Transient administration of estradiol-17β establishes an autoregulatory loop permanently inducing estrogen receptor mRNA

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Communicated by Robert T. Schimke, June 13, 1988

ABSTRACT A single transient dose of estradiol-17β is sufficient to elicit the permanent induction of hepatic estrogen receptor mRNA, which is induced 18-fold (from 0.13 to 2.4 molecules per cell) and then remains fully induced for at least 125 days. In primary liver cultures, extremely low concentrations of estradiol-17β, which are below the Kd of the Xenopus laevis estrogen receptor, maintain persistent induction of estrogen receptor mRNA but not of estrogen-inducible vitellogenin mRNA. These data and the ability of the antiestrogen, hydroxytamoxifen, to reverse persistent induction of estrogen receptor mRNA, support a model in which transient doses of estradiol-17β induce the estrogen receptor and thereby establish an autoregulatory loop. The low levels of estradiol-17β normally circulating in male X. laevis and the elevated level of receptor provide sufficient hormone–receptor complex to permanently maintain the induced level of expression of the estrogen receptor gene. The permanent induction of the estrogen receptor may be the regulatory switch that results in the persistent expression of a recently identified class of proteins that exhibit long-term responses to estrogen.

A central problem in the molecular biology of development is the question of how the expression of genes encoding gene regulatory proteins is controlled. Variations in the levels of these regulatory proteins initiate a cascade of long-term changes in the expression of genes subject to developmental regulation and tissue-specific expression. The recent observations that the retinoic acid receptor, which is likely to play a key role in vertebrate morphogenesis, and the transcription factor SP1, are members of the superfamily of DNA-binding proteins containing metal-binding fingers (1–4), reinforces the conclusion that this superfamily includes many eukaryotic gene regulatory proteins. Since other members of this superfamily mediate the effects of steroid hormones (5–15) and are oncogenes (14), they are currently generating intense research interest. The steroid hormone receptors currently represent some of the best-understood models for nuclear receptor proteins containing metal-binding fingers (reviewed in refs. 3, 5, 6, and 14). Although a great deal of progress has been made in both the identification of DNA sequences that interact with steroid hormone receptors and in the cloning of hormone receptors, very little is known about control of the expression of steroid hormone receptors and other nuclear receptor genes (3, 5, 6, 14). We have exploited an experimentally tractable steroid hormone receptor system to study one gene regulatory protein, the estrogen receptor. Our studies indicate that induction of the estrogen receptor mRNA by its ligand estradiol-17β results in the establishment of a stable, and probably permanent, regulatory loop. Induced levels of the estrogen receptor mRNA and protein maintain this autoregulatory loop long after serum estradiol levels in male Xenopus laevis have returned to an extremely low basal level. Our experiments show the very long-term induction of estrogen receptor mRNA in vivo, reproduce this effect in primary Xenopus liver cultures maintained in levels of estradiol that are below the Kd of the estrogen receptor, and use the antiestrogen hydroxytamoxifen to demonstrate an in vivo requirement for residual estradiol–estrogen receptor complex in maintaining the autoinduction of estrogen receptor mRNA. These studies, combined with detailed studies showing the long-term induction of type II nuclear estrogen binding sites and apolipoproteins in human HepG2 cells (16) and in avian liver (17), and of retinol-binding protein (RBP) in Xenopus liver (18), suggest that the administration of estradiol results in long-term effects that persist far beyond the period when the added hormone is present.

MATERIALS AND METHODS

Hormone Administration and Isolation of RNA Samples. Male X. laevis (Xenopus I, Ann Arbor, MI) received injections of 2 mg of estradiol-17β (dissolved in propylene glycol) on days 0, 2, 4, and 6 of the 14-day study of induction in vivo. Two animals were used for each time point. For the study of the long-term effects of estradiol in vivo, male X. laevis received a single 2-mg dose of estradiol-17β on day 0.

Primary X. laevis liver fragment cultures were prepared from untreated frogs essentially as described (19). Hormones were added to the culture medium in dimethyl sulfoxide. At the indicated times, total liver RNA was isolated by phenol/chloroform extraction (20). Poly(A) + mRNA was selected by batchwise adsorption to oligo(dT)-cellulose (Collaborative Research, Waltham, MA) followed by elution (21).

Quantitation of X. laevis Estrogen Receptor (xER) mRNA Levels. In quantitative RNA dot hybridizations, 5-μl aliquots of each sample containing 2 and 4 μg of poly(A) RNA were spotted onto GeneScreen, and fixed to the membrane by cross-linking with ultraviolet light. The amount of poly(A) RNA in each spot was standardized by hybridization to a β-actin cDNA clone. Actin mRNA levels are not regulated by estrogen (data not shown).

[32P]DNA hybridization probes (specific radioactivity, 5–10 × 106 cpm/μg) were prepared by hexamer-primed synthesis (22) from the isolated insert of the xER cDNA clone, which encompasses the entire protein coding region of xER mRNA (13). Hybridizations were performed essentially as described for genomic sequencing by Church and Gilbert (23) with the following modifications: the prehybridization period was 6–8 hr, the first wash contained 1% bovine serum albumin, and a total of three washes were performed.

The absolute amount of xER mRNA in each sample was determined by comparison to an xER complementary RNA (cRNA) standard curve. A tritiated xER RNA standard was prepared by T7 polymerase transcription of the xER cDNA insert subcloned into the vector pGEM3 (Promega Biotec, Madison, WI) (24). The tritiated cRNA standards and poly(A) RNAs were spotted onto GeneScreen and hybridized

Abbreviations: xER, Xenopus laevis estrogen receptor; RBP, retinol-binding protein.
to probes as described above. The spots were visualized by autoradiography. The autoradiograms were scanned by densitometry, and the spots were cut out and counted. The results obtained by scintillation counting of the spots and by densitometry were repeated four or five times for each time point and were very similar. The absolute amount of xER mRNA in each sample was therefore calculated by averaging the measurements. The number of molecules of xER mRNA per cell was calculated from the absolute amount of xER mRNA per sample, as we have described for vitellogenin mRNA (20). Despite the extremely low levels of xER mRNA in Xenopus liver, the accuracy and reproducibility of measurements made by this protocol are very high. For example, the level of xER mRNA in control liver was determined in two independent hybridizations of two samples from different animals. One sample yielded values of 0.13 and 0.13 molecules of xER mRNA per cell in the two hybridizations, and the other sample yielded values of 0.15 and 0.09 molecules per cell [mean, 0.125 ± 0.015 (SEM) molecules of xER mRNA per cell].

RESULTS AND DISCUSSION

Estrogen Induction of xER mRNA in Vivo. In this study, we used a xER cDNA clone (13) to investigate the regulation of estrogen receptor gene expression. Several types of evidence indicate that this clone encodes the xER. It encodes a protein both identical in size and exhibiting extremely high amino acid sequence similarity with the human estrogen receptor. In the DNA-binding domains of the two proteins, 82 of 83 amino acids are identical (13). The hormone-binding domains of the two proteins contain 44 and 46 amino acid tracts that exhibit 100% sequence identity (13). The Xenopus clone induces estrogen-dependent vitellogenin transcription upon transfection into Xenopus fibroblasts (T. C. Chang, D. Lew, and D.J.S., unpublished observations) and encodes a protein whose affinity for estradiol-17β is similar to that of the X. laevis estrogen receptor (data not shown; ref. 25). In RNA blot hybridizations of Xenopus poly(A) mRNA carried out under highly stringent conditions, both the intact cDNA clone and the hormone-binding domain (M. Hiken and D.J.S., unpublished observations) of the xER hybridize to the same family of four estrogen-recognizable mRNA species (mRNAs) (ref. 13; data not shown). To provide a convenient method of quantitating the levels of these four extremely rare mRNAs, we carried out high stringency quantitative RNA dot hybridizations with the xER cDNA insert. The basal level of xER mRNA in control Xenopus liver is only 0.13 molecule per cell (1 molecule per 10^{6} molecules of mRNA). This is perhaps the lowest level ever measured for an mRNA in a higher euakaryotic cell. In contrast to the transient down-regulation of the glucocorticoid (26) and progesterone (27) receptor mRNAs by their hormone ligands, estradiol-17β elicits an 18-fold induction of xER mRNA, which increases over a 2-week period from 1 molecule for every 6 or 7 hepatocytes to >2 molecules per cell (Fig. 1). The time course of induction of xER mRNA is similar to the time course of estrogen induction of vitellogenin mRNA (28). These data demonstrate that a steroid hormone induces the mRNA encoding the receptor to which it binds. While this ≈20-fold induction of xER mRNA represents a large change in an extremely rare message population, the absolute number of xER molecules per hepatic cell remains low. Even after induction to 2.4 molecules per cell, xER mRNA comprises only 0.002% of cellular mRNA.

Induction of xER mRNA in Primary Cultures. To determine whether the activation of xER gene expression was a direct effect of the hormone within the liver or resulted from extrahepatic production of other hormones or factors, we investigated the effects of estradiol-17β in primary Xenopus liver cultures. Two separate studies of the effects of estrogen on these cultures illustrated that the estradiol-17β induction of estrogen receptor mRNA is a direct response that is reproducible in culture (Fig. 2). The similarity of the induction in vivo and in liver cultures suggests that estrogen exerts a direct effect on hepatic xER gene expression. These studies also illustrated a second major difference between the regulation of estrogen receptor mRNA levels and those of glucocorticoid receptor. Down-regulation of glucocorticoid receptor mRNA by dexamethasone is a transient response in cultured cells. Glucocorticoid receptor mRNA levels return to a basal level within 3 days after addition of dexamethasone to the culture medium (26). The induction of xER mRNA by estradiol continues for 12–14 days both in vivo (Fig. 1) and in primary cultures (Fig. 2). The extremely low levels of xER mRNA make it impossible for us to definitively state whether xER mRNA accumulation is nearly linear or exhibits a modest increase in rate late in induction when the level of xER is increased.
Long-Term Stimulation of xER mRNA in Vivo. To examine long-term effects of estrogen on xER mRNA levels, we analyzed the RNA isolated from male animals that had received a single injection of estradiol up to 125 days earlier. The surprising finding was that hepatic estrogen receptor mRNA remains at a fully induced level for at least 4 months after a single injection of estradiol (Fig. 3). The persistent induction of xER mRNA is not due to residual estradiol from the injection, since estrogen levels in serum return to basal levels within 1 day after injection (29). Several years ago, we used hormone-binding studies to demonstrate that estradiol-17β evoked a long-term increase in high-affinity estrogen binding sites (25). The significance of those data was obscure, as long-term effects of steroid hormones had not been proposed.

We propose that a transient dose of estradiol, which elicits the long-term, and probably permanent, induction of estrogen receptor mRNA and protein (25), establishes an auto-regulatory loop resulting in the essentially permanent induction of estrogen receptor mRNA. Since the estrogen receptor protein induces the synthesis of its cognate mRNA, an elevated level of xER mRNA could be maintained indefinitely by the residual level of circulating estradiol in the male animal. The observation that the circulating estradiol level in control male Xenopus serum (0.2 nM) is sufficient to load ~25% of the receptor is consistent with this model (29). Although only a fraction of the estrogen receptor will be loaded with hormone, the elevated receptor level provides enough estradiol–estrogen receptor complex to maintain the induced rate of estrogen receptor gene expression. The observation that only 10–20% of cellular estrogen receptor may be required for some hormone responses is supported by studies that showed estrogen induction of uterine growth required only a small fraction of the cell’s complement of estrogen receptor (30).

This model predicts that a low level of residual hormone–receptor complex plays a central role in maintaining persistent activation of estrogen receptor gene expression. To more directly evaluate the role of the estrogen receptor in long-term induction, we used the antiestrogen, hydroxytamoxifen, a competitive inhibitor of the binding of estradiol-17β to the estrogen receptor (31–33). Hydroxytamoxifen was administered during the final week of a 60-day "withdrawal" period to whole animals given the single dose of estradiol used to establish persistent induction. Hydroxytamoxifen reduces the level of xER mRNA (Fig. 3, 60:7d HT sample) in the presence of inhibitor-saturated receptor protein. We have also recently shown that hydroxytamoxifen blocks the persistent estrogen induction of serum RBP mRNA in Xenopus liver (18). These data establish a requirement for estradiol–estrogen receptor complex in the maintenance of persistent induction and provide direct support for a key prediction of our model.

Subsaturating Concentrations of Estradiol Maintain Fully Induced Levels of xER mRNA. The estradiol required to form hormone–receptor complexes during withdrawal must be derived from the extremely low concentration of estradiol-17β circulating in the serum of male X. laevis. To directly test this idea, we used our primary culture system to establish a dose-response curve for persistent estrogen induction of xER mRNA (Fig. 4). Vitellogenin mRNA induction provides an appropriate control for these experiments, as detailed studies have unambiguously shown that induction of vitellogenin gene transcription and its mRNA requires the continuous presence of exogenous estradiol (19). An initial induced level of xER and vitellogenin mRNAs was established by maintaining the cultures in medium containing a saturating level of estradiol (1 μM) for 1 week. Separate cultures were then maintained for an additional 5 days in medium containing various concentrations of hormone including 0.5 nM, the Ks of the estrogen receptor, and 0.2 nM, the concentration of serum estradiol in control male Xenopus. All levels of estradiol added to the medium, even 0.2 nM, continued to induce xER mRNA to a level similar to that observed for the saturating 1 μM level. When no estradiol was added to the medium after the initial induction, the continued induction of xER mRNA was halted but not reversed. We observed no decline to the basal level during this 5-day period even in the absence of exogenous estrogen.

Comparison of the levels of xER and vitellogenin mRNA in these samples illustrates the dramatic differences that the vitellogenin and estrogen receptor genes display upon exposure to the same levels of hormone. No level of hormone below the full 1 μM saturating level is capable of continuing the induction of vitellogenin mRNA. The level of vitellogenin mRNA declines in a dose-dependent manner when lower concentrations of hormone are present (Fig. 4). Since vitellogenin transcription stops soon after estrogen is removed from the culture medium (19), the dose-dependent decline in vitellogenin mRNA levels is likely to result from dose-dependent differences in the time course with which vitellogenin mRNA shifts from the estrogen-stabilized (t50, 500 hr) to the destabilized (t16, 16 hr) state (19). Clearly, there are vast differences in the threshold levels of hormone needed for regulation of these two estrogen-responsive genes. Maintaining vitellogenin gene expression requires a concentration of estradiol-17β >1000 times higher than is required for estrogen receptor gene expression.

Other Genes Exhibit Long-Term Responses to Estradiol. In our in vivo studies on the estrogen-inducible RBP mRNA (18) indicate that this gene also displays a persistent induction by estradiol. Run-on transcription studies demonstrate that the persistent estrogen induction of RBP mRNA in Xenopus liver is due to a specific long-term increase in the rate of RBP gene transcription (18). The extremely low levels of xER mRNA, which is present in liver cells at a level lower than the level of RBP (21) and very low density apolipoprotein II mRNAs by a factor of >1000 (17), precludes direct measure-
ments of the contributions of gene transcription and mRNA stability to long-term induction by currently available methods. However, the observation that persistent estrogen induction of RBP mRNA in the same cells is caused by a specific increase in the rate of RBP gene transcription suggests a similar mechanism is responsible for long-term effects on estrogen receptor gene expression.

The phenomenon of long-term induction of proteins by estrogen is not restricted to Xenopus liver. In a series of studies, Deeley and coworkers have established that estrogen elicits a long-term induction of apolipoproteins in cultured human cells and in avian liver (16, 17). In contrast to Xenopus liver, they find that the avian liver high-affinity type I nuclear estrogen receptor is only transiently induced by estrogen (17). Since they observe the long-term estrogen induction of a lower affinity type II estrogen-binding protein, they propose that this protein mediates the persistent effects of estrogen by serving as a constitutive estrogen-independent activator of gene transcription (17). Three lines of evidence leads us to conclude that the persistent effects of estradiol on gene expression in our system are directly mediated by the well-characterized high-affinity estrogen receptor, and not by a secondary effector. (i) We find that protein synthesis is not required for the initial induction of vitellogenin (35) and RBP gene transcription (21). (ii) Our earlier hormone binding studies established that the induced estradiol-17β binding protein, present 70 days after estrogen stimulation, was the high-affinity estrogen receptor with the same Ks for estradiol as the estrogen receptor in control male liver cells (25). (iii) Each member of the family of xER mRNAs is estrogen inducible and hybridizes at high stringency to our cDNA clone of the "classical" high-affinity estrogen receptor (ref. 13; data not shown).

Two Classes of Estrogen-Regulated Genes Exist. The striking differences between the effects of estrogen on genes such as vitellogenin and on the estrogen receptor and RBP genes lead us to propose the existence of two classes of hormone-regulated genes and the model shown in Fig. 5. The most widely studied class of hormone-regulated genes, typified by the vitellogenin genes, requires the presence of exogenous hormone and a high level of loaded hormone-receptor complex to induce and maintain their transcription. A second class of target genes exemplified by RBP and xER exhibits long-term induction by estradiol. The seminal event that allows these genes to exhibit persistent estrogen induction is the establishment of an autoregulatory loop that maintains xER mRNA levels via a combination of high levels of induced estrogen receptor protein and residual estrogen circulating even in the serum of male animals (25, 29). This additional class of hormone-regulated genes presumably exhibits a lower threshold response to loaded hormone–receptor complex. The very different threshold levels exhibited for estrogen-regulated transcription by these two classes of genes suggests their promoters may contain different hormone-responsive elements. Further studies are necessary to determine whether the estrogen receptor gene contains a single class of estrogen response element in its 5' flanking region or multiple copies of the same estrogen response elements found in the vitellogenin genes.

A detailed understanding of the apparent differences in threshold levels of hormone receptor complex required for transcription of the two classes of genes requires additional experimentation. Our simplified schematic model does not consider the potential contribution of negative regulation to control of the short-term response class of genes such as vitellogenin. The possibility that unloaded native estrogen receptor binds to the estrogen response element without activating transcription is raised by the recent estrogen receptor deletion studies of Kumar et al. (36). This unoccupied receptor could act as a negative regulator, competing with the estradiol–estrogen receptor complex for binding to the response elements of short-term regulated genes, and making the ratio of unoccupied to occupied receptor critical for the activation of these genes. The possibility that activating the transcription of some genes requires concentration-dependent association of receptors to form estrogen receptor dimers or multimers also must be considered in evaluating threshold levels of receptors required for induction.

The demonstration of the long-term, and probably permanent, induction of a gene regulatory protein provides an interesting and experimentally accessible model system for the study of irreversible switch systems in development. One switch system in which the autoregulation of estrogen receptor gene expression might play an important role is related to

![Graph showing the induction of xER mRNA by estradiol](image1)

**FIG. 4.** Trace concentrations of estradiol maintain full induction of xER mRNA. The levels of xER mRNA (solid bars) and vitellogenin mRNA (hatched bars) were determined in cultures of liver cubes maintained for 1 week in medium containing 1 µM estradiol-17β (days 0–7). Separate portions of the cultures were then maintained in medium containing the indicated concentrations of estradiol for 5 days (days 7–12). Vitellogenin and xER mRNA concentrations were then determined as described. The induced level of vitellogenin mRNA (=50,000 molecules per cell) is similar to the maximum level we reported previously (34).
sex determination in *Xenopus*. When *Xenopus* tadpoles are incubated in water containing estradiol-17β during a brief developmental window, complete conversion of males to females is observed, and 100% of the resulting adults are fertile females (37, 38). The recent demonstrations in another developmental system that the *fushi tarazu* gene, which functions as a modulator of *Drosophila melanogaster* development, regulates its own expression (39) and that the *Drosophila* engrailed gene product binds to its own 5' flanking region (40) suggest that autoregulatory loops may provide a common mechanism by which cells control the expression of key regulatory protein genes and thereby provide long-term modulation of the expression of large sets of target genes.

We thank B. S. Katzenellenbogen for helpful comments on the manuscript and W. Soo Hoo for artwork. This work was supported by National Institutes of Health Grant HD 16720. M.C.B. is a National Institutes of Health predoctoral trainee.