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Estradiol Effect on Type C Viral Gene Expression in the Uterus of the Ovariectomized Mouse (40845)

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Type C RNA viruses (oncornaviruses, retroviruses) have been isolated from almost all mouse strains studied to date (1). Whereas some oncornaviruses are clearly associated with lymphoreticular neoplasms and sarcomas, evidence has not supported such an association for the majority of viral isolates (2). The presence and conservation of proviral DNA (virogenes) in the genome of many, if not all, mammals (1) and its natural expression in many tissues in the absence of malignancies is strong circumstantial evidence for a biological role for type C RNA viruses. A specific function of these viruses, however, remains to be identified. Studies of the factors and physiological phenomena which modulate the expression of viral antigens and complete virions would, therefore, be helpful in approaching this problem.

One of the major effects of steroid hormones is to enhance the synthesis and accumulation of RNA in target organs (3). We have used the NIH Swiss mouse, a randomly bred strain, to demonstrate that estrogens modulate the synthesis of viral proteins in the uterus in a dose-dependent manner and that the magnitude of the response correlates with the biological potency of the estrogens (4). As these observations implied the presence of viral mRNA, it was of interest to study the kinetics of estradiol induction of viral-specific RNA in the estrogen-deprived uterus of the NIH Swiss mouse.

For this purpose we have synthesized ^{3}H-labeled cDNAs from high molecular weight viral RNAs and used them in molecular hybridization assays with uterine RNA obtained at different time intervals after injection of a single dose of estrogen. We have been able to detect low levels of viral-specific RNA in the estrogen-withdrawn uterus and a progressive increase in the amounts of viral RNA up to 8 hr after treatment. Thereafter, there was a gradual decrease, though at 24 hr the amounts of viral RNA still exceeded control values. Our results are consistent with transcriptional control of virogenes by estradiol in the mouse uterus, though other mechanisms remain to be explored.

Materials and methods. Cells and viruses. The Rauscher murine leukemia virus (R-MuLV) (5), grown in the BALB/c mouse embryo cell line JLS-V9 was obtained through the Office of Program Resources and Logistics, NCI. The M55 strain of MuLV, isolated from cell-free uterine extracts of normal adult NIH Swiss mice (6), was grown in the CCL64 mink cell line. Viruses were purified by isopycnic gradient centrifugation.

[^{3}H]cDNA preparation. High molecular weight RNA was prepared from purified virus by lysis with 0.5% sodium dodecyl sulfate (SDS) and centrifugation on 15 to 30% (w/w) sucrose gradients. ^{3}H-Labeled cDNA was synthesized in the presence of 50 μg/ml actinomycin D either in an endogenous reaction (7) or using purified viral RNA with oligo(dT) as primer and purified avian myeloblastosis virus (AMV) reverse transcriptase (8). Specific activity of the purified [^{3}H]cDNA was approximately 2×10^7 cpm/μg.

Animals. NIH Swiss female mice, bilaterally ovariectomized at age 18 to 24 days, were given a single injection of 1 μg 1,3,5(10)-estratrien-3,17β-diol (17β-estradiol) in peanut oil at age 2 to 3 months. Mice were killed by cervical dislocation at the indicated times, and uteri were excised for

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RNA extraction, essentially according to (9). Nucleic acids were quantified according to (10). The A_{260}/A_{280} ratio of purified uterine RNA was approximately 2.

RNA/DNA hybridizations. Hybridizations between [^{3}H]cDNA and either viral or tissue RNA were done in 10 m*M* Tris–HCl (pH 7.5), 0.5 *M* NaCl, 0.002 *M* EDTA, 0.1% SDS. Nucleic acid concentrations in hybridization reactions were: viral RNA, 6–10 μg/ml; tissue RNA, 10–20 mg/ml; [^{3}H]cDNA (approximately 800 cpm per tube), 1–3 ng/ml. After 5 min at 90°C, hybridization reaction tubes were incubated at 68°C for 90–170 hr. The degree of hybridization was determined by analysis with single-strand specific nuclease S_1 (11). Data are presented as percentage hybrid versus ER_0t (12). R_0t values were the product of absorbance at 260 nm times incubation time (hr) divided by 2, corrected for monovalent cation concentration to give equivalent R_0t (ER_0t). The percentage of viral specific RNA in a sample was calculated as

$$\frac{ER_0t_{1/2}\text{ (viral RNA)}}{ER_0t_{1/2}\text{ (sample RNA)}} \times 100,$$

where $ER_0t_{1/2}$ is the value at which 50% of the input cDNA was hybridized.

For thermal stability studies, samples were hybridized to ER_0t values 5–10 times the $ER_0t_{1/2}$ value, incubated 15 min at the indicated temperatures, quick cooled, and assayed with S_1 nuclease for percentage hybridization remaining. The melting temperature (T_m) is the point at which 50% of the hybridized [^{3}H]cDNA was dissociated. These data measure the degree of relatedness of the hybridized sequences (13).

Results. Characterization of the [^{3}H]-cDNAs. R-MuLV cDNA, synthesized in an endogenous reverse transcriptase reaction (7), was used in experiment 1 (Table I). Two R-MuLV cDNAs and one M55 cDNA, synthesized in exogenous reactions (8) were used in experiments 2, 3, and 4, respectively. On alkaline sucrose gradients (not shown) the [^{3}H]cDNA had sedimentation coefficients of 8–10 S, implying a length of about 1100 nucleotides. A small portion (about 10%) consisted of longer copies; however, no attempts were made to fractionate the products. The specificity of the products as hybridization reagents was tested by several criteria. Maximum hybridization of 80–90% was obtained with their respective templates (Fig. 1). The hybridization reaction was essentially completed within 2 log periods, as expected for a pseudo-first-order reaction in RNA excess (14). $E\bar{R}_0t_{1/2}$ values were 4 to 6 × 10^{-2} in agreement with published results (7, 15). The cDNAs also hybridized to an equal extent with cytoplasmic RNA extracted from virus-producing cells (Fig. 1). Additional hybridizations with poly(A), AMV 70 S RNA, tRNA, and human placental RNA (the latter two in concentrations equivalent to those used with uterine RNAs) gave values 2–4% above background (Fig. 1). Thus, the [^{3}H]cDNA did not detect cellular poly(A)-containing RNAs or tRNAs and did not self-anneal under the conditions of high RNA concentrations and long incubation times used in the reactions with uterine RNA. Lastly, thermal denaturation (Fig. 2) of the hybrids formed between cDNAs and viral RNA templates showed a T_m of 88°C, indicating high nucleotide sequence homology. The R-MuLV [^{3}H]cDNA cross-reacted to approximately 40% with M55 virus RNA and showed a ΔT_m of approximately 6°C in comparison with the homologous reaction. Similar results were obtained with M55 cDNA hybridized to R-MuLV RNA (data not shown). These observations are in agreement with results obtained with R-MuLV and ATl24, another NIH Swiss viral isolate (16). Thus, both reagents could be applied to a study of RNA metabolism in the NIH Swiss mouse tissues.

Hybridization of [^{3}H]cDNAs to uterine RNAs. Cellular RNA was extracted by a procedure designed to eliminate contaminating DNA and tRNA (9). Using hybridization conditions as described under Materials and Methods, we could detect the presence of viral-specific RNA in the tissues. Under these conditions, self-annealing of the [^{3}H]cDNA and nonspecific hybridization were excluded by the control experiments described above. The extent of base-pairing was high as indicated by a T_m of 3–6°C below that of the duplex with cDNA and homologous viral RNA (Fig. 2),

TABLE I. HYBRIDIZATION OF MuLV [^{3}H]cDNAs TO UTERINE RNA

Expt No.	$ER_0t_{1/2}$ of Homologous hybrid ([^{3}H]cDNA-MuLV RNA) ($\times 10^{-2}$)	Time (hr)	Percentage hybridization at ER_0t = 60,000	$ER_0t_{1/2}$ of [^{3}H]cDNA hybrid with uterine RNA ($\times 10^3$)	Percentage viral-specific uterine RNA[a]	n-Fold increase relative to control
1	2	Control	62	7.4	2.7×10^{-4}	—
	(R-MuLV)	4	76	2.2	9.1×10^{-4}	3
		8	76	0.8	2.4×10^{-3}	9
		16	74	1.8	1.1×10^{-3}	4
		24	70	2.6	7.7×10^{-4}	3
2	3	Control	60	7.0	4.2×10^{-4}	—
	(R-MuLV)	4	68	2.2	1.3×10^{-3}	3
		8	68	1.0	2.9×10^{-3}	7
		16	67	2.0	1.5×10^{-3}	4
		24	64	2.5	1.2×10^{-3}	3
3	5	Control	62	8.0	6.3×10^{-4}	—
	(R-MuLV)	4	62	2.7	1.9×10^{-3}	3
		8	70	0.3	1.7×10^{-2}	27
		16	72	1.1	4.4×10^{-3}	7
		24	68	1.3	3.8×10^{-4}	6
4	6	Control	N.D.[b]	N.D.[b]	N.D.[b]	—
	(M55-MuLV)	4	60	2.1	2.9×10^{-3}	—
		8	64	1.0	6.0×10^{-3}	—
		16	56	3.0	2.0×10^{-3}	—
		24	53	4.0	1.5×10^{-3}	—

[a] Calculated as ($ER_0t_{1/2}$ template RNA)/($ER_0t_{1/2}$ tissue RNA) $\times$ 100

[b] Control (time zero) was not done. Therefore, n-fold increase values were not calculated for this experiment.

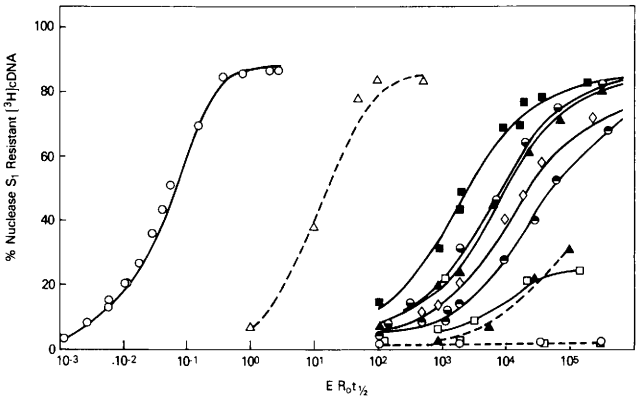

FIG. 1. Kinetics of hybridization of viral [^{3}H]cDNA to uterine RNA. The results of a representative experiment are shown. [^{3}H]cDNA was hybridized to viral 70S RNA (○——○), RNA from JLS-V9 cells producing R-MuLV (△- - -△), tRNA (○- - -○), human placental RNA (□- - -□), mouse brain RNA (□——□), liver RNA (▲- - -▲), and uterine RNA obtained at different time intervals after estradiol treatment: 0 (◓——◓), 4 (▲——▲), 8 (■——■), 16 (◒——◒), and 24 hr (◇——◇).

showing that the RNA sequences detected were virus specific. Results of a representative experiment with an R-MuLV [^{3}H]cDNA are shown in Fig. 1, and data from four separate experiments are summarized in Table I.

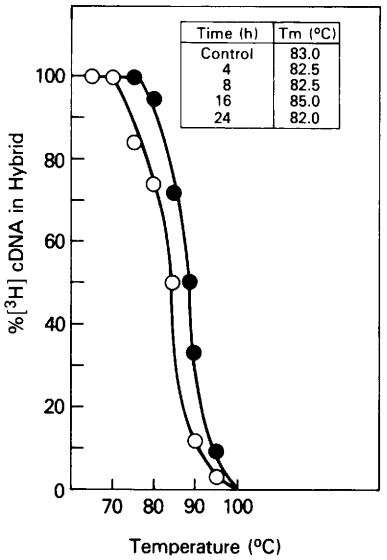

FIG. 2. Thermal stability of hybrids between [^{3}H]-cDNA and template viral RNA (●——●) or uterine RNAs (○——○). Formation of hybrids and thermal denaturation were done as described under Materials and Methods. The T_m values of hybrids between [^{3}H]cDNA and uterine RNAs obtained at different intervals after estradiol treatment are shown in the inset.

A comparison of the $ER_0t_{1/2}$ of these reactions to that of the homologous reaction with the viral RNA template is an index of the relative concentrations of viral RNA in the tissue samples. The $ER_0t_{1/2}$ of RNA from untreated uteri was 7 to 8 × 10^3, indicating that the viral-specific sequences represented 0.0003 to 0.0006% of the cellular RNA (Table I). Using RNA isolated from uteri of mice treated with a single injection of 1 μg estradiol, the rate of the hybridization reaction increased (Fig. 1) due to an increase in the concentration of viral-specific RNA (Table I). Thus, at 4 hr, the earliest time point studied, there was a threefold increase in viral RNA content. Maximal response was observed at 8 hr and represented a 7- to 27-fold increase over basal values. This occurred at a time when total uterine RNA and DNA were not significantly increased (Table II). After 8 hr there was a decrease in the relative concentrations of viral-specific RNA at 16 and 24 hr. However, at 24 hr viral-specific RNA was still three- to sixfold times control levels.

Hybridization of [^{3}H]cDNA with RNA from other tissue. In nontarget tissues such as muscle and brain, removed 24 hr after estradiol injection, lower rates of hybridization were obtained between viral cDNA and cellular RNAs, and only 20–30% of the cDNAs hybridized at the

TABLE II. CALCULATION OF VIRAL 35 S RNA EQUIVALENTS PER ENDOMETRIAL CELL

Uterine RNA (hr after estradiol treatment)	$ER_0t_{1/2}$ with uterine RNA ($\times 10^3$)	mg RNA/g tissue[a]	mg DNA/g tissue[a]	RNA/DNA	35 S Equivalents per cell[b]	35 S Equivalents per endometrial cell[c]
Control	7.5	11.3	7.5	1.6	10	250
4	2.3	11.5	8.4	1.4	30	750
8	0.8	9.2	5.4	1.7	100	2000
16	2.0	12.2	7.6	1.9	50	830
24	2.6	17.3	8.2	2.1	40	400

[a] Mean values, determined in total homogenate as described under Materials and Methods.

[b] Calculated according to the formula: No. copies = $(6.022 \times 10^{23})/(3 \times 10^6) \times$ (RNA)/(DNA) $\times$ 6 pg DNA/cell $\times$ ($ER_0t_{1/2}$ template RNA)/($ER_0t_{1/2}$ tissue RNA) $\times 10^{-12}$ g/pg. where 6.022×10^{23} is Avogadro's number; 3×10^6 is molecular weight of 35 S RNA (34); $ER_0t_{1/2}$ template RNA is 4×10^{-2}; and 6 pg DNA/murine cell is a value from the literature (35). Sample calculation for control RNA: $(6.022 \times 10^{23})/(3 \times 10^6) \times 1.6 \times 6 \times (4 \times 10^{-2})/(7.5 \times 10^3) \times 10^{-12} = 10.2$.

[c] Assuming 4% epithelial cells at 0 and 4 hr, 5% at 8 hr, 6% at 16 hr, and 10% at 24 hr as reported in (26).

highest values tested (Fig. 1), whereas at similar ER_0t values uterine RNA hybridized to over 65%.

Discussion. We have followed the kinetics of the estrogen-induced appearance of viral-specific RNA in the uterus of the NIH Swiss mouse by molecular hybridization with specific [^{3}H]cDNAs prepared from MuLV 70 S RNAs. We chose R-MuLV because earlier studies had demonstrated the presence of homologous sequences in NIH Swiss mouse DNA (17), and this virus was available in adequate quantity for our requirements. Following the completion of experiments with R-MuLV cDNA, we were able to obtain sufficient amounts of NIH Swiss mouse virus M55 (6) to synthesize a cDNA and repeat the kinetics experiment. The results (Table I, experiment 4) were similar to those obtained with the R-MuLV probe. The hybrids of uterine RNA with R-MuLV cDNA at all time points had T_m values 3–6°C lower than the hybrids with purified viral RNAs (Fig. 2). It has been estimated that a 1°C lowering of the T_m indicates about 0.7% mismatching of base pairs (13). Therefore, these T_m values imply a 2–4% nucleotide sequence mismatching. In preliminary experiments to characterize the M55 virus we observed a similar level of mismatching in heterologous hybrids between R-MuLV and M55 RNAs and their [^{3}H]cDNAs (data not shown). Since the virus-specific RNA expressed in the uterus in response to estrogen is presumably that of the NIH Swiss endogenous, xenotropic virus M55, a degree of mismatching between R-MuLV cDNA and uterine RNA comparable to that seen between the same cDNA and M55 RNA is to be expected. Furthermore, our hybridization control studies with muscle and brain RNA showed that the appearance of viral-specific RNA sequences in response to estrogen was target-organ specific, and the kinetics were consistent with the interpretation that these sequences were mRNAs for viral proteins that we have previously shown to be synthesized in response to estrogen treatment (4).

In the estrogen-deprived uterus, viral-specific RNA sequences were detected at low levels, comprising 0.0003 to 0.0006% of the total cytoplasmic RNA (Fig. 1 and Table I). Since we have shown low levels of the major viral core protein, p30, in the unstimulated uterus (4), we believe that proviral gene expression is not totally repressed.

Following administration of estradiol there was a three- to fourfold increase in the concentration of viral-specific RNA at 4 hr and a maximal increase of 7- to 27-fold at 8 hr. Neither total DNA nor total RNA was significantly different from control levels at 4 or 8 hr (Table II). In addition, it has been reported that at these time intervals after estrogen treatment the number of mitoses were not significantly increased in the mouse uterus (18). Thus, the response at these time points cannot be explained by an

increase in the number of target cells or by changes in total RNA. Such an increase in the cellular concentration of viral RNA could be explained either by increased rate of transcription or by a slower rate of degradation of viral RNA. We cannot at present directly assess the relative role of each of these processes in our experiments. In studies with established mammary tumor cell lines (19, 20), glucocorticoids stimulated mouse mammary tumor virus (MMTV) RNA synthesis. Furthermore, the accumulation of ovalbumin mRNA in the chick oviduct has been shown to be due to increased transcription (3, 21). By analogy with these systems, a likely explanation for our results is enhanced transcription of viral RNA. At least some of these sequences are probably mRNAs for viral proteins since our previous studies demonstrated higher than basal levels of viral proteins at 8 hr but not at 4 hr (4). Studies in other laboratories (22–24) of the incorporation of labeled precursors into newly synthesized RNA in the rodent uterus following estradiol have shown that before 6 hr most of the label was present in rRNA, tRNA, and heteronuclear RNA, probably rRNA precursor. There was only a small amount of incorporation into DNA-like RNA (presumably mRNA). We conclude, therefore, that viral-like RNA is among the earliest nonribosomal RNAs to accumulate in the uterus after hormone treatment.

Following the maximal response there was a decline in the relative concentration of viral-specific RNA sequences at 16 and 24 hr, the last time points examined. Since we showed in earlier studies (4) that levels of viral proteins in the mouse uterus did not peak until 48 hr after estrogen injection, it is unlikely that the decline in viral RNA during the 12–24 hr time period resulted principally from degradation. In addition, the sedimentation rate profile of cellular viral-specific RNA, constructed by hybridizations with each fraction of a sucrose gradient, gave major RNA peaks of approximately 14, 22, and 35 S at all time points following estrogen stimulation with no apparent increase in low molecular weight RNA (data not shown). The most likely explanation for the decline in relative concentration of viral-specific RNA is a proportionately greater increase in total organ RNA in comparison with viral RNA that occurred during this period (3), resulting in an apparent dilution of the latter. It should be noted that a similar decline in relative MMTV RNA levels occurred in the mammary tumor system noted above despite the continuous presence of dexamethasone (25).

Calculations of viral 35 S equivalents per mouse cell, based on the relative concentration of RNA to DNA in uteri following hormone treatment, are presented in Table II, column 6. From ten 35 S equivalents per cell in controls, the number increased to 100 copies per cell at 8 hr and then dropped to 38 by 24 hr. These calculations, however, may underestimate the actual number of viral sequences in responsive cells for the following reasons. Our studies were performed with total uterine RNA, but it has been shown that only luminal and glandular uterine epithelia respond to estrogen in the ovariectomized rodent (18, 26) during the first 24 hr after estradiol injection and that 4–6% of total uterine cells up to 16 hr and 8–12% at 24 hr are epithelial cells (26). Moreover, immunofluorescence studies by us (27) and others (28) have shown that viral antigens are localized in uterine epithelial cells. We therefore calculated that a much higher number of viral sequences was expressed in epithelial cells (Table II, column 7) when target cell specificity was considered. Values ranged from two hundred fifty 35 S RNA equivalents per epithelial cell in controls to a high of 2000 copies per epithelial cell at 8 hr post-treatment with estradiol.

An important observation from these experiments is that the increase in viral RNA levels occurred very early after hormone treatment, at a time when other *in vivo* kinetic studies (22–24) have not shown a significant increase in mRNA accumulation. The biologic significance of this event is not known. Increased synthesis of endogenous murine retroviruses has been observed in other rapidly proliferating tissues, such as lymphoid and hemopoietic organs (29), regenerating liver (30), and early embryos (31), and on the basis of these observations, it has been hypothesized that these viruses

play a regulatory role in tissue growth and differentiation (2, 31).

We conclude from the present studies that estradiol increases the accumulation of viral RNA in the mouse uterus early after its administration. Since a small amount of viral RNA may be continuously present in the uterus of the unstimulated, ovariectomized animal, enhancement of transcription of active proviral genes by estradiol is the most likely explanation for the apparent increase, in analogy with other experimental systems. However, since the NIH Swiss mouse genome contains 10 or more copies of proviral sequences (32), we cannot exclude the possibility that estradiol also unmasks inactive genes similar to the mechanisms reported for the chick oviduct (21, 33).

Summary. Kinetic studies using hybridization with complementary DNAs synthesized from high molecular weight type C viral RNAs showed that virus-specific sequences were among the earliest nonribosomal RNAs to accumulate in the mouse uterus after estrogen treatment. Following hormone administration to ovariectomized animals, significant increases above the low level of viral-specific RNA continuously present in the uterus were observed as early as 4 hr, with maximum levels at 8–16 hr. Subsequently, there was a decrease in the relative concentration of viral RNA. However, levels were still higher than basal at 24 hr, the last time point examined. The increase in viral RNA occurred before a detectable increase in protein synthesis, suggesting that this viral-specific RNA was probably mRNA.

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