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PROGESTERONE CONTROL OF NUCLEAR ESTROGEN RECEPTOR: DEMONSTRATION IN HAMSTER UTERUS DURING THE ESTROUS CYCLE AND PSEUDOPREGNANCY USING A NEW EXCHANGE ASSAY

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Summary—Our previous studies showed that total nuclear estrogen receptor (Re) can be extracted and measured by exchange using 10 mM pyridoxal-5′-phosphate (PLP) at low temperature (0–4°C). In order to further validate the PLP assay, we measured the Re concentration in uterine cytosol and nuclei by this method under physiological conditions, i.e. during the hamster estrous cycle and pseudopregnancy. In addition, we compared the Re results obtained by the PLP method with those obtained with two other assay procedures, i.e. the KCl and NaSCN methods.

During the follicular phase of the estrous cycle, all three methods showed an elevation of nuclear Re in parallel with the increase in serum estradiol (E). However, the quantity of nuclear Re obtained with the PLP method was significantly greater than with the KCl method during the follicular phase. The surge of serum progesterone (P) during the ovulatory phase of the estrous cycle was followed by a dramatic fall in nuclear Re, and the greatest loss of nuclear Re during the ovulatory phase of the cycle was detected with the PLP and NaSCN methods. On a DNA basis, cytosol Re increased significantly between Day 3 and proestrus and subsequently fell during the ovulatory phase of the cycle. P treatment of proestrus hamsters resulted in a rapid (< 4 h) loss of nuclear Re with little or no change in cytosol Re. Chronic P exposure during pseudopregnancy with serum E maintenance, resulted in a significant suppression of both cytosol and nuclear Re. Following P withdrawal, both cytosol and nuclear Re rose significantly (< 4 h), indicating that this effect of P was readily reversible. The results demonstrate that the Re detected under physiological conditions by the PLP method responds to both E action and P action, and that the PLP assay provides a greater recovery of Re as compared to the KCl assay.

INTRODUCTION

Reliable methods are needed to study target tissue receptors under physiological conditions. Various procedures have been used to measure uterine steroid receptors during the reproductive cycle in several species including the hamster [1, 2], human [3], monkey [4], mouse [5], rat [6, 7] and sheep [8]. However, few of these methods permit the simultaneous analysis of estrogen receptor (Re) and progesterone receptor (Rp) in target cell nucleus and cytosol. The original Re assay employed TE (Tris–EDTA) buffer which does not provide adequate Rp recovery. Rp stability is improved by addition of glycerol and monothioglycerol to the TE buffer system [1, 5]. In addition, the nuclear exchange procedure developed originally for the assay of total Re is performed with a suspension of intact nuclei [9], and this approach is not suitable for the assay of nuclear Rp since hormone–receptor complex is lost from nuclei during the assay [10, 11]. Thus, nuclear Re and Rp can be measured after KCl extraction from the nuclear fraction in Tris buffer containing glycerol [2, 10]. However, comparison of the KCl method with a new exchange assay using pyridoxal 5′-phosphate (PLP) revealed that greater recovery of nuclear Re was achieved with the PLP assay than with the KCl procedure [12]. Furthermore, our previous studies of hormonal control of the Re system showed that progesterone (P) specifically down regulates nuclear Re [13]. Since the PLP assay appeared to provide higher estimates of nuclear Re, it became important to determine whether different assay methods detected different populations of Re. Therefore, in the present study, we have evaluated the quantity and pattern of Re detected under physiological conditions by three different exchange assays, e.g. the PLP, NaSCN and KCl methods. Total Re was measured in uterine cytosol and nuclear fractions during the hamster estrous cycle and pseudopregnancy, and similar patterns of Re were found with all methods. However, evidence is presented to indicate that the PLP assay is the method of choice for improved recovery of nuclear Re particularly under conditions of hormone action during the estrous cycle and pregnancy.

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EXPERIMENTAL

Chemicals and buffer

[2,4,6,7-$^3H_4$] Estradiol-17β ([^{3}H]E; 110 Ci/mmol) was obtained from New England Nuclear Corp.,

Boston, MA, and stored in ethanol (100 μCi/ml) at −10°C. Radioinert steroid and pyridoxal 5′-phosphate (PLP) were from Sigma Chemical Co. (St Louis, MO). Sodium barbital was from Mallinckrodt (St Louis, MO). All other chemicals were obtained from standard commercial sources and were reagent grade or better.

Saline was buffered with 10 mM Tris–HCl (pH 7.4). Buffer A_{30} contained 50 mM Tris–HCl, 1 mM EDTA, 12 mM monothioglycerol, and 30% glycerol (v/v), pH 7.5; barbital buffer contained 20 mM sodium barbital and 5 mM dithiothreitol, pH 8.0; buffer TED contained 10 mM Tris–HCl, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.5. Dextran-coated charcoal (DCC) contained 0.5 g Norit A (Sigma) and 50 mg Dextran-70 (Pharmacia Fine Chemicals, Piscataway, NJ) in 100 ml of 10 mM Tris–HCl and 1 mM EDTA, pH 7.5. Scintillation counting solution was toluene–Triton X-100 (2:1, v/v) with 5 g diphenyloxazole (PPO) and 50 mg 1,4-*bis*-[2-(5-phenyl-oxazolyl)] benzene (POPOP) per liter.

Animal preparation

Adult female golden hamsters (Engle Labs, Farmersburg, IN) were housed under controlled conditions with a 14-h light, 10-h dark photoperiod (lights on from 0500–1900 h). The regularity of estrous cycles was established according to the appearance of the postestrous vaginal discharge (morning, cycle day 1).

Pseudopregnancy (PS) was induced by sterile mating with vasectomized males at estrus (PM cycle day 4), and the following day was considered day 1 of PS. On day 4 of PS, animals were anesthetized with pentobarbital (90 mg/kg BW) and ovariectomized. Decidualization was induced by mechanical traumatization of the endometrium by insertion of a 3-cm length of nylon filament into the lumen of each uterine horn. Estradiol (E) and progesterone (P) implants were prepared by packing crystalline steroid into 1 cm (E) or 2.5 cm (P) lengths of Silastic tubing (Type C; od, 2.50 mm; bore, 1.50 mm; New Brunswick Scientific, Inc., New Brunswick, NJ) and the ends were sealed with polymerized Silastic plugs. After 24 h of incubation in saline, E and P implants were inserted subcutaneously in opposing flank regions at the time of ovariectomy. P withdrawal on day 7 of PS was performed by removal of P implants under light ether anesthesia. Control animals were anesthetized in a similar manner.

Preparation of cytosol and nuclear extract

Animals were killed by cervical dislocation or by decapitation when trunk blood was collected. Uteri were removed rapidly, stripped of fat and mesentery, slit longitudinally, blotted, weighed, and placed in ice-cold buffered saline. In PS hamsters, myometrial tissue was harvested by gently scraping away decidual tissue with a spatula. All subsequent procedures were carried out at 0°C, unless otherwise indicated.

Tissues were rinsed and homogenized (1:6, w/v) in buffer (KCl method, A_{30} buffer; NaSCN method, TED buffer; PLP method, barbital buffer) with a Polytron Pt-10 homogenizer (Brinkman Instruments, Westbury, NY). The homogenate was centrifuged at 800 *g* for 15 min and the supernatant (cytoplasmic fraction) centrifuged at 170,000 *g* for 1 h to yield the cytosol fraction. The cytosol fraction was then diluted 1:1 with A_{30} buffer (KCl method), TED with 1 M NaSCN (NaSCN method), or barbital buffer with 20 mM PLP (PLP method). The nuclear fraction was washed twice by resuspension in the homogenization buffer with intervening centrifugations at 800 *g* for 15 min. Nuclear Re was extracted from the washed pellet by resuspension (1:12, w/v) in the following buffers; A_{30} plus 0.5 M KCl, TED buffer plus 0.5 M NaSCN, or barbital buffer plus 10 mM PLP. The nuclear suspension was incubated for 1 h with mixing at 15-min intervals, and centrifuged at 170,000 *g* for 1 h to remove nuclear debris. The resultant supernatant fraction was the nuclear extract used for the assay of Re.

Assay of Re in cytosol and nuclear fractions

Receptor concentrations were determined by Scatchard plot analysis of specific binding data as previously described [14]. Aliquots (300 μl) of cytosol or nuclear extract were incubated in a total volume of 500 μl with increasing concentrations of [^{3}H]E (0.19–3 nM) for determination of total binding. A parallel set of samples was incubated with unlabelled diethylstilbestrol (0.088–1.14 μM) for determination of non-specific binding. A final concentration of 0.5 M NaSCN and 10 mM PLP was used in the samples assayed by the NaSCN method and PLP method, respectively. Samples assayed by the KCl method were incubated for 1 h at 30°C to measure total Re (occupied and unoccupied), as previously described [2]. Total Re was assayed by the NaSCN [15] and PLP [12] methods by incubation of samples at 0°C for 24 h. After treatment of cytosol or nuclear extract, free steroid was removed from each sample by incubation with 500 μl DCC for 10 min, followed by centrifugation at 1500 *g* for 4 min. Radioactivity was counted in the supernatant. Specific binding was calculated (total binding minus nonspecific binding) and plotted according to Scatchard [16].

General methods

In samples containing PLP, 50 μl of 120 mM sodium borohydride was added to blanch the characteristic yellow color of PLP in order to prevent color quenching during scintillation counting.

DNA was determined according to Burton [17], using calf thymus DNA as standard.

Statistical analysis of results was performed by

analysis of variance and the Student–Neuman–Keuls' test.

RESULTS

We compared the Re detected in cytosol and nucleus of hamster uterus during the estrous cycle using three different exchange methods (Fig. 1). The concentration of nuclear Re detected by the PLP method (Fig. 1A) was significantly greater than that obtained with the KCl method during periods of elevated serum E titers, i.e. Day 3 and Day 4 (1100 and 1700 h). The NaSCN method showed a level of nuclear Re greater than the KCl method only on the morning of proestrus [Day 4, 1100 h] (Fig. 1A). The PLP and NaSCN methods gave a significantly higher level of cytosol Re on Day 1 (Fig. 1B). Although quantitative differences were observed between the Re results obtained with the KCl method and the PLP and NaSCN methods, the patterns of nuclear and cytosol Re during the estrous cycle were similar with all methods.

We further compared these methods with respect to the Re levels obtained following P treatment of the proestrous hamster and after P withdrawal during pseudopregnancy. The net loss of nuclear Re (pmol/g tissue) in response to P treatment of proestrous was greater at 4 h with the PLP and NaSCN assays (PLP, 1.31 and NaSCN, 1.32 vs KCl, 0.46) (Fig. 2A). Significantly higher levels of cytosol Re were detected with the PLP method at 0 and 4 h (Fig. 2B). At 4 h following P withdrawal in the pseudopregnant hamster, nuclear Re levels as measured by the PLP and NaSCN methods were significantly greater than the value obtained with the KCl method (Fig. 3A). Similar results were seen with cytosol Re levels (Fig. 3B).

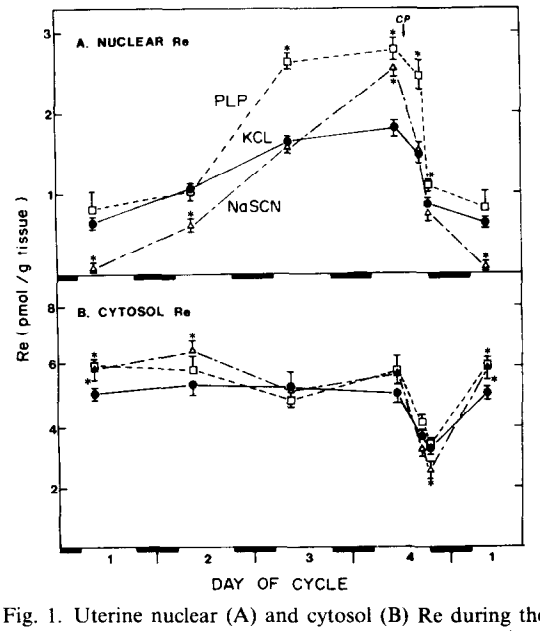

Fig. 1. Uterine nuclear (A) and cytosol (B) Re during the hamster estrous cycle. Re levels were measured by three assay methods: KCl (●); PLP (□); and NaSCN (△). Each point represents the mean ± SEM ($N = 6$–9). The arrow indicates the critical period (CP) for gonadotropin release. *Significantly different ($P < 0.05$) vs the result from the KCl method.

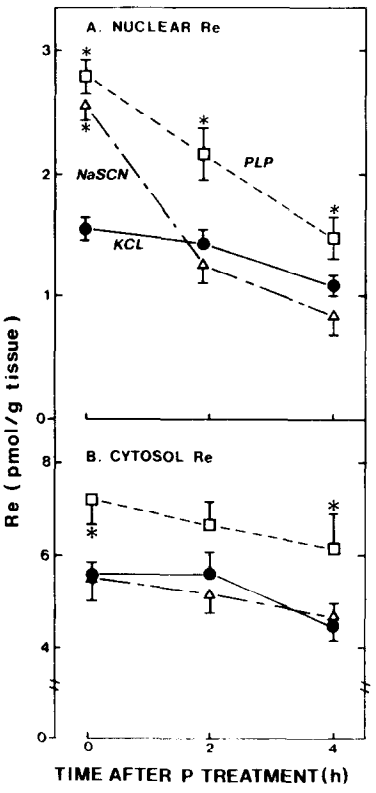

Fig. 2. The time course of uterine nuclear (A) and cytosol (B) Re response to P treatment. Proestrous hamsters (AM) were treated with P (5 mg/100 g BW, sc) and sacrificed 2 or 4 h after treatment. Control animals received corn oil vehicle. Cytosol and nuclear Re were assayed in individual uteri by three methods: KCl (●); PLP (□); and NaSCN (△). Each point represents the mean ± SEM ($N = 6$). *Significantly different ($P < 0.05$) vs the result from the KCl method.

In order to evaluate Re data on a cellular basis, the results were expressed according to DNA (pmol per mg DNA) [Table 1]. During the estrous cycle, the pattern of nuclear Re observed with each assay method was similar to that seen in Fig. 1, i.e. nuclear Re rose as serum E increased during the follicular phase of the cycle (Day 2 to Day 4) and subsequently declined between Day 4 and Day 1 (Table 1A). Cytosol Re was relatively steady between Day 1 and the morning of Day 3, increased between Day 3 and Day 4, and subsequently fell back to Day 3 levels on the afternoon of Day 4. During the ovulatory phase of the estrous cycle on Day 4, there was a rapid down regulation of both cytosol and nuclear Re (Table 1A). Cytosol Re dropped significantly between 1100 and 1900 h on Day 4. Total nuclear Re during this period paralleled the change in cytosol Re and continued to decline through the morning of Day 1.

Following P-treatment at proestrus, both the PLP and NaSCN assays detected a significant decline in

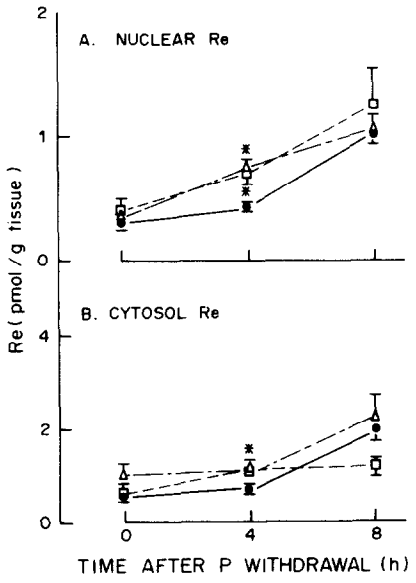

Fig. 3. Myometrial nuclear (A) and cytosol (B) Re response to P withdrawal in the pseudopregnant decidualized hamster. Pseudopregnant hamsters were ovariectomized at the time of deciduomal induction (Day 4), and Silastic E and P implants were placed sc. P implants were removed on Day 7, and Re responses were measured at the times indicated thereafter. Re was assayed in individual uteri by three methods: KCl (●); PLP (□); and NaSCN (△). Each point represents the mean ± SEM ($N = 3$–9). *Significantly different ($P < 0.05$) vs the result from the KCl method.

nuclear Re at 2 h, and all three assays demonstrated a significant reduction of nuclear Re at 4 h (Table 1B). In contrast, no differences were observed in cytosol Re after P treatment using either the PLP or the NaSCN methods. Thus, the rapid and selective loss of nuclear Re characteristic of P action [2, 18] was observed with all three assays.

Ovariectomized pseudopregnant hamsters were prepared with subcutaneous P and E implants. Following removal of the P implant (P withdrawal) on Day 7 of pseudopregnancy, all assays showed that nuclear Re increased significantly at 4 and 8 h above the control level (implants maintained) [Table 1C]. Thus withdrawal of P when serum E is maintained results in the rapid (<4 h) recovery of nuclear Re. Similarly, chronic P treatment during pseudopregnancy resulted in suppression of cytosol Re which was readily reversed when P was withdrawn for 4–8 h (Table 1C).

DISCUSSION

Several methods have been employed to measure the target tissue concentration of cytosol and nuclear Re [9, 14, 19, 20]. However, this is the first report to evaluate different methods under physiological conditions. Thus, we have compared three Re exchange assays in order to determine which one is most appropriate for the study of hormonal control of uterine Re [13], and our results indicate that the PLP assay has several advantages over the KCl and NaSCN methods. More nuclear Re is detected by the PLP method as compared to the KCl procedure perhaps because Re sites are lost during the exchange incubation at elevated temperature in the KCl method [12]. PLP may also serve to stabilize Re [21] so that PLP assays conducted at elevated temperature (30°C, 1 h) or low temperature (0–4°C) give the same nuclear Re values [12]. The NaSCN assay cannot be used for Re at elevated temperature [12], and Rp is unstable in NaSCN at low temperature (unpublished results). Thus, another advantage of the PLP method is that nuclear Rp can be assayed in the same PLP extract [22]. In addition, Isomaa *et al.* [23] have shown that PLP can be used to measure androgen receptor. Therefore, PLP appears to be a useful and versatile reagent for the extraction and measurement of steroid hormone receptors in general.

Table 1. Total uterine cytosol and nuclear Re during the estrous cycle, after P-treatment, and following P withdrawal during pseudopregnancy in the hamster

Condition		Re (pmol/mg DNA) KCl		PLP		NaSCN	
		Cytosol	Nuclear	Cytosol	Nuclear	Cytosol	Nuclear
A.	Estrous cycle						
	Day 1	1.39 ± 0.05	0.36 ± 0.03	1.64 ± 0.07	0.46 ± 0.12	1.63 ± 0.11	0.05 ± 0.02
	Day 2	1.16 ± 0.08	0.62 ± 0.4[a]	1.26 ± 0.08	0.62 ± 0.07[a]	1.39 ± 0.07	0.35 ± 0.04[a]
	Day 3	1.26 ± 0.12	0.91 ± 0.4	1.16 ± 0.03	1.47 ± 0.05	1.24 ± 0.03	0.88 ± 0.04
	Day 4						
	1100 h	1.76 ± 0.10[a]	1.16 ± 0.07[a]	2.02 ± 0.13[a]	1.74 ± 0.09[a]	2.01 ± 0.11[a]	1.48 ± 0.19[a]
	1700 h	1.27 ± 0.06[b]	0.93 ± 0.08[b]	1.43 ± 0.07[b]	1.54 ± 0.11[b]	1.13 ± 0.06[b]	0.96 ± 0.06[b]
	1900 h	1.13 ± 0.07[b]	0.54 ± 0.05[b]	1.16 ± 0.04[b]	0.68 ± 0.05[b]	0.89 ± 0.05[b]	0.47 ± 0.05[b]
B.	P. Treatment, AM Cycle Day 4						
	0	1.91 ± 0.09	0.97 ± 0.05	2.31 ± 0.18	1.74 ± 0.09	1.89 ± 0.17	1.37 ± 0.15
	2 h	1.93 ± 0.14	0.90 ± 0.06	2.29 ± 0.18	1.35 ± 0.13[c]	1.81 ± 0.12	0.80 ± 0.09[c]
	4 h	1.54 ± 0.12[c]	0.68 ± 0.05[c]	2.12 ± 0.27	0.92 ± 0.10[c]	1.64 ± 0.07	0.53 ± 0.10[c]
C.	Pseudopregnancy						
	0	0.33 ± 0.04	0.35 ± 0.04	0.45 ± 0.07	0.49 ± 0.11	0.68 ± 0.07	0.41 ± 0.09
	4 h	0.47 ± 0.06[c]	0.52 ± 0.06[c]	0.72 ± 0.10[c]	0.88 ± 0.12[c]	0.76 ± 0.06	0.90 ± 0.13[c]
	8 h	1.32 ± 0.17[c]	1.26 ± 0.13[c]	0.73 ± 0.10[c]	1.54 ± 0.37[c]	1.48 ± 0.42[c]	1.30 ± 0.21[c]

Receptor levels were determined as described in the Experimental section. Each value represents the mean ± SEM ($N = 3$–9).
[a]Significantly different ($P < 0.05$) from Day 3.
[b]Significantly different ($P < 0.05$) from Day 4 (1100 h).
[c]Significantly different ($P < 0.05$) from control (0).

In the present study, we used animal preparations which would allow the assessment of E and P effects on uterine Re levels. In the cyclic hamster, as serum E rises during the follicular phase there is a corresponding increase in uterine nuclear Re retention which reaches a peak on Day 4 (proestrus) [13]. All three assays produced similar patterns of nuclear Re during the follicular phase of the estrous cycle (Fig. 1, Table 1). However, nuclear Re accumulation was greater when assayed by the PLP method during times of high serum E (Day 3–4), but all methods gave comparable values for nuclear Re when serum E was lower (Day 1–2). During the ovulatory phase of the cycle, nuclear Re declines in response to P action [2, 18], and all three assays detected this response (Fig. 1, Table 1). Thus, hormonal control of nuclear Re during the estrous cycle can be attributed to a positive effect of E and a negative effect of P.

The cytosol Re level can be affected by two opposing processes: (1) depletion (E-dependent Re translocation), and (2) replenishment (recycling of Re from the nuclear compartment and Re synthesis). As serum E titers rise during the estrous cycle, accumulation of nuclear Re would be expected to coincide with cytosol Re depletion. However, depletion of cytosol Re during the estrous cycle may be offset by E induction of its own receptor [24] and concurrent cytosolic Re replenishment [25]. The data in Table 1 support the concept that cytosol Re is maintained at fairly constant levels by these processes during the estrous cycle. The increase in cytosol Re observed between Day 3 and Day 4 (AM) is most likely attributable to E induction of Re replenishment and the rise in serum E on the afternoon of proestrus results in the depletion of cytosol Re [2]. This Re depletion is subseqently tempered by the fall of serum E, and cytosol Re is replenished by the morning of Day 1. As serum P rises during the ovulatory phase of the cycle, P action results in a selective reduction of nuclear Re with no immediate effect on cytosol Re [18] as is evident by the results of the experiment in which P treatment of the proestrous hamster caused a rapid reduction in nuclear Re with no change in cytosol Re (Table 1). Thus, hormonal regulation of cytosol Re during the estrous cycle can be ascribed primarily to E action with little or no effect of P. In addition to hormone-dependent regulation of cytosol Re which includes E-dependent Re depletion (PM, Day 4) and Re synthesis (Day 3 to Day 4), hormone-independent processes appear to maintain a basal or constitutive Re synthesis in the uterus. Chronic P treatment would be expected to suppress hormone (E)-dependent synthesis of Re without altering hormone-independent Re. Such a mechanism for cytosol Re regulation is similar to that recently reported for E-dependent and E-independent levels of Rp in the hamster uterus [26].

High titers of circulating P during pregnancy and pseudopregnancy suppress the concentration of myometrial Re to very low levels [13, 27]. The recovery of the myometrial Re system in response to P withdrawal may provide an important clue to the hormonal control of parturition in the hamster and other rodent species which have a prepartum decline in circulating P. The results from the present study show that when serum E is maintained, P withdrawal leads to the recovery of nuclear Re within 4 h. In contrast to short-term P action during the estrous cycle, chronic P exposure during pseudopregnancy results in a significant down regulation of cytosol Re. However, upon P withdrawal, E action is no longer inhibited, and nuclear and cytosol Re recover and approach the levels observed in the proestrous uterus. These results confirm recent work from our laboratory on the mechanism of P regulation of the Re system in the uterus. Studies employing the KCl assay indicated that P rapidly and selectively inhibits retention of nuclear Re in the uterus [13]. This has now been confirmed in the hamster with three different assay techniques. Furthermore, the rapid recovery of nuclear Re in response to P withdrawal in the decidualized uterus was observed with all assay methods, demonstrating that the effect of P is readily reversible. We have postulated that this effect of P depends on the induction of an Re-regulatory factor (ReRF) [28, 29]. ReRF can be extracted from uterine nuclei and its activity measured *in vitro* at 37°C according to the inactivation of nuclear Re as determined by a subsequent ligand exchange assay [29, 30]. The present results suggest that the assay for ReRF can be improved by employing PLP to measure Re at low temperature in an effort to prevent nonspecific receptor inactivation during the Re assay.

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