Endocrine (1995) 3, 429-437

Turtle oviduct progesterone receptor: radioligand and immunocytochemical studies of changes during the seasonal cycle

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In order to determine the regulation of the oviduct progesterone receptor in *Chrysemys picta*, radioligand binding studies were performed to determine changes in the high and lower affinity binding sites during the seasonal cycle. Lower affinity sites were present in both cytosolic and nuclear fractions during the cycle and peaked during the peri-ovulatory/early luteal periods. The high affinity sites, present exclusively in the nuclear fraction, increased following the preovulatory peak in plasma estradiol, remained elevated during the early luteal phase following the post-ovulatory peak in progesterone, and declined to non-detectable levels just before egg-laying. DNA-cellulose affinity chromatography showed that both high and low affinity binding sites were integral to both progesterone receptor B and A isoforms. Western blot analysis confirmed the binding studies and showed that PR-B (115 kDa) was present in greatest amounts during the peri-ovulatory and luteal periods, whereas PR-A (88 kDa) increased during those periods and was present following egg-laying. Immunocytochemical analysis revealed increased progesterone receptor immunostaining from the winter to the peri-ovulatory period in the three major zones (luminal epithelium, submucosal glands and the myometrium) following the preovulatory peak in estradiol, a decrease in all three zones, especially the myometrium, in the late luteal period following the post-ovulatory peak in progesterone, and an increase again during fall recrudescence. Competition studies demonstrated that progesterone was the most effective competitor followed by pregnenolone, R5020 and deoxycorticosterone. RU 486 does not bind to the high affinity site, but binds quite well to the lower affinity site. This study suggests that progesterone receptor isoforms in the turtle oviduct may be under the regulation of changing estrogen/progesterone ratios during the cycle.

Keywords: progesterone receptor; oviduct; reproductive cycle turtle

Introduction

The painted turtle, *Chrysemys picta*, is a seasonally breeding reptile which, in the northern part of its range, commonly ovulates one clutch of eggs ($n = 10$) over a period of 12-24 h in the late spring (May/June). The ovulatory cycle is characterized by cyclic fluctuations in ovarian and oviduct weight which correlate with preovulatory increases in estrogen (E) and progesterone (P) titers. A short luteal phase (14-21 days) follows ovulation during which eggs are held in the oviduct for addition of albumen and a shell (Harless & Morlock, 1979). This period is somewhat longer than in avian species where the luteal phase is largely suppressed and eggs rapidly oviposited (Rothchild, 1981). After oviposition, ovarian and oviduct weights regress through the summer, and ovarian weight begins to increase with the onset of vitellogenesis in the fall in preparation for the next year's clutch (Callard *et al.*, 1978). Seasonal changes in oviduct weight, structure, contractile activity and extensibility suggest control by ovarian steroids (Motz & Callard, 1988, 1991).

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Received 9 December 1994; accepted 3 April 1995

The oviduct is characterized as having five distinct regions: the infundibulum, the glandular or albumen-secreting region, the intermediate region, the uterus, and the vaginal or cervical region (Giersberg, 1923). The glandular region is homologous with the avian magnum which is known to secrete albumen (Aitken & Solomon, 1976). Seasonal histological changes in the oviduct of the garter snake (Mead *et al.*, 1981) and the lizard, *Anolis pulchellus* (Ortiz & Morales, 1974), also suggest control by ovarian steroids in these species.

Our hypothesis is that luteal P is a key steroid in the regulation of reproductive tract function in all vertebrates, specifically controlling the interval between ovulation and oviposition in oviparous species. In support of this, we have previously partially characterized the progesterone receptor (PR) in the oviduct of the turtle, *Chrysemys picta*, a seasonally breeding reptile (Ho & Callard, 1984; Reese & Callard, 1989), but changes associated with the reproductive cycle have not been defined. Turtle PR appears to be up-regulated by estrogen. Thus, Reese & Callard (1989) demonstrated up-regulation of the PR-B isoform by E in *Chrysemys picta* in winter using DNA-cellulose affinity chromatography. Further, in a related species, *Trachemys scripta*, Selcer & Leavitt (1991) provided evidence of E up-regulation of PR. Changes in snake (*Natrix*) oviduct PR associated with follicular development and pregnancy have also been described by Kleis-San Francisco & Callard (1986). Recently Paolucci & Di Fiori (1994) have shown changes in PR correlated with estrogen receptor (ER) during the reproductive cycle of the lizard, as well as PR induction following E treatment of castrates. In the present study, we have extended our observations of the turtle, *Chrysemys picta*, to analyse the relationship between oviductal high and lower affinity PR and plasma steroid levels during the annual cycle; radioligand binding studies are supported by Western blot analysis in order to determine related changes in PR isoforms. In addition, we describe the tissue distribution of oviduct PR using immunocytochemistry.

Results

Seasonal cycle of oviduct progesterone binding sites determined by Scatchard analysis (Figures 1, 2A and B)

Saturation analysis for diluted nuclear (1:5) and cytosol (1:10-1:40) extracts ranging from 1-100 nM ^{3}H-P and 1 μM corticosterone was performed in the presence (non-specific binding) and absence (total binding) of 400-fold excess radioinert P. To determine PR values for the high and low affinity binding components over the seasonal cycle (Figure 1A and B), Scatchard analysis was obtained by the Lundon ReceptorFit Saturation Two-Site program, and the molar values of the two binding components were converted into fmol/mg protein. Typical biphasic (two site) and single site (low affinity only) Scatchard plots are shown for samples where either both or only the lower affinity site are present (Figure 2A and B). The K_d values were: $K_{d1} = 3.78 \times 10^{-9}$ M and $K_{d2} = 2.88 \times 10^{-8}$ M (Figure 2A); and $K_d = 5.34 \times 10^{-8}$ M (Figure 2B).

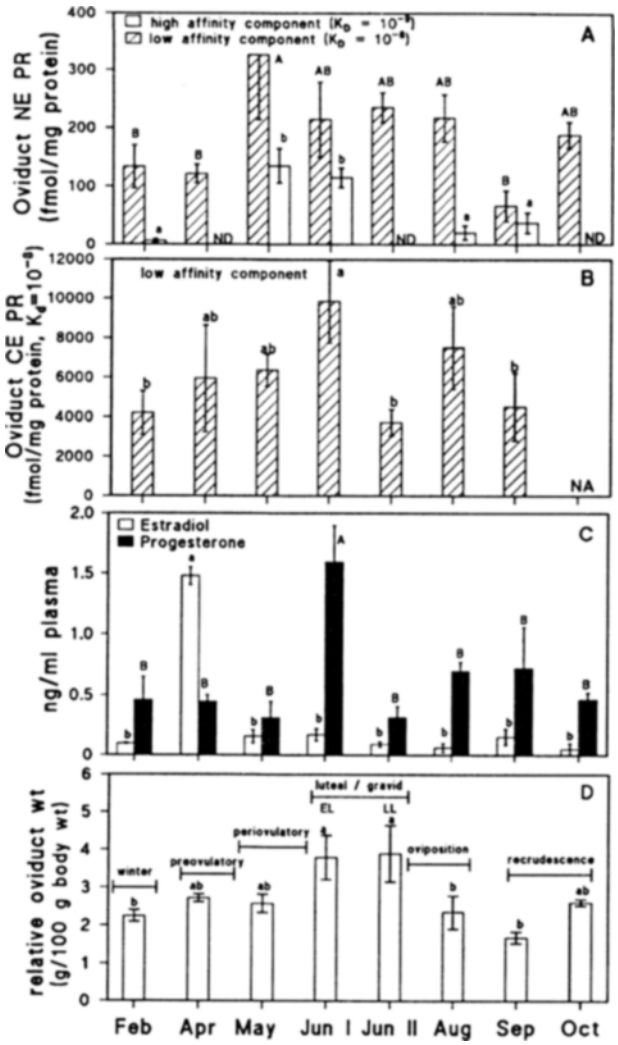

Figure 1 Seasonal changes in nuclear (**A**) and cytosolic (**B**) oviduct PR binding levels, plasma steroid levels (**C**) and oviduct weight (**D**). Values were expressed as the mean ± SEM. One-way ANOVA followed by Duncan's multiple range test ($P<0.05$) were used to determine any significant changes (indicated by differing letters, i.e. **A**, a *vs* **B**, b) over the cycle for oviduct PR, plasma steroid levels, and oviduct weight. For the nuclear PR (**A**) and steroid levels (**B**), statistical analysis was performed on each variable separately. ND = non-detectable. NA = not analysed

The high affinity component was only observed in nuclear extracts (Figure 1A) in May (peri-ovulatory period) following the preovulatory plasma E peak (April); the high affinity component remained elevated ($P<0.05$, May and June early luteal) during the post-ovulatory peak in plasma *P* (early luteal/gravid period). By the end of the gravid period (late luteal), the high affinity PR could not be detected. After egg laying (August and September) the high affinity component reappeared in parallel with increased plasma E and P levels. The lower affinity PR component was always detectable in nuclear extracts and was significantly increased during May ($P<0.05$ *vs* February, June late luteal and September) after the preovulatory peak of plasma E. Levels were lowest in the winter (February) and the fall (September). Based on Scatchard analysis, only the lower affinity PR component was present in oviduct cytosol (Figure 1B). Levels started to rise during follicular growth in April through the early luteal period (peak plasma P). Near the end of the gravid period (late luteal), levels dropped significantly ($P<0.05$, June early luteal *vs* June late luteal). As can be seen, the lower affinity site was always in excess of the high affinity site in the nucleus (approximately 2:1 in May, June early luteal and September *vs* 1:10 in August and 1:20 in February); cytosolic lower affinity sites were also in great excess over nuclear lower affinity sites (16:1, June early luteal–68:1, September) and high affinity sites were not detectable in the cytosol.

Seasonal changes in plasma steroid levels and oviduct weight (Figure 1C and D)

Oviduct weight (Figure 1D) was relatively constant prior to ovulation during the months of February to May, and increased significantly in June (early luteal and late luteal stages) ($P<0.05$, June early luteal and late luteal *vs* February and August) associated with egg shell deposition prior to laying. Following oviposition and the fall months, oviduct weight returned to pre-gravid levels. Radioimmunoassay results revealed two significant steroid peaks (Figure 1C) over the seasonal cycle. The peak in E levels, 1478 ± 208 pg/ml plasma ($P<0.05$, April *vs* the remaining periods of the cycle), occurred during the preovulatory period in April and dropped to basal levels in May, peri-ovulatory period. A peak in P, 1595 ± 591 pg/ml plasma ($P<0.05$, June early luteal *vs* remaining periods of the cycle), occurred during the post-ovulatory, early luteal period. Near the end of the gravid period (late luteal), P levels returned to peri-ovulatory (May) values and started to increase again in August following egg-laying.

DNA-cellulose affinity chromatography (Figure 2A and B)

To determine whether high or lower affinity sites were preferentially associated with PR-A or B isoforms, de-salted nuclear extracts containing both the high and lower affinity binding components and diluted cytosol extracts containing only the low affinity binding component were chosen for DNA-cellulose affinity chromatography on the basis of Scatchard analysis. Both nuclear (Figure 2A) and cytosol (Figure 2B) extracts revealed two specific peaks, the first peak eluted between 0.075–0.1 M NaCl, and the second peak eluted at 0.295 M NaCl.

Binding Specificity of the lower affinity binding site (Table 1)

The specificity of ^{3}H-P binding activity of the cytosolic lower affinity binding component determined by competition assay in the presence of 10-, 100- and 1000-fold radioinert competitor. P was the best competitor, followed by pregnenolone; R5020, and deoxycorticosterone. Other steroids competed less well (dihydrotestosterone > testosterone = estradiol- > RU 486 > corticosterone > mibolerone). Specificity of the high affinity site as previously published by Reese & Callard (1989) with additional data from this study is shown for comparison.

Seasonal changes in oviduct PR determined by Western blot analysis (Figures 3A and B and 4A and B)

Western blot analysis of nuclear and cytosol extracts was conducted throughout the seasonal cycle using the anti-PR 6 antibody. In nuclear extracts (Figure 3A), a specific band of 115 kDa (PR-B) was seen during the peri-ovulatory and two luteal periods (early and late luteal). A specific band was also seen at 88 kDa (putative PR-A) between the late luteal and post-egg laying periods. In cytosol extracts (Figure 4A), only one specific band of 115 kDa (PR-B) was observed during the months of June through August (the gravid and post-egg laying periods). No reactive bands were seen at these molecular weights in the mouse IgG controls (Figure 3B and 4B).

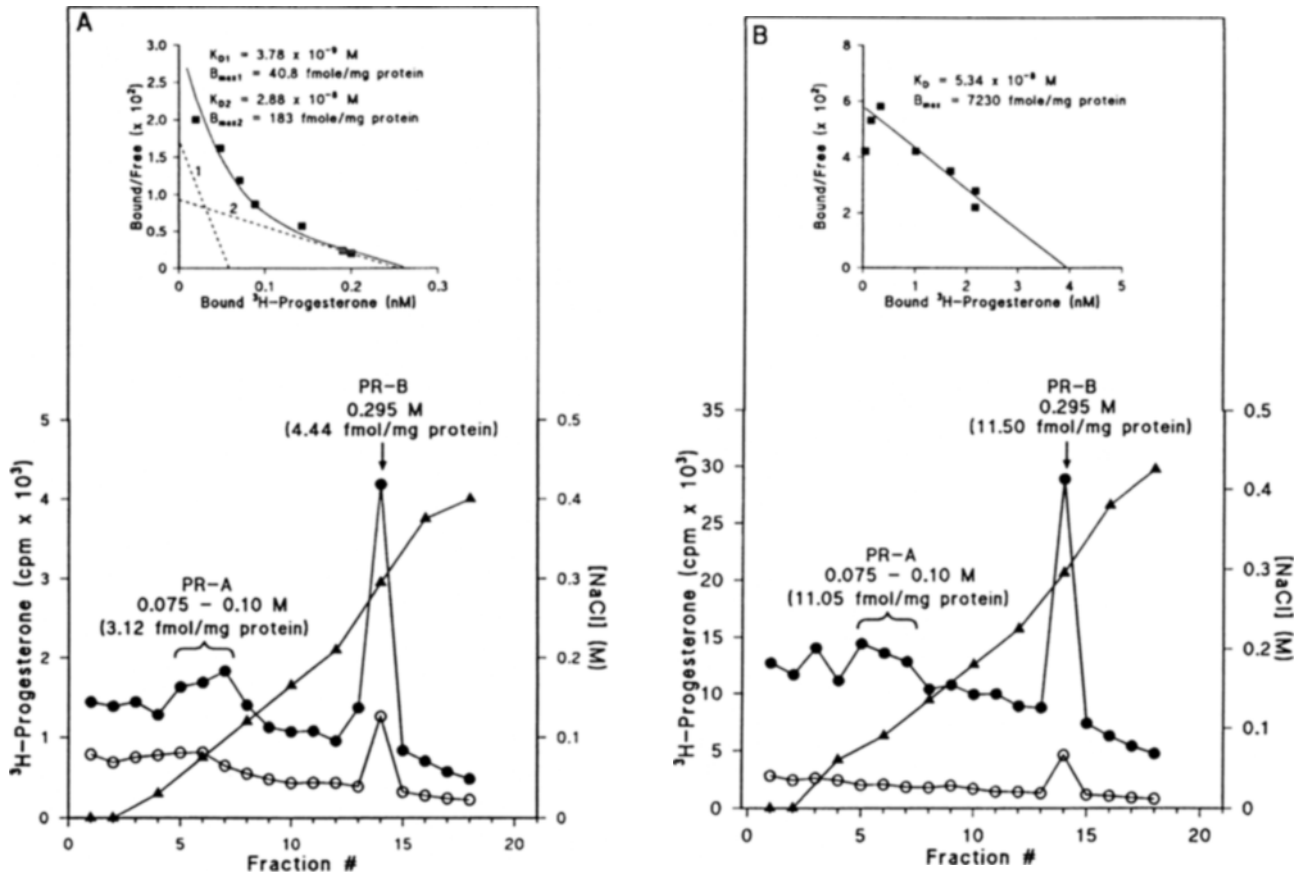

Figure 2 DNA-cellulose affinity chromatography of oviduct PR using nuclear extracts containing high and lower affinity sites (**A**) or cytosol extracts containing only the lower affinity sites (**B**), as determined by Scatchard analysis (**A** and **2** inserts). The Lundon ReceptorFit Saturation Two-Site program revealed a Scatchard plot with either two parameters (insert **A**, dashed lines $K_{d1} = 3.78 \times 10^{-9}$ M, $B_{max1} = 40.8$ fmol/mg protein; $K_{d2} = 2.88 \times 10^{-8}$ M, $B_{max2} = 183$ fmol/mg protein) or one parameter (insert 2B: $K_d = 5.34 \times 10^{-8}$ M, $B_{max} = 7230$ fmol/mg protein). In nuclear extracts containing two binding sites (**A**) and cytosol extracts containing only the lower affinity binding site (**B**), two specific peaks eluted from DNA-cellulose at 0.075–0.10 M NaCl (nuclear: 3.12 fmol/mg protein; cytosol: 11.05 fmol/mg protein) and 0.295 M NaCl (nuclear: 4.44 fmol/mg protein; cytosol: 11.50 fmol/mg protein). Total binding (●); non-specific binding (○); NaCl gradient (▲)

ICC analysis of PR distribution in turtle oviduct (Figure 5)

The same monoclonal antibody, PR 6, was used to monitor the changes in PR distribution throughout the tissue. Both ICC and structural changes were observed in the three major zones in uterine cross-sections during the seasonal cycle.

Immunocytochemical PR was localized in the nuclei of the epithelium, submucosal glands, and myometrium. During the winter (February, Figure 5C–D), the receptor was mainly present in the nuclei of the epithelial cells with minor staining in the submucosal glands and myometrium. Associated with the April E peak, mucosal gland PR became more apparent as did the intensity of reaction product in myometrial layers (Figure 5E–F). By May (Figure 5G–H), the amount of epithelial and submucosal glands increased in mass along with the amount of PR staining. Parallel to the increase in PR staining in May, a significant increase in PR binding was also first observed (Figure 1A). After ovulation (early luteal) (Figure 5I–J), the epithelium was intensely stained, the submucosal glands and staining were reduced, and myometrial staining appeared to be maintained. Near the end of the gravid period (late luteal) (Figure 5K–L), the epithelium and submucosal glands were reduced; a reduced level of epithelial staining was evident, and although the glandular region was reduced, nuclei remained stained and less dispersed, and no visible staining was observed in the myometrium. In August through October (Figure 5M–R), the epithelial and submucosal glands began to re-develop and PR reaction product was strong; myometrial staining reappeared.

Discussion

These results demonstrate seasonal changes in PR by radioligand and immunochemical methods. The observed changes correlate with the seasonal fluctuations in plasma steroid levels and oviduct structure and weight in the turtle, and are consistent with a role for P in the final stages of egg formation (secretion of proteins and shell) through a mucosal site of action. Although changes in myometrial immunocytochemical PR are less marked than in the mucosa, the apparent disappearance of PR from the myometrium just prior to egg-laying is consistent with the postulated role of P in retaining eggs in the oviduct during the luteal phase (Callard *et al.*, 1992). In the chick, regulation of lysozyme, ovalbumen, and avidin production by steroids has been extensively investigated (Kohler *et al.*, 1968; Oka & Schimke, 1969a,b; Mester & Baulieu, 1984). Of these proteins, only avidin, a marker for P action in the chick (Joensuu *et al.*, 1990), has been identified in the reptile (Botte *et al.*, 1974). These studies support the concept of a functional PR involved in both secretory and myometrial activity in the oviduct of chelonian species, which evolved around 200 million years before present.

PR binding in the turtle consists of two binding components comprised of high (3.78×10^{-9} M) and lower (2.88×10^{-8} M) affinity sites. Earlier biochemical studies have shown that the PR in the avian oviduct (Maggi *et al.*, 1984) and human breast cancer cell line (T47D cells; Sarup *et al.*, 1988) contain both the high (10^{-9} M) as well as the lower

Table I Competition study on oviduct lower affinity PR sites

Cold steroid	*% Inhibition lower affinity site* 10×	100×	1000×	*% Inhibition high affinity site* 10×	100×	1000×
Progesterone	46.1	81.6	98.2	75.4	83.1	85.6
Promegestone (R5020)	0	63.4	93.1	79*	93*	
RU 486	0	23.7	73.6	0.1	1.2	13.1
Pregnenolone	20.5	73.2	92.1	79*	90*	
Deoxycorticosterone	19	66.3	94.1	73*	93*	
Dihydrotestosterone	0	56.1	85.7	10*	55*	
Testosterone	0	35.2	87.5	4	33.3	
Mibolerone	0	13.2	64.2	18	48.6	
Estradiol	6.5	32.1	81.9	0	0	25.9
Corticosterone	0	26.5	74.6	3*	30*	

One hundred μl of DCC stripped cytosol extracts, diluted 1:30, were incubated overnight at 4°C with 80 nM ^{3}H-P in the presence of 10×, 100× and 1000× competitors. Data is presented as % inhibition of ^{3}H-P by competitor. In addition, a competition study for the high affinity site as previously published by Reese & Callard (1989), using 1 nM ^{3}H-P, with additional data from this study, using 8 nM ^{3}H-P, is shown for comparison (*)

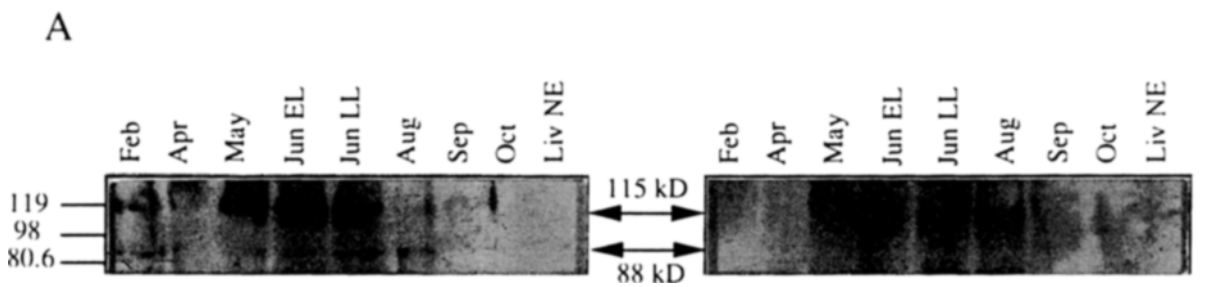

Figure 3 Western blot analysis of oviduct PR in nuclear extracts during the seasonal cycle. Equal volumes (26 μl) of each sample were run on 5–20% gradient SDS–PAGE gels. Samples were then transferred onto nitrocellulose, incubated with 12.5 μg/ml of either anti-PR 6 (**A**) or mouse IgG (**B**). Early luteal, EL; late luteal, LL

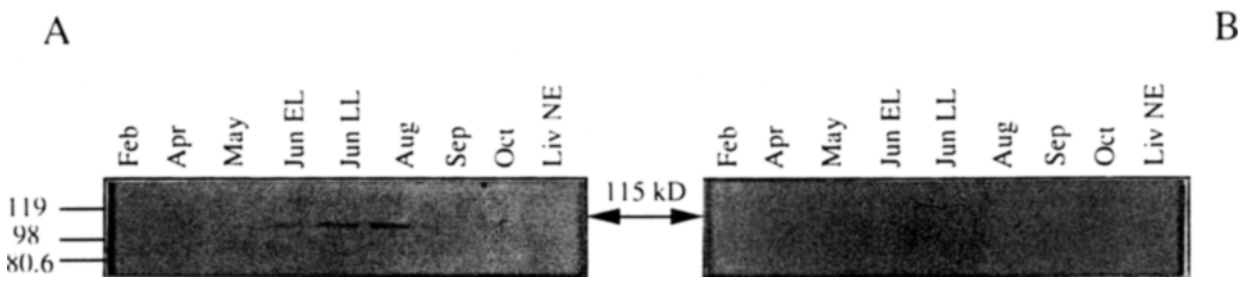

Figure 4 Western blot analysis of oviduct PR in cytosol extracts during the seasonal cycle. Equal volumes (40 μl) of each sample were run on 5–20% gradient SDS–PAGE gels. Samples were incubated with 12.5 μg/ml of either anti-PR 6 (**A**) or mouse IgG (**B**). Early luteal, EL; late luteal, LL

(10^{-8} M) affinity binding components. The high affinity sites have been shown to be the active receptor, but the role of the lower affinity binding sites is not clear. Attempts were made to separate the two sites in the avian system (DEAE-cellulose and DNA-cellulose chromatography, and Sephadex G-100 gel filtration chromatography; Maggi *et al.*, 1984) and in T47D cells (non-ionic detergent cell extraction *vs*/tissue homogenization; Sarup *et al.*, 1988) with no success. In the avian system, the low affinity sites could be selectively destroyed with the use of alkaline phosphatase and chronic stimulation of chick oviduct with DES resulted in an increase of lower affinity sites which disappeared after DES withdrawal. In the T47D cells, high ionic strength buffer converted low affinity binding to high affinity binding.

As demonstrated by Riley *et al.* (1988), the lower and high affinity sites of the turtle hepatic PR cannot be separated using affinity chromatography or DEAE Sepharose. A typical biphasic Scatchard plot was obtained from eluates of both A and B isoforms. Although Reese & Callard (1989) did not verify this for the turtle oviduct, this was the most likely due to methodological and procedural differences in the two studies. In the present study, quantitation of the two binding sites revealed significant differences within and between the nuclear and cytosolic compartments. Thus, high affinity sites can only be detected in the nucleus, and at three times of the year: (a) in the peri-ovulatory and luteal phases, (b) during the late summer–early fall ovarian recrudescent phase and (c) winter hibernation period. The luteal phase corresponds to the time during which eggs are held in the oviducts for about 2 weeks in which they accumulate albumen, ovalbumen, avidin and a shell. The presence of high affinity PR in the nucleus at this time is consistent with exposure of the oviduct to high E in the previous follicular phase and the induction of PR; further, the high affinity PR is consistent with its suggested physiologic importance in inhibition of turtle myometrial activity associated with egg retention (Callard & Hirsch, 1976; Callard *et al.*, 1994) and oviductal secretion (Motz & Callard, 1991; see Discussion). The disappearance of the high affinity sites in the late luteal phase is further consistent with down-regulation of PR by

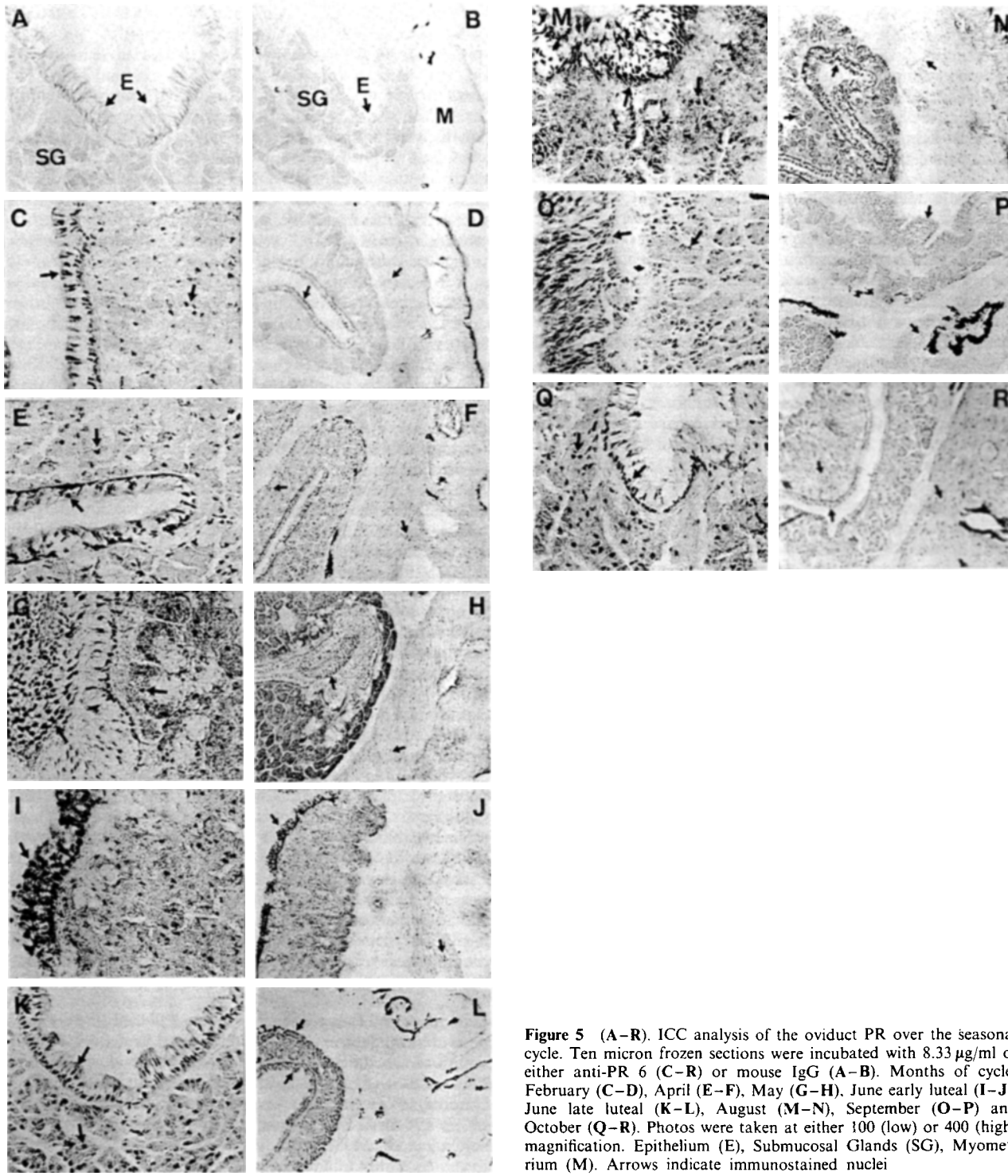

Figure 5 (**A–R**). ICC analysis of the oviduct PR over the seasonal cycle. Ten micron frozen sections were incubated with 8.33 μg/ml of either anti-PR 6 (**C–R**) or mouse IgG (**A–B**). Months of cycle: February (**C–D**), April (**E–F**), May (**G–H**), June early luteal (**I–J**), June late luteal (**K–L**), August (**M–N**), September (**O–P**) and October (**Q–R**). Photos were taken at either 100 (low) or 400 (high) magnification. Epithelium (E), Submucosal Glands (SG), Myometrium (M). Arrows indicate immunostained nuclei

high levels of luteal P and is necessary to allow mature eggs to be laid under the influence of neurohypophyseal hormones (Callard *et al.*, 1992). The reappearance of high affinity sites during the ovarian recrudescent phase correlates with the presence of both E and P in the circulation as vitellogenesis begins again.

Lower affinity binding sites were detected in nuclear extracts at all times of the year, and the number of sites was double the number of high affinity sites when both were present in the nucleus. Lower affinity, but not high affinity sites, could also be detected in the cytosol at all times; however, the amount of the lower affinity sites in the cytosol was far in excess of either nuclear high or lower affinity sites (cytosol: nuclear lower affinity sites, 16 to 68:1; nuclear low: high affinity sites, 2 to 20:1). Even though high affinity sites may be undetectable by Scatchard analysis in cytosol, both a high salt and a low salt binding component (putative B and A isoforms) can be eluted from DNA-cellulose affinity columns; the same is true of nuclear fractions in which both the high and lower affinity components are detected by Scatchard analysis. Since both high and low salt forms of receptor exhibit high and lower affinity sites (Riley *et al.*, 1988), we have to assume that PRs are present even when high affinity nuclear sites are undetectable by Scatchard analysis. The reason for their undetectability by Scatchard analysis is not yet clear but may relate to both amount of receptor, its form (activated/unactivated), and in ICC studies, availability of antibody binding sites.

As seen with high affinity sites, nuclear lower affinity sites peak in the peri-ovulatory/luteal period and may be regulated by fluctuating plasma steroid levels. However, based on immunocytochemistry, the PR is present at all times of year (see Discussion). The year round presence of nuclear and cytosolic lower affinity sites, as determined by radioligand binding studies, would account for the presence of the PR (though not in all oviductal tissues simultaneously), but the high affinity sites can only be detected by radioligand studies when most abundant or in the appropriate configuration (i.e. 'activated' receptor). Because lower and high affinity sites are difficult to separate, suggesting they reside on the same molecule, these studies support a change in conformation of the PR which influences the availability of high affinity binding sites. Since the latter can be demonstrated only at or around the time of peak steroid levels, and only in the nucleus, this suggests a physiological role for the high affinity binding site. The role of the lower affinity binding site is not clear, but based on its binding specificity and affinity it could act by maintaining adequate levels of P in the immediate vicinity of the high affinity site and ensure saturation and activation. When P levels are low, the large size of this pool of P binding sites may ensure a certain degree of activation of high affinity sites.

The results of Western blot analysis of oviduct PR support the idea that a single protein or protein complex is responsible for both high and lower affinity binding, but suggest some differences in A and B isoform distribution from nuclear to cytosolic fractions during the annual cycle. Thus Western blots reveal a band migrating at 115 kDa (PR-B) in cytosol extracts when no high affinity component is revealed by Scatchard analysis. Western blots show that the major nuclear and cytosolic form is PR-B since the 115 kDa band is seen in both. However, a less distinct 88 kDa (PR-A) band is seen in nuclear extracts from June and August animals. Although this appears to conflict with the result one might expect from the use of the avian B isoform-specific anti-PR 6 antibody, this may be explained on the basis of differences in length of turtle and chicken PR-A (truncated isoform). Thus the estimated size for turtle PR-A is 88 kDa *vs* 79 kDa for the chicken PR-A (Coty *et al.*, 1979) and suggests the possibility that at least part of the reactive immunogenic epitope (which enables a differentiation between chicken PR-B and PR-A to be made) is present at the amino terminus region of turtle PR-A. Failure to detect any PR-A in cytosol by Western blots despite large numbers of low affinity sites may reflect sensitivity differences of radioligand vs blotting techniques. Cytosolic PR-B can only be seen by blotting in June and August despite the presence of lower affinity binding sites (Scatchard analysis) at all times of the year. That PR 6 reactive material (PR-B and possibly PR-A) can be seen in tissue by ICC in all samples supports the presence of PR year around, and that variations in A/B isoforms may occur in a tissue specific and seasonal manner. These observations serve to underscore the need for a combination of methods to be used simultaneously for an understanding of seasonal fluctuations in PR in different tissues.

As indicated above, using immunocytochemical detection, PR is seen in the turtle oviduct at all times of the year. The principal tissues of interest are the lumenal epithelium, the sub-epithelial glandular mucosa, consisting of tubuloalveolar glands, and the myometrium, consisting of circular and longitudinal layers. Although there appear to be differences in intensity of reaction, all layers are immunostained to a certain degree with the exception of the myometrium which does not appear to be immunostained just prior to egg laying (late luteal). Given the density of nuclei in the two regions, it is clear that most of the receptor is present in the epithelial and sub-mucosal zones, where it presumably is involved in the transcriptional regulation of the secretion of ovalbumen, avidin and shell material (Motz & Callard, 1991). Sub-mucosal gland immunostaining increases from February (reproductively inactive) to April and May (follicular/estrogenic phase) and secretory granules, plentiful in May, (preovulatory) are discharged after ovulation (early luteal); by the late luteal phase, the sub-mucosal zone has significantly reduced in diameter. PR immunostaining remains in the sub-muscosal region, although the tubuloalveolar glands do not begin to reorganize morphologically until October. The epithelium has variable numbers of PR immunostained nuclei, but epithelial immunostaining is particularly strong in the preovulatory and early post-ovulatory stages. This correlates with the functional role of the epithelium in the secretion of albumen and mucus, essential for lubrication of the passage of the eggs down the oviduct (Motz & Callard, 1991). PR changes in different oviduct cells and tissues were not synchronous, suggesting selective regulation of PR by P and other tissue factors. Selective down regulation of this kind has been observed by ICC in the Rhesus monkey (Okulicz *et al.*, 1993) and cat (Li *et al.*, 1992). The low levels of E present in the circulation may be adequate to maintain PR at these stages of the cycle.

Although there are differences in the spectrum of steroid binding affinities between the lower and high affinity binding sites, they are quite similar. At 100 × competitor, P is the most effective in displacing label from the lower affinity site. In contrast, the high affinity site has a similar affinity for all four steroids (P, pregnenolone, deoxycorticosterone and R5020). Affinity for androgens (testosterone, dihydrotestosterone), characteristic of PR, is moderate, with mibolerone being a somewhat better ligand for the high affinity than lower affinity site. Mibolerone, a synthetic androgen, has been shown to have high affinity for the PR in the human prostate and rabbit uterus (K_d = 5.9 and 1.1 nM, respectively; Murthy *et al.*, 1986), but has no apparent affinity for the elasmobranch PR (Cuevas & Callard, 1992). Interestingly, RU 486 does not bind to the high affinity site to any significant degree, although its binding to the low affinity site is in the same range as for the androgens, E and corticosterone. The turtle PR thus can be added to the chick (Moudgil *et al.*, 1986), hamster (Gray & Leavitt, 1987), and tammar wallaby (Fuller *et al.*, 1994) in not binding this synthetic steroid which has such high affinity for the human PR and glucocorticoid receptor (Baulieu, 1989). Although the possibility of an intracellular steroid binding protein (SBP) should be considered in the context of the lower affinity site, it is highly unlikely that they are one and the same. The binding specificity of turtle SBP is quite different; further, SBP does not bind to DNA-cellulose (Salhanick *et al.*, 1979).

In summary, changes in oviduct PR occur during the normal reproductive cycle of the turtle. The use of a combination of techniques appears to indicate the presence of a PR in the oviduct at all times of the year tested (February to October). Based on this and prior work (Reese & Callard, 1989), PR exists as PR-A and PR-B in turtle oviduct, these being low (88 kDa) and high salt (115 kDa) forms, respectively. The high affinity site can only be detected by radioligand studies in luteal phase animals when P is considered important in the retention of eggs and concomitant oviduct secretory activity, and when follicular growth resumes in the fall. The role of the cytosolic lower affinity binding site, present in such large numbers, is enigmatic at present; however, it may represent the processed receptor at a different phosphorylation state, or the receptor in combination with a chaperoning molecule such as hsp 70 or 90 (Frudman *et al.*, 1994). This may maintain the cytosolic receptor in a folded conformation which either alters or partially obscures the high affinity binding site depending upon ligand availability. These results are consistent with the hypothesis that progesterone and the progesterone receptor have been key elements in the evolution of vertebrate reproductive tract secretory and contractile functions.

Materials and Methods

Animals

Animals were freshly caught during February ($n = 6$), April ($n = 4$), May ($n = 4$), June (early luteal, $n = 4$; late luteal, $n = 6$), August ($n = 4$), September ($n = 4$) and October ($n = 3$) (Lemberger, WI) and shipped to Boston. Experimental animals were maintained in fresh running water aquarium tanks at 24°C (water), 22°C (air) on a 12 h L/12 h D photoperiod. Animals were fed Wardley reptile 'Total essential nutrition floating food sticks' (Wardley Corp., Secaucus, NJ).

Reproductively active animals (April–October) were classified on the basis of ovarian and follicular size at autopsy and the presence/absence and condition of oviductal eggs as determined by this laboratory. April animals were considered preovulatory on the basis of follicular size (15–17 mm) and plasma E levels. May–August animals were segregated based on (a) absence of eggs in the oviduct and size of ovaries, (b) eggs in the oviduct and (c) having laid eggs in the laboratory (average clutch size: 9–12 eggs in a single egg-laying episode). These were referred to, respectively, as peri-ovulatory (maximal follicular size ~18 mm) (a), post-ovulatory/gravid (b), and post-oviposited (eggs laid) (c). For group b (gravid animals), several criteria were used in order to assign animals to one of two post-ovulatory, but pre-oviposited, groups: early luteal and late luteal. Animals in these two groups were initially assigned on the basis of hardness of egg shell (i.e. flexibility and thickness based on direct visual inspection) and subsequently on the basis of plasma P (high P being indicative of luteal function) and oviduct receptor content (positive or negative high affinity binding).

Chemicals and Reagents

Radioactive [1,2,6,7-^{3}H] progesterone, P, (specific activity = 80.2–111.1 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Radioactive [2,4,6,7-^{3}H] estradiol-17β (E) (specific activity = 103–108 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Radioinert steroids used were P (Nutritional Biochemicals Corporation, Cleveland, OH); R5020 and mibolerone (New England Nuclear, Boston, MA); RU 486 (Roussel Uclaf, France); corticosterone and pregnenolone (Sigma, St Louis, MO); E, 5α-dihydrotestosterone, testosterone and deoxycorticosterone (Steraloids, Inc., Wilton, NH). Radioactive P and E were repurified before use by thin layer chromatography using either a benzene-acetone (4:1, vol/vol) system for P, or an ether-hexane (3:1, vol/vol) system for E. Stock solutions of hormones were prepared in absolute ethanol and stored at −20°C. Estradiol-17β and progesterone antisera for radioimmunoassay (RIA) analysis were provided by Dr G Niswender (Colorado State University, Fort Collins, CO). A monoclonal antibody, anti-PR 6, raised against the chick progesterone receptor B isoform was provided by Dr D.O. Toft (Mayo Clinic, MN). Vectastain ABC Kit (biotinylated mouse IgG) was purchased from Vector Laboratories, Inc. (Burlingame, CA). Sephadex LH-20, Sephadex G25 and Dextran T-70 were purchased from Pharmacia (Piscataway, NJ). Acrylamide, N-N'-methylene-bis-acrylamide and other electrophoretic materials were from Bio-Rad (Richmond, CA). Norit A charcoal, prestained protein molecular weight markers, heparin sodium salt from bovine intestinal mucosa, mouse IgG purified immunoglobulin from serum, rabbit anti-mouse IgG (whole molecule) peroxidase conjugate and 3', 5' diaminobenzidine were from Sigma (St Louis, MO). Enhanced chemiluminescence (ECL) Western blot detection reagents were purchased from Amersham (Arlington Heights, IL). Optifluor scintillation cocktail was obtained from Packard (Meriden, CT). Kodak X-OMAT film was purchased from Eastman Kodak Company (Rochester, NY). Other chemicals were reagent grade or better.

The buffers used were as follows: TEMG buffer (10 mM Tris-Base, 1 mM EDTA, 1 mM 2-mercaptoethanol, 10% glycerol, pH 7.5); homogenizing buffer (50 mM Tris-HCl, 1 mM EDTA, 12 mM monothioglycerol, 30% glycerol, pH 7.5). The nuclear extraction buffer used was 0.7 M KCl in homogenizing buffer according to Chen & Leavitt (1979). The nuclear washing buffer consisted of 10 mM Tris-Base, 12 mM monothioglycerol, 3 mM $MgCl_2$, 0.25 M sucrose at pH 7.5. Buffers for DNA cellulose were as follows: 200 mg/l BSA + 10 mM sodium molybdate in TEMG (Buffer A); 0.5 M NaCl + 200 mg/l BSA + 10 mM sodium molybdate in TEMG (Buffer B). Dextran-coated charcoal (DCC) was made up in TEMG (0.5% washed Norit-A charcoal and 0.05% Dextran T-70). Western buffer consisted of 20 mM Tris-Base, 150 mM NaCl, 0.5% Tween 20, 1.0% BSA with a pH of 7.5 (Sullivan *et al.*, 1988). PBS buffer consisted of 154 mM NaCl, 9.15 mM sodium phosphate dibasic anhydrous and 33.3 mM sodium phosphate monobasic anhydrous, pH 7.2. RIA buffer contained 5.9 mM K_2HPO_4, 3.97 mM KH_2PO_4, 139.9 mM NaCl, 15.4 mM NaN_3 and 0.1% gelatin (g/vol), pH 7.4.

Preparation of cytosol and nuclear extracts

All procedures were carried out at 0–4°C. After decapitation, the ventral plastron was removed to expose the body cavity. The entire oviducts (glandular, intermediate and uterine portions) were removed and washed three times in 0.65% NaCl TEMG buffer. They were weighed, minced and homogenized in 4 vol (vol/g) homogenizing buffer using 5 bursts (5 s each) with a Polytron PT10/35 (Brinkmann Instruments, Westbury, NY) on a rheostat setting of 5. The homogenate was centrifuged at 1000 *g* for 15 min. The surface lipid was removed, and the supernatant was centrifuged at 100 000 *g* for 1 h (37 000 r.p.m. rotor T-865.1, Sorvall OTD-65 centrifuge, Du Pont Instruments, Wilmington, DE) to obtain crude cytosol, which was frozen at −70°C. The pellet was washed 3× in washing buffer with centrifugation at 1000 *g* for 15 min at 4°C between each wash. The pellet was then re-suspended in 4 vol (vol/g) of nuclear extraction buffer and incubated for 1 h on ice with vortexing every 15 min. The final nuclear suspension was centrifuged at 100 000 *g* for 60 min to yield a clear nuclear extract. Samples were stored at −70°C until analysis.

Sephadex LH-20 Chromatography

Sephadex LH-20 was swollen in distilled water, packed into 5 × 105 mm acid washed columns, and equilibrated with TEMG (cytosol) or 0.5 M KCl TEMG (nuclear extracts) buffer at 4°C. Chromatography was performed according to Mak *et al.* (1983). Briefly, 100 µl cytosol (or 200 µl nuclear extract) was applied and allowed to adsorb onto the equilibrated columns. A further 200 µl buffer was added to columns and allowed to adsorb. The columns were sealed and incubated 30 min at 4°C followed by elution of sample with 800 µl of TEMG (cytosol) or 0.5 M KCl TEMG (nuclear extracts) buffer. Optifluor (4 ml) was added and radioactivity measured in a Delta Liquid Scintillation Counter (Tracor, Elk Grove, IL; efficiency for tritium, 60%). Values were expressed as fmole steroid bound per mg protein in extract as determined by method of Lowry *et al.* (1951).

Saturation analysis of [^{3}H] progesterone binding

To remove endogenous steroids, cytosol was charcoal stripped prior to analysis by incubating for 10 min at 0°C with a DCC pellet derived from a suspension equivalent to sample volume followed by centrifugation (2800 r.p.m. × 5 min at 4°C). Stripped cytosol was diluted 1:10–1:40 in TEMG buffer, and nuclear extracts were diluted 1:5 in 0.5 M KCl

TEMG. Aliquots of diluted cytosol (100 µl) and nuclear extracts (200 µl) were incubated with 1–100 nM ^{3}H-P (total binding) and 1–100 nM ^{3}H-P plus 400-fold radioinert P (non-specific binding) for 16 h at 4°C. Samples contained 1 µM corticosterone to prevent P binding corticosterone binding globulin-like components (Chen & Leavitt, 1979). Bound and free steroids were separated by LH-20 chromatography. Specific binding was calculated by subtracting non-specific from total binding. Appropriate cytosol dilution of each sample was determined prior to saturation analysis by a mini-Sephadex LH-20 assay as follows: cytosol was diluted 1:10–1:40 and 100 µl of each dilution was incubated with 8 nM ^{3}H-P (previously determined concentrations for saturation of high affinity P binding component) plus 1 µM corticosterone in the presence (non-specific binding) or absence (total binding) of 400-fold radioinert P for 16 h at 4°C followed by Sephadex LH-20 chromatography. The dilution exhibiting the highest ^{3}H-P binding was chosen for subsequent saturation analysis. Data was analysed by the Lundon ReceptorFit Saturation Two-Site program (Lundon Software, Inc., Chagrin Falls, OH).

Competition analysis

One hundred µl of diluted and DCC stripped cytosol and 200 µl nuclear extract were incubated with 8 nM or 80 nM ^{3}H-P with increasing concentrations (10-, 100- and 1000-fold excess) of radioinert competitors for 16 h at 4°C. Bound and free steroids were separated by Sephadex LH-20 chromatography. Percent inhibition was calculated by subtracting the percent specific ^{3}H-P bound in the presence of each competitor from total ^{3}H-P (100%).

Sephadex G-25 de-salting

Sephadex G-25 was swollen in distilled water and packed into a 1 × 10 cm column (Pharmacia) and equilibrated with 10 vol of TEMG buffer. Nuclear extracts were applied to the column and eluted with TEMG. A Beckman DU-70 Spectrophotometer (UV_{280}) was used to isolate fractions containing the receptor, and the conductivity of the samples was then monitored. Samples having a conductivity of 0–0.05 M KCl were pooled and used for DNA-cellulose affinity chromatography.

DNA-cellulose affinity chromatography

DNA-cellulose was prepared according to Alberts & Herrick (1970), and procedures similar to those by Salhanick *et al.* (1979) were used with modifications. The post-labelling method was used. Diluted cytosol (1 ml) or de-salted nuclear extract (1 ml) was applied and allowed to adsorb onto DNA-cellulose columns (5 × 120 mm) equilibrated with DNA-cellulose buffer A. The columns were incubated at 22°C for 30 min followed by 4°C for 10 min. The columns were washed with buffer A for 3 h using a Rainin Rabbit peristaltic pump (Rainin Inst. Co., Inc., Boston, MA) set at a 4 ml/h rate. Twenty nM (nuclear extract) or 80 nM (cytosol) ^{3}H-P plus 1 µM corticosterone in the presence (non-specific binding) or absence (total binding) of 400-fold radioinert P in 1 ml buffer A was added and adsorbed onto the columns followed by a 16 h incubation at 4°C. The columns were then washed for 6.5 h with buffer A before 1 ml fractions were eluted with a linear 0–0.5 NaCl gradient using buffers A and B. Salt concentration was determined by Conductivity Meter CDM3 (The London Co., Cleveland, OH) and radioactivity by scintillation counting.

Immunocytochemistry (ICC)

Oviducts were removed and the uterine portion was infiltrated with 10% sucrose/PBS buffer for 6 h followed by freezing in Tissue Tek OCT Compound (Miles Inc., Elkhart, IN) and stored at −70°C. Ten micron fresh frozen sections were obtained on an AO Cryo-Cut Cryostat Microtome Model 840C (American Optical Corporation, Buffalo, NY) and air dried on gel coated slides for 10 min. The sections were fixed in 4% paraformaldehyde/PBS buffer for 10 min, washed 10 min in PBS, followed by 2 min in 0.05% H_2O_2/PBS. The sections were washed 10 min in PBS followed by 20 min in blocking solution (Vector ABC Kit). Application of primary antibody, 8.3 µg/ml anti-PR 6 or mouse IgG, for 24 h was terminated by a 15 min PBS wash followed by the application for secondary antibody labelled with biotinylated complexes (Vector ABC Kit) for 30 min. A 10 min PBS wash was followed by a 30 min application of avidin complexed with peroxidase. The sections were washed 10 min in PBS followed by a 2 min exposure of 3′, 5′ diaminobenzidine (5 mg/ml in Tris-HCl, pH 7.2) in the presence of 0.025% H_2O_2 which resulted in the precipitation of a brown chromogen. The reaction was stopped by distilled water (10 min) followed by a series of 2 min ethanol washes: 70%, 2x; 95%, 2x; 100%, 2x. The sections were dehydrated with 2, 5 min washes in xylene and cover slips mounted with Permount (Fisher, Fair Lawn, NJ).

Ammonium sulfate precipitation of progesterone receptor (PR)

Cytosol and nuclear PR was precipitated with 50% ammonium sulfate incubated for 30 min at 4°C on a rocker platform. Extracts were centrifuged at 4000 *g* (IEC centra MP4R, Fisher Scientific, Pittsburg, PA). The pellet was washed with distilled water and reconstituted in 100 µl distilled water. Protein concentration was determined by method of Lowry *et al.* (1951).

Western blotting procedure

Equal volumes of cytosol and nuclear extracts were precipitated and resolved by electrophoresis on discontinuous polyacrylamide gels according to the method of Laemmli (1970) using a 5–20% gel gradient. The Western procedure was according to Sullivan *et al.* (1988) with modifications as follows: the proteins were transferred onto nitrocellulose (Schleicher and Schuell, Keene, NH) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio Rad, Richmond, CA) for 45 min at 15 V. The nitrocellulose was blocked for 30 min at 37°C in Western buffer. The nitrocellulose was incubated with primary antibody (anti-PR 6 or mouse IgG) at a concentration of 12.5 mg/ml Western buffer overnight at 4°C on a rocker platform. The nitrocellulose was washed 3 times (15 min, 1x; 5 min, 2x) in Western buffer and incubated with horse radish peroxidase (HRP) conjugated rabbit anti-mouse IgG diluted 1:1000 or 1:5500 in Western buffer at 22°C for 4 h on a rocker platform. The nitrocellulose was washed 3 times (15 min, 1x; 5 min, 2x) in Western buffer followed by 1 min incubation in ECL Western blotting reagents. Bands were exposed on Kodak film.

Plasma steroid extraction procedure

Animals were sacrificed by decapitation and blood was collected into heparinized test tubes. Blood was centrifuged at 2800 r.p.m. for 10 min to obtain plasma and subsequently stored at −20°C until extraction. Steroids were extracted from plasma by methods routinely used in this lab (Callard *et al.*, 1978). Briefly, P and E were extracted from 0.5 ml turtle plasma with 2, 10 vol diethyl ether. To check recovery of steroids from plasma, 1000 c.p.m.s. of each radiolabelled tracer were extracted from an equal volume of DCC stripped turtle plasma (Venkatesh *et al.*, 1989). Plasma samples were vortexed for 30 sec and centrifuged for 15 min at 2800 r.p.m. at 4°C. After a clear separation of the two phases, the lower aqueous phase was quick frozen by immersing the test tube in a dry-ice methanol mixture. The organic layer was decanted into clean 16 × 100 mm test tubes and dried under

a stream of nitrogen. The steroids were resuspended in the original 0.5 ml volume with RIA buffer.

Steroid Radioimmunoassay (RIA)

P and E levels were determined as previously described by Callard *et al.* (1978) using antibodies obtained by Dr G. Niswender. Aliquots of extracted steroids (2–50 µl) diluted to a final volume of 100 µl were incubated with 100 µl of the appropriate radiolabelled tracer (10 000–15 000 c.p.m.s.) and 200 µl of diluted antisera for a final incubation volume of 400 ml, overnight at 4°C. The final incubation dilution of antisera was as follows: P 1:35–1:54, maximum binding 32–57%; and E 1:30–1:35, maximum binding 30–48%. Bound and free steroids were separated using 400 µl DCC suspension in RIA buffer. Bound radioactivity was measured in a Delta Liquid Scintillation Counter (Tracor, Elk Grove, IL; efficiency for tritium, 60%). Assay sensitivity was 3.9 pg for P and E. The interassay coefficient of variation was 13.5% for P and 13.75% for E.

Acknowledgements
Supported by NIHRR 06633 to IPC.

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