Estrogen Regulation of Epidermal Growth Factor Receptor Messenger Ribonucleic Acid

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Previous studies have demonstrated that 17 β-estradiol (E_2) causes a 3-fold increase in epidermal growth factor (EGF) receptors in uterine membranes. We now report that the increase in uterine EGF receptor levels is due to an increase in the steady-state levels of EGF receptor mRNA. After a single E_2 injection, EGF receptor mRNA levels, as determined by RNA blots, increase 3- to 4-fold between 1 and 3 h, remain elevated at 6 h, and decline between 12 and 18 h. The effect is specific for E_2 since the nonestrogenic hormones progesterone, dexamethasone, 5α-dihydrotestosterone, and the inactive stereoisomer of E_2, 17α-estradiol, are without effect. E_2-Mediated increases in EGF receptor mRNA levels are blocked by actinomycin D but not by puromycin. Taken together, these results indicate that E_2 regulates the level of EGF receptor by increasing the steady-state concentration of EGF receptor mRNA *in vivo*. (Molecular Endocrinology 2: 230–235, 1988)

INTRODUCTION

Estrogens regulate cell growth and division by initially binding to high affinity nuclear receptors which, in turn, bind to specific DNA sequences which regulate gene transcription (1). The subsequent events that mediate the mitogenic effect of estrogen are unknown. We are examining the hypothesis that estrogens promote growth by regulating the levels of certain growth factors and/or their respective receptors. We have used the immature rat uterus as an *in vivo* model for tissue growth and have focused on epidermal growth factor (EGF) and its cognate receptor.

EGF responses are mediated through activation of a membrane-bound receptor which is a tyrosine-specific protein kinase (2). EGF binding to its receptor promotes the synthesis of proteins (3–6) and the rapid stimulation of gene transcription (7–10). We have previously shown that 17β-estradiol (E_2) increases the level of functional uterine EGF receptors (11). This increase is prevented by cycloheximide or actinomycin D, suggesting that this regulation occurred, at least in part, at the level of transcription.

The study reported here was undertaken to determine if the increase in EGF receptor protein could be ascribed to increases in the levels of uterine EGF receptor mRNA after E_2 treatment *in vivo*. The results indicate that E_2 increases the steady-state levels of the EGF receptor mRNA as early as 3 h after E_2 administration and this increase in EGF receptor mRNA precedes the appearance of functional EGF receptors.

RESULTS

To monitor the steady-state levels of uterine EGF receptor mRNA we used a rat EGF receptor cDNA the structure of which is shown in Fig. 1. T7 RNA polymerase was used to synthesize a 500 nucleotide antisense ^{32}P-RNA probe, extending to the internal *Hind*III site of the cDNA, which was used in RNA blot analysis.

In initial experiments, animals were treated with E_2 for 6 h before isolation of RNA. This time was chosen based on previous studies demonstrating that EGF receptor levels increased 12–18 h after E_2 treatment (11) and we anticipated that increases in EGF receptor mRNA would precede increases in EGF receptor protein (see Fig. 5B). A 3- to 4-fold increase in the steady-state levels of uterine EGF receptor mRNA was observed 6 h after E_2 treatment (Fig. 2). The antisense RNA probe hybridizes to two transcripts with apparent sizes of 9.5 kilobases (kb) and 6.6 kb which are similar in size to reported values for EGF receptor mRNAs from A431 cells (12–15) and from WB cells, a rat hepatoma cell line (16). Although E_2 increases the level of both transcripts the effect is most clearly visualized for the 9.5-kb species. Actinomycin D completely abolishes the induction by E_2 as well as decreasing basal levels of EGF receptor mRNA (Fig. 2). These data suggest that hormonal regulation of EGF receptor mRNA occurs at the level of message synthesis. For comparison, total RNA prepared from A431 cells and

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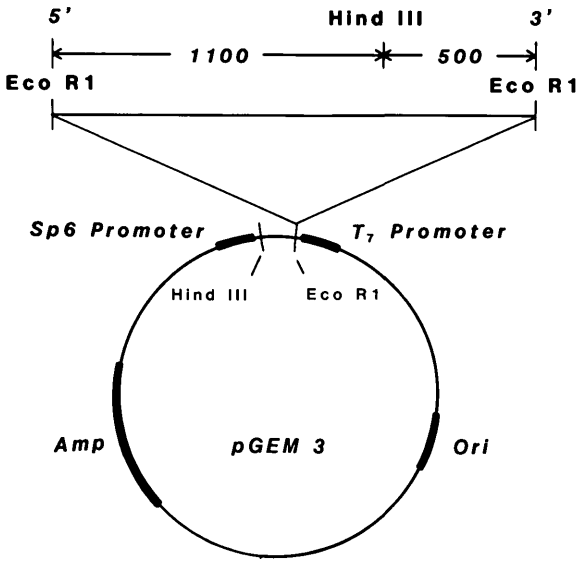

Fig. 1. Structure of Rat EGF Receptor cDNA

The 1600 base pair rat EGF receptor cDNA was subcloned into the *Eco*RI site of the cloning vector, pGEM3 (Promega) transformed into AG1 bacteria (Stratagene) as described by Hanahan (40) and DNA was prepared according to standard procedures (Progmega). The cDNA recognizes coding regions in the tyrosine kinase domain of the mature mRNA (A. Ullrich, personal communication) and has an internal *Hind*III site 500 base pairs from the 3′-end. *Hind*III was used to linearize the plasmid and T7 RNA polymerase, starting at the 3′-end of the insert and stopping at the internal *Hind*III site, was used to synthesize a 500 nucleotide ^{32}P-labeled antisense probe as described by Melton *et al.* (41). Template DNA was removed by digestion with DNase (Promega) and the antisense probe was purified by column chromatography and used in RNA blot analysis.

blotted with the rat antisense RNA probe is also shown (Fig. 2).

To determine whether protein synthesis was required for the E_2 induction of the EGF receptor mRNA animals were treated for 6 h with the hormone in the presence or absence of 100 mg/kg puromycin, a dose of drug that inhibits protein synthesis in rat uterus (17) and liver (18). The results (Fig. 3) indicate that puromycin decreases basal levels of EGF receptor mRNA but does not prevent the induction of EGF receptor mRNA after E_2 treatment (see *Discussion*).

The hormonal specificity of the induction of uterine EGF receptor mRNA was studied by examining the uterine response to several nonestrogenic steroids. Animals were treated for 6 h before killing with E_2 (40 μg/kg), progesterone (4 mg/kg), dexamethasone (600 μg/kg), or 5α-dihydrotestosterone (400 μg/kg). The nonestrogenic steroids do not elevate levels of EGF receptor mRNA (Fig. 4A). In a similar manner we compared the effects of E_2, the naturally occurring estrogen, with its stereo-isomer, 17α-estradiol, which binds to the estrogen receptor with a much lower affinity as compared to E_2 (19). E_2 (4 μg/kg), but not 17α-estradiol (4 μg/kg), increases steady-state levels of EGF receptor mRNA (Fig. 4B). Higher doses of E_2 and 17α-estradiol (40 μg/kg) produce a 3- to 4- and 0.2-fold increase, in uterine EGF receptor transcript levels, respectively (results not shown).

These results indicate that E_2 can increase uterine EGF receptor mRNA levels within 6 h and the hormonal specificity observed implied that the effect was mediated by the estrogen receptor system. Since occupation of the estrogen receptor occurs within 60 min or less after E_2 administration *in vivo*, we examined EGF receptor mRNA levels as a function of time.

The results (Fig. 5A) illustrate that the steady-state levels of the EGF receptor transcripts increase within 3 h of hormone administration and remain elevated for an additional 3 h. Messenger RNA levels then decrease by 12 h and are actually lower than control values 18 h after E_2 treatment.

The temporal relationship between E_2 effects on EGF receptor mRNA and the appearance of functional EGF receptors (as assessed by ligand binding) is presented in Fig. 5B. The data points for mRNA levels were obtained by densitometric measurements of the 9.5-kb transcript from a number of different experiments. The data points for EGF receptor levels were obtained in a previous study (11) and are redrawn in Fig. 5B for comparison. The increase in peak EGF receptor mRNA

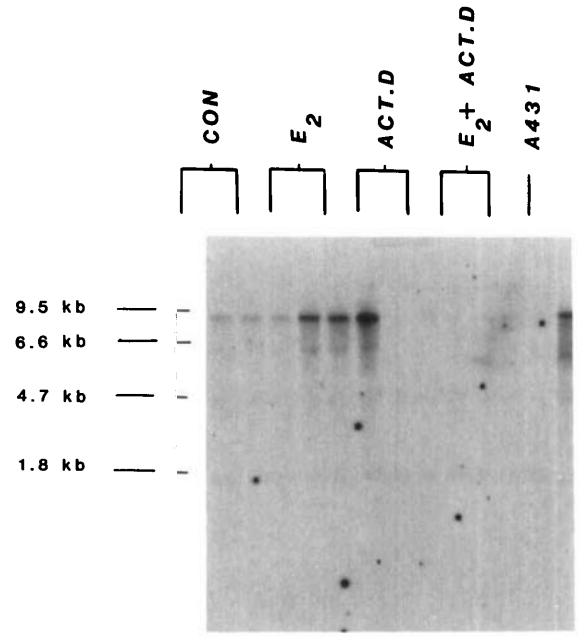

Fig. 2. E_2 Induction of Uterine EGF Receptor mRNA

Animals (four to six per group) were treated with vehicle, E_2 (40 μg/kg), actinomycin D (4 mg/kg), or E_2 (40 μg/kg) plus actinomycin D (4 mg/kg) for 6 h before killing; each RNA sample was prepared from three to six pooled uteri as described in *Materials and Methods*. Total RNA (12 μg) from each sample was electrophoresed, transferred to nitrocellulose, and the blot was hybridized with the ^{32}P-labeled antisense RNA probe (2.5 × 10^6 cpm/ml) as described in *Materials and Methods*. The blot was washed at high stringency (0.1× SSC, 0.1% SDS at 55 C) and exposed to Kodak XAR-5 film at −70 C. Each lane represents a separate RNA sample prepared from four to six pooled uteri. A sample of total A431 RNA (12 μg) is also shown for comparison.

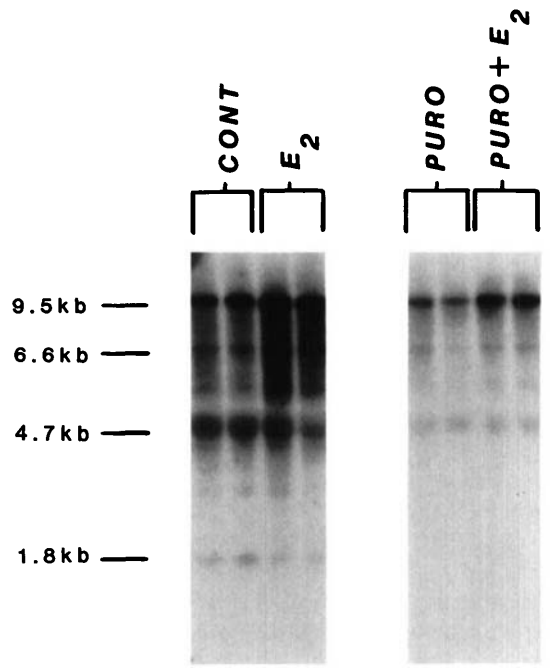

Fig. 3. The Effect of Puromycin on the Induction of EGF Receptor mRNA by E_2

Animals (four to six per group) were treated with vehicle, E_2 (40 μg/kg), puromycin (100 mg/kg), or E2 (40 μg/kg) plus puromycin (100 mg/kg) for 6 h before killing. Total RNA was prepared from pooled uteri, electrophoresed, and transferred to nitrocellulose as described. Each lane represents a separate RNA sample prepared from four to six pooled uteri.

levels precedes the peak increase in receptor levels (see *Discussion*) by about 9–12 h.

DISCUSSION

Target tissues, such as the uterus and mammary gland, respond rapidly to estrogen with enhanced tissue growth and development. However, the processes that result in growth after estrogen treatment are obscure. We have examined the hypothesis that some of the mitogenic effects of estrogen are mediated by EGF and have previously reported that E_2 can modulate the level of functional EGF receptors *in vivo* (11). E_2 produced a 3-fold increase in EGF receptors as measured by ^{125}I-EGF binding, EGF-stimulated kinase activity, and chemical cross-linking of ^{125}I-EGF to its receptor with disuccinimidyl suberate (11). Furthermore, the E_2 induction of EGF receptor levels was sensitive to both cycloheximide and actinomycin D (11).

We now report that E_2 increases the steady-state levels of uterine EGF receptor mRNA. The induction of EGF receptor mRNA by E_2 apparently occurs through an estrogen receptor mechanism since this regulation displays the appropriate hormonal specificity (Fig. 4). The time course of EGF receptor mRNA accumulation is rapid (Fig. 5), especially since maximal occupancy of nuclear estrogen receptors required 30–60 min after *in vivo* hormone treatment (1). These data, as well as the complete inhibition of the E_2-induction by actinomycin D (Fig. 2), suggest that estrogens regulate the EGF receptor gene by altering the level of mRNA synthesis.

The studies performed with puromycin are more difficult to interpret. In the absence of hormone, puromycin

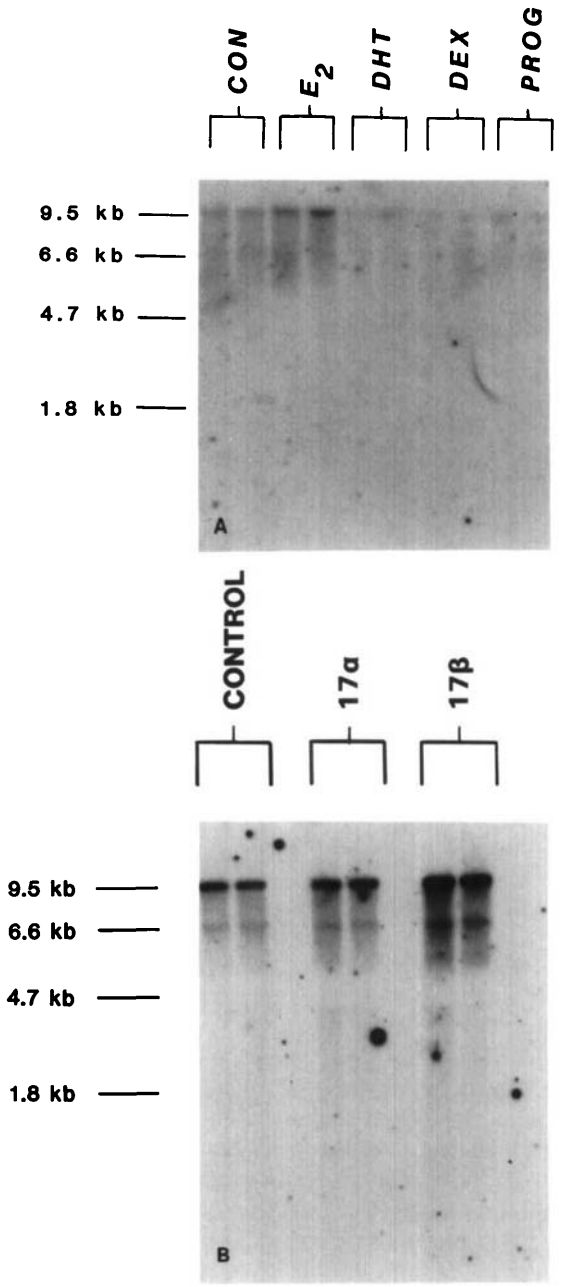

Fig. 4. Hormonal Specificity of EGF Receptor mRNA Induction

A, Animals (four to six per group) were treated with vehicle, E_2 (40 μg/kg), progesterone (4 mg/kg), dexamethasone (600 μg/kg), or 5α-dihydrotestosterone (400 μg/kg) for 6 h before killing. Total RNA was prepared from pooled uteri and RNA blots were run as described in *Materials and Methods*. Each lane represents a separate RNA sample prepared from four to six pooled uteri. B, Animals (four to six per group) were treated with vehicle, 17α-estradiol (4 μg/kg), or E_2 (4 μg/kg) for 3 h. Total RNA was prepared and analyzed as described in Fig. 2. Each lane represents a separate RNA sample prepared from four to six pooled uteri.

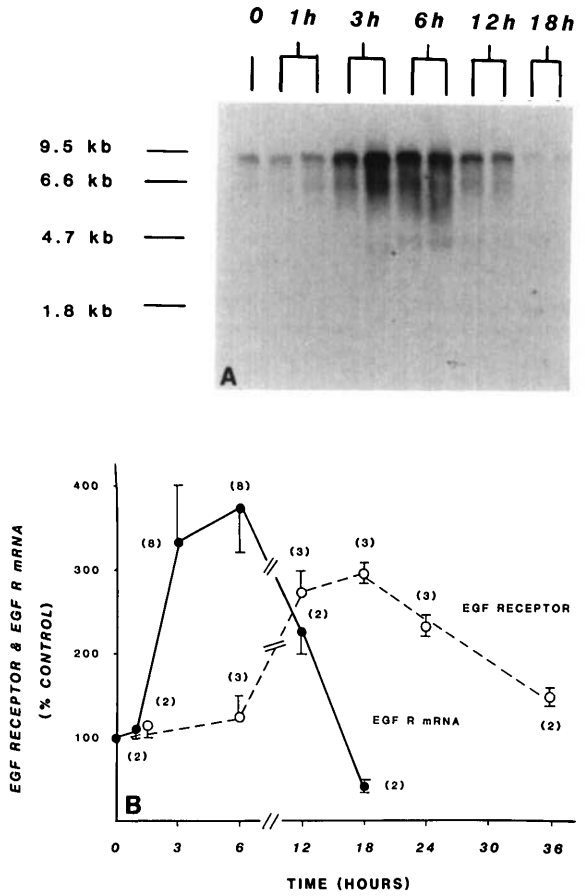

Fig. 5. Time Course for E_2 Induction of EGF Receptor mRNA
A, Animals (four to six per group) were treated with E_2 (40 μg/kg) for the indicated periods of time before killing. Total RNA was prepared and RNA blots were run as described. Each lane represents a separate RNA sample prepared from four to six pooled uteri. B, The film obtained from A, as well as other films, were scanned with a laser densitometer. The data shown represent the means ± SE of the indicated number of different experiments. The binding of ^{125}I-EGF to the EGF receptor was obtained in a previous study (11) and is also presented in B.

decreases basal levels of EGF receptor mRNA (Fig. 3). This is probably related to the mechanism of action of puromycin. This drug inhibits protein synthesis by causing premature termination of the nascent polypeptide chain with the release of the mRNA as well as the peptide chain from the ribosome (20). A likely consequence is that the free EGF receptor mRNA is more rapidly degraded resulting in a lower steady-state level of EGF receptor mRNA as seen in Fig. 3. E_2, in the presence of puromycin, still elicits an increase in the steady-state levels of the EGF receptor mRNA indicating that protein synthesis is not required for hormonal induction of the EGF receptor mRNA.

Although the results of this study demonstrate that estrogens can increase levels of EGF receptor mRNA by apparently regulating expression of the EGF receptor gene, alternative explanations exist that could explain these data. For example, estrogens have been shown to increase the rate of transcription for vitellogenin mRNA as well as stabilizing this mRNA against degradation (21, 22). The precise mechanism underlying the changes in half-life are not known. It is therefore possible that the hormonal control of the EGF receptor mRNA observed in this study occurs at two levels, namely, increases in transcription and mRNA stabilization.

The rat uterus expresses two EGF receptor mRNAs of 9.5 and 6.6 kb which are similar in size to those observed in WB cells, a rat hepatoma cell line (16), human placenta (13), A431 cells (12–15), and chick embryos (23). Although E_2 increases the level of both mRNAs the effect is most clearly visualized for the 9.5-kb transcript. The relevance of multiple mRNA transcripts for the EGF receptor is unknown (12–14).

Various factors including hormones, other growth factors, phorbol esters, vitamins, as well as cell density, cell differentiation, cell cycle position, and partial hepatectomy have been reported to affect cellular EGF receptor levels (24). Relatively few studies have examined the regulation of EGF receptor mRNA levels (16, 25, 26). The results reported here are thus the first direct demonstration that EGF receptor mRNA levels are under hormonal control in an intact animal model.

EGF regulates the level of mRNA for its own receptor in cultured cells (16, 25, 26). This raises the possibility that the E_2-mediated effects observed in this study occur secondarily in response to an initial increase in the synthesis and/or release of EGF from estrogen-sensitive tissues. This possibility cannot be discounted since EGF is present in uterine homogenates (27), and luminal fluid (28, 29). In addition, estrogens have been reported to increase the levels of EGF and EGF-like peptides in uterus (27, 29), and in MCF-7 cells which are estrogen sensitive (30, 31).

Estrogens, by unknown mechanisms, increase uterine DNA synthesis approximately 24 h after hormone administration (32, 33). The role of EGF and its receptor in this growth response in normal uterine tissue is unclear. One early event that occurs between 1 and 3 h after E_2 treatment is an increase in EGF receptor mRNA (Fig. 5A). This temporally precedes the appearance of functional receptors (Fig. 5B) (11), which in turn precedes the increase in DNA synthesis (32, 33). It is, therefore, possible that increases in EGF receptor levels are related to estrogen-stimulated uterine DNA synthesis. This possibility is supported by several observations. EGF can clearly stimulate the growth of many cells in culture, including cells derived from uterine myometrium (34) and uterine epithelium (35). In another recent study, antibodies to EGF were reported to decrease estrogen stimulated uterine growth in an organ culture system (36). While these observations are highly suggestive, a role for EGF and its receptor in the normal physiological control of uterine growth by estrogens has not yet been established directly.

The mechanism by which EGF regulates growth is unclear. Since EGF binding to its membrane receptor precedes its nuclear actions, several possibilities for control of gene transcription and DNA synthesis may be considered. For example, EGF, acting via its recep-

tor-linked protein kinase, could cause changes in intracellular ion fluxes, phosphoinositol turnover, internalization of receptors, and/or production of metabolites, all of which may represent potential mechanisms for regulation of gene expression and DNA replication.

In summary, this study demonstrates that E_2 increases uterine EGF receptor mRNA levels *in vivo*. This increase in the steady-state level of mRNA is likely to be the basis, at least in part, for the previously observed estrogen-mediated increase in functional EGF receptor levels (11). While several observations suggest a role for EGF in estrogen-stimulated uterine growth *in vivo*, further studies will be required to unequivocally evaluate this possibility.

MATERIALS AND METHODS

Unless noted below all chemicals and reagents were as previously described (11, 37). Restriction endonucleases were from either Boehringer Mannheim Biochemicals (Indianapolis, IN) or International Biotechnologies (New Haven, CT). The cloning vector, pGEM 3, and T7 RNA polymerase, were obtained from Promega Biotech (Madison, WI). The rat EGF receptor cDNA was generously provided by Dr. Axel Ullrich (Genentech, Inc., South San Francisco, CA). α-^{32}P-UTP (800 Ci/mmol) was obtained from Amersham (Arlington Heights, IL), and nitrocellulose (SA, 85, 0.45 μm) was obtained from Schleicher & Schuell (Keene, NH). E_2 and 17α-estradiol were obtained from Steraloids (Wilton, NH), and progesterone, 5α-dihydrotestosterone, dexamethasone, actinomycin D, puromycin, salmon sperm DNA, and sodium *N*-lauroylsarcosine were obtained from Sigma (St. Louis, MO). Guanidine thiocyanate was obtained from Fluka (Ronkonkoma, NY) and cesium chloride was purchased from Schwarz/Mann (Cleveland, OH). Agarose was from FMC (Rockland, ME). All other materials were the highest grade commercially available.

Animals

Immature female Sprague-Dawley rats (35–40 g, Harlan-Sprague Dawley, Houston, TX) were ovariectomized 4–7 days before use. Animals were injected sc with E_2 (40 μg/kg) dissolved in 95% saline, 5% ethanol; control animals received the vehicle alone. The nonestrogenic steroids progesterone (4 mg/kg), 5α-dihydrotestosterone (400 μg/kg), and dexamethasone (600 μg/kg) were administered in propyleneglycol containing 2% acetone due to the limited solubility of these drugs in saline. In experiments employing metabolic inhibitors, actinomycin D (4 mg/kg) or puromycin (100 mg/kg) were administered 30 min before E_2.

RNA Extraction and Agarose Gel Electrophoresis

RNA was prepared essentially as described by Chirgwin *et al.* (38) using the guanidine thiocyanate and cesium chloride procedure. Samples of total uterine RNA (12 μg) from control or E_2-treated animals were denatured in 15 mM methyl mercury hydroxide and electrophoresed through 1% agarose gels containing 20% (vol/vol) formaldehyde and stained with ethidium bromide to ensure that a constant amount of ribosomal RNA was present in each lane. RNA was transferred to nitrocellulose as described by Maniatis *et al.* (39). The filter was baked at 72 C for 2 h and prehybridized in 10 mM piperazine-*N*,*N*′-bis[2-ethanesulfonic acid], pH 6.5, 800 mM NaCl, 2 mM EDTA, 50% formamide, 0.5% sodium dodecyl sulfate (SDS), 0.1 mg/ml salmon sperm DNA, 0.04% polyvinyl pyrrolidone, 0.04% Ficoll 400 and 0.04% BSA for 6–12 h at 55 C.

Antisense Probe Synthesis

The rat cDNA was subcloned into the *Eco*RI site of the pGEM 3 vector according to standard procedures (39). Ag1 bacteria (Stratagene, San Diego, CA) were transformed as described by Hanahan (40) and DNA was prepared by standard procedures provided by Promega Biotech. T7 RNA polymerase was used to synthesize ^{32}P-labeled antisense RNA probes as described by Melton *et al.* (41) and hybridized to the filter for 12–18 h at 55 C. Filters were washed three times with 2× SSC (sodium standard citrate, 1× = 150 mM NaCl, 15 mM Na citrate, pH 7.0), and 0.1% SDS for 15 min at 55 C and then twice with 0.1× SS C plus 0.1% SDS for 15 min at 55 C. The filters were blotted dry and exposed to Kodak XAR-5 film at −70 C. Films were scanned with a Zeineh soft laser scanning densitometer.

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