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Oncornaviral Protein Modulation in Mouse Uterine Tissue by Estrogen (38467)

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Part or perhaps all of the genome of C-type RNA tumor viruses is present in the DNA of cells in many animal species. In most instances, however, this information remains dormant until derepressed by an appropriate stimulus such as irradiation (1–3), chemicals (4), graft-versus-host reactions (5), hormonal alterations (3, 6–8) or the aging process (9).

We have recently demonstrated that estrogen or compounds having an estrogen mimicking action induce in the uteri of certain mice the expression of a protein that has serological identity to the group specific (gs) proteins of the murine and feline C-type RNA tumor viruses (8). Similarly, estrogen treatment causes the activation of an RNA-directed DNA polymerase that is specifically inhibited by antibody made in rabbits against the purified DNA polymerase of Rauscher leukemia virus (RLV) (3).

Since hormones are tissue-specific effectors capable of modifying gene expression, these observations suggest a physiological mechanism regulating expression of viral information. We now present data characterizing the time sequence of the appearance of gs protein and an RNA-directed DNA polymerase in uterine tissue of mice following a single estrogen treatment of near physiological dosage.

Materials and Methods. Mice. Female NIH-Swiss mice were ovariectomized prior to sexual maturity and aged to approximately 100 days. Unless otherwise indicated, mice treated with hormone received a single intramuscular injection containing 1.0 μg estrogen in 0.1 ml peanut oil. Control mice received only the vehicle. At appropriate time intervals following injection mice were killed by cervical fracture, the uteri excised, carefully trimmed, weighed, and examined for gs protein of the C-type RNA viruses and for RNA-directed DNA polymerase activity.

Group specific antigen assays. Two serological techniques were utilized: complement fixation (CF) and radioimmune precipitation assay (RIPA). Complement fixation tests were conducted on 10% uterine extracts (w/v) in phosphate-buffered saline (PBS) as previously described (6). For RIPA gs antigen was purified from banded RLV as described by Scolnick, *et al.* (10). Briefly, the virus was disrupted by Triton X-100, extracted with ether, and chromatographed on either Sephadex G-100 or G-150 and then isoelectrically focused. Polyacrylamide gel electrophoresis of the purified antigen yielded a single band. Iodination of the gs protein was performed by the method of Greenwood, *et al.* (11). For quantitation of gs protein two- or threefold dilutions of uterine extracts (centrifuged at 40,000 *g* for 24 min) were mixed with 0.01 *M* EDTA, 0.01 *M* potassium phosphate (pH 7.8), and normal sera. To these mixtures either goat, porcine or guinea pig antimurine leukemia virus (MuLV) sera that had been demonstrated to precipitate 50% of the labelled purified antigen was added. The mixtures were incubated 1 to 2 hr at 37° and then 100 μl of iodinated antigen was added to each tube. The components were incubated for an additional hr at 37° and then overnight at 4°. Subsequently, the gs antigen–antibody complex was precipitated either by saturated $(NH_4)_2SO_4$, for which ^{22}Na was added as a nonspecific precipitation control (12), or by a second antibody (10).

After centrifugation either the precipitates or the supernatants were counted in an automatic gamma spectrometer. Quantitation was made by comparison with appropriate standard curves. Both procedures of precipitation gave similar results.

Protein assay. Protein determinations of the uterine extracts in PBS were performed according to Bramhall *et al.* (13) using bovine serum albumin as a standard. In brief, 10 μl of a 1:2 dilution of uterine extract was

placed on 1 cm² discs of Whatman No. 42 filter paper. The filters were washed four times with 7% TCA, twice with ethanol: ether (1:1, v/v) and once with ether. Dry discs were stained at 50° with xylene brilliant cyanin G (K&K Laboratories, Inc., Plainview, NY—10 mg/ml made in 7% acetic acid) and destained at 50° with 7% acetic acid. The stained protein was eluted from the discs with methanol:H_2O:NH_4OH (66:34:1) and absorbance read in a Gilford 2400 spectrophotometer at 610 nm.

RNA-directed DNA polymerase assay. Uterine tissue was homogenized in a mixture containing a vol of cold 0.85% NaCl equal in microliters to the number of milligrams of tissue and 2 vol of a solution containing 20 m*M* Tris–HCl (pH 8), 1.0% Triton X-100, 8 m*M* magnesium acetate, 4 m*M* dithiothreitol, 1.0 *M* KCl, and 10% glycerol. A 100 μl vol of a high speed supernatant (100,000 *g*, 1 hr) was layered onto a 4 ml 10–30% glycerol gradient containing 500 m*M* KCl, 2 m*M* dithiothreitol, 10 m*M* Tris–HCl (pH 8), 2 m*M* magnesium acetate, and 0.05% Triton X-100. Gradients were centrifuged 15 hr at 50,000 rpm in a Beckman SW 56 titanium rotor. Approximately 20 fractions were collected from the bottom of each gradient, and fractions were surveyed for DNA polymerase activity in response to $(rA)_n \cdot (dT)_{12-18}$ (poly rA·oligo dT) and $(dA)_n \cdot (dT)_{10}$ (poly dA·oligo dT) template-primers. Assay mixtures contained 50 m*M* Tris–HCl (pH 8.2, measured at 37°), 0.01% Triton X-100, 0.1 m*M* manganese acetate, 2 m*M* dithiothreitol, and 75 μ*M* [³H]TTP (1.0 Ci/mmole Schwarz/Mann). Assays with poly rA·oligo dT contained 6 μg/ml $(rA)_n$ (P-L Biochemicals) and 10 μg/ml $(dT)_{12-18}$ (Collaborative Research). These components were mixed before being added to the reaction mixture. Reactions with poly dA·oligo dT (P-L Biochemicals) contained a template concentration of 0.25 A_{260} units/ml.

Results. In preliminary experiments, the ability of several estrogen metabolites-estradiol-17β ($E_2\beta$), estradiol-17α ($E_2\alpha$), estrone (E_1), and estriol (E_3)—to promote the accumulation of gs protein in the uteri of ovari-

TABLE I. VIRAL GROUP SPECIFIC ANTIGEN TITER IN POOLED UTERINE EXTRACTS FROM OVARIECTOMIZED NIH SWISS MICE.

Treatment	Level (μg)	Time after treatment (hour) 24 No.	24 Mean uterine weight (mg)	24 Anti MSV pool 1 × 322 gs	24 Anti MSV pool 1 × 322 1:40	24 Anti MSV pool 1 × 322 1:80	72 No.	72 Mean uterine weight (mg)	72 Anti MSV Pool 1 × 322 gs	72 Anti MSV Pool 1 × 322 1:40	72 Anti MSV Pool 1 × 322 1:80
Sham		5	5.0	−	<2[a]	<2	5	5.8	−	<2	<2
Estradiol-17β	1.0	5	14.4	+	2	2	5	14.2	+	4	2
	0.1	5	12.4	+	4	2	5	11.0	+	4	<2
	0.01	5	8.2	+	2	<2	5	9.8	+	2	<2
	0.001	5	5.8	−	<2	<2	5	6.2	−	<2	<2
Estrone	1.0	5	12.0	+	4	2	5	11.6	+	2	<2
	0.1	5	7.4	−	<2	<2	5	7.6	+	2	<2
	0.01	5	6.0	−	<2	<2	5	5.4	+	2	<2
	0.001	5	5.2	−	<2	<2	5	5.4	−	<2	<2
Estradiol-17α	1.0	5	8.8	−	<2	<2	5	8.4	+	2	<2
	0.1	5	5.8	−	<2	<2	5	6.6	+	2	<2
	0.01	5	5.0	−	<2	<2	5	5.6	−	<2	<2
	0.001	5	5.4	−	<2	<2	5	4.8	−	<2	<2
Estriol	1.0	5	7.2	+	2	<2	5	6.0	+	2	<2
	0.1	5	7.4	−	<2	<2	5	6.0	+	2	<2
	0.01	5	4.8	−	<2	<2	5	5.0	+	2	<2
	0.001	5	5.4	−	<2	<2	5	6.2	−	<2	<2

[a] Reciprocal of antigen dilution giving a 3+ positive reaction in the complement-fixation test.

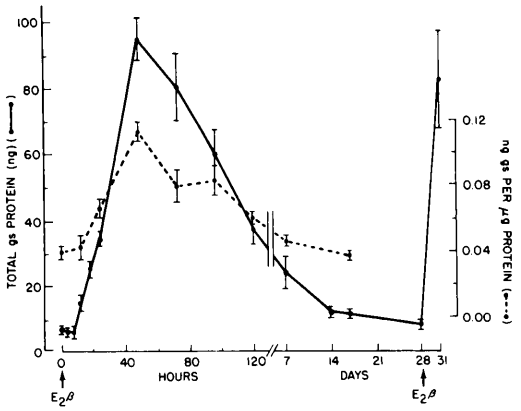

FIG. 1. Changes in the group specific antigen level (mean ± SE) in pooled uterine extracts from ovariectomized NIH Swiss mice following im injection of estradiol-17β. Treatments of 1.0 μg per mouse are indicated ($E_2\beta$).

ectomized NIH Swiss mice was compared (Table I). Each metabolite increased the level of uterine gs protein in a manner which, within the limitations of the CF assay employed, reflected their relative biological potency. $E_2\beta$, the more potent estrogen, was the most effective activator of gs protein. In mice receiving as little as 0.01 μg $E_2\beta$, gs protein was detectable after 24 hr. By comparison, 1.0 μg E_1 or E_3 was required to induce detectable levels of gs protein at 24 hr, although a lesser amount (0.01 μg) was effective by 72 hr. $E_2\alpha$, the least active estrogen examined, was the weakest inducer of gs protein. By this method of assay, gs protein was not detected in the uteri of sham-treated mice or of mice receiving 0.001 μg of the estrogens.

To examine more critically the possibility that gs protein is present in the uteri of unstimulated mice, but at a level below CF detection, as well as to characterize the kinetics of estrogen-promoted accumulation of gs protein, a radioimmunoassay with a sensitivity of 2 ng gs protein was used. Using this technique a low amount of gs protein (5 ng) was found in the uteri of control mice (Fig. 1). Following estrogen administration the first detectable increase in uterine gs protein content was evident between 8 and 12 hr after treatment. The twofold increase at 12 hr was significant ($P < 0.05$) and a further increase was seen up to 48 hr. At this time gs protein level was maximal (94 ng/uterus) and reflected not only a general estrogen induced protein synthesis of the uterus but also a specific elevation of tissue gs protein concentration, 0.04 ng gs protein/μg uterine protein *vs* 0.11 ng gs protein/μg uterine protein for control and estrogen treated mice, respectively. After 48 hr, the level of gs protein declined until at approximately 3 wk it was not significantly different from prestimulation levels ($P > 0.05$). The involuted uterus following one estrogen treatment was not refractive to a second estrogen-initiated increase (Fig. 1). The content of uterine gs protein in mice 72 hr after receiving either a single or a repeated estrogen treatment, 28 days subsequent to the first, was similar (approximately 80 ng).

Changes in the uterine DNA polymerase patterns following estrogen stimulation are shown in Fig. 2. It is evident that the steroid treatment results in the increased activity of at least two DNA polymerases having different template specificities and sedimentation velocities on 10–30% glycerol gradients. The enzyme responding to the poly dA·oligo dT template-primer sedimented as a single narrow peak near the top of the gradient. In control mice this polymerase exhibited moderate activity but increased considerably (fourfold) following estrogen stimulation. Enzymatic activity with poly rA·oligo dT was distributed over a much wider portion (fractions 4–16) of the gradient. In samples prepared from unstimulated mice the activity with this template was low with the major peak near the bottom of the gradient (fraction 6). After estrogen stimulation little, if any, shift in this profile was evident for 24 hr. Between 24 and 48 hr after treatment, however, a large increase in activity that peaked in fractions 8 and 9 was observed. The activity of the enzyme responding to poly dA·oligo dT persisted at 72 hr after estrogen stimulation, while the activity of the polymerase detected with poly rA·oligo dT had declined markedly by that time.

Discussion. Previous studies have demonstrated the presence of viral-related gs protein and an RNA-directed DNA polymerase in uterine extracts from NIH Swiss mice (3, 6–8). Data from these earlier studies led to the suggestion that in this tissue the level of

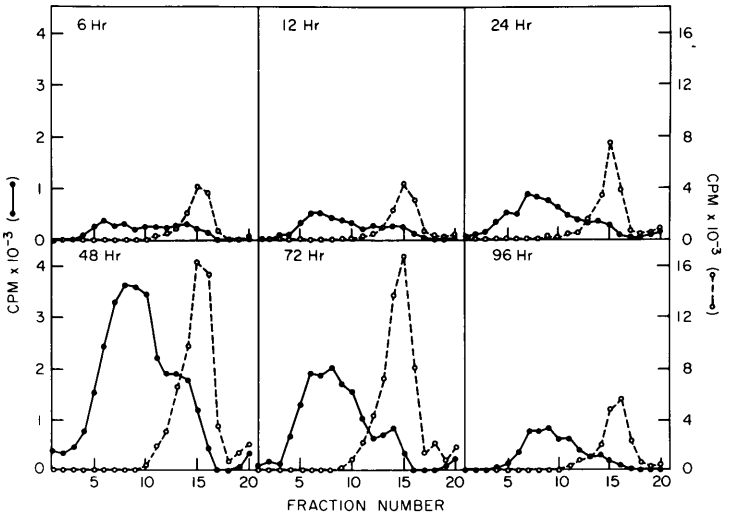

FIG. 2. Changes in DNA polymerase activity with poly rA·oligo dT(●) and poly dA·oligo dT(○) template-primers in pooled uterine extracts from ovariectomized NIH Swiss mice following im injection of 1.0 μg estradiol-17β.

these proteins is regulated, in part, by a mechanism controlled by hormone milieu. The present studies provide additional evidence demonstrating hormonal influence on uterine virogene-marker expression. This is supported by the observations that the efficacy of several natural estrogen metabolites to activate these markers is associated with their relative biological potency and further, that the kinetics of the estrogen-initiated response follows a highly predictable temporal sequence.

In the experiments reported here the RNA-directed DNA polymerase activity and the level of gs protein, both previously demonstrated to be related to viral proteins (8), clearly have been elevated by estrogen administration. This elevation, first seen approximately 12 hr after stimulation, coincides with the whole organ protein synthesis which occurs in the uterus in response to estrogen and appears to be highly dependent on this class of hormone. To date the only nonestrogen mimicking treatments found that potentiate uterine virogene marker expression have been irradiation (3) and inhibitors of protein synthesis (unpublished data). Significantly, irradiation treatment is well known for its capacity to activate the synthesis and release of C-type RNA tumor viruses (1, 2) and further, it has been reported recently that inhibitors of protein synthesis also induce virus release (14). From evidence gathered to date it appears that in the NIH Swiss mouse gs protein and RNA-directed DNA polymerase are often expressed in other tissues, independently of estrogen (unpublished data). This occurs, however, only in rapidly proliferating tissues such as the thymus, lymph nodes, spleen, and intestine but not in nonproliferative muscle or nerve tissues. Whether the tissues that exhibit virogene activation do so in response to specific stimulating factors such as immunological challenge or other hormones, similar to the estrogen effect on the uterus, has yet to be determined. That other hormones can potentiate virogene expression has been shown by a recent report that prolactin activates gs protein in the thymus, spleen, and kidney of DW/J mice and again is probably the result of a general growth stimulation (15).

It is well recognized that steroid hormones influence a variety of target tissue activities, including the synthesis, activation, and possibly the degradation of enzymes and structural proteins (16). Although the mechanism of this influence is complex and only partially characterized, it appears to involve the uptake, binding, and transport of the hormone to the nucleus with the subsequent activation of the transcriptional and translational

phases of the genetic "read-out" and protein synthetic processes. Viewed in this manner steroids may function as inducers or derepressors of genetic information.

Numerous genetic studies have clearly shown the involvement of host genes in the partial or complete expressions of C-type RNA tumor viruses (7, 17–19). Currently, the mechanisms by which these genes are controlled is poorly understood. Similarly the mechanism of hormonal influence of virogene expression is unknown. Data collected to date favor the interpretation that estrogen induces the *de novo* synthesis of uterine virogene protein, however additional studies must be performed to verify this, as well as to determine the specific mode of control. If estrogens are found to regulate virogene expression at the transcriptional level, this may provide a useful model to study not only the physiological control mechanism(s) of oncogenesis but also steroid-gene interaction.

Summary. Treatment of ovariectomized NIH Swiss mice with estrogens elevated the level of the murine leukemia virus group specific protein and the activity of an RNA-directed DNA polymerase in the uterus. The extent that these markers were raised was dependent on the relative biological potency of the estrogen and on the time interval following treatment. Increases in the levels of both viral marker proteins were evident within 24 hr of treatment and were highest at 48 hr. Subsequently, viral protein levels declined to pretreatment levels.

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