High Level Expression of a Truncated Chicken Progesterone Receptor in Escherichia coli*

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Steroid hormones exert their biological effect on eukaryotic cells via specific intracellular high affinity receptors (for reviews see Yamamoto, 1985; Beato, 1989). We have been studying the structure-function relationship of the chicken progesterone receptor A (Mr = 72,000) and B (Mr = 86,000). Previous work from this laboratory has shown that both A and B forms of the receptor are derived by alternate initiation of translation from two in-frame start codons located on a single mRNA transcript (Conneely et al., 1987).

Extensive structure-function analysis of the chicken progesterone receptor (cPR) by deletion studies and site-directed mutagenesis has helped to dissect and define three major functional domains in the receptor molecule (Carson et al., 1987; Dobson et al., 1989). These consist of a variable amino-terminal domain that is thought to exert a modulatory effect on transactivation of target genes; a short cysteine-rich central DNA-binding domain that is highly conserved among all steroid receptors; and a carboxyl-terminal ligand-binding domain. A similar structural organization has been found in all of the members of this receptor superfamily, which has now grown to include other trans-acting transcription factors and receptor-related proteins (Evans, 1988; Wang et al., 1989).

Conventional isolation techniques can provide only small quantities of purified receptor from target tissue for biochemical analysis. Methods such as transient transfection of mammalian cells and in vitro transcription and translation have greatly facilitated the functional dissection of the respective receptor domains, but the amount of receptor material which these techniques provide is not enough for certain structural and biochemical analyses. To circumvent this problem, we have devised a protocol for overexpression of receptor protein from complementary DNA (cDNA) in Escherichia coli. Although complex multidomain proteins such as recombinant tissue plasminogen activator have been isolated from E. coli in large quantities and in a biologically active form (Sarmiento et al., 1989), expressing heterologous genes in this organism has often been characterized by such technical problems as low yields, insolubility, and short half-lives (Gross, 1989). Often, these problems can be minimized when the foreign proteins are produced in E. coli as fusion peptides (Shine et al., 1980; Stanley and Luzio, 1984). Receptor fragments have been shown recently to be produced as fusion constructs in E. coli (Freedman et al., 1988; Dahlman et al., 1989; Eul et al., 1989), and we have used an E. coli expression system in which the carboxyl terminus of ubiquitin is fused with the desired region of the chicken progesterone receptor. Ubiquitin, a 76-amino acid polypeptide, is one of the most conserved proteins in nature and is found in all eukaryotic cells (Hershko and Ciechanover, 1986). Ubiquitin is not produced in prokaryotes. Although it is not fully understood why the yield and activity of unstable cloned gene products can be enhanced in E. coli by fusing them to ubiquitin, this system has been used previously to express a ubiquitin-metallothionein fusion protein at levels up to 20% of total cell protein (Butt et al., 1989). High level expression of chimeric ubiquitin fusion proteins has also been reported in the yeast Saccharomyces cerevisiae (Sabin et al., 1989).

In the present study, we report the production of significant amounts of a truncated progesterone receptor using this system. The overexpressed region of the receptor, which we term C1C2, contains both the DNA- and hormone-binding domains. Receptor material produced in this bacterial system is virtually indistinguishable from authentic chick oviduct receptor.

MATERIALS AND METHODS

Biochemicals and Buffers—Restriction enzymes were obtained from Promega Biotec (Madison, WI), Boehringer Mannheim, and Amersham Corp. Klenow DNA polymerase and T4 DNA ligase were...
purchased from Promega Biotech and Boehringer Mannheim, respectively. Triticated steroids were from Amersham or Du Pont-New England Nuclear. 125I-Protein A and [α-32P]dGTP were purchased from ICN (Irvine, CA). Radioinert steroids were from Sigma. Buffers were: A (10 mM Tris-HCl, 1 mM Na2EDTA, 12 mM 1-thioglycerol (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 1 mM L-1-tosylamide-2-phenylethyl chloromethyl ketone), and B (50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 12 mM 1-thioglycerol, 10% [v/v] glycerol).

**Bacterial Strains and Plasmids**—The construction of pUBF (see Fig. 1) has been described previously (Jonnalagadda et al., 1987). It is a bacterial expression vector in which a synthetic penta-ubiquitin gene is regulated by a lambda inducible AP1 promoter. The E. coli strain AR68 is a cryptic X-lysogen that carries the N gene with a truncated chicken progesterone receptor cDNA, was constructed using as an initial starting plasmid pSP86(3'), which has been described previously (Jonnalagadda et al., 1987). It is a bacterial expression vector in which a synthetic penta-ubiquitin gene is placed under the regulation of an inducible XPL promoter. The truncated chicken progesterone receptor which we constructed with a ubiquitin a-protein hydrolase. The truncated progesterone receptor which we constructed was used for immunoblotting as described previously (Tsai et al., 1987). Gel Retardation Assays—GRE/PRE oligonucleotides (Tsai et al., 1988) were end labeled with Klenow DNA polymerase and [α-32P]dGTP to a specific activity of >1 x 10^6 cpm/μg. Labeled GRE/PRE (0.2 ng) was incubated with C2 receptor material that had been prepared as described above. Also included in each reaction was 50 ng of HifI-digested pBR322 and varying concentrations of specific competitor DNA as described previously (Tsai et al., 1988). Incubation mixtures were run on 5% polyacrylamide gels as described elsewhere (Tsai et al., 1987).

**Sucrose Gradient Centrifugation**—Linear gradients of 0-30% sucrose in TESH buffer (20 mM Tris-HCl, 1 mM Na2EDTA, 12 mM 1-thioglycerol, pH 7.4) with no NaCl added or in TESH buffer containing 0.3 M NaCl were prepared in 4 ml heat-sealable polycarbonate tubes (Beckman) and handled as described previously (O’Malley et al., 1970). The gradients were centrifuged for 2 h at 65,000 rpm in a Beckman V Ti 65 rotor at 0-2°C. 

**RESULTS**

The portion of the chicken progesterone receptor which we expressed using the ubiquitin fusion vector pUBFC2 (Fig. 1) extends from amino acid 410 to amino acid 787 and contains both DNA- and hormone-binding domains. This fragment, termed C1C2, is produced by an in-frame fusion of ubiquitin cDNA and receptor cDNA via a short oligonucleotide linker that codes for the last 6 amino acids of ubiquitin.
The linker also restores a ubiquitin amino acid sequence that is the substrate for an ubiquitin α-protein hydrolase (Butt et al., 1989; Miller et al., 1989), thereby allowing optional cleavage of the fusion protein by this enzyme after production.

We expressed this plasmid fusion protein by two induction methods as shown in Fig. 2A. Both heat induction in AR68 cells and nalidixic acid induction in AR120 cells produced recombinant receptor protein of the expected size (approximately 47 kDa) that was detectable on Coomassie Blue-stained SDS-polyacrylamide gels.

We confirmed that these induced proteins were indeed Ub-C1-C2 fusion proteins using Western immunoblotting of a duplicate gel and anti-cPR IgG, RAP-2 (Fig. 2B, lanes 3 and 5). This antibody recognizes a region of the receptor within the DNA-binding domain. RAP-2 is a low sensitivity antibody, and will not detect authentic receptor in oviduct cytosol (lane 1) and only poorly detects 1 µg of partially purified B subunit receptor (lane 2). Additional immunoreactive bands seen on this Western blot arise from nonspecific interaction of the antibody with three endogenous E. coli proteins (compare induced AR120 cell lysate in lane 3 with uninduced lysate in lane 4). An immunoreactive band corresponding to the Ub-C1-C2 fusion peptide detected in uninduced AR68 cells (lane 6) results from a low basal level of transcription from the λPr promoter.

A large proportion of the ubiquitin-receptor protein was present in a soluble form (Fig. 2B, right panel). Crude lysate from nalidixic acid-induced AR120 cells was clarified by ultracentrifugation (100,000 × g), and Western analysis of soluble (lane 8) and particulate (lane 7) fractions was performed using antibody RAP-2. This revealed that about 30–40% of the Ub-C1-C2 protein was present in the soluble supernatant fraction.

Immunodot analysis (Fig. 2C) of crude bacterial lysates using antibody 907 (Weigel et al., 1989), which does not react with endogenous E. coli proteins, indicates that the Ub-C1-C2 receptor peptide represents approximately 1–4% of total cell protein in nalidixic acid-induced AR120 cells. The recombinant receptor peptide represents approximately 5% of total cell protein in heat-induced AR68 cells (data not shown).

Bacterially Produced Ubiquitin-C1-C2 Receptor Binds Progesterone with High Affinity and High Specificity—We performed hormone-binding titration assays using [3H]R5020 and soluble fractions from induced cells to determine if the bacterially produced C1-C2 receptor binds hormone with wild-type affinity. Using C1-C2 receptor from heat-induced AR68 cells, it was found that none of the truncated receptor material bound hormone even when the induction time was shortened to 30 min (data not shown), reflecting the sensitivity of the receptor to heat. This loss of hormone-binding activity may also be due in part to the induction of proteases at high growth temperature (see “Discussion”). The results obtained using receptor material from nalidixic acid-induced AR120 cells are shown in Fig. 3. This receptor material bound hormone with

FIG. 2. A, Coomassie Blue-stained SDS-polyacrylamide gel of bacterial extracts from both induced (+) or uninduced (−) AR120 and AR68 cells. The position of the induced Ub-C1-C2 receptor is indicated (arrowhead). The molecular weights of marker proteins are also indicated. OV, oviduct cytosol; B Std, B subunit receptor. B, left panel, Western immunoblot analysis of duplicate samples as shown in A. Lane 1 contains crude chick oviduct cytosol, and lane 2 contains a sample of partially purified receptor B protein (1 µg). Lanes 3–6 contain extracts from induced (+) versus uninduced (−) AR120 and AR68 cells. The position of recombinant receptor material is indicated (arrowhead). B, right panel, Western immunoblot of soluble (100,000 × g supernatant) versus particulate (100,000 × g pellet) material from nalidixic acid-induced AR120 cells. C, Western dot blotting of crude cell sonicates from induced and uninduced AR120 cells using anti-cPR IgG, 907. Receptor standards were diluted in a 10 µg/ml bovine serum albumin solution and were spotted onto nitrocellulose filters in concentrations ranging from 11.5 to 460 ng. Aliquots of crude lysate (equivalent to between 1 and 100 µl of cells) from induced and uninduced AR120 cultures were also spotted onto the same filter. Total cell protein was estimated using the Bio-Rad protein determination reagent system.

FIG. 3. A, saturation analysis of cell extracts; lysates from nalidixic acid-induced AR120 cells were incubated with 0–20 nM [3H] R5020 as described under “Materials and Methods.” Nonspecific binding was determined by the inclusion of a 100-fold M excess of unlabeled progesterone. B, Scatchard analysis of data from A was performed.
a high affinity. Scatchard analysis of this hormone-binding activity indicated a nonlinear plot that was resolved into two binding components (Fig. 3B). The higher affinity component had a $K_d$ of 0.6 nM, whereas the lower affinity component had a $K_d$ of 4.0 nM. These binding forms were present in approximately equal amounts, and both $K_d$ values are close to the $K_d$ for either authentic chick oviduct receptor ($K_d = 1-2$ nM) or in vitro synthesized receptor ($K_d = 1.4$ nM) (Schrader et al., 1977; Carson et al., 1987). From these binding data, we estimate that there are approximately 300 pg of high affinity hormone-binding protein/liter of cells, representing approximately 10% of the soluble $C_1C_2$ material present in induced AR120 cells.

The ligand specificity of the Ub-$C_1C_2$ peptide was measured by incubating soluble extracts from induced cells with tritiated progesterone and 20-fold excesses of various radioinert steroids. As shown in Fig. 4, progesterone itself was the most effective competitor (~90% competition). Estradiol and dexamethasone did not compete significantly, nor did RU486, a strong anti-progestin in many species but not in chickens (Moudgil et al., 1986). The significant level of competition (~60%) observed with testosterone correlates well with previous data obtained using either authentic oviduct receptor (Schrader and O'Malley, 1972) or in vitro synthesized receptor (Carson et al., 1987). Thus, we conclude that the $C_1C_2$ receptor produced in $E. coli$ exhibits binding affinities and specificities that are very similar to native oviduct receptor. By these criteria, the biological activity of the prokaryotic protein retains the same activity as the authentic oviduct receptor.

Specific Interaction of the Ub-$C_1C_2$ Receptor with Its Hormone Response Element—Using gel retardation assays, we analyzed the binding of partially purified Ub-$C_1C_2$ preparations to a labeled oligonucleotide containing the progesterone response element (PRE). Protein-DNA complexes were readily detected when a $^{32}$P-labeled oligonucleotide containing the GRE/PRE of the tyrosine amino transferase gene (Jantzen et al., 1987) was incubated with the Ub-$C_1C_2$ preparations (Fig. 5). This binding was sequence specific as shown by the ability of a 20-fold excess of unlabeled GRE/PRE oligonucleotide to compete for binding (5th–7th lanes). Furthermore, migration of this complex was retarded after incubation with anti-cPR 907 (Weigel et al., 1989), indicating that the oligonucleotide was interacting with the $C_1C_2$ peptide and not a contaminant in the crude preparation (4th lane). No competition for binding was observed when even a 100-fold excess of unrelated oligonucleotides containing either chicken ovalbumin upstream promoter element sequence (Hwang et al., 1988) or randomized PRE sequence (Rodriguez et al., 1989) was used (8th–13th lanes).

Additional bands, migrating faster than the main protein-DNA complexes, are also visible on the autoradiogram. These appear to represent partial degradation products of the Ub-$C_1C_2$ receptor material as indicated by the ability of unlabeled PRE oligonucleotide to compete for binding to these complexes. It is also conceivable that these faster migrating complexes may represent alternate forms of the GRE/PRE-protein complex (see “Discussion”). Taken together, the above results indicate that receptor produced in bacteria binds specifically to its GRE/PRE element in a manner identical to authentic chick oviduct receptor (Tsai et al., 1988). Thus, this biological activity is also retained by the prokaryotic protein.

Characterization of the Truncated Progesterone Receptor Complex Produced in $E. coli$—In an effort to characterize the basic physical characteristics of the $C_1C_2$ receptor, we subjected extracts from induced bacteria to sucrose gradient centrifugation. Soluble extracts were labeled with 20 nM $[^{3}H]$progesterone, and identical aliquots of radioactive bound frac-

![Fig. 4. Specificity of $[^{3}H]$progesterone binding to Ub-$C_1C_2$. Truncated receptor material produced in AR120 cells was incubated with 10 nM $[^{3}H]$progesterone and 200 nM samples of each indicated competitor as described under “Materials and Methods.” $[^{3}H]$Progestosterone counts associated with receptors were measured using a hydroxylapatite assay. Mean values from duplicate experiments are displayed. Prog., progesterone; $E_2$, estradiol; DEX, dexamethasone; Testo., testosterone.](image)

![Fig. 5. Binding specificity of $C_1C_2$ receptor to its GRE/PRE. Each binding reaction contained $^{32}$P-labeled GRE/PRE (0.4 ng), pBR322/HindIII (60 ng), and 5 ng of partially purified receptor material. The molar ratio of competitor to probe is indicated. Competitors were unlabeled oligonucleotides containing glucocorticoid/progesterone response element (GRE/PRE) sequence, chicken ovalbumin upstream promoter element (COUP) sequence, or randomized (RAND) PRE sequence. The 1st lane contains free probe. The 2nd lane contains no competitor. The 4th lane contains antibody 907 (4 µg). The arrowhead indicates the bands of interest.](image)
Expression system that is capable of producing high levels of T. R. Butt, W. T. Schrader, and B. W. O'Malley, unpublished results.

Fig. 6. Sucrose gradient analysis of C1C2 receptor material and oviduct cytosol receptor material. Extracts were layered with 20 nM [3H]progesterone and were analyzed on 0–30% linear sucrose gradients under conditions of low or high ionic strength. The samples are labeled chick cytosol on a high ionic strength gradient (A—A) and a low ionic strength gradient (C—O), and labeled Ub-C2 peptide on a high ionic strength gradient (A—A) and a low ionic strength gradient (A—A).

The data presented in this study describe a novel bacterial expression system in which an identical receptor fragment was expressed in E. coli, yielding 100 µg of recombinant material/liter of cells (Eul et al., 1989). Of this amount, over 60% was composed of the β-galactosidase protein itself. Thus, this expression system produced only 38 µg of biologically active C1C2 receptor/liter of culture.

Several regions for the ability of the small ubiquitin molecule to stabilize even such labile proteins as steroid hormone receptors have been suggested (Butt et al., 1989). As well as protecting the amino terminus of fused proteins from degradation, the possibilities for stabilization include the facilitation of correct protein folding and the formation of a stable translation initiation complex; the latter possibility is due to the fact that ubiquitin is one of the most conserved proteins existing in nature and has probably evolved excellent codon usage for efficient translation.

The Ub-C1C2 material produced in this expression system was analyzed for its binding activity. It is noteworthy that none of the material produced by heat induction of AR68 cells bound hormone. Apart from the well-documented heat lability of hormone-binding activity, it is known also that a variety of E. coli proteases are heat inducible; for example, the ion protease is controlled by the heat shock protein regulator gene (Guff et al., 1984). Our results demonstrate the attractiveness of a well-regulated gene expression system that is inducible at low temperatures (≤30 °C).

The receptor induced by naldixic acid binds hormone with an affinity very close to that of authentic chick oviduct progesterone receptor. Of particular interest to us was the fact that two affinity binding forms were detected by Scatchard analysis, one having a Kd value of 0.6 nM, and the other a Kd value of 4 nM. When this same truncated receptor was expressed in yeast, similar high and lower affinity binding sites for progesterone were detected. The presence of these two binding sites or forms may not be an artifact due to the use of a truncated receptor species, since a similar result has been obtained both in chick cytosol and in partially purified receptor preparations (Maggi et al., 1984). It is quite possible that the two different affinities do not represent separate sites as such but may be due to a covalent modification of certain receptor molecules.

The production of high affinity binding forms of the progesterone receptor in E. coli demonstrates that bacteria possess a complement of any necessary enzymes, such as kinases, for correct post-translational modification of the receptor to a form capable of binding hormone with high affinity. Furthermore, it is known that in eukaryotic cells, glucocorticoid (Catelli et al., 1985; Housley et al., 1985; Sanches et al., 1985; Mendel et al., 1986), and progesterone (Dougherty et al., 1984; Renoir et al., 1984, 1986) receptors are associated with heat shock protein 90 (hsp90). This protein is not found in prokaryotic cells. It has been postulated that these steroid receptors are maintained in the untransformed 8 S state through selective interaction with hsp90. Upon binding hormone, the complex dissociates, yielding free hsp90 and transformed receptor (for review, see Pratt, 1987). An interesting question arising from these studies is whether the interaction of receptor with hsp90 is a prerequisite for the receptor to bind its ligand (Denis and Gustafsson, 1989). The production of a receptor in E. coli which displays wild-type binding affinity argues against an obligatory requirement for eukaryotic hsp90, at least in the case of progesterone receptor.

As well as binding progesterone with high affinity and specificity, the C1C2 receptor produced in bacteria binds preferentially to its cognate progesterone response element (Fig. 5). In addition to the expected protein-DNA complex, addi-

DISCUSSION

The data presented in this study describe a novel bacterial expression system that is capable of producing high levels of a soluble stable biologically active truncated progesterone receptor containing both DNA- and hormone-binding domains. We have found previously that unfused steroid receptors as expressed in E. coli are extremely unstable and insoluble (Butt et al., 1989). By fusing steroid receptor sequences in-frame with a synthetic ubiquitin gene and using low temperature naldixic acid induction, we can produce 10 mg of intact receptor fusion protein/liter of AR120 cells. As much as 40% of this protein is soluble, and we estimate that at least 0.3 mg of the soluble receptor material is biologically active, as assessed by DNA-binding and hormone-binding assays.

Although previous studies have shown that receptor fragments encoding just the DNA-binding domain can be produced in mg quantities in E. coli (Freedman et al., 1988; Dahlman et al., 1989), larger fragments containing both DNA- and hormone-binding domains have not been expressed in significant amounts in this organism. A comparable expression study in which an identical receptor fragment was ex-

tional faster migrating complexes were seen by gel retardation
assay, which may be attributable to partial degradation of the
Ub-CC2 material. However, the level of degradation observed
by Western analysis of even crude AR120 extracts (Fig. 2)
seems insignificant. Recently, work in this group has shown
that the binding of truncated receptor to its GRE/PRE can
occur in a two-step cooperative process whereby one receptor
molecule binds first to a half-site of the PRE followed by
binding of a second molecule to the other half-site (Tsai et al.,
1988). Such interactions may contribute to the lower
molecular weight complexes observed in the gel retardation
assay shown in Fig. 5.

We observed anomalous sedimentation characteristics of
the C14, C26 peptide on sucrose gradients. It has been noted
that high level expression of even fusion peptides in E. coli
may result in the expressed products being sequestered into
inclu.
sion bodies or protein aggregates (Goeddel et al., 1979;
Williams et al., 1982). The broad sedimentation profile observed
in low salt conditions may result from self-aggregation or
association of the Ub-CC2 receptor with endogenous bacterial
protein(s). Either event could explain the low relative per-
centage of receptor material produced which is capable of
binding of a second molecule to the other half-site (Tsai et
al., 1989).

In conclusion, the expression system that we have described
is capable of producing large amounts of functionally active
receptor species produced at levels of up to 10 mg/liter. It
is capable of producing large amounts of functionally active
receptor material.

A. Phosphorylation

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