000 kilodalton (kDa)] and the other contains A-proteins (94,000 kDa) (15). Each receptor subtype binds DNA and each, independently of the other, can exert a biological effect (our unpublished observations). Using anti-PR antibodies and PR cDNAs, we have now compared the levels and hormonal regulation of PR mRNAs, with the progestin-mediated down-regulation of receptor proteins in human breast cancer cell lines. The antibody used, AB-52, cross-reacts with both A- and B-receptors of hPR (11). The cDNA, hPR-50, codes for the steroid binding domain and part of the DNA binding domain characteristic of both proteins (9). Here we describe the existence of at least six mature hPR mRNAs, quantify their levels, and compare PR message and protein regulation in breast cancer cell lines after estrogen, progestin, and antiprogestin treatment. We also estimate message turnover rates and conclude that at least one level of PR control involves autoregulation of the receptors by progestins at the level of PR gene transcription. This negative feedback loop is inoperative when the receptors are occupied by the antagonist RU 486.

RESULTS

Characterization of Human PR mRNAs

Northern transfer hybridization analyses of mRNA from chick oviducts detect three PR mRNA species of approximately 4.5, 4.0, and 3.9 kb (9); rabbit uterine RNAs contain a PR message doublet of 5.9 kb and 6.6 kb (10); and hPR were recently reported to be encoded by five messages of 5.9, 5.1, 4.3, 3.7, and 2.9 kb (12). We have been studying PR regulation in the ER-negative $T47D_{co}$ human breast cancer cell subline (14). To determine the size of the mRNAs encoding PR in these cells, Northern blot hybridization experiments were performed using total cellular polyadenylated RNA. In $T47D_{co}$ cells, hPR-50 hybridized to at least six mRNA species of approximately 11.4, 6.1, 5.2, 4.5, 3.2, and 2.5 kilobases (kb) under high stringency washing conditions (Fig. 1). The 11.4-kb species, which had not been previously described, is the most abundant, and

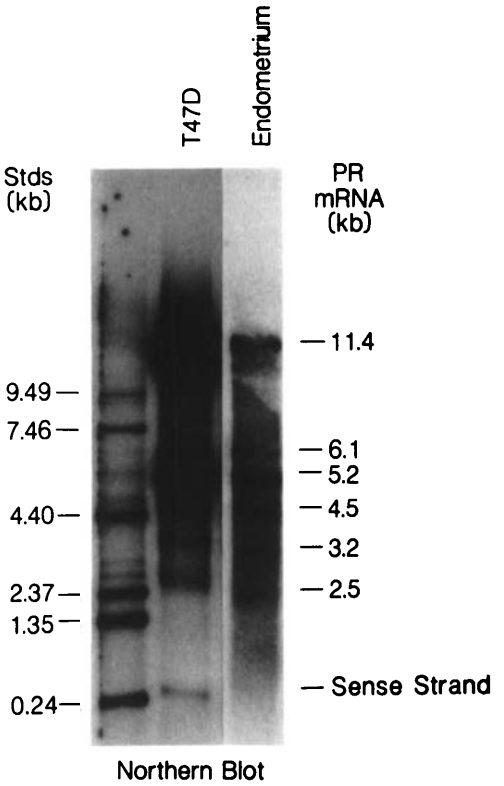

Fig. 1. Characterization of hPR mRNA in $T47D_{co}$ Cells

Total cellular poly(A^+) RNA was isolated from approximately 5×10^7 $T47D_{co}$ cells and from 1.5 cm^3 of endometrial tissue using guanidine thiocyanate as described in *Materials and Methods*. Seven and a half micrograms of each poly(A^+) RNA sample were separated by electrophoresis on 0.8% agarose-6% formaldehyde gels; the RNA was transferred to nitrocellulose and hybridized with nick-translated hPR-50 insert DNA (1×10^9 cpm/μg DNA). In a parallel lane (*far left*), 0.6 μg RNA ladder was used for size standards.

in some autoradiograms exhibits a triplet composition (see Fig. 6, for example). Thus, there are six to eight hPR messages detected with hPR-50, a probe complementary to 3′-protein coding sequences. With hPR-54, a longer cDNA probe which comprises the complete coding region except for 200 base pairs (bp) at the 5′-end, the same eight bands are hybridized (data not shown), suggesting that all of the major RNA species are being detected. Since the 11.4-kb band had not been previously reported and since $T47D_{co}$ cells are tumor-derived cells, we confirmed that this was a normal message by Northern blot analysis of poly(A^+)RNA isolated from human endometrium. The 11.4-kb band, as well as the smaller bands, are all represented in normal tissue (Fig. 1), demonstrating that they are neither tumor nor breast specific. The pattern of six bands is qualitatively and quantitatively reproducible; densitometric analysis shows that the 11.4- and 5.2-kb messages are most abundant representing, respectively, ~65% and ~15% of total PR mRNA.

Since PR are believed to be encoded by a single copy gene, (15a), possible explanations for the supernumerary PR messages include variable 3′-extensions arising from alternative polyadenylation sites; differ-

ences in the 5′-untranslated region; alternate exon splicing; or stable poly (A^+) RNA processing intermediates. The last would be expected to be confined to nuclei. To show whether any of the messages are processing intermediates, $T47D_{co}$ cells were homogenized, the cytoplasm was separated from nuclei, poly (A^+) RNA was isolated from both fractions, and their PR message content was compared to the pattern obtained from total cell lysates by Northern blot analysis (Fig. 2). Clearly none of the six major hPR mRNAs were restricted to nuclei strongly suggesting that they are all mature cytoplasmic forms, and additional studies are required to explain their origin and function. Since human PR consist of two hormone binding proteins, it is conceivable that at least two messages are requires for their synthesis, though recent cDNA cloning (9) and *in vitro* translation (16) data suggest that a single mRNA codes for both A- and B-receptors by use of alternate translation start sites.

Quantitation of mRNAs and Their Regulation by Estrogen

$T47D_{co}$ cells are exceptionally PR rich and their receptors are not estrogen regulated (14). This is in contrast to other progesterone target cells, including some human breast cancer cells of which MCF-7 cells are the prototype, in which PR are low but are estrogen inducible. Even after estrogen treatment, the PR protein levels in MCF-7 cells are below the levels found in T47D cells (1). In Fig. 3 we compare the levels of the hPR mRNAs in $T47D_{co}$, in MCF-7 cells before and after treatment for 5 days with 10 nM estradiol, in PR-negative MDA-231 human breast cancer cells, and in PR-negative HeLa human cervical carcinoma cells. Untreated MCF-7 cells contain the same six major message species but at ~18% of the level in $T47D_{co}$ cells, as quantified by scanning densitometry; the levels of all six increase 2- to 3-fold with estrogen treatment. No hPR-50 hybridizable bands were detectable in either of the two PR-negative cell lines. These data suggest that all six messages are PR specific since only PR, among the steroid receptors, are up-regulated by estradiol.

The exceptionally high PR levels in T47D cells may be due in part to unusual message abundance. To quantify PR mRNA levels accurately, we constructed a standard curve by slot-blot hybridization using defined amounts of PR-specific RNA synthesized *in vitro* from pGEM-4 containing the hPR-50 insert, using SP6 polymerase. The inset in Fig. 4 shows the band intensities of increasing concentrations (7.8–500 pg) of hPR-50

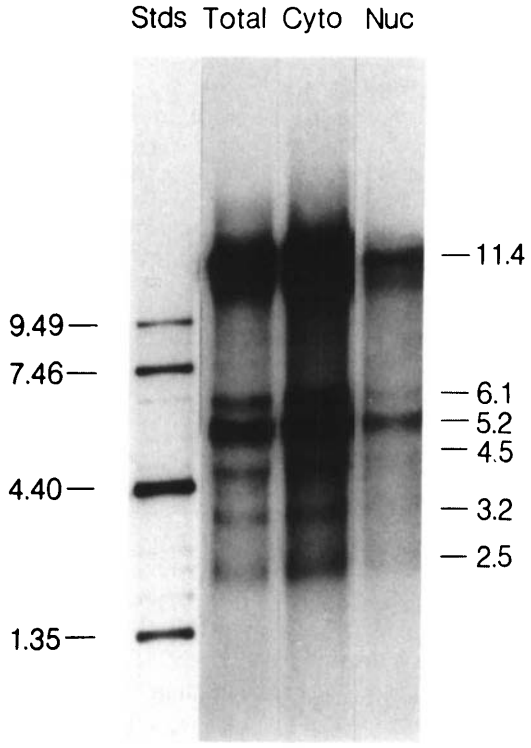

Fig. 2. Cellular Compartmentalization of the Six PR Messages

$T47D_{co}$ cells were homogenized and the low speed cytoplasm was resuspended in 10 vol guanidine thiocyanate. The crude nuclear pellet was further purified by rehomogenization in detergent-containing buffer, then washed, and the pellet was solubilized in 10 vol guanidine thiocyanate. Total RNA was isolated from intact $T47D_{co}$ cells. Poly(A^+) RNA was purified from total, cytoplasmic (Cyto), and nuclear (Nuc) compartments, and 7.5 μg were separated by electrophoresis on 0.8% agarose-6% formaldehyde gels. The RNA was transferred to nitrocellulose and hybridized as described in Fig. 1. An RNA ladder was used for size standards.

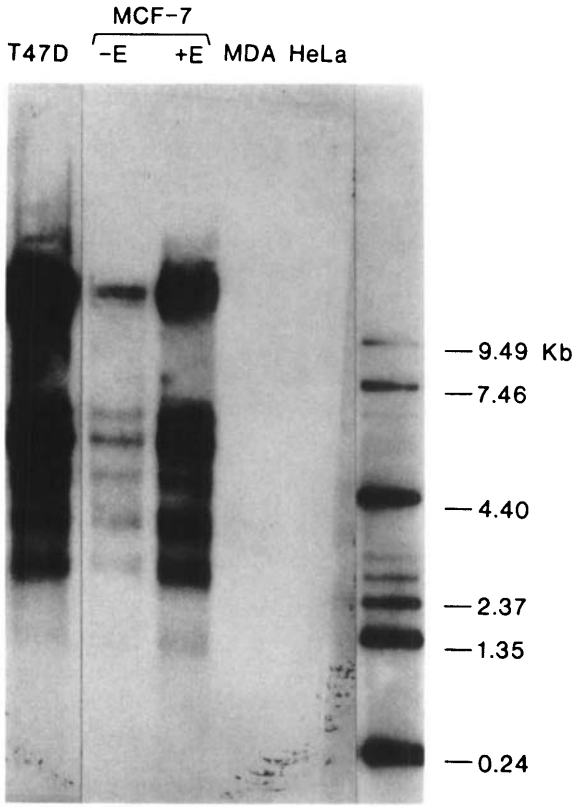

Fig. 3. Northern Analysis of hPR mRNA in Several Cell Lines

Total cellular poly(A^+) RNA (7.5 μg) from $T47D_{co}$ cells, from MCF-7 cells treated 5 days with (+E) or without (−E) 10 nM 17β-estradiol, from MDA-231 cells, and from HeLa cells, was separated by electrophoresis on 0.8% agarose-6% formaldehyde gels. The RNA was transferred to nitrocellulose and hybridized with nick-translated hPR-50 (~1.96×10^9 cpm/μg DNA). In the far right lane 0.6 μg RNA ladder was used for size standards.

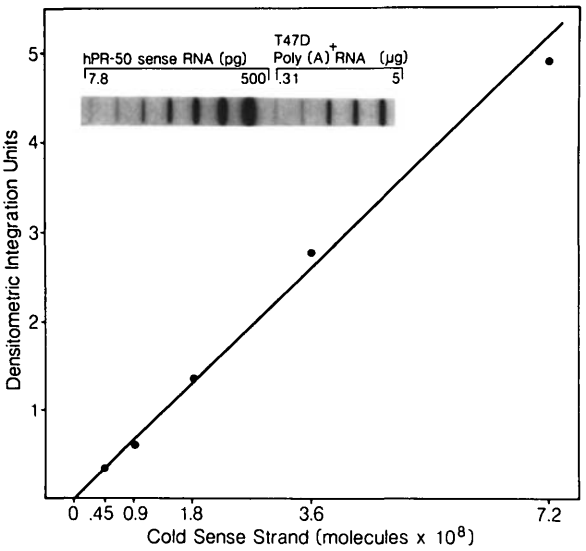

Fig. 4. Quantitation of hPR mRNAs in T47D$_{co}$ Cells by Slot Blot

Duplicate samples of total cellular poly(A$^+$) RNA from T47D$_{co}$ cells (0.31–5 μg) were applied by vacuum filtration onto a nitrocellulose sheet held in a slot blot apparatus. As a standard, duplicate 7.8- to 500-pg samples of unlabeled *in vitro* synthesized sense strand RNA were also applied. RNA samples were denatured with formaldehyde, baked, and then hybridized to nick-translated hPR-50 cDNA (1 × 10^9 cpm/μg DNA). The blot was washed and used to expose x-ray film. One set of duplicate samples is shown in the *inset*. The autoradiogram was analyzed densitometrically and the sense strand RNA signals used to calculate the standard curves. At 7.5 and 15.6 pg sense strand RNA, the signal was below the limits of sensitivity for accurate detection by the densitometer. The ordinate is expressed as arbitrary densitometric integration units and the abscissa as molecules of sense strand RNA. Conversion of RNA mass to number of molecules is described in *Materials and Methods*.

sense strand RNA hybridized to nick-translated hPR-50 cDNA. Densitometric quantitation was performed from duplicate experiments and the number of RNA molecules (×10^8) was calculated. As shown in Fig. 4, there is a linear relationship between increasing amounts of synthetic PR mRNA and arbitrary densitometric integration units. (RNA levels less than 15 pg were below the sensitivity of the densitometer.) On the same slot blot varying amounts (0.31 to 5 μg) of poly(A$^+$)RNA from T47D$_{co}$ cells were hybridized to labeled hPR-50 (Fig. 4, *inset*) and the number of PR mRNA molecules (×10^8) in the total mRNA pool was determined from the standard curve after densitometry. (For calculation details, see *Materials and Methods*.) We calculate that T47D$_{co}$ cells contain approximately 90 PR messages per cell and untreated MCF-7 cells contain ~16 messages per cell (not shown). This difference in message number could in part explain the high PR levels of T47D$_{co}$ cells, though it does not explain their estrogen and antiestrogen resistance (14).

Progestin Regulation of PR Protein and Message Levels

Although the PR in T47D$_{co}$ cells are estrogen insensitive, we have previously shown by steroid binding assays, by photoaffinity labeling, and by immunoblotting that their levels can be down-regulated by progestins (14, 15, 17, 18). This down-regulation or processing of receptors is not due to a loss of hormone binding affinity or capacity, or an inability to exchange unlabeled hormone for labeled hormone, but is due to a loss of absolute receptor protein mass (15). To determine the mechanisms by which progestins autoregulate their receptors, we undertook a quantitative comparison of PR mRNA and PR protein levels after progestin treatment. T47D$_{co}$ cells were incubated either with progesterone (Fig. 5) or with the synthetic progestin R5020 (see Fig. 7) for 30 min to 48 h. One set of cells was homogenized and their A- and B-receptor protein levels were assayed in cytosols and in 0.4M salt extracts of nuclei by immunoblotting. The antibody used was AB-52, one of our anti-PR MAbs that recognizes both A and B receptors (11). The immunoblots were scanned densitometrically to assess relative amounts of cytosolic and nuclear PR. Simultaneously, poly(A$^+$) RNA was purified from a parallel set of T47D$_{co}$ cells and analyzed for PR mRNA levels by hybridization to hPR-50. To normalize for variabilities in poly(A$^+$)RNA isolation, gel loading, or transfer efficiency, each blot was stripped and reprobed with a cDNA for human β-actin, whose mRNA is not affected by hormonal status. Northern blot data are expressed as the ratio of PR mRNA/β-actin mRNA determined by densitometric scanning. All hormone treatments and analyses were done in duplicate and the data from two or three experiments were combined to obtain RNA levels (see Figs. 5 and 7).

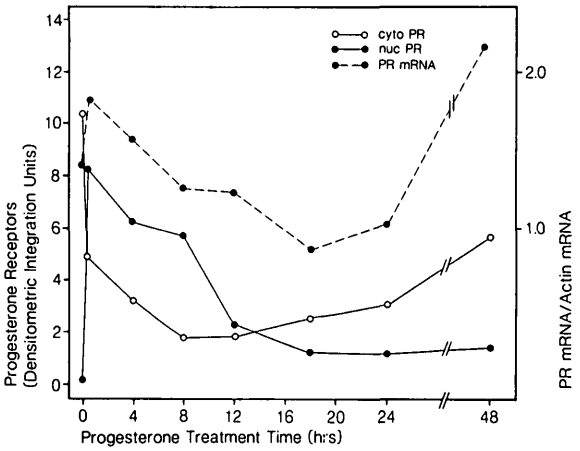

Fig. 5. Changes in hPR mRNA and hPR Protein during 48 h of Progesterone Treatment

Total cellular poly(A$^+$) RNA was isolated from T47D$_{co}$ cells treated 0–48 h with a single pulse of 80 nM progesterone, and 7.5 μg/time point were analyzed by Northern blotting using the hPR-50 cDNA probe. The autoradiogram was scanned densitometrically and the sum of the signals from all six mRNA bands was expressed as arbitrary densitometric integration units. RNA values normalized to β-actin are shown by (– – –) and are the mean of three experiments done in duplicate. Cells from parallel progesterone-treated flasks were homogenized and immunoblots of A- and B-receptors, isolated from cytosols (○) and nuclei (●), were analyzed densitometrically. Nuclear and cytosol receptor levels are the mean of three Western blots.

Figure 5 shows the typical changes in hPR levels that occur after progesterone treatment; there is a rapid increase in chromatin-associated nuclear receptors and a corresponding decrease in soluble receptors. After the initial rise, nuclear PR steadily decrease and remain low, while soluble cytosolic receptors are gradually restored. Total receptor protein levels, which are the sum of receptors in the two compartments, fall to approximately 50% of control at 18 h before replenishment occurs. Replenishment rates are somewhat variable; receptors are usually restored to control levels by 48 h (19) but are incompletely restored in this study. The changes in PR mRNAs after progesterone treatment parallel the protein changes, first steadily decreasing between 30 min and 18 h, then returning to control values. A typical Northern blot is shown in Fig. 6 and demonstrates that all 6 PR message bands decrease synchronously during down-regulation, then increase during replenishment. The maintenance of their relative abundance suggests that the large species are not processing intermediates of the smaller ones.

A similar study is shown in Fig. 7 except that the synthetic progestin R5020, rather than progesterone, was used to treat the cells. Within 30 min of R5020 addition, receptors transform and bind tightly to chromatin with a parallel decrease in soluble cytosolic receptors. Thereafter there is a continuous loss of nuclear PR but, unlike progesterone-treated cells, this is not

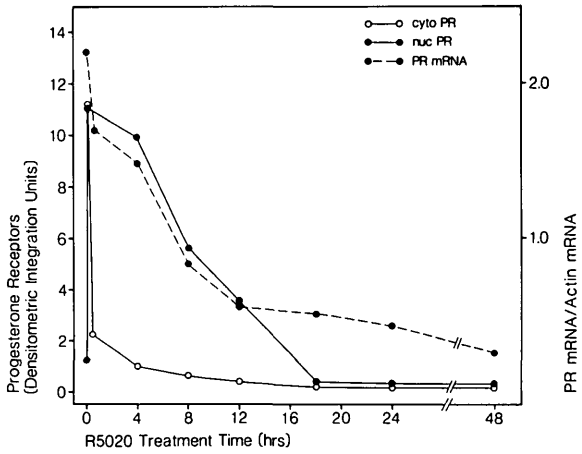

Fig. 7. Changes in hPR mRNA and hPR Protein during 48 h of R5020 Treatment

Total cellular poly(A$^+$) RNA was isolated from T47D$_{co}$ cells treated 0–48 h with 80 nM R5020 and 7.5 μg/timepoint was analyzed by Northern blotting using the hPR-50 cDNA probe. The autoradiogram was scanned densitometrically and the data expressed as densitometric integration units. RNA values normalized to β-actin are shown by (– – –) and are the mean of three experiments. Cells from parallel R5020-treated flasks were homogenized, and immunoblots of A- and B-receptors, isolated from both cytosols (O) and nuclei (●), were analyzed densitometrically. Nuclear and cytosol receptor levels are the mean of two Western blots.

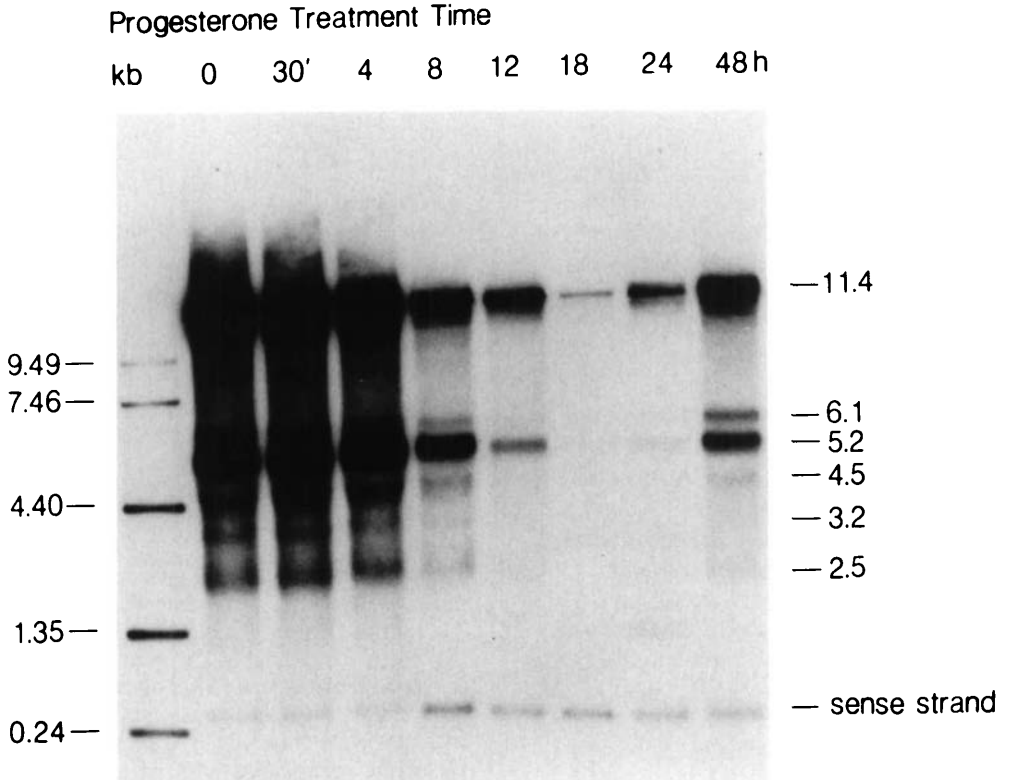

Fig. 6. Synchronous Down-Regulation of All hPR Messages after Progesterone Treatment

Total cellular poly(A$^+$) RNA was isolated from T47D$_{co}$ cells treated 0–48 h with 80 nM progesterone, and 7.5 μg/time point were denatured and separated by electrophoresis on 0.8% agarose-6% formaldehyde gels. The RNA was transferred to nitrocellulose, hybridized with nick-translated hPR-50 (~1.96 × 10^9 cpm/μg DNA), and the blot was exposed to film. RNA standards are shown on the *right*.

accompanied by cytoplasmic replenishment. Thus there is a profound loss of total cellular receptors. PR mRNA levels also fall continuously for 48 h after R5020 treatment and, like the proteins, are not restored in this time period. Generally, when $T47D_{co}$ cells are treated with progestational agonists, PR mRNA levels parallel total PR levels. Message levels fall during progesterone or R5020 mediated receptor down-regulation; they are restored after progesterone treatment but are chronically suppressed by R5020 treatment.

To compare the effects of the two agonists with those of an antagonist, we next incubated $T47D_{co}$ cells for 30 min to 48 h with the antiprogestin RU 486. This agent has high affinity for PR and drives PR to the tight chromatin binding state, but it has mixed agonist effects when given alone (20), and it antagonizes the actions of R5020 when the two hormones are given together (21). After treatment of $T47D_{co}$ cells with RU 486, total cellular PR were analyzed by immunoblotting, and mRNAs were quantified (Fig. 8). We find that unlike the case with the agonists, with the antagonist, protein and message levels do not change in parallel. As with the agonists, RU 486 binds unoccupied PR and transforms them to the tight chromatin-binding state. However, unlike the subsequent down-regulation that occurs when progesterone or R5020 is the ligand, when RU 486 is the ligand, there is no absolute protein loss; receptors remain elevated in nuclei for as long as 48 h. Surprisingly, despite the elevated protein levels, message levels do decrease before returning to control. Thus, there appears to be a transient agonist-like suppression of PR mRNA levels which cannot, however, be chronically sustained by the chromatin-bound receptors; this failure may be related to their antagonist actions.

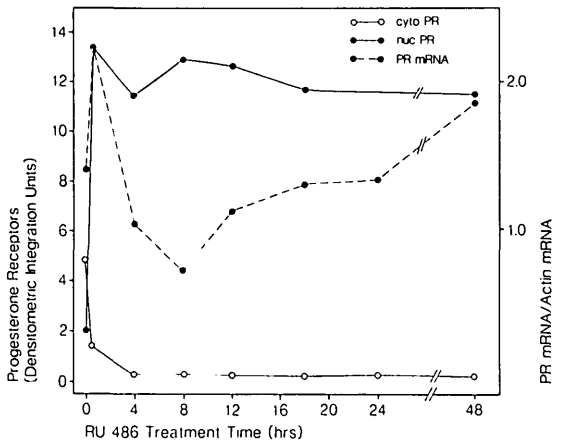

Fig. 8. Effects of RU 486 on hPR mRNA and hPR Protein Levels

Total cellular poly(A^+) RNA was isolated from $T47D_{co}$ cells treated 0–48 h with 80 nM RU 486, and 7.5 μg/time point were analyzed by Northern blotting using the hPR-50 cDNA probe. The autoradiogram was scanned densitometrically and the data expressed as densitometric integration units. RNA values normalized to β-actin are shown by (– – –) and are the mean of four experiments. Cells from parallel RU 486 treated flasks were homogenized, and immunoblots of A- and B-receptors, isolated from both cytosols (O) and nuclei (●), were analyzed densitometrically. Nuclear and cytosol receptor levels are the mean of two Western blots.

PR Message Turn-Over Rates

All three progestins led to an initial decrease in the levels of PR messages. The rate varied, having a half-life of approximately 7 h after RU 486 and R5020, and approximately 14 h after progesterone. To see whether we could assign a transcriptional or post-transcriptional mechanism to this mRNA loss we estimated the average rate of PR message decay in untreated cells in which *de novo* transcription was blocked 2–12 h with 5 μg/ml actinomycin D, a concentration that preferentially inhibits RNA polymerase II directed RNA synthesis in these cells (22). Poly(A^+)RNA was purified from 1 mg total RNA and its absolute amount was estimated spectrophotometrically. The levels of PR mRNAs were then analyzed by Northern and/or slot blotting and quantitated densitometrically. The results are show in in Fig. 9. The total cellular RNA per flask remained constant despite actinomycin D treatment (not shown) as has been previously reported (22). However, the mRNA fraction of the total RNA pool decreased with a biphasic decay rate: one population of messages had a half-life of 3–4 h, and a second more stable population had a half-life of 10–12 h. The existence of two pools of mRNAs with different half-lives has also been documented (23). The rate of loss of specific PR mRNAs appears to parallel the decay rate of the slow

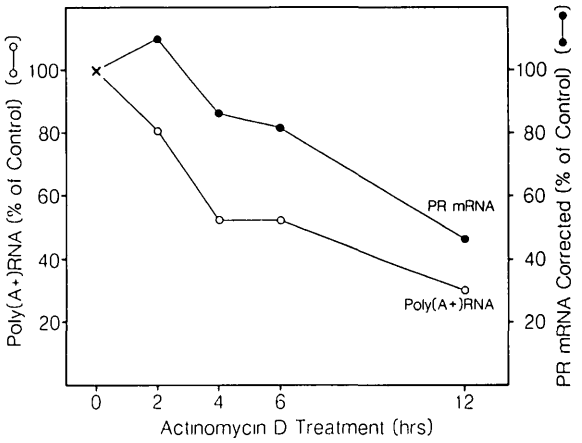

Fig. 9. The Decay Rate of hPR mRNAs and Total Messages after Actinomycin D Treatment

$T47D_{co}$ cells were treated with actinomycin D (5 μg/ml) for 0–12 h and total cellular poly(A^+) RNA was isolated from 1 mg total RNA/timepoint. Poly(A^+) RNA (4 μg) was analyzed by slot blot, and by Northern analysis (7.5 μg) using the hPR-50 cDNA probe. Autoradiograms were scanned densitometrically. The total poly(A^+) RNA isolated from 1 mg total RNA from control and actinomycin D-treated cells per time point is expressed as a percent of poly(A^+) RNA purified from control $T47D_{co}$ cells (100%). PR mRNA levels were normalized to the concentration of poly(A^+) RNA isolated from total RNA per treatment point and data are expressed as a percent of PR mRNA in untreated cells. Each *point* for PR mRNA represents the mean of two experiments done in duplicate. Each point for total poly(A^+) RNA is the mean of two experiments.

poly(A$^+$)RNA pool, with a half-life of approximately 12 h. It appears then that the natural PR mRNA decay rate is similar to the rate of PR mRNA decrease after progesterone treatment (Fig. 5), but that this rate is accelerated after treatment with the synthetic progestins (Figs. 7 and 8). This suggests that progestins down-regulate PR messages through transcriptional inhibition but that an effect on message stability by the synthetic progestins may also be involved.

DISCUSSION

Message Size and PR Synthesis

What is the origin and function of these multiple hPR mRNAs? They are clearly not restricted to man, since multiple PR messages have been detected in tissues from three species. In chick oviducts, there are at least three messages ranging in size from 3.9–4.5 kb (9); in the rabbit uterus two messages of 5.9 and 6.6 kb are seen (10); in human cells five mRNAs of 2.9–5.9 kb have been reported (12) and we find an additional triplet at 11.4 kb. The 6–8 hPR messages we describe here are all mature cytoplasmic mRNAs (Fig. 2), are not mammary gland specific, and are found in normal and malignant cells (Fig. 1). The largest 11.4-kb messages are the most abundant in breast tumor cell lines (Fig. 3) though this may not be the case for normal human tissues (Fig. 1). That all of the mRNA species code for hPR or closely related proteins is suggested by several indirect observations including 1) their estrogen inducibility, 2) their down-regulation by progestins, 3) their absence in PR-negative cells that contain other steroid receptors, 4) their size in comparison to messages for other steroid receptors, and 5) their hybridization to PR-specific probes under stringent conditions.

With the exception of ER for which only one mRNA has been detected (24, 25), more than one message encodes other steroid receptors. There are two glucocorticoid receptor mRNAs of 4.8 and 6.5 kb in the rat (26) and three (5.6 to 7.1 kb) in man (27). Sequence analysis of human GR cDNAs suggests that two of the messages code for two proteins that differ at their carboxy termini and result from alternate precursor RNA splicing, while the third results from alternate poly(A$^+$) site selection (27). Two messages have been identified for the vitamin D receptors (28), and at least four for the placental thyroid hormone receptors encoded by the *c-erb A* genes (29). Since some of these receptors are believed to be products of single copy genes (15a), mRNA heterogeneity has usually been ascribed to polyadenylation at different sites, but this has not always been proved, and large variations in length are uncommon. Examples of multiple messages arising from differences at the 5′-terminus are also described (30, 31). Whether all, some, or only one of the PR mRNAs are translationally competent is unknown. The existence of multiple polyadenylation sites has been characterized in other systems including rat insulin-like growth factor II messages (32) and mouse dihydrofolate reductase messages (33, 34). Only one of multiple insulin-like growth factor II messages, and that not the most abundant, was found to be translationally competent in an *in vitro* system (35), whereas all four mouse dihydrofolate reductase messages were translatable *in vitro* and produced an identical sized protein (33).

For hPR, the existence of two hormone binding forms adds another level of complexity not found in other steroid receptors. Are B- and A-receptors the products of two messages or one? Two in-frame AUG codons, satisfying the Kozak consensus sequence for translational initiation (36), are present 495 bases apart in the rabbit and human PR mRNA deduced from partial cDNA clones (10, 12). These AUGs could initiate translation of two homologous proteins with the small protein truncated at the N-terminus by approximately 20,000 daltons. We have preliminary evidence for such a mechanism for chick and human A- and B-protein synthesis (unpublished). The largest open reading frame in the hPR cDNA contains 2799 nucleotides (12). If it codes for the B protein, and if A is initiated at its downstream AUG, then the 2.5-kb band we see on Northern blots could not encode protein A and is either untranslated or codes for a C-terminal truncated protein lacking a complete hormone binding domain. Besides the well characterized A- and B-receptors, we find by photoaffinity labeling and by immunoblotting, that the B-receptors are themselves microheterogenous proteins consisting of two or three isoforms. These probably do not arise from multiple phosphorylation events since they are uniformly labeled with [^{32}P]orthophosphate; we must therefore look to other mechanisms for the origin of these size variants, including multiple messages. Clearly additional studies are needed to understand the structure and synthesis of hPR and their mRNAs.

Hormonal Regulation of PR Messages

We conclude from the study shown in Fig. 3 that estradiol induces PR synthesis in MCF-7 cells (37) and by analogy, in other estrogen target cells, by increasing the number of hPR messages. Whether this is a transcriptional or posttranscriptional effect is not known since estrogens can operate through both mechanisms to regulate protein levels (38). The failure of estradiol to regulate PR in T47D$_{co}$ cells remains unexplained, however. Occult estrogens do not account for the high PR message levels since T47D$_{co}$ cells do not synthesize estradiol (our unpublished data), nor does removal of phenol red (unpublished) or addition of antiestrogens (14) alter PR levels. We have preliminary data from DNA blot hybridization and cytogenetic analysis that a duplication of chromosome 11 in T47D$_{co}$ may have increased the PR gene copy number (39), accounting in part for the high PR message levels in these cells. This duplication does not explain the anomalous estrogen independence of hPR protein and message.

The mechanisms by which progestins autoregulate PR may also turn out to be complex. For the two agonists we tested, changes in hPR mRNA levels par-

alleled the PR proteins. Progesterone treatment resulted in transient PR down-regulation followed by replenishment, and hPR mRNA levels also decreased and then increased (Figs. 5 and 6). R5020 treatment led to chronic PR down-regulation, which was mirrored by PR mRNA levels (Fig. 7). Based on these direct relationships between messages and protein, and the rate of message decay, we propose that hormone-occupied hPRs autoregulate their levels by binding to, and inhibiting transcription of the PR gene. Using the method of dense amino acid labeling it has recently been reported that R5020 increases the rate of receptor protein turnover compared to that of unoccupied receptors (40). Taken together the protein and mRNA data suggest that the negative feedback of progestins on their own receptors occurs on at least two levels: 1) by decreasing the half-life of occupied receptors leading to their more rapid decay, and 2) by decreasing the rate of PR gene transcription, preventing synthesis of additional receptors. The rapid down-regulation or processing of nuclear PR must be physiologically desirable as a means of terminating the actions of the hormone-occupied receptors on regulatable genes. Because progesterone is promptly metabolized in cells ($t_{½} \sim 4$ h), and R5020 is not (19), unoccupied receptors are restored more rapidly after progesterone treatment, thereby reversing the transcriptional inhibition. The rapid metabolism of progesterone, which results in regeneration of unoccupied receptors, may also explain the lesser effect of progesterone on mRNA decay rates as compared to R5020 and RU 486, which are not metabolized and chronically occupy their receptors.

Hormone-induced autologous receptor down-regulation is a common property of most steroid hormones. Estrogen receptors and GR are also down-regulated after homologous hormone treatment (41–44), and the protein loss is associated with a reduction in message levels. Diethylstilbestrol, an estrogen analog, decreases ER mRNA in chick oviducts 8–24 h after the start of treatment (43); dexamethasone, after a transitory increase, decreases GR mRNA for 18–48 h (44). An exception is the vitamin D_3 receptors, where hormone treatment leads to an increase in protein and message levels in certain species (28). It has been speculated that the down-regulation of GR mRNA by glucocorticoids is due to a direct interaction of the receptors with their own gene, since *in vitro* DNase I protection studies show three potential binding sites for GR in the 3′-noncoding region of a GR cDNA (44). Whether these intragenic GR binding domains are functional and regulate GR mRNA synthesis remains to be determined.

What about receptors occupied by an antagonist? $T47D_{co}$ cells contain undetectable levels of GR as measured by standard steroid binding assays and as shown by introduction into $T47D_{co}$ cells of a plasmid containing mouse mammary tumor virus-long terminal repeat linked to chloramphenicol acetyl transferase. Such transfectants do not respond to dexamethasone with increased chloramphenicol acetyl transferase activity (45). Thus, the antiprogestational properties of RU 486 can be studied in $T47D_{co}$ cells without interference from its antiglucocorticoid actions. We find that, like progesterone and R5020, RU 486 leads to an initial decrease in PR mRNA levels, which are then quickly restored. However, this initial decline in PR mRNA is not coupled to receptor processing. Our observations with PR proteins resemble the effects of RU 486 on GR where, compared to dexamethasone, it fails to down-regulate and shorten receptor half-life (46). The discrepancy between the levels of PR proteins and PR messages in RU 486-treated $T47D_{co}$ cells is interesting. It has been speculated that the antagonistic actions of RU 486 result from its inability to effect proper conformational changes in the receptors necessary to bring about their full biological activity (47, 48). Our results suggest that the interaction of RU 486 with PR causes a conformational change which is transiently effective for the interaction of receptors with the regulatory regions of the PR gene and leads to the initial decline in message levels, but that the antagonist-bound receptors are unable to bring about a sustained effective interaction, so that transcription resumes despite continued occupancy of the gene by the receptors.

MATERIALS AND METHODS

Cell Culture

$T47D_{co}$, described by Horwitz *et al.* (14), are human breast cancer cells in permanent culture that are exceptionally PR rich. HeLa cervicocarcinoma cells and MDA-231 human breast cancer cells are PR negative. They were obtained from D. Edwards (Denver, CO). Cells were plated in Falcon (Becton-Dickinson, Oxnard, CA) plastic flasks (175 cm^2) and grown in humidified 5% CO_2 and air at 37 C. The growth medium consisted of minimum essential medium (Earle's basal salts) supplemented with 0.1 mM nonessential amino acids, 2 mM L-glutamine, 5% heat-inactivated (30 min; 56 C) fetal calf serum, 25 μg/ml gentamycin sulfate, and 6 ng/ml bovine insulin. Cell culture reagents were purchased from Grand Island Biological Co. (Grand Island, NY). MCF-7, another human breast cancer cell line which has low but estrogen-inducible PR (37), were grown in the same medium as $T47D_{co}$ cells except that 5% charcoal-stripped fetal calf serum was used as previously described (14). To induce PR, MCF-7 cells were treated for 5 days with 10 nM 17β-estradiol dissolved in ethanol (0.1% final concentration), or with ethanol alone. For time-course studies, progesterone, R5020 (New England Nuclear, Boston, MA) or RU 486 (a gift from Roussel Uclaf, Romainville, France) were used at 80 nM final concentration, after which cells were harvested and PR protein and message levels were assayed as described below.

Complementary DNA Probes

Two human PR cDNA clones hPR-50 and hPR-54 were isolated from a T47D-pcD library containing 2×10^6 recombinants. The library was screened with a 3.3-kb chicken PR probe (CPR 19), containing the highly conserved C1 and C2 regions (9). Human PR-50 consists of 1075 bp, of which 960 bp represent C-terminal protein coding sequences and the remainder are 3′-untranslated region. Its authenticity was verified by DNA sequence analysis (O'Malley, B. W., unpublished data) and by its homology to chicken PR cDNA. The translated segment of hPR-50 cDNA contains the 185-bp C2 domain, which has 98% homology to the corresponding region in the chicken PR cDNA. An additional sequence located at the extreme C-terminal coding region is 90% homologous with

chicken PR. Human PR-50 was subcloned into pGEM-4 (Promega Biotec, Madison, WI). Human PR-54 includes the entire coding region except approximately 200 bp from the 5′-end and was subcloned into pGEM-3. A human cDNA probe specific for β-actin containing bases 133 to 537 of the 3′-untranslated region inserted into pHP34 was purchased from L. Kedes (Stanford, Palo Alto, CA) (49). Complementary DNA probes were nick translated using a commercial kit (Bethesda Research Laboratories, Gaithersburg, MD) and [^{32}P]dCTP] (~3200 Ci/mmol; ICN, Irvine, CA). The specific activity of the probes was typically 1×10^9 cpm/μg DNA. For use as a standard on slot blots and as a hybridization control, unlabeled RNA was synthesized *in vitro* from the hPR-50 insert-containing pGEM-4 plasmids, using SP-6 polymerase (Promega Biotec).

Northern Analysis and RNA Slot Blots

Total RNA was isolated from cell pellets by lysis in 4 M guanidine thiocyanate (Ultrapure; Boehringer Mannheim Biochemicals; Indianapolis, IN) according to the procedure of Chirgwin *et al.* (50). RNA was separated from DNA and protein by sedimentation through 3.5 ml 5.7 M cesium chloride (Ultrapure; International Biotechnologies Inc., New Haven, CT). Centrifugation was at 200,000 × *g* for 5 h at room temperature using a Beckman 70.1Ti rotor. The RNA pellet was dissolved in ETS (10 mM EDTA, 10 mM Tris-HCl, 0.2% sodium dodecyl sulfate (SDS), pH 7.5) and after addition of 1/10 vol 3 M sodium acetate, pH 5.2, it was precipitated at −20 C with 2.5 vol absolute ethanol. Normal endometrial tissues (~1.5 cm^3) were lysed in guanidine thiocyanate and solubilized with 5 × 10-sec bursts of a polytron at a medium setting. After sedimentation through a cesium chloride cushion, RNA samples were dissolved in ETS and frozen at −20 C. Poly(A$^+$) RNA was separated from total RNA by affinity chromatography on oligo(dT)-cellulose (type 7; Pharmacia; Piscataway, NJ) under high salt conditions (0.4 M NaCl in ETS), and mRNAs were eluted with ETS at 65 C. For Northern analysis, aliquots of poly(A$^+$) RNA (5–10 μg) were denatured with formaldehyde at 65 C for 5 min, separated by electrophoresis on 0.8% agarose gels containing 6.0% formaldehyde (51), and transferred to nitrocellulose (52). RNA molecular weight standards (0.6 μg), purchased from Bethesda Research Laboratories, were run in a parallel lane; the ladder consists of six species of 9.49, 7.46, 4.40, 2.37, 1.37, and 0.24 kb in size. The nitrocellulose blots were prehybridized for approximately 16 h at 42 C in 50% (vol/vol) formamide, containing 5× SSC (0.75 M NaCl, 75 mM Na citrate, pH 7.0), 20 mM HEPES, pH 7.5, 500 μg/ml denatured salmon sperm DNA, and 0.02% (wt/vol) each of BSA, Ficoll, and polyvinylpyrrolidone, and then hybridized for 48 h at 42 C with approximately 8×10^6 cpm/ml nick-translated hPR-50 cDNA insert. After hybridization, filters were washed three times for 5 min at room temperature in 2× SSC, 0.1% SDS, then three times for 5 min at 50 C in 0.2× SSC, 0.1% SDS, and air dried. For autoradiography the blot was exposed to Kodak XAR-5 film held in a metal cassette with two Dupont Intensifying Screens at −70 C for 1–3 days. For slot blots, poly(A$^+$) RNA was denatured with formaldehyde according to White and Bancroft (53) and applied to nitrocellulose using a commercial apparatus (Minifold II; Schleicher & Schuell; Keene, NH). The filter was dried, baked under vacuum for 2 h at 78 C, prehybridized, and hybridized with the probe as described above. Northerns and slot blots were later stripped of probe according to Thomas (52) and reprobed with β-actin cDNA to normalize all message levels to those of β-actin. Autoradiograms of Northern and slot blots were analyzed densitometrically using an LKB (Gaithersburg, MD) Ultro Scan 2202 coupled to a Varicam VISTA 401 computer. The area under the curves was calculated with an integrator program. The number of PR messages per cell was calculated from the standard curve generated using unlabeled sense strand RNA synthesized *in vitro*. To correct for the differences in message size, the number of molecules determined from the standard curve was multiplied by the average size of PR mRNAs based on their relative abundance. This number was normalized to the amount of poly(A$^+$) RNA and total RNA isolated from a known number of cells.

Cytoplasmic and Nuclear RNA Fractionation

T47D$_{co}$ cells were differentially homogenized in a glass-teflon homogenizer with either 5, 10, 20, or 40 strokes in 10 mM Tris, pH 7.4, 1 mM $MgCl_2$. The crude nuclei were sedimented at 3000 × *g* for 15 min and the cytoplasm was removed and mixed with 10 volumes of guanidine thiocyanate and 1/20 vol β-mercaptoethanol. One milliliter of 10 mM Tris, pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, 1% Tween-40, 0.5% sodium deoxycholate was added to each crude nuclear pellet, and after further homogenization (54, 55), nuclei were pelleted and washed. Microscopic examination showed that they were 90–95% free of cytoplasmic tags. Nuclei were then immediately dissolved in guanidine thiocyanate and β-mercaptoethanol, as described above.

Immunoprecipitation

Progesterone receptors were concentrated by a two-stage immunoprecipitation procedure in order to lower backgrounds. First, cytosols or nuclear extracts (1 ml) prepared as previously described (14) were mixed with 20 μg/ml (final concentration) of a MAb specific to ovalbumin (56) and incubated at 4 C for 2 h. In a separate reaction, precoated Protein-A Sepharose was prepared by mixing well washed Protein-A Sepharose in a 1:1 ratio with TEDG (10 mM Tris, pH 7.4, 1 mM EDTA containing 1 mM dithiothreitol, and 10% glycerol). This slurry was incubated for 1 h at 4 C with rabbit antimouse immunoglobulin G (0.3 mg/ml final concentration) using end-over-end rotation. After three additional washes in TEDG the slurry was diluted with TEDG (1:2) and stored at 4 C. One milliliter of the antiovalbumin MAb-protein complex was mixed with the pellet from 1 ml precoated Protein-A Sepharose slurry and incubated 2 h at 4 C with end-over-end rotation. The resin was then sedimented (5 min, 1000 × *g*) and the receptor-containing supernatant was aspirated and incubated at 4 C as described above with 20 μg/ml anti-hPR specific antibody AB-52. This Mab was generated as previously described (11). After overnight incubation receptor antibody complexes were mixed with the pellet from 1 ml precoated Protein-A Sepharose and incubated for 2 h at 4 C as described. After centrifugation (5 min, 1000 × *g*), the supernatant was removed, and the pellet was washed three times with TEDG + 20 mM sodium molybdate (M), three times with TEDG + M + 0.3 M KCl, three times with TEDG + M + 0.2% Nonidet P-40, and finally with TEDG + M. The pellet was then mixed with 50 μl TEDG + M, 100 μl 10% SDS, 0.1 M dithiothreitol, and 0.02% bromphenol blue in 50% glycerol), boiled 5 min, centrifuged (3000 × *g*, 1 min), and the supernatants were subjected to polyacrylamide gel electrophoresis immediately or stored frozen for later use.

Gel Electrophoresis

One hundred to 150-μl sample of immunoaffinity purified receptors were analyzed by electrophoresis on discontinuous 1 mm-polyacrylamide slab gels (model 220, Bio-Rad Laboratories, Richmond, CA). Resolving gels consisted of either linear 7.5–19% acrylamide linear gradients or 7.5% gels as described by Laemmli (57) and 0.08–0.2% *N,N′*-methylene-*bis*-acrylamide (0.3% for 7.5% gels); stacking gels contained 3% acrylamide and 0.08% *N,N′*-methylene-*bis*-acrylamide. Gels were run for 4 h at 40 mA/gel or until the dye front reached the end of the gel. ^{14}C-Labeled molecular weight standards and prestained standards which were run in parallel, were purchased from Bethesda Research Laboratories and had sizes of: myosin, 200,000; phosphorylase B, 97,400; BSA, 68,000; ovalbumin, 43,000; chymotrypsinogen, 25,700; β-lactoglobulin, 18,400; and cytochrome *c*, 12,300 daltons, respectively.

Immunoblotting

After electrophoresis, gels were incubated 30 min at room temperature in 20 mM sodium phosphate, 20% methanol, 0.05% SDS, pH 6.5, then transferred to nitrocellulose filters (Schleicher & Schuell, 0.45 μm, Research Products International, Inc., Prospect, IL) using a Transphor apparatus (Hoeffer Scientific, San Francisco, CA) operating at 0.4 A for 5 h (58). Prestained molecular weight markers (Bethesda Research Laboratories) were cotransferred to the filters. After transfer, nonspecific sites on the nitrocellulose membrane were blocked by a 1- to 3-h incubation at room temperature in wash buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, 30 mM NaCl, 0.5% Triton X-100, 0.25% gelatin) containing 3% BSA. The filter was incubated at 4 C overnight with 10 μg/ml AB-52 in wash buffer containing 1% BSA, then washed three times for 10 min with wash buffer using a rocker platform at room temperature. The filter was then incubated at room temperature for 3 h with a 1:1000 dilution of horseradish peroxidase-conjugated goat antimouse immunoglobulin G (heavy and light chain specific, Cappel, Malvern, PA). Unbound antibody was removed with wash buffer, followed by three washes in substrate buffer (50 mM HEPES, 150 mM NaCl, pH 7.4), and immunoreactive bands were visualized by incubation with substrate-buffer containing 0.5 mg/ml 4-chloro-1-naphthol in 15% (vol/vol) methanol; 0.025% (vol/vol) H_2O_2. After achieving the desired band intensity the filter was washed with distilled water and photographed.

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