Estrogen Regulation of *c-fos* Messenger Ribonucleic Acid

David S. Loose-Mitchell, Constance Chiappetta, and George M. Stancel

Department of Pharmacology
The University of Texas Medical School at Houston
Houston, Texas 77225

Acute administration of 17β-estradiol to immature female rats elicits a rapid and striking increase in the size of the uterus. This increase in size to caused by both hypertrophy and hyperplasia in the epithelial, stromal, and myometrial cells in the uterus. Previous studies have shown that induction of mRNA for the epidermal growth factor receptor, the cellular homolog of the erb-B oncogene, occurs early during estrogen-stimulated uterine growth. We report here that estradiol causes a very rapid induction of the mRNA for the cellular oncogene c-*fos* in immature rat uterus. Steady state levels of c-*fos* mRNA reach a maximum 3 h after 17β-estradiol administration and slowly return to low basal levels in 15 h. Dexamethasone, progesterone, and 5α-dihydrotestosterone had no effect on uterine c-*fos* mRNA expression. The induction of c-*fos* mRNA by estrogen was unaffected by the protein synthesis inhibitor puromycin but completely abolished by the RNA synthesis inhibitor actinomycin D. (Molecular Endocrinology 2: 946–951, 1988)

INTRODUCTION

Acute administration of estradiol (E_2) to immature female rats elicits a rapid and dramatic growth response of the uterus. This response involves an initial (within 1–3 h) E_2-induced hyperemia, which is followed by hypertrophy and hyperplasia within 24 h of hormone administration (1–3). The combination of these three effects results in a massive overall growth of the organ. Within the immature uterus the three predominant cell types, epithelial, stromal, and myometrial, all undergo a growth response after estrogen treatment (4–6). This massive, yet controlled, uterine growth provides an ideal model for elucidating the events and mechanisms underlying hormone-mediated proliferation of normal mammalian cells.

We and others have been interested in the possibility that the activation of cellular protooncogenes might play a role in estrogen-stimulated growth. Previous studies from our laboratories have established that the uterine epidermal growth factor (EGF) receptor, the cellular homolog of the erb-B oncogene, is induced by E_2 (7, 8). E_2 induction of the receptor as measured by the appearance of EGF receptor mRNA is very rapid and reaches a peak within 3–6 h (8), while an increase in functional receptor occurs between 6 and 12 h after steroid administration (7). Similarly, Murphy *et al.* (9) demonstrated recently that N-*myc* and c-*myc* uterine in RNA levels are rapidly increased after estrogen treatment *in vivo*, and Travers and Knowler (10) also reported that c-*myc* and c-*ras*^{Ha} mRNA levels are increased in the uterus after E_2 treatment. Taken together these findings thus suggest that it may be important to investigate the precise pattern of expression of a variety of cellular oncogenes to fully understand estrogen-stimulated growth.

Numerous studies have associated expression of the cellular oncogene c-*fos* with proliferation *in vitro* (11, 12) and *in vivo* (13), differentiation (14, 15), and development (15). Although the precise function of the c-*fos* gene product is not known, recent reports that the 55 kilodalton c-*fos* nuclear protein activates specific promoters suggest that c-*fos* may be a trans-acting regulatory factor (15–17). In this work we sought to determine if increased c-*fos* expression also occurred in the uterus after estrogen stimulation. This report demonstrates that mRNA from the cellular oncogene c-*fos* is rapidly increased in the immature rat uterus *in vivo* after a single injection of E_2. This response is specific for estrogenic steroids, occurs at E_2 levels which stimulate normal tissue growth, and is blocked by actinomycin but not puromycin.

RESULTS

In ovariectomized rats there is a low but detectable level of c-*fos* mRNA as shown in the RNA blot analysis in Fig. 1. After a single injection of E_2 there is a marked induction of the steady state level of a 2.2 kilobase (kb) c-*fos* mRNA. This induction is apparent within 1 h and reaches maximum levels at approximately 3 h. This rapid increase is followed by a rapid decline in c-*fos* mRNA over the next 3 h. However, the level of c-*fos* mRNA remains significantly above uninduced levels for at least 15 h (Fig. 1). These observations have been

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reproducibly observed in at least four separate experiments similar to those illustrated in Fig. 1. In other experiments, initial increases (2- to 3-fold) in c-*fos* mRNA levels have been observed within 30 min of E_2 administration. Densitometric analysis of a number of time course studies was performed to quantify this increase in c-*fos* mRNA levels. These results are illustrated in Table 1. This increase in c-*fos* RNA thus represents one of the earliest reported increases in a specific mRNA mediated by estrogens in the uterus.

The same RNA samples hybridized with the c-*fos* probe in Fig. 1 were also analyzed by hybridization to an antisense cRNA β-tubulin and the results are presented in Fig. 2. E_2 causes minor changes in the steady state levels of the 2.65 and 1.8 kb β-tubulin transcripts in the period examined; but the magnitude of these changes is minimal and the time course is different than that observed for the 2.2 kb c-*fos* transcript. This indicates that the massive changes in the c-*fos* signal observed in Fig. 1 are due to a specific increase in the sequence abundance of c-*fos* transcripts in uterine mRNA.

To determine if this increase in c-*fos* mRNA requires RNA and protein synthesis we employed the inhibitors actinomycin D and puromycin, respectively. For actinomycin D studies, animals were treated with the inhibitor (4 mg/kg) 3 h before estrogen treatment. Animals then received a second injection of the inhibitor (4 mg/kg) at the same time that E_2 was administered and tissues were removed and RNA was prepared 3 h after

Table 1. Time Course of c-*fos* mRNA Inhibition after Estradiol Administration

Time (h)	c-*fos* mRNA	n
0	1.00 ± .22	5
½	2.12 ± .07	2
1	7.02 ± 1.55	6
3	31.5 ± 8.53	6
6	8.64 ± 1.33	4
12	10.4 ± 0.51	2
15	2.64 ± 0.08	2
18	1.83 ± 0.10	2

Ovariectomized rats were treated with E_2 (40 μg/kg) and killed at the indicated times. RNA was prepared and blot analysis performed as described in *Materials and Methods* and the legend to Fig. 1. The 2.2 Kb c-*fos* bands of the resultant films were scanned with a laser densitometer. c-*fos* mRNA levels are expressed in relative units based upon control samples present on the same film. Values represent means with the indicated SEM. N represents the number of different samples analyzed at each time point.

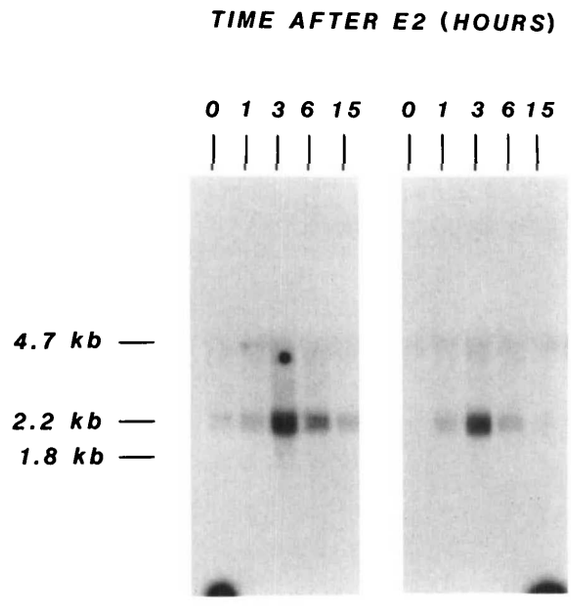

Fig. 1. Time Course of E_2 Induction of c-*fos* mRNA in Rat Uterus

Rats were ovariectomized 5 days before use and then treated with either E_2 (40 μg/kg, injected sc) or vehicle (5% ethanol) for the periods indicated. Uteri were then removed and RNA prepared using guanididium isothiocyanate and purified through CsCl. Each lane contains RNA from four animals, the two panels represent independent experiments. Ten-microgram samples of total RNA were denatured in methylmercuric hydroxide, separated on a 1% agarose/formaldehyde gel, and electroblotted to Gene Screen Plus. Membranes were hybridized with ^{32}P antisense c-*fos* RNA (1×10^6 cpm/ml) prepared with Sp6 RNA polymerase. Blots were hybridized 16–20 h at 62 C in 50% formamide, 0.8 M NaCl, 20 mM PIPES, 2 mM EDTA, 0.5% SDS with 100 μg/ml salmon sperm DNA and 100 μg/ml yeast tRNA, and then washed (twice in 2× SSC, 0.1% SDS at room temperature, twice for 30 min in 0.1× SSC, 0.1% SDS at 65 C), treated with RNase A, and exposed to x-ray film. The position of the 2.2 kb c-*fos* transcript is indicated.

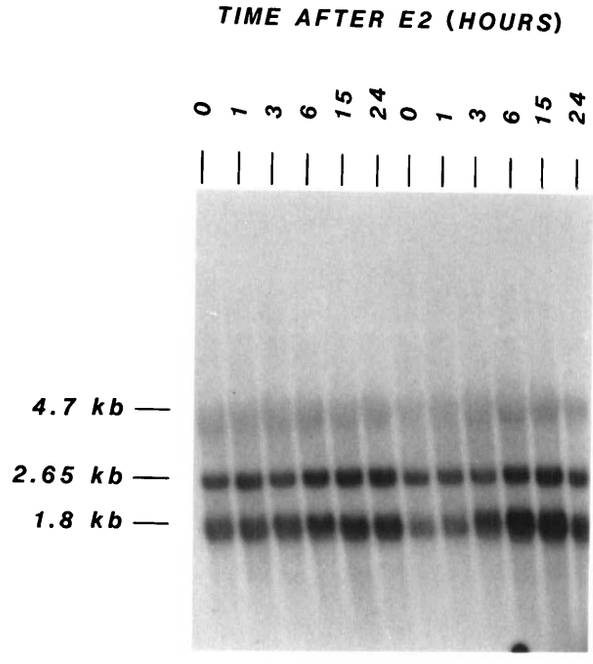

Fig. 2. Effect of E_2 on β-Tubulin Expression in Uterine RNA

The same samples analyzed in Fig. 1 were hybridized with a ^{32}P-RNA β-tubulin probe under the conditions described in the legend to Fig. 1.

steroid treatment. As shown in Fig. 3 actinomycin D abolished totally the increase in c-*fos* RNA seen after estrogen administration.

To determine if this increase in c-*fos* mRNA requires protein synthesis, a separate experiment was performed with the metabolic inhibitor puromycin. Animals were injected with puromycin (100 mg/kg) 30 min before E_2 administration. This dose of puromycin effectively inhibits protein synthesis in the liver (18) and uterus (19). As shown in Fig. 4 and confirmed by densitometric analysis, treatment of rats with puromycin did not impair the E_2 mediated increase of c-*fos* in the uterus. These results suggest that the induction of c-*fos* mRNA by E_2 is a direct effect which does not require the prior synthesis of other proteins.

We next examined the increase in uterine c-*fos* expression as a function of the dose of E_2 administered. For these studies animals were injected sc with various amounts of E_2 and the steady state level of c-*fos* mRNA was determined 3 h after injection. At the lowest doses tested (0.004–0.04 μg/kg) there is little or no increase in c-*fos* mRNA; between 0.4–4.0 μg/kg there is a marked increase in the level of the 2.2 kb transcript; and a higher dose of the hormone does not cause a further elevation (Fig. 5). This increase in c-*fos* mRNA by E_2 thus parallels the occupancy of nuclear estrogen receptors (20, 21) and increase in tissue DNA synthesis (22, 23) as a function of hormone dose. A more quantitative analysis of the data in Fig. 5 was obtained by densitometric measurements of the 2.2 Kb transcript. These results are shown in Table 2.

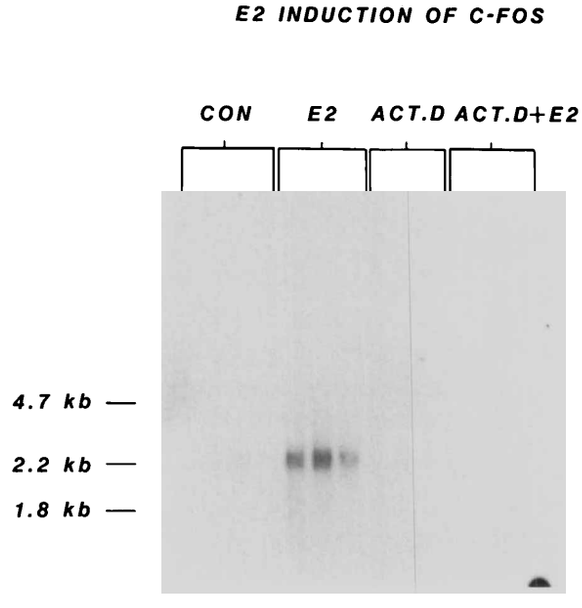

Fig. 3. Effect of Actinomycin D on E_2 Induction of c-*fos*

Total RNA was prepared from animals (three per sample) receiving the vehicle (con) or 40 mg/kg E_2 (E_2) 3 h before killing. Actinomycin D was administered in two doses (4 mg/kg each), 3 h before and at the same time as vehicle (Act. D) or E_2 (Act. D + E_2). Uterine RNA was prepared and blotted as described in *Materials and Methods* and in the legend to Fig. 1.

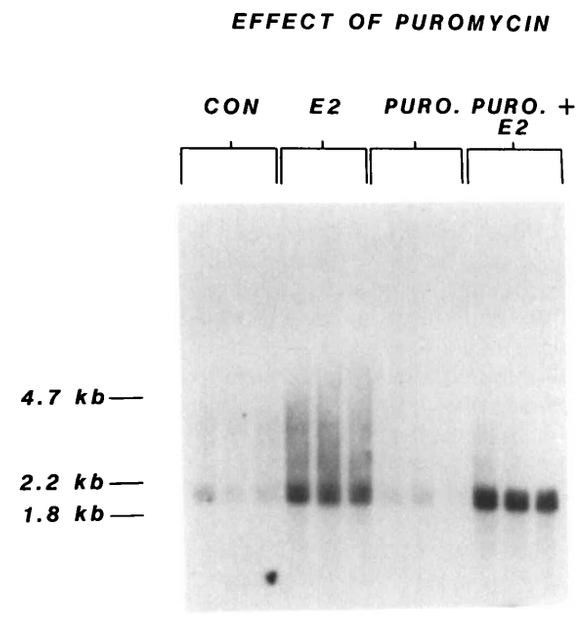

Fig. 4. Effect of Puromycin on E_2 Induction of c-*fos*

Total RNA was prepared from animals (three per sample) 3 h after treatment with vehicle, 40 μg/kg E_2, 100 mg/kg puromycin, or 100 mg/kg puromycin given 30 min before E_2. Uterine RNA was prepared and blotted as described in *Materials and Methods* and in the legend to Fig. 1.

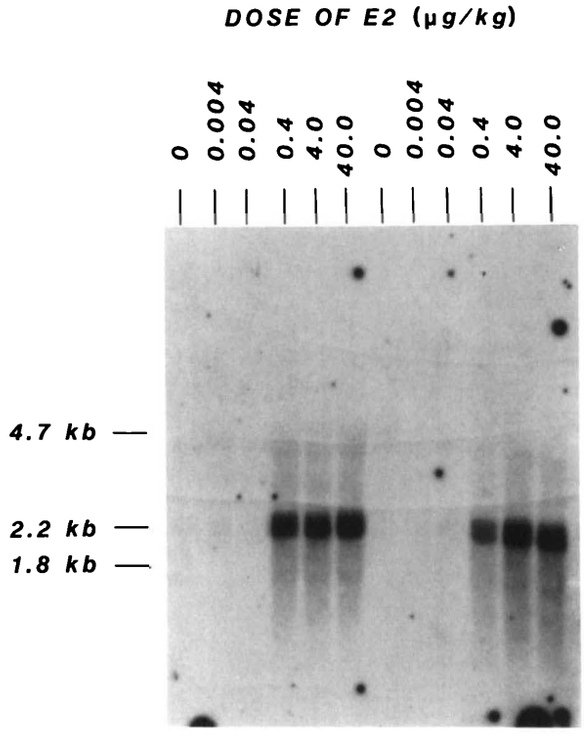

Fig. 5. Effect of Various Doses of E_2 on the Expression of c-*fos* mRNA in Rat Uterus

Animals (three per sample) were given E_2 at the indicated doses; total uterine RNA was prepared 3 h after E_2 treatment and hybridized with the murine c-*fos* probe. Hybridization conditions were as described in the legend to Fig. 1.

In another series of studies, several nonestrogenic steroids were tested for their ability to increase c-*fos* mRNA levels in the uterus. As shown in Fig. 6, the glucocorticoid dexamethasone, the androgen 5α-dihydrotestosterone, and progesterone were without effect while E_2 caused the expected elevation in uterine c-*fos* mRNA. This indicates that the induction of this transcript in the uterus is highly specific for estrogens under the conditions we have investigated, *i.e.* 3 h after acute administration of steroid. This result does not, however, rule out the possibility that other nonestrogenic steroids might affect c-*fos* expression under different conditions or treatment regimens.

Table 2. Induction of c-*fos* mRNA as a Function of E_2 Dose

Dose E_2 (μg/kg)	c-*fos* mRNA
0	1.0
0.004	1.44
0.04	1.51
0.40	32.3
4.00	43.1
40.0	43.7

The blots illustrated in Fig. 5 were scanned with a laser densitometer to quantify the 2.2 Kb c-*fos* transcripts. c-*fos* mRNA values are given in relative units and represent the mean for the two samples in Fig. 5 at each dose level.

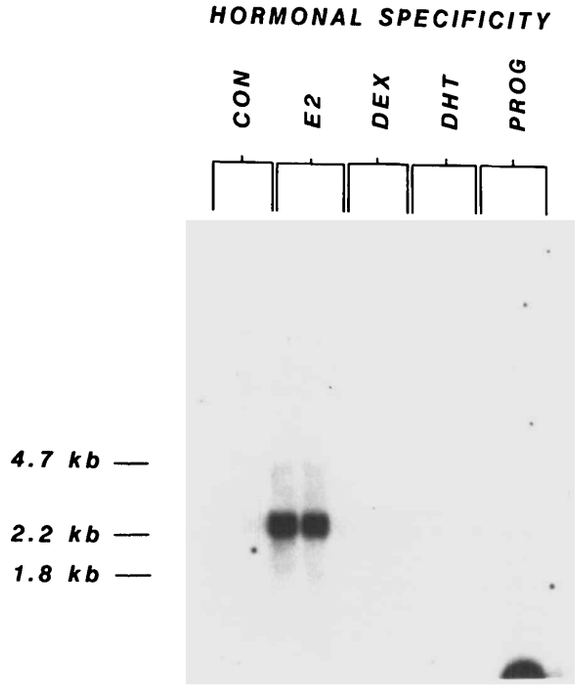

Fig. 6. Hormonal Specificity of the Induction of c-*fos* mRNA in Rat Uterus

Animals (three per sample) were injected sc with vehicle (CON), 40 μg/kg (E_2), 600 μg/kg dexamethasone, (DEX), 400 μg/kg 5α-dihydrotestosterone (DHT), or 40 mg/kg progesterone (PROG). Total uterine RNA was prepared 3 h after steroid treatment and subsequently hybridized to the c-*fos* antisense RNA as described in *Materials and Methods* and the legend to Fig. 1.

DISCUSSION

We have demonstrated that E_2 causes a rapid and pronounced increase in the steady state level of c-*fos* mRNA in the immature rat uterus *in vivo*. After a single injection of E_2, c-*fos* mRNA is elevated as early as 30 min and routinely reaches peak levels at 3 h. The increase of c-*fos* is thus one of the most rapid *in vivo* markers of estrogen action reported in the uterus and is in good agreement with the time course of occupancy of nuclear estrogen receptors which occurs rapidly and reaches peak levels in 30–60 min after hormone treatment (20, 21). After the peak at 3 h, levels of c-*fos* mRNA fall rapidly over the next 3 h but remain above basal levels for an additional 15 h. This time course is much more extended than most cases of c-*fos* induction. For example, in various types of cultured cells, agents such as phorbol esters (24), nerve growth factor (25, 26), or serum (27, 28) induce c-*fos* mRNA within 15–20 min and expression then returns to basal levels in 60–90 min. However, in placental tissue *in vivo* (28) and at various periods during fetal development (29) c-*fos* levels remain elevated for extended times. The pattern of c-*fos* mRNA in the uterus after estrogen treatment, however, is generally similar to the pattern for the EGF receptor (8), N-*myc* (9), and c-myc (9, 10) transcripts.

Since estrogens regulate the transcription of several genes, it was of interest to examine the sequence of the c-*fos* gene for potential estrogen responsive elements. Sequence analysis of the c-*fos* gene (27) reveals a 12 base pair sequence in the 5′-flanking region which has the nucleotide sequence 5′-GGTCTAGGAGACC-3′. This is similar to the palindromic estrogen responsive element identified in the Xenopus vitellogenin (5′-GGTCANNNTGACC-3′) (30) and PRL (31) genes. This region is located at -219/-207 with respect to the start site of transcription. Also, internal to the start site of c-*fos* transcription (at base 2102 to 2114) is a similar sequence 5′-GGTCTGCCTAGGC-3′. While this is an interesting comparison, it has not been determined which, if either, of these sequence elements is responsible for c-*fos* induction by estrogen in the uterus.

The rapid time course of E_2 induction and the studies using the metabolic inhibitors puromycin and actinomycin D suggest that this induction is mediated by directly increasing the rate of transcription of the c-*fos* gene. This interpretation would also be consistent with the presence of sequences homologous to the estrogen regulatory element of the Xenopus vitellogenin A2 gene (30). However, other interpretations are also possible. For example, estrogens are known to stabilize mRNA against degradation in other systems (32). Additional studies (*e.g.* nuclear run-on experiments) will therefore be required to establish unequivocally the mechanism(s)

by which E_2 increases levels of c-*fos* mRNA in the uterus.

Another unanswered question at present is the identity of the uterine cell types in which E_2 increases c-*fos* mRNA levels. In the immature animal, E_2 elicits a growth response in all three major uterine cell types (epithelium, stroma, and myometrium) (4–6), but we have no information at present about c-*fos* expression in these cell types or the role of the c-*fos* protein in their growth.

The induction of uterine c-*fos* mRNA is very specific for E_2. Given the wide array of agents reported to induce c-*fos*, it is almost surprising that none of the other steroids tested had any effect on expression of this oncogene. This is especially true since the uterus has receptors for progesterone (33), androgens (34, 35), and glucocorticoids (36), as well as a complement of estrogen receptors. It is possible, however, that these other steroids may play a role in the regulation of c-*fos* in other target tissues, or the uterus under different conditions, or at different times.

The precise function of the c-*fos* protein in any system studied is not yet clear. While c-*fos* induction is correlated in many cases with proliferation and cell division (11–15), there are clear examples of its induction upon cessation of cell division and commitment to a differentiated state (14, 15). Recent reports of c-*fos* protein interaction with specific promoters in mammalian (15, 17) and yeast (16) cells suggest that the protein may be a trans-acting regulatory molecule. The events mediated by induction of c-*fos* in the uterus and their association, if any, with uterine growth or differentiation thus pose interesting questions for further study.

Studies from our laboratories have now shown that E_2 mediates increases in the mRNA levels of two cellular oncogenes c-*fos* and erb-B (8), *i.e.* the EGF receptor, in the uterus. Others have made the important observations that N-*myc* (9), c-*myc* (9, 10), and c-*ras*^{Ha} (10) transcripts are also increased in the uterus after estrogen treatment. Given the increasingly recognized numbers of oncogenes and their normal cellular counterparts, it is not unlikely that the expression of other protooncogenes in normal tissues may be regulated by estrogens. Systems such as the uterus may thus be well suited to investigating the role of protooncogenes in the *in vivo* proliferation of normal tissues, and may provide possible insights into the pathological basis for abnormal growth.

MATERIALS AND METHODS

Materials

All chemicals and reagents were obtained from Sigma Chemical Corp. (St. Louis, MO) unless otherwise indicated. Restriction enzymes, calf intestinal alkaline phosphatase, and T4 DNA ligase were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Sp6 and T7RNA polymerases were obtained from Promega Biotech (Madison, WI). These reagents were used following the manufacturer's recommendations. The c-*fos* probe used in these studies was subcloned from a mouse c-*fos* construct (pc-fos-3) described by Miller *et al.* (37) which was obtained from the American Type Tissue Collection. pc-*fos*-3 was digested to completion with *Eco*RI and *Sst*I and the 1.5 kb fragment containing the 3′-three exons was inserted into pGEM3 (Promega). Antisense c-*fos* transcripts were produced from this insert using Sp6 RNA polymerase. The β-tubulin probe used in some experiments was prepared from a Chinese hamster ovary cDNA isolated by Boggs and Cabral (38); a 5′ 1.3 kb *Bam*HI fragment of this clone was ligated into pGEM3 and antisense probe was synthesized T7 RNA polymerase. The ^{32}P-UTP (800 Ci/mmol) was obtained from Amersham Radiochemicals (Arlington Heights, IL) and diluted to 400 Ci/mmol with radioinert UTP (Boehringer Mannheim) for the RNA polymerase reactions. Hybridization membranes were obtained from Dupont (Gene Screen Plus). E_2 was obtained from Steraloids (Wilton, NH) and progesterone, 5α-dihydrotestosterone, dexamethasone, puromycin, salmon sperm DNA, and yeast tRNA were from Sigma. Guanidine isothiocynate, cesium chloride, and formamide were obtained from International Biotechnologies (New Haven, CT).

Animals

Immature female Sprague-Dawley rats (21 days old, 40–45 g, Harlan Sprague Dawley, Houston, TX) were ovariectomized 5 days before use. Animals were injected sc in the peri-scapular region with 0.5 ml E_2 dissolved in 5% ethanol, 95% normal saline; control animals received only vehicle. The nonestrogenic steroids, progesterone, 5α-dihydrotestosterone, and dexamethasone were also administered sc. In certain experiments the metabolic inhibitor puromycin was given 30 min before estrogen treatment (8) and actinomycin D was given in two equal doses, 3 h before and then concomitantly with estrogen.

RNA Blot Analysis

RNA was prepared as described by Chirgwin *et al.* (39), as described previously (8). Briefly, uteri were removed from decapitated animals and immediately disrupted in 5 M guanidium isothiocynate using a Polytron (Brinkmann, Westbury, NY) set at maximum speed for 60 sec. RNA was pelleted through 5.7 M CsCl, extracted twice with phenol-chloroform (1:1) once with chloroform, and precipitated with ethanol. RNA was quantified by absorption at 260 nm. Samples of total RNA (10 μg) from control or treated animals were denatured for 15 min in 15 mM methylmercuric hydroxide (Alfa, Salt Lake City, UT) and separated on a 1% agarose gel containing 20% (vol/vol) formaldehyde. RNA was transferred to Gene Screen Plus by electroblotting (16 h at 35 V in 40 mM Tris, 1 mM EDTA). Membranes were allowed to dry at room temperature and then prehybridized in 0.8 M NaCl, 2 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), 20 mM piperizine-*N*-*N*′-bis[2-ethanesulfonic acid], 50% deionized formamide, 100 μg/ml denatured salmon sperm DNA, and 100 μg/yeast tRNA at pH 6.2 for 3 h at 62 C. ^{32}P-Labeled antisense RNA probes were synthesized following the manufacturer's recommendation with Sp6 or T7 RNA polymerase and radiolabeled RNA was added directly to the prehybridization mixture. Blots were hybridized for 16–24 h at 62 C and then washed twice with 2× SSC, 0.1% SDS (1× SSC is 0.15 M NaCl, 0.015 mM NaCitrate pH 7.0) at room temperature for 30 min, twice with 0.1× SSC, 0.1% SDS at 65 C for 30 min and rinsed with 2× SSC. After the rinse, blots were treated with RNase A (0.5 g/ml) in 2× SSC for 6 min, washed in 0.1 SSC, 0.1% for 10 min, and exposed to x-ray film. Where indicated in the text, films were scanned with a Zeineh Soft Laser scanning densitometer.

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Address requests for reprints to: Dr. David S. Loose-Mitchell, Department of Pharmacology, The University of Texas Medical School at Houston, P.O. Box 20708, Houston, Texas 77225.

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