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Evidence of a Progesterone Receptor in the Liver of the Green Frog *Rana esculenta* and Its Down-Regulation by 17β Estradiol and Progesterone

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ABSTRACT Progesterone is a versatile hormone showing an ample variety of effects. One of the numerous functions attributed to progesterone is the modulation of vitellogenesis in oviparous vertebrates. As a prerequisite for the possible involvement of progesterone in vitellogenesis modulation, we investigated the presence of a progesterone receptor (PR) in the liver of the female green frog *Rana esculenta*. ^{3}H-Progesterone (^{3}H-P) binding activity was found in both cytosol and nuclear extract of the liver of *Rana esculenta*. The progesterone-binding moiety showed the typical characteristics of a true receptor, such as high affinity, low capacity, and specificity for progesterone. It also bound to DNA-cellulose and was eluted with a linear salt gradient at a concentration of 0.05 M of NaCl. The progesterone-binding moiety was down regulated by steroid hormones, in that ovariectomy resulted in a significant increase, in both cytosol and nuclear extract, of ^{3}H-P binding activity with respect to intact females. On the contrary, ^{3}H-P binding activity was almost undetectable after estradiol and/or progesterone treatment. The progesterone binding moiety of *Rana esculenta* was analyzed by Western blotting with the aid of a monoclonal antibody raised against the subunits A and B of the chicken PR. An immunoreactive band of about 67 kDa was observed in the liver of both intact and treated females. The 67 kDa band showed an increased intensity in ovariectomized animals, while it was faint following treatment with estradiol and/or progesterone.

This is the first report on the presence of a progesterone receptor (PR) in the liver of an amphibian. PR of *Rana esculenta* is down regulated by estradiol and/or progesterone and shows peculiar immunological and biochemical characteristics, which make it rather different from the PR of other vertebrates. *J. Exp. Zool. 284:765–775, 1999.*

Progesterone is defined as the "pregnancy hormone" of mammals. However, in lower vertebrates, progesterone is a highly versatile hormone that exerts an ample variety of effects on diversified target organs (Paolucci et al., '98; for review). Among other functions, it has been proposed that progesterone may have a physiological significance for the suppression of vitellogenesis during the ovarian cycle, therefore, redirecting energy reserves once vitellogenesis and ovulation are achieved (Callard and Ho, '87; Callard et al., '92). In support of this hypothesis, it has been shown that vitellogenin synthesis in the liver of vertebrates is induced not only by estradiol, but that several other hormones are involved in the modulation of vitellogenin synthesis. In the iguanid lizard *Diplosaurus dorsalis*, estradiol is synergized by pituitary factors in vitellogenin regulation (Callard et al., '72) and in the turtle *Chrysemys picta* and in the green frog *Rana esculenta*, vitellogenin synthesis is under a multi-hormonal control (Ho et al., '82a,b, '85; Gobbetti et al., '85; Ho, '87; Carnevali et al., '92a,b). It has been reported that progesterone specifically inhibits estrogen-induced vitellogenesis in reptiles and elasmobranchs. In the turtle *Chrysemys picta*, a single injection of progesterone simultaneously with estradiol delays and diminishes vitellogenesis in a dose-dependent manner (Ho et al., '81). In the little skate *Raja erinacea*, progesterone administration inhibits vitellogenin synthesis in estrogen-treated females, either intact or hypophysectomized (Perez and Callard, '89). How progesterone inhibits vitellogenesis, either directly via the vitellogenin gene or indirectly via estrogen receptor down-regulation, or both, is unknown. Certainly, progester-

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one action requires the presence of a specific receptor. A PR, whose characteristics recall the subunits A and B of the PR of mammals and birds, has been identified for the first time in the liver of a reptile, the turtle *Chrysemys picta* (Riley et al., '88). Successively, a PR has been identified and characterized in the liver of the elasmobranch *Raja erinacea*, where its presence in those periods of the reproductive cycle during which vitellogenesis does not occur sustains the hypothesis that the PR may play a role in inhibiting the vitellogenesis (Paolucci and Callard, '98). In spite of several studies carried out on vitellogenin synthesis and regulation in amphibians, the presence of a PR in the liver has never been investigated in this class of vertebrates.

For this study we used the oviparous frog *Rana esculenta*, a species characterized by a prolonged recovery period, ranging form September to March, during which vitellogenin is synthesized in the liver and accumulated by growing oocytes. Oocytes start increasing their size quite slowly at the end of summer and the beginning of fall. From November to March, their growth proceeds at a fast rate, interrupted only by a period of stasis coinciding with lethargy, which at this latitude takes place in December (Rastogi et al., '83). We undertook the present study to investigate the presence of a PR in the liver of the female of the green frog *Rana esculenta*. Progesterone binding activity characteristics have been determined by classic binding assay studies and DNA-cellulose chromatography. Additional information about its receptorial nature has been provided by using monoclonal antibodies raised against both the subunits A and B of the chicken PR. Further, the effect of steroid hormone treatment on ^{3}H-P binding activity level has been evaluated in an experimental design with intact and gonadectomized females.

MATERIAL AND METHODS

Chemicals and reagents

Radioactive [1,2,6,7-^{3}H] Progesterone (^{3}H-P) (SA = 80.2–111.1 Ci/mmol) was purchased from Amersham Radiochemical Center (Amersham Bucks, UK). Radioinert steroids used were progesterone, 17β-estradiol, testosterone, deoxycorticosterone (DOC), corticosterone, 17α-hydroxyprogesterone (Sigma, St. Louis, MO), R5020 (New England Nuclear, Boston, MA), RU486 and RU26988-5 (Roussel Uclaf, France), and 2914R-2 (provided by Dr. P.N. Rao, Southwest Foundation for Biomedical Research, San Antonio, TX). Sephadex G-25, DNA-Cellulose and Dextran T-70 were from Pharmacia (Piscataway, NJ). Acrylamide, N-N′-methylene-bis-acrylamide and other electrophoretic materials were from Bio-Rad (Richmond, CA). MS222 (3-aminobenzoic acid ethyl ester), Norit A charcoal, protein molecular weight markers, goat anti-mouse IgG alkaline phosphatase conjugated were from Promega, mouse IgG purified immunoglobulins were from Sigma. Maxifluor scintillation cocktail was obtained from Packard (Packard, Milan, Italy).

Buffers

The buffers used were as follows: Homogenization buffer (50 mM Tris-HCl, 1 mM EDTA, 12 mM monothioglycerol, 10% glycerol, pH 7.5). Washing buffer (10 mM Tris-HCl, 3 mM $MgCl_2$, 2 mM monothioglycerol, 0.25 M sucrose, pH 7.5). The extraction buffer for nuclear pellets was 0.7 M KCl in homogenization buffer. The standard buffer for receptors assay was 10 mM Tris-HCl, 1 mM 2-mercaptoethanol, and 10% glycerol, pH 7.5 (TEMG). Buffers for DNA-cellulose columns consisted of 200 mg/liter BSA in TEMG (buffer A); 0.5 M NaCl + 200 mg/liter BSA in TEMG (buffer B). Western blot buffer consisted of 20 mM Tris-Base, 150 mM NaCl, 0.5% Tween-20, 1.0% BSA, pH 7.5. Substrate buffer was 100 mM Tris-Base, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5.

Homogenization buffer, washing buffer, extraction buffer, and TEMG were added with a cocktail of protease inhibitors consisting of: Trypsin-Chymotripsin inhibitor 1 μM (Sigma); Pefabloc SC (AEBSF) 1 mM, Aprotin 0.1 μM, E64 Protease inhibitor 1 μM (Boehringer Mannheim, GmbH, Germany).

Experimental design

Sixty adult females of *Rana esculenta* were captured from the outskirts of Naples during the month of October when circulating vitellogenin is low (Giorgi et al., '82). Animals were separated into seven groups and underwent the following experimental design:

Group 1 (seven animals): Females sacrificed soon after the arrival (intact);
Group 2 (eight animals): Intact females kept in captivity for the whole duration of the experiment (captivity);
Group 3 (nine animals): Females sham operated soon after the arrival and kept in cap-

tivity for the whole duration of the experiment (sham).

The following 36 females were ovariectomized and allowed to recover for two weeks. Afterwards they were gathered into four groups (nine animals per group) and treated, any other day, for two weeks as follows:

Group 4: Animals injected with 0.1 ml of saline solution (NaCl 0.64%) (ovx + ss);
Group 5: Animals injected with 17β-estradiol (0.1 μg) in 0.1 ml of saline solution (ovx + E_2);
Group 6: Animals injected with progesterone (0.1 μg) in 0.1 ml of saline solution (ovx + P);
Group 7: Animals injected with 17β-estradiol (0.1 μg) and progesterone (0.1 μg) in 0.1 ml of saline solution (ovx + E_2 + P). Steroids were previously dissolved in absolute ethanol, then added to the saline solution at the required concentration.

Animals were fed on meat worms ad libitum. The mortality within groups was: 11% within group 2; 18% within group 3; and 21% within groups 4, 5, 6, 7. At the end of the experiment, each group consisted of seven animals (n = 7). Animals were sacrificed the day after the last injection. Upon the sacrifice, the animals were anaesthetized by immersion in water containing 1% MS222. After sacrifice, the liver was processed as follows.

Tissue preparation

The liver was perfused with saline solution, excised, weighed, minced, and homogenized in buffer in a ratio of 2:1 (buffer:tissue). The homogenate was filtered through several layers of cheesecloth and centrifuged at 1500*g* for 10 min. The supernatant was centrifuged at 100,000*g* for 1 hr to yield the cytosol. The nuclear pellet was washed twice with washing buffer and resuspended in nuclear extraction buffer. The suspension was kept on ice for 1 hr with low stirring to extract nuclear proteins and centrifuged at 100,000*g* to yield a clear nuclear extract. Samples were stored in liquid nitrogen until further analysis.

^{3}H-P binding assay

To remove endogenous steroids, cytosol was charcoal stripped prior to analysis by incubating for 10 min at 4°C with a Dextran coated charcoal (0.5% charcoal, 0.05% Dextran T-70) pellet derived from a suspension equivalent to sample volume, followed by centrifugation (800*g* for 10 min at 4°C). Charcoal-stripped cytosol and nuclear extracts were used undiluted or diluted with TEMG. For Kd determination, aliquots of 200 μl were incubated with increasing amounts (1–100 nM) of ^{3}H-P with, or without, a 200-fold excess of unlabeled progesterone for 16 hrs at 4°C. Samples were supplemented with 1 μM corticosterone to prevent binding to corticosterone binding globulin-like components (Chen and Leavitt, '79). After incubation, 0.6 ml of Dextran coated charcoal suspension was added. The mixture was vortexed and kept in ice for 5 min, followed by centrifugation at 800*g* for 10 min. The supernatant was decanted in counting vials with 5.0 ml Maxifluor scintillation fluid. Radioactivity was measured in a liquid scintillation counter (Packard 1600-CA) at 45% counting efficiency. For single point assay 200 μl of either cytosol or nuclear extract (at a protein concentration of 1 mg/ml) were incubated with 20 nM of ^{3}H-P in absence or presence of 200-fold excess of unlabeled progesterone. All samples were supplemented with 1 μM of corticosterone to saturate potential glucocorticoid receptor. Incubation and separation of bound and unbound steroids were performed as previously reported. For binding specificity evaluation, 200 μl of sample were added to 20 nM of ^{3}H-P and 1 μM corticosterone, with or without 1-, 10-, 100-, 1000-fold excess of various unlabeled steroids. Incubation and separation of bound and unbound steroids were performed as previously reported. The relative binding affinity (RBA) of each steroid was determined from the concentration of unlabeled competitor at the 50% level of competition (Leavitt et al., '74).

Desalting on Sephadex G-25

Sephadex G-25 was swollen in TEMG and packed into a 1 × 10 cm column (Pharmacia) at 4°C and washed with 10 vol of TEMG. Nuclear extract was applied to the column and eluted with TEMG. Fractions containing receptor were pooled and used for further analysis.

DNA-cellulose affinity chromatography

DNA-cellulose procedure was similar to that described in Salhanick et al. ('79), except for a few changes. The post-labeling method was used. Charcoal stripped cytosol (1 ml) and nuclear extract (1 ml), previously de-salted by chromatography on G-25 Sephadex columns, were applied to DNA-cellulose columns (0.5 × 10 cm) and equili-

brated overnight with DNA-cellulose buffer A. The columns were incubated at 22°C for 30 min. The following procedures were carried out at 4°C. The columns were washed with buffer A for 3 hr. 20 nM 3H-P plus 1 μM corticosterone in the presence or absence of 200-fold excess unlabeled progesterone in 1 ml of buffer A was added and absorbed onto the columns, followed by a 16 hr incubation at 4°C. The columns were then washed for 6.5 hr with buffer A, and 1 ml fractions were eluted with a linear 0–0.5 M NaCl using buffers A and B at a flow rate of 2 ml/hr. Salt concentration was determined by conductivity meter Econo Gradient Monitor (Bio-Rad) and radioactivity by scintillation counting.

DEAE-Sepharose chromatography

DEAE-Sheparose chromatography was performed according to Reese and Callard ('89). Analytical columns, 5 mm × 100 mm, were packed with DEAE-Sepharose in TEMG at 4°C and washed overnight with the same buffer. Charcoal stripped cytosols (0.5 ml) or de-salted nuclear extracts (0.5 ml), were incubated with 20 nM ^{3}H-P and 1 μM corticosterone, with or without 200-fold excess of unlabeled progesterone. After 16 hr incubation at 4°C, an equal volume of Dextran coated charcoal solution was added to the samples, which were vortexed, incubated at 4°C for 10 min, and centrifuged at 1000*g* for 10 min. A 0.4 ml aliquot of supernatant was added to the columns. The columns then were washed with 15 vol of TEMG and eluted with 20 ml linear 0–0.5 M NaCl gradient. Fractions (0.5 ml) were collected at a flow rate of 2 ml/hr and 4 ml of scintillation cocktail were added before counting. Salt concentration was determined by Conductivity Meter Econo Gradient Monitor (Bio-Rad) and radioactivity by scintillation counting.

Electrophoresis and Western blotting

Samples of cytosol and nuclear extract from whole tissue eluted from DNA-cellulose chromatography (40 μg total protein each) were run on discontinuous polyacrylamide gradient gel (5–20%), under denaturing conditions as described by Laemmli ('70). The Western procedure was according to Sullivan et al. ('88), with modifications as follows: after electrophoresis the proteins in gels were transferred onto nitrocellulose membrane (Schleicher and Schuell, Keene, NH) using a Mini Trans Blot Cell (Bio-Rad) (60 min run, with 250 V at the beginning and 350 V at the end). The nitrocellulose membranes were blocked for 30 min at 37°C in Western buffer. Nitrocellulose membranes were incubated with antibody to chicken PR (PR22, provided by Dr. D.O. Toft, Medical School, Rochester, MN) or mouse IgG (Sigma) at a concentration of 10 μg/ml in Western buffer overnight at 4°C. Nitrocellulose membranes were washed three times in Western buffer and incubated with alkaline phosphate conjugated antimouse IgG (from Promega) diluted 1:500 in Western buffer at 22°C for 4 hr. Nitrocellulose membranes then were washed three times in Western buffer. The antibody complex on nitrocellulose membranes were stained with 0.03% nitroblue tetrazolium (wt/vol, Sigma) and 0.03% 3 bromo-4-cloro-5-indolyl phosphate (wt/vol,

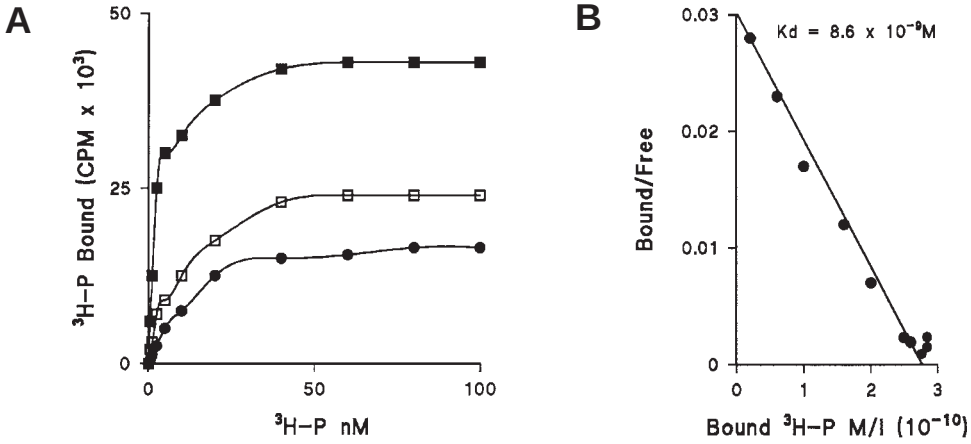

Fig. 1. Saturation (**A**) and Scatchard analysis (**B**) of ^{3}H-P binding in the cytosol of the liver of *Rana esculenta*. Only the specific binding is shown in the Scatchard plot. The experiment was repeated three times with similar results. ● = specific binding; ■ = total binding; □ = non-specific binding.

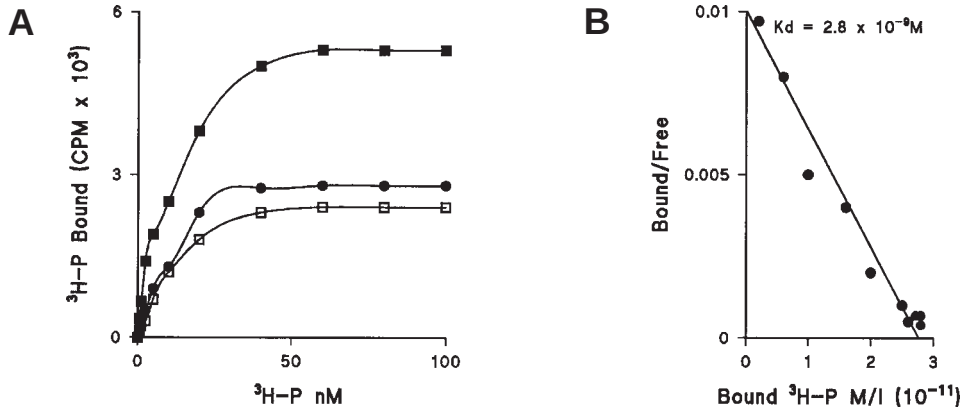

Fig. 2. Saturation (**A**) and Scatchard analysis (**B**) of ^{3}H-P binding in the nuclear extract of the liver of *Rana esculenta*. Only the specific binding is shown in the Scatchard plot. The experiment was repeated three times with similar results. ● = specific binding; ■ = total binding; □ = non-specific binding.

Sigma) in substrate buffer. Molecular markers from Sigma were used as standards.

Protein determination

Protein concentration was determined by the method of Lowry et al. ('51), using BSA as a standard.

Statistical analysis

Numerical data were analyzed by a one-way ANOVA method, followed by Duncan's multiple range test. Values were expressed as means ± SD.

RESULTS

^{3}H-P binding characteristic

^{3}H-P binding activity was detected in both cytosol and nuclear extract. It showed high affinity and low capacity for the ligand. The average Kd was 6.8 ± 2.0 nM in the cytosol (Fig. 1) and 2.0 ± 1.1 nM in the nuclear extract (Fig. 2). Only one binding component was present and the saturation was reached at a concentration of 20 nM of ^{3}H-Progesterone in both cytosol and nuclear extract.

The specificity of ^{3}H-P binding activity is reported in Table 1. Competition curves of unlabeled steroids for ^{3}H-P binding sites in cytosol and nuclear extract are reported in Fig. 3. In the cytosol, P, testosterone, and deoxycorticosterone, all competed to the same extent. The other competitors did not compete effectively. In the nuclear extract, P was the best competitor, followed by R5020. 17β-estradiol, corticosterone, testosterone, deoxycorticosterone, 17-hydroxyprogesterone, and the antagonists 2914-R2, RU486 and RU266998-5, all competed less effectively.

Cytosol and nuclear extract from the liver were subjected to DNA-cellulose affinity chromatography under post-labeling conditions. Figure 4 shows the DNA-cellulose chromatography profiles after the columns were washed free of DNA non-adhering components. A single peak of radioactivity was eluted at about 0.05 M NaCl.

When both cytosol and nuclear extract were analyzed by DEAE-Sepharose chromatography, a single peak of radioactivity was eluted at a salt concentration between 0.04 and 0.09 M NaCl (Fig. 5).

Effect of estradiol and/or progesterone treatment on ^{3}H-P binding in ovariectomized females

Figure 6 shows the effects of hormonal treatment on ^{3}H-P binding activity level. Ovariectomized females treated with saline solution showed the highest levels of ^{3}H-P binding activity in both cytosol and nuclear extract. Indeed, ^{3}H-P binding activity levels were significantly higher in ovariectomized

TABLE 1. Competitive binding activity of various steroids for ^{3}H-progesterone binding sites in the cytosol and nucelar extract of the liver of Rana esculenta

Steroid	RBA[1] Cytosol	RBA[1] Nuclear extract
Progesterone	100	100
Testosterone	100	1.8
17β-estradiol	<1	10.0
R5020	<1	69.0
Corticosterone	<1	10.0
Deoxycorticosterone	100	8.5
17α-Hydroxyprogesterone	<1	7.8
Antagonist 2914-R2	<1	6.8
Antagonist RU26998-5	<1	13.7
Antagonist RU486	<1	6.5

[1]RBA = relative binding activity.

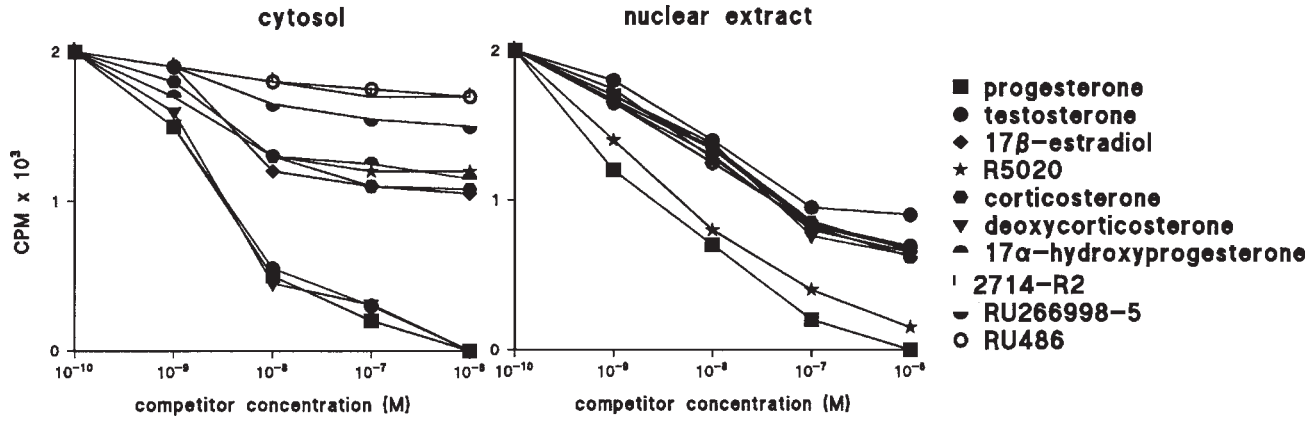

Fig. 3. Competition of unlabeled steroids for ^{3}H-P binding sites in the cytosol and nuclear extract of the liver of *Rana esculenta.* Each point represents the mean of three different determinations. The relative binding assay (RBA) of each steroid was determined from the concentration of unlabeled competitor at the 50% level of competition.

females than in intact females sacrificed soon after capture, in intact females kept in captivity for the whole duration of the experiment, and in sham operated females. Treatment with estradiol and/or progesterone brought about a decrease in ^{3}H-P binding activity levels in both cytosol and nuclear extract. Statistical analysis (ANOVA and Duncan test) results are given in Table 2 and Fig. 6, respectively.

Western blotting

Western blot analysis was performed with monoclonal antibodies against purified chicken PR subunits A and B. When liver cytosol and nuclear extract were resolved on SDS polyacrylamide gel and then transferred by blotting, the monoclonal antibody cross-reacted with a band corresponding to a molecular weight of about 67 kDa (Fig. 7). Western blotting of putative PR after DNA-cellulose chromatography of cytosol and nuclear extract preparations provided further support for the identity of the DNA-adhering fraction as PR (Fig. 8). Western blotting of cytosol and nuclear extract of liver of females subjected to the experimental design showed one immunore-

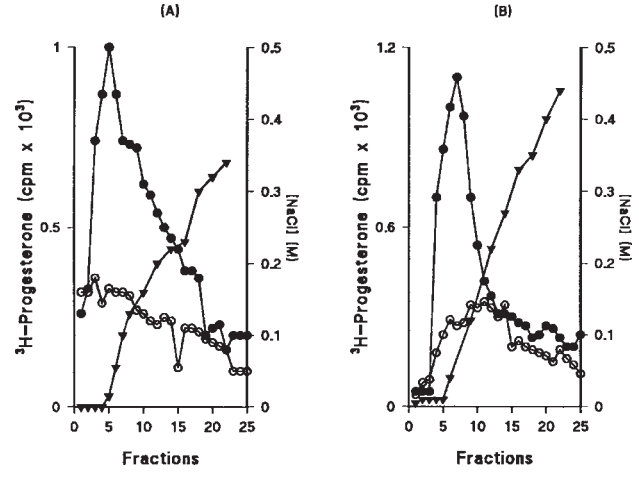

Fig. 4. DNA-cellulose chromatography profile of ^{3}H-P binding in the liver cytosol (**A**) and nuclear extract (**B**) of *Rana esculenta*. The linear gradient 0–0.5 M NaCl is reported on the right (▼). Total binding (●); non-specific binding (○). Data were similar in three separate experiments. Samples were from non-laying skates.

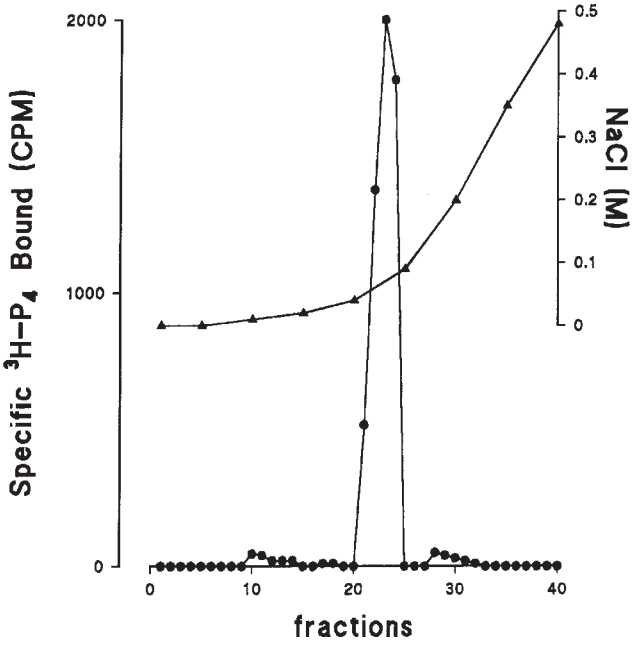

Fig. 5. DEAE-Sepharose chromatography of ^{3}H-P binding in the nuclear extract of the liver of *Rana esculenta*. Only specific binding is reported. The linear gradient 0–0.5 M NaCl is reported on the right (▲).

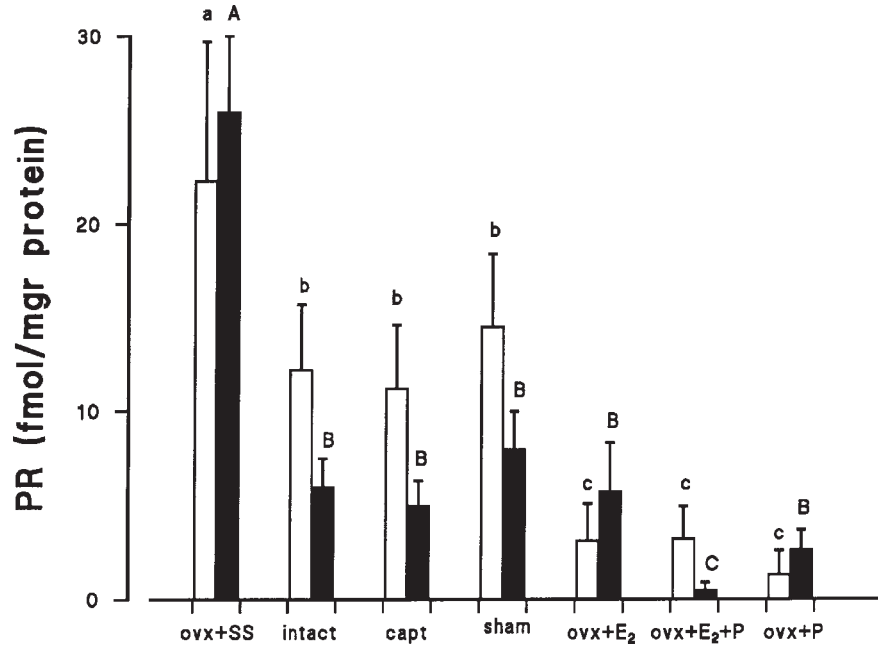

Fig. 6. ^{3}H-P binding activity levels (fmoles/mg protein) in cytosol (□) and nuclear extract (■) of liver of *Rana esculenta* females subjected to different experimental treatment (see Material and Methods). Values are shown as means ± SD. Data for cytosol and nuclear extract were analyzed separately with Duncan's test and identified with italic and capital letters, respectively. Different letters over bars indicate statistically significant differences at $P < 0.01$. Intact = non-operated females sacrificed soon after capture; capt = non-operated females kept in captivity for the whole duration of the experiment; sham = sham operated; ovx+SS = ovariectomized injected with saline solution; ovx+E_2 = ovariectomized injected with 17β-estradiol; ovx+E_2+P = ovariectomized injected with 17β-estradiol and progesterone; ovx+P = ovariectomized injected with progesterone.

active band of about 67 kDa (Fig. 9). The intensity of the immunoreactive band in females ovariectomized and treated with saline solution was stronger than the immunoreactive band of the other groups (Fig. 9, lane a). In any of the above cited cases, the immunoreactive band of 67 kDa was undetected when mouse IgG were used as a control in place of PR22 antibodies.

DISCUSSION

In this study we identified a PR in the liver of an amphibian, the female of the green frog *Rana esculenta*. ^{3}H-P binding activity was present in both cytosol and nuclear extract, showing high affinity and low capacity for the ligand. Scatchard analysis revealed only one binding site for progesterone, with a Kd of about 10^{-9} M, a value similar to the one reported for PR in the oviduct of the watersnake *Nerodia sipedon* (Kleis-San Francisco and Callard, '86a) and in both the oviduct and liver of the little skate *Raja erinacea* (Paolucci and Callard, '98).

TABLE 2. ANOVA analysis

Variability	FD	Deviation	Variation	F
		Cytosol		
Total	48	2827	—	
Among groups	6	2759	459	286.8*
Within groups	42	68	1.6	
		Nuclear extract		
	48	2856	—	
Among groups	6	2748	458	176.1*
Within groups	42	108	2.6	

*$P < 0.01$.

The nuclear PR in the liver of *Rana esculenta* was specific for progesterone, while the cytosolic PR also bound testosterone and DOC other than progesterone. The low specificity of binding in the cytosol could be due to an interference exerted by other binding molecules. Indeed, an androgen binding moiety has been identified in the liver of *Rana esculenta*, which binds both androgens and 17β-estradiol, but not progesterone (De Fiore et al., '98). Although in the dogfish testis Cuevas and Callard ('92) were able to separate the androgen receptor from the PR on the basis of their different specificity for mibolerone and R5020, respectively, we made no attempt to distinguish between these two recep-

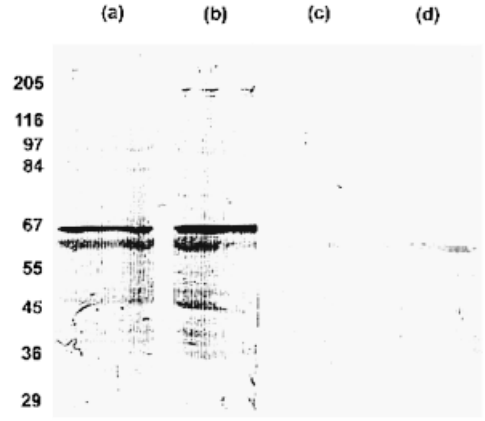

Fig. 7. Western blots of *Rana esculenta* liver cytosol (lanes a and c) and nuclear extract (lanes b and d) incubated with PR22 antibodies (lanes a and b) and with mouse IgG as a control (lanes c and d). The experiment was performed at least three times on cytosol and nuclear extract from different animals and gave similar results. Molecular weight markers are reported on the left.

tor types in the liver of *Rana esculenta*. The low specificity of the cytosolic PR in the liver of *Rana esculenta* could also be attributed to a contamination of sex steroid binding proteins (SSBPs). However, SSBPs characterized in *Rana esculenta* bind both testosterone and estradiol, but not progesterone (Paolucci and Di Fiore, '94a). DOC was a potent competitor for the cytosolic binding site and suggests a possible interference with corticosteroid binding sites. Although it is not known whether corticosteroid binding molecules are present in *Rana esculenta* liver, we chose to saturate potential corticosteroid receptors by adding 1 μM of corticosterone to our samples,

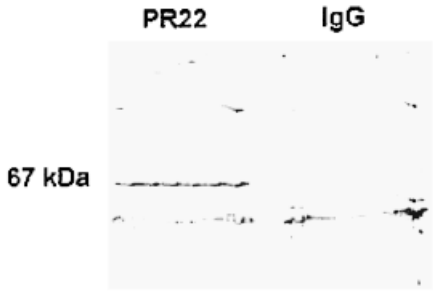

Fig. 8. Western blot of liver nuclear extract after DNA-cellulose chromatography. Cytosol gave similar results. Western blots were performed three times on cytosol and nuclear extract from different samples with consistent results. PR22 = DNA-cellulose eluted nuclear extract incubated with PR22 antibodies; IgG = DNA-cellulose eluted nuclear extract incubated with IgG as a control. The molecular weight marker is reported on the left.

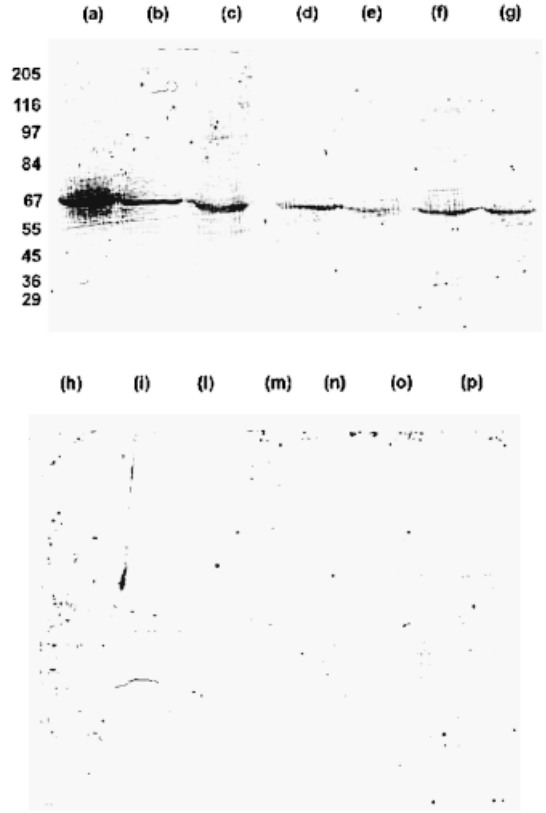

Fig. 9. Western blot of liver nuclear extract of *Rana esculenta* females after experimental treatment. a and h = ovariectomized injected with saline solution; b and i = intact; c and l = intact females kept in captivity; d and m = sham operated; e and n = ovariectomized injected with progesterone and 17β-estradiol; f and o = ovariectomized injected with 17β-estradiol; g and p = ovariectomized injected with progesterone. Lanes from a to g were incubated with PR22 antibodies; lanes from h to p were incubated with mouse IgG as a control. Western blots were repeated three times with consistent results. Cytosol gave similar results. Molecular weight markers are reported on the left.

on the basis of corticosteroid receptor characteristics in mammals, which are known to bind progestins (Chen and Leavitt, '74).

Western blot analysis of the hepatic PR of *Rana esculenta* sustains, along with binding studies, the presence of only one form of PR. Indeed, one immunoreactive band with a Mw of about 67 kDa was present in both cytosol and nuclear extract. This value is rather low, although literature has reported an ample variety of PR forms with diverse Mw. Indeed, several truncated forms of PR, lacking the amino-termini and with a Mw comprised between 110 and 65 kDa, have been described in the human uterus (Vu-Hai et al., '89). Moreover, a putative PR isoform C with a Mw of 64 kDa has been detected throughout the repro-

ductive cycle in the liver of *Chrysemys picta* (Giannoukos and Callard, '95). It is possible that the PR form in *Rana esculenta* represents a proteolytic fragment of a larger PR form. However, we are confident that this possibility can be ruled out in this case, since protease inhibitors were added to our samples.

In agreement with the immunological evidence of the presence of only one form of PR in *Rana esculenta*, only one peak of ^{3}H-P binding activity was eluted on both ionic-exchange chromatography and DNA-cellulose chromatography. Moreover, the DNA-cellulose eluted peak cross-reacted with antibodies against the chicken PR, showing an immunoreactive band of the same Mw of that one present in the crude preparation of cytosol and nuclear extract. On the contrary, in the majority of vertebrates, PR is formed by two subunits or isoforms A and B (Krett et al., '88; Savouret et al., '89; Carbajo et al., '96). The only ^{3}H-P binding site that we have detected in *Rana esculenta*, shows an affinity value for progesterone comprised between the high affinity and low affinity values reported in other vertebrates. Thus, this situation poses the problem of ascribing the PR of *Rana esculenta* to either PR-A or PR-B. According to the data presented here, it seems that the PR in *Rana esculenta* is not present with two isoforms or subunits. Certainly, we cannot exclude that specific phases of the reproductive cycle may accompany with distinct forms of PR, which could be absent in the period we have taken into consideration (beginning of recovery period). Thus, in the liver of *Chrysemis picta*, the isoform PR-B was detected only during the early and late luteal stages (Giannoukos and Callard, '95), and in the oviduct the expression of PR isoforms or subunits is modulated by steroids (Giannoukos and Callard, '96). Our data show that no additional immunoreactive bands were observed in *Rana esculenta* following hormonal treatment. Instead, the intensity in the 67 kDa immunoreactive band increased after ovariectomy and decreased after hormonal treatment suggesting a direct influence of estradiol and/or progesterone on PR levels.

Studies carried out on lower vertebrates report that PR fluctuates throughout the reproductive cycle, along with the plasma steroids estradiol and progesterone (Kleis-San Francisco and Callard, '86b; Paolucci and Di Fiore, '94b; Giannoukos et al., '95; Giannoukos and Callard, '95; Paolucci and Callard, '98). This behavior suggests that the internal hormone environment may influence PR. Although PR regulation exerted by estradiol and/or progesterone has been investigated in several species of vertebrates, it is difficult to draw a clear-cut conclusion. Among lower vertebrates, PR regulation has been studied only in reptiles, with results not always in agreement (Selcer and Leavitt, '91; Paolucci and Di Fiore, '94b; Giannoukos and Callard, '96). In this study we observed that in the liver of *Rana esculenta*, ovariectomy is followed by an increase in PR levels. Further, PR levels decrease in estradiol and/or progesterone injected animals. Thus, our data suggest that in *Rana esculenta* the ovary may exert a negative regulation on PR. In light of the hypothesized role of progesterone as a suppressor of vitellogenesis during the ovarian cycle (Callard et al., '92), we can attempt an interpretation regarding the physiological role of PR in the liver of *Rana esculenta*. Although speculative, we can hypothesize that during the reproductive cycle, when the levels of circulating estradiol and progesterone are low, as occurs at the beginning of the recovery period (Paolucci et al., '90), PR levels in the liver are high and, therefore, vitellogenin synthesis is kept at its minimum. As follicular growth proceeds, estradiol and progesterone levels increase (Wallace, '85), causing a decrease in PR levels with a consequent augmentation of the rate of hepatic synthesis of vitellogenin. Incidentally, estradiol receptor fluctuates in the liver of *Rana esculenta* throughout the reproductive cycle, increasing from the beginning of the recovery period to attain a maximum level around the time of ovulation (Paolucci and Botte, '88). In agreement with ER fluctuations, vitellogenin levels in the plasma are low during the post-reproductive period and increase at the beginning of the recovery period with a climax shortly before ovulation (Giorgi et al., '82).

In summary, we have identified and partially characterized a PR in the liver of the green frog *Rana esculenta*. According to the immunoreactivity and binding assay, it seems that PR characteristics are quite different from those described so far in other vertebrates. The down regulation exerted by estradiol and/or progesterone provides indirect evidence of the possible involvement of the PR in the modulation of vitellogenesis. The mechanism whereby vitellogenin production is modulated during the reproductive cycle of oviparous species has important evolutionary implications. During the evolution from the oviparous to the viviparous mode of reproduction, PR might have played a role in the gradual elimination of

yolk (Callard et al., '92). Our data on the presence of a PR in amphibians further sustains this theory. Moreover, the evidence that only one form of PR is expressed in amphibians provides a useful indication to trace a phyletic pattern of PR whose specific function seems to be carried on by only one subunit in ancient vertebrates.

LITERATURE CITED

Callard IP, Ho S-M. 1987. Vitellogenesis and viviparity. In: Chester-Jones I, Ingleton PM, Phillips JG, editors. Fundamentals of comparative vertebrate endocrinology. New York: Plenum Press. p 257–281.

Callard IP, Bayne CG, McConnel WF. 1972. Hormones and reproduction in the female lizard, *Sceloporus cyanogenys*. Gen Comp Endocrinol 18:175–194.

Callard IP, Fileti LA, Perez LE, Sorbera LA, Giannoukos G, Klosterman L, Tsang P, McCracken J. 1992. Role of the corpus luteum and progesterone in the evolution of vertebrate vivipary. Am Zool 32:264–275.

Carbajo P, Christensen K, Edwards DP, Skafar DF. 1996. Binding of ^{3}H-progesterone to the human progesterone receptor: differences between individual and mixed isoforms. Endocrinol. 137:2339–2346.

Carnevali O, Mosconi G. 1992a. In vitro induction of vitellogenin synthesis in *Rana esculenta*: role of the pituitary. Gen Comp Endocrinol 86:352–358.

Carnevali O, Mosconi G, Yamamoto K, Kobayashi T, Kikuyama S, Polzonetti-Magni AM. 1992b. Hormonal control of in vitro vitellogenin syntesis in *Rana esculenta* liver: effects of mammalian and amphibian growth hormone. Gen Comp Endocrinol 88:406–414.

Chen TJ, Leavitt WW. 1979. Nuclear progesterone receptor in hamster uterus: measurement by ^{3}H-progesterone exchange during the estrous cycle. Endocrinology 104:1588–1597.

Cuevas ME, Callard LP. 1992. Androgen and progesterone receptors in shark (*Squalus*) testis: characteristics and stage-related distribution. Endocrinology 130:2173–2182.

Di Fiori MM, Assise L, Botti V. 1998. Aromatase and testosterone receptor in the liver of the female green frog *Rana esculenta*. Life Sci 62:1949–1958.

Giannoukos G, Callard LP. 1995. Reptilian (*Chrysemys picta*) hepatic progesterone receptors: relationship to plasma steroids and the vitellogenic cycle. J Steroid Biochem Mol Biol 55:93–106.

Giannoukos G, Callard LP. 1996. Radioligand and immunochemical studies of turtle oviduct progesterone and estrogen receptors: correlation with hormone treatment and oviduct contractility. Gen Comp Endocrinol 101:63–75.

Giannoukos G, Coho DW, Callard LP. 1995. Turtle oviduct progesterone receptor: radioligand and immunocytochemical studies of changes during the seasonal cycle. Endocrinology 3:429–437.

Giorgi F, Gobbetti A, Polzonetti-Magni A. 1982. Variations in the vitellogenin titre during the reproductive cycle of *Rana esculenta*. Comp Biochem Physiol 72B:501–506.

Gobbetti A, Polzonetti-Magni A, Zerani M, Carnevali O, Botte V. 1985. Vitellogenin hormonal control in the green frog, *Rana esculenta*: interplay between estradiol and pituitary hormones. Comp Biochem Physiol 82A:855–858.

Ho S-M. 1987. Endocrinology of vitellogenesis. In: Norris EO, Jones RE, editors. Hormones and reproduction in fishes, amphibian and reptiles. New York: Plenum Press. p 145–159

Ho S-M, Danko D, Callard LP. 1981. Effect of exogenous estradiol-17β on plasma vitellogenin levels in male and female *Chrysemys picta* and its modulation by testosterone and progesterone. Gen Comp Endocrinol 43:413–421.

Ho S-M, Kleis S, McPherson R, Heisermann GJ, Callard LP. 1982a. Regulation of vitellogenesis in reptiles. Herpetologica 38:40–50.

Ho S-M, Taylor S, Callard LP. 1982b. Effect of hypophysectomy and growth hormone on estrogen-induced vitellogenesis in the freshwater turtle, *Chrysemys picta*. Gen Comp Endocrinol 48:254–260.

Ho S-M, Wangh LJ, Callard LP. 1985. Sexual differences in the in vitro induction of vitellogenesis in the turtle: role of pituitary and growth hormone. Comp Biochem Physiol 81:467–472.

Kleis-San Francisco SM, Callard LP. 1986a. Identification of a putative progesterone receptor in the oviduct of a viviparous watersnake (*Nerodia*). Gen Comp Endocrinol 61:490–498.

Kleis-San Francisco SM, Callard LP. 1986b. Progesterone receptors in the oviduct of a viviparous watersnake (*Nerodia*): correlation with ovarian function and plasma steroid levels. Gen Comp Endocrinol 63:220–229.

Krett NL, Edwards DP, Horwitz KB. 1988. Progesterone action and receptors. In: Cooke BA, King RJB, van der Molen HJ, editors. Hormones and their actions, part I. 14:241–267.

Laemmli UK. 1970. Clearage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Leavitt WW, Toft DO, Strott CA, O'Malley BW. 1974. A specific progesterone receptor in the hamster uterus: physiologic properties and regulation during the estrous cycle. Endocrinology 94:1041–1053.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275.

Paolucci M, Botte V. 1988. Estradiol-binding molecules in the hepatocytes of the female water frog, *Rana esculenta*, and plasma estradiol and vitellogenin levels during the reproductive cycle. Gen Comp Endocrinol 70:466–476.

Paolucci M, Di Fiore MM. 1994a. Estrogen and progesterone receptors in lizard *Podarcis s. sicula* oviduct: seasonal distribution and hormonal dependence. J Exp Zool 269: 432–441.

Paolucci M, Di Fiore MM. 1994b. Sex steroid binding proteins (SSBP) in the plasma of the green frog *Rana esculenta*: changes during the reproductive cycle and dependence on pituitary gland and gonads. Gen Comp Endocrinol 96:401–411.

Paolucci M, Callard LP. 1998. Characterization of progesterone-binding moieties in the little skate *Raja erinacea*. Gen Comp Endocrinol 109:106–118.

Paolucci M, Esposito V, Di Fiore MM, Botte V. 1990. Effects of short postcapture confinement on plasma reproductive hormone and corticosterone profiles in *Rana esculenta* during the sexual cycle. Boll Zool 57:253–259.

Paolucci M, Custodia N, Callard IP. 1998. Progesterone: effects and receptors (subavian species). In: Encyclopedia of reproduction, vol. 4. Academic Press. p 454–461.

Perez LE, Callard LP. 1989. Evidence for progesterone inhibition of vitellogenesis in the skate (*Raja erinacea*). Am Zool 29:357.

Rastogi RK, Izzo-Vitiello I, Di Meglio M, Di Matteo L, Franzese R, Di Costanzo MG, Minucci S, Iela L, Chieffi G. 1983. Ovarian activity and reproduction in the frog, *Rana esculenta*. J Zool Lond 200:233–247.

Reese JC, Callard LP. 1989. Two progesterone receptors in the oviduct of the freshwater turtle *Chrysemys picta:* possible homology to mammalian and avian progesterone receptors systems. J Steroid Biochem 33:297–310.

Riley D, Reese JC, Callard LP. 1988. Hepatic progesterone receptors: characterization in the turtle *Chrysemys picta*. Endocrinology 123:1195–1201.

Salhanick AR, Vito CC, Fox TO, Callard IP. 1979. Estrogen binding proteins in the oviduct of the turtle, *Chrysemys picta*: evidence for a receptor species. Endocrinology 105:1388–1395.

Savouret JF, Misrahi, M., Loosfelt, H., Atger, M., Bailly, A., Perrot-Applanat, M., Vu Hai, M.T., Guiochon-Mantel A, Jolivet A, Lorenzo F, Logeat F, Pichon MF, Bouchard P, Milgrom E. 1989. Molecular and cellular biology of mammalian progesterone receptors. In: Recent progress in hormone research. San Diego: Academic Press, Inc. p 65–118.

Scatchard G. 1949. The attractions of proteins for small molecules and ions. Ann NY Acad Sci, 51:660–672.

Selcer KW, Leavitt WW. 1991. Progesterone downregulates progesterone receptor, but not estrogen receptor, in the estrogen-primed oviduct of a turtle (*Trachemys scripta*). Gen Comp Endocrinol 83:316–323.

Sullivan WP, Smith DF, Beito TG, Krco CJ, Toft DO. 1988. Hormone-dependent processing of the avian progesterone receptor. J Cell Biochem 36:103–109.

Vu-Hai MT, Jolivet A, Ravet V, Lorenzo F, Perrot-Applanat M, Citerne M, Milgrom E. 1989. Novel monoclonal antibodies against human uterine progesterone receptor. Biochem J 260:371–376.

Wallace RA. 1985. Vitellogenesis and oocyte growth: nonmammalian vertebrates. In: Browder LW, editor. Developmental biology: a comprehensive synthesis, vol. 1. New York: Plenum Press. p 127–177.