Persistent Estrogen Induction of Hepatic Xenopus laevis Serum Retinol Binding Protein mRNA*

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Administration of estradiol-17β to male Xenopus laevis induces the hepatic mRNA coding for the serum retinol binding protein (RBP) approximately 10-fold, both in vivo and in primary liver cultures. Estrogen induction of RBP mRNA is completely blocked by the anti-estrogen, hydroxytamoxifen. Testosterone administration reduces the elevated level of RBP mRNA observed in livers of female X. laevis to the constitutive level seen in livers of control male animals, and partially blocks the estrogen induction of RBP mRNA. Intracellular RBP mRNA levels therefore represent a balance between the opposing effects of estradiol-17β and testosterone.

In marked contrast to the estrogen induction of vitellogenin mRNA, which requires the continuous presence of exogenous estrogen, induction of RBP mRNA persists for at least 4 months after a single injection of estrogen. Runoff transcription measurements demonstrate that persistent induction of RBP mRNA is due to an increased rate of RBP gene transcription. Administration of hydroxytamoxifen abolishes persistent induction of RBP mRNA, suggesting that residual hormone receptor complex plays a role in the persistent induction of RBP gene transcription. The persistent estrogen induction of RBP mRNA provides the first demonstration of long-term activation of the transcription of a hormone-responsive gene in response to a transient dose of a steroid hormone.

Estrogen induces liver cell differentiation and the synthesis of massive amounts of egg yolk precursor protein vitellogenin in Xenopus laevis. The estrogen induction of vitellogenin mRNA has emerged as a major model for the study of estrogen action at the gene level (reviewed in Refs. 1–3). In an effort to determine whether the estrogen regulation of vitellogenin gene expression was achieved through control mechanisms unique to genes expressed at an extremely high level, we set out to identify and clone other members of the set of liver proteins synthesized under estrogen control in Xenopus liver for secretion and storage in oocytes. We employed plus-minus hybridization to identify a clone coding for an estrogen inducible serum protein (4). Isolation and sequencing of a full-length cDNA clone of this protein demonstrated that the clone encodes the mRNA for serum retinol binding protein (RBP)† (5). We demonstrated that estradiol-17β induces hepatic RBP mRNA 10-fold in vivo from approximately 1,800 to 18,000 molecules/cell, and showed that estrogen induces a specific increase in the rate of RBP gene transcription (5).

In this work we have analyzed in detail the hormone regulation of RBP mRNA levels in primary Xenopus liver cultures and in vivo. The estrogen induction of RBP mRNA is shown to be mediated by the estrogen receptor as it is completely blocked by the anti-estrogen, hydroxytamoxifen. The ability of testosterone to both reduce the elevated level of RBP mRNA seen in female Xenopus, and to partially block estrogen induction of hepatic RBP mRNA suggests that the level of RBP mRNA represents a balance between the inducing effect of estrogen, and the repressive effect of testosterone. In the absence of additional estrogen, the estrogen induction of hepatic RBP mRNA is remarkably persistent. In contrast to vitellogenin mRNA, which totally disappears from cells in the absence of exogenous estradiol-17β, RBP mRNA levels remain elevated for at least 4 months after a single injection of estrogen. The persistent induction of RBP mRNA is shown to be due to an increased rate of RBP gene transcription, and to be dependent on estradiol-17β-estrogen receptor complex.

EXPERIMENTAL PROCEDURES

Induction of RBP mRNA in Vivo—Male X. laevis received injections of 2 mg of estradiol-17β dissolved in propylene glycol on days 0, 2, 4, and 6 when maximum induction was desired. In studies of the effects of anti-estrogens, the animals received 1 mg of hydroxytamoxifen dissolved in dimethyl sulfoxide and 0.25 mg of estrogen 1-2 h after injection of the hydroxytamoxifen. Testosterone was administered to female animals at 2 mg/injection on days 0, 2, 4, and 6. In studies of the persistent induction of RBP mRNA the only injection of hormone was 2 mg of estradiol-17β on day 0.

Liver Explant Cultures—These experiments were conducted essentially as we have described (6), except that hormones were dissolved in dimethyl sulfoxide rather than ethanol. Hormone concentrations in media were: 1 μM estradiol-17β, 1 μM testosterone, 5 μM hydroxytamoxifen. All incubations included dexamethasone at 10 nM, triiodothyronine at 10 nM, and insulin at 0.5 μg/ml. In order to randomize variation between individual animals, each culture dish contained liver cubes from two toads.

RNA Isolation—RNA was isolated by phenol-chloroform extraction as we have described (7). Following precipitation of nucleic acids by ethanol and dissolution in aqueous buffer, the RNA was selectively precipitated by addition of LiCl to 2.5 M and overnight incubation at 4 °C (5). The isolated RNA was shown to be intact and free of DNA by fractionation on an agarose gel, followed by staining in 2 μg/ml acridine orange.

Quantitation of RBP mRNA Levels—Hybridization probes were either derived from transcription of a PXRB1 cDNA subclone (in pGEM3) with T7 RNA polymerase (U. S. Biochemicals) in the presence of [32P]UTP (following the supplier’s recommended conditions), or from hexamer-primed synthesis of the gel-purified cDNA insert (8). Quantitative RNA dot hybridization was carried out as we have recently described (5). Briefly, RNA was heat denatured, spotted

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† The abbreviation used is: RBP, serum retinol binding protein.
onto the membrane by cross-linking in ultraviolet light. At least three dilutions of each sample were spotted. All samples were made to the same RNA concentrations by addition of yeast tRNA. After scanning the autoradiograms with a soft laser densitometer, the absolute amount of RBP mRNA in samples was determined by comparison to a standard curve obtained from spotting, hybridizing, and counting of five concentrations of 3H-labeled antisense RBP cRNA (5). The amount of RBP mRNA in samples was then used to calculate the number of molecules of RBP mRNA/cell as we have described (7).

Gels for blot hybridizations were performed as described in McKearin et al (5). Hybridizations using cRNA were carried out at 60°C as described (9). For hexamer-primed DNA probes, the conditions for the hybridization and washes were as described by Church and Gilbert (10), except that occasionally nonfat dry milk was substituted for bovine serum albumin.

RESULTS

Hydroxytamoxifen Blocks the Estrogen Induction of RBP mRNA—In previous work we demonstrated that estrogen induces a single species of RBP mRNA in vivo (5). In order to determine whether or not the estrogen induction of RBP mRNA is mediated by the nuclear estrogen receptor, we examined the effect of the synthetic anti-estrogen, hydroxytamoxifen. Hydroxytamoxifen is a very weak estrogen which binds tightly to the estrogen receptors of numerous species and serves as a specific antagonist of estrogen binding (11). Hydroxytamoxifen completely blocks the estrogen induction of vitellogenin mRNA (data not shown), a response which shows a high degree of correlation with estrogen-receptor loading (12, 13). When hydroxytamoxifen is injected into unstimulated control male Xenopus, which have not previously been exposed to exogenous estrogen, it neither induces RBP mRNA (Fig. 1, panel A (□)), nor abolishes constitutive expression of RBP mRNA (Fig. 1, panel A (□)). However, the low level of estradiol-17β used in this experiment (0.25 mg/animal), resulted in only a modest induction of RBP mRNA (Fig. 1, panels A and B (○)). This made it difficult to reach a definitive conclusion on the ability of hydroxytamoxifen to block estrogen induction of RBP mRNA by in vivo studies. These data also suggested that the expression of the RBP gene in the withdrawn animals (which received a single injection of estrogen for 40 days previously, but are no longer transcribing the vitellogenin genes at the time of restimulation (7)), differed significantly from RBP gene expression in the control animal. The 40-day withdrawn animal exhibited an elevated level of RBP mRNA (Fig. 1, panel B (●)). The elevated level of RBP mRNA in the withdrawn animal was not further induced by estrogen (Fig. 1, panel B (□)), and was repressed by hydroxytamoxifen (Fig. 1, panel B (○)).

Since the in vivo study did not provide unequivocal data on the effect of hydroxytamoxifen on the estrogen induction of RBP mRNA, we examined its effect in primary Xenopus liver cultures. In a preliminary experiment we used Northern blot hybridization to demonstrate that estradiol-17β induces RBP mRNA by approximately 10-fold, and that RBP mRNA represents a single species in primary liver cultures (Fig. 2). These data demonstrated that the induction of RBP mRNA in primary Xenopus liver cultures was similar to the induction we had previously observed in the intact animal (5). Quantitative RNA dot hybridizations demonstrated that estradiol-17β induced RBP mRNA from approximately 350 molecules/cell to 4000 molecules/cell in liver cultures from control male animals (Fig. 3 (□)). The level of RBP mRNA in the primary cultures on day 0 is lower than is observed in vivo because placing the liver in primary culture elicits a stress response which results in the degradation of most pre-existing mRNA (6). When estradiol-17β and hydroxytamoxifen were both added to the culture medium, the induction of RBP mRNA was completely blocked (Fig. 3 (△)), indicating that estrogen receptor mediates the induction of RBP mRNA. In a separate experiment, in which RBP mRNA levels were not quantitated, hydroxytamoxifen did not alter the basal level of RBP mRNA (data not shown). Since the in vivo data of Fig. 1, panel A, also indicates that hydroxytamoxifen does not substantially alter the basal level of RBP mRNA in control animals, it seems probable that the RBP gene is transcribed at a basal level which is independent of the presence of estrogen.

**RBP mRNA Induction Is Repressed by Testosterone**—The observation that hydroxytamoxifen, the antagonist of the principle female hormone, blocked induction of RBP mRNA...
raised the question of what effect the male sex steroid hormone, testosterone, might have. Female *X. laevis*, whose constitutive level of hepatic RBP mRNA is 12-fold higher than the level in control males, were injected with testosterone, RNA was isolated at various times following the first injection, and RBP mRNA levels were analyzed by Northern blot hybridization. Administration of testosterone causes the levels of RBP mRNA in the females to decline 12-fold over the 8 days of the experiment (Fig. 4), which reduces the level of RBP mRNA to approximately the constitutive level prevailing in control male animals.

The observation that testosterone could repress the high level of RBP mRNA which normally prevails in female animals led us to examine its effect on the estrogen induction of RBP mRNA in cultures from male animals. Addition of equal concentrations of testosterone and estradiol-17β to the culture medium resulted in a significant decrease in the extent of estrogen induction of RBP mRNA in cultures from the control male animals (Fig. 3 (Δ)). The antagonistic relationship of these two hormones indicates that intracellular levels of RBP mRNA are likely to represent a balance between the inductive effects of estrogen and the repressive effects of testosterone.

**Fig. 3. Hormone regulation of RBP mRNA levels in primary liver cultures.** Primary liver cultures from control male animals were maintained for the stated periods of time in culture medium containing the indicated hormones: 1 μM estradiol-17β (○), 1 μM testosterone (◇), 1 μM hydroxytamoxifen (Δ), and 1 μM estradiol-17β plus 1 μM testosterone (▲). RNA isolation, quantitative RNA dot hybridization, and calculation of the number of molecules of RBP mRNA/cell were as described under "Experimental Procedures" and the legend to Fig. 1.

**Fig. 4. Testosterone reduces hepatic RBP mRNA levels in female X. laevis.** Female animals received 2 mg of testosterone on days 0, 2, 4, and 6. Liver RNA was isolated at the indicated times. Aliquots of total RNA (400 ng) were fractionated on a denaturing formaldehyde-agarose gel, blotted, transferred to GeneScreen, and the blot was hybridized to RBP cRNA as described under "Experimental Procedures." The autoradiogram (shown at the bottom) was scanned with a soft laser densitometer and the relative values for RBP mRNA levels were plotted.

summarized in Fig. 5 demonstrate that a single injection of estradiol-17β resulted in a long-term induction of RBP mRNA at all times tested, even 60 and 125 days after injection. Since estrogen levels in serum return to basal levels within 1 day after injection of estrogen (14), the persistent induction of RBP mRNA is not the result of a continued high serum level of estrogen.

In previous work we demonstrated that short-term estrogen induction of RBP mRNA is accompanied by an increase of 2–3-fold in the relative rate of RBP gene transcription (5). In order to evaluate the role of gene transcription in the persistent induction of RBP mRNA, we determined the relative rate of RBP gene transcription in control male *Xenopus* liver, and in the livers of animals which had received a single injection of estrogen 85 or 90 days earlier. In two experiments, each involving two animals/data point, the rate of RBP gene transcription averaged 2.5-fold greater than was observed in the control animals (Table I). These data are in good agreement with the approximately 2.5-fold increase in the level of RBP mRNA seen in persistent induction (Fig. 5), and with the average 2.3-fold increase in the relative rate of RBP gene transcription we reported 3 days after estrogen administration (5). These data indicate that the persistent estrogen induction
FIG. 5. Estrogen induction of RBP mRNA persists in the absence of additional estrogen. Male animals received 2 mg of estradiol-17β on day 0 and were killed at the indicated times. Liver mRNA was isolated and quantitated by dot hybridization. Dot hybridizations were carried out after spotting the samples on the same membrane used to obtain the data shown in Figs. 1 and 5. The data for days 0 and 6 is the average of data from two animals. The longer time points represent data from single animals. All animals used in this experiment were from the same group of male X. laevis.

TABLE I

Persistent estrogen induction of RBP gene transcription

Nuclei were isolated, and runoff transcription measurements were made exactly as we have recently described (5). The data of experiments 1-3 are summarized from our previous study (5). The withdrawn animals used in experiments 4 and 5 received a single injection of 2 mg of estradiol-17β on day 0 and were used 85 or 88 days later. In the experiments on withdrawn animals, we hybridized 2.4·10^6 cpm of labeled nuclear RNA to each filter. Recent experiments in our laboratory indicate that the Xenopus RBP gene contains at least 2 very large intervening sequences near its 5' end. Since the precise size of the gene appears to be greater than 20 kb, and is unknown, we cannot calculate the transcription rate in parts/million. In a typical experiment on the withdrawn animals (experiment 5), we hybridized 3.8·10^6 cpm of RNA from the control animals, and 2.4·10^6 cpm of RNA from the withdrawn animals to the RBP and control filters. Hybridization efficiency was 33%. Specific RBP hybridization was 6.7 cpm/10^6 cpm of input nuclear RNA for the control animals, and 12.0 cpm/10^6 cpm for the nuclear RNA from withdrawn animals. This yields the 1.5-fold increase in specific RBP gene transcription in the long-term withdrawn animals shown in the table. The withdrawn animals used in experiments 4 and 5 exhibited an average increase of approximately 2-fold in their levels of hepatic RBP mRNA.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Runoff transcription</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.4</td>
</tr>
<tr>
<td>Control +E2</td>
<td>3.3</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.8</td>
</tr>
<tr>
<td>Control +E2</td>
<td>3.2</td>
</tr>
<tr>
<td>Experiment 5</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.8</td>
</tr>
<tr>
<td>Control +E2</td>
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</table>

of RBP mRNA is due to a long-term increase in the rate of RBP gene transcription.

The elevated level of RBP mRNA appears to be somewhat refractory to further induction by estrogen. The magnitude of the estrogen induction of RBP mRNA in withdrawn liver is less than in control liver (Fig. 1, panels A and B, O; and data not shown). In contrast, estrogen induction of vitellogenin mRNA in withdrawn Xenopus liver is both more rapid and reaches a higher level than in control animals (6, 7, 12).

DISCUSSION

Multihormone Regulation of RBP mRNA Levels—RBP mRNA is induced approximately 10-fold by estrogen in primary Xenopus liver cultures (Figs. 2 and 3) and in vivo (5). The estrogen induction of RBP mRNA is accompanied by a selective increase in the rate of RBP gene transcription. Our observation that estrogen induced RBP mRNA severalfold in the presence of cycloheximide suggests that the induction of RBP mRNA is a direct effect of estrogen (as is the activation of vitellogenin gene transcription (14)).

The anti-estrogen, hydroxytamoxifen, which binds with high affinity to the estrogen receptor and prevents binding of estradiol-17β to the receptor, completely blocks the estrogen induction of RBP mRNA, indicating that the estradiol-17β estrogen receptor complex is essential for induction. Hydroxytamoxifen and testosterone do not significantly repress the basal level of RBP mRNA (Figs. 1 and 3; and data not shown), indicating that transcription of the RBP gene proceeds at a basal level which is independent of the presence of male or female sex steroid hormones. The intracellular level of hepatic RBP mRNA appears to represent a balance between the opposing effects of circulating estradiol-17β and testosterone. Thus female animals, which exhibit an elevated circulating level of estradiol-17β (13), exhibit a level of RBP mRNA which approaches that seen in livers of chronically estrogen-stimulated male animals (Fig. 4; Ref. 5). Injection of testosterone reduces the level of RBP mRNA in female Xenopus liver, so that it becomes similar to the hormone-independent basal level seen in male Xenopus. Testosterone also partially antagonizes the estrogen induction of RBP mRNA in primary liver cultures (Fig. 3 A). The difference between the complete repression seen in the intact female animal and the effect of testosterone in liver cultures may be due to the much greater level of estrogen to which the cultures are exposed, or to sex differences in the response to the hormones.

Since testosterone does not compete with estradiol-17β for binding to the Xenopus estrogen receptor (13, 15-17) its repressive effect must be mediated through other mechanisms. Although it is possible that testosterone-receptor complex acts directly to repress RBP gene transcription, direct repression of gene transcription by steroid hormones appears to be relatively rare (18). It is possible that the testosterone-testosterone receptor complex binds at or near an estrogen response element in the RBP gene without producing an effect on transcription, and thereby blocks estrogen receptor binding. An alternative hypothesis is that testosterone exerts its effect by altering estrogen metabolism on binding to cellular components. However, the cytoplasmic middle affinity estrogen binding protein we have previously identified cannot play such a role, since testosterone does not compete with estrogen for binding to this protein (17).

Persistent Estrogen Induction of RBP mRNA—The induction of RBP mRNA by estrogen, the developmental regulation of RBP gene expression (19), and the identification of RBP in chicken eggs (20) all support the view that RBP and vitellogenin are members of a class of proteins induced by estrogen in Xenopus liver for storage in the developing oocyte. It is therefore surprising that the induction of RBP mRNA is strikingly different from that of vitellogenin mRNA. Vitellogenin gene expression exhibits a "memory effect," in which induction of new vitellogenin mRNA is more rapid and reaches a higher final level in withdrawn animals (1, 7). In contrast, the induction of RBP mRNA may actually be less

1 R. McKinney and D. J. Shapiro, unpublished observations.
efficient in withdrawn animals (Fig. 1, compare panels A and B, C)).

Estrogen induction of RBP mRNA results in a long-term alteration in the pattern of RBP gene expression. The persistence of the induction of RBP mRNA for at least 4 months after levels of estrogen have declined to basal levels (14) suggests that the estrogen activation of RBP gene transcription may be irreversible. This is fundamentally different from the induction of transcription of vitellogenin and most other hormone-inducible genes, which appear to require the continuous presence of an elevated level of the inducing steroid hormone (6, 7, 12, 18). At this time, the mechanism for the persistent estrogen induction of RBP mRNA is therefore mediated by a long-term elevation of the rate of RBP gene transcription. These data provide the first demonstration of the long-term activation of gene transcription and induction of a specific hormone-regulated mRNA in response to a transient dose of a steroid hormone. Our studies both extend earlier studies which used hormone binding assays to report a long-term induction of type I (16) and type II (21) estrogen receptors in Xenopus liver (16) and in human liver cells (21), and suggest which of the several possible explanations for this phenomenon seem most plausible. Although it remains possible that estrogen induction of RBP gene transcription induces a permanent change in the structure of RBP chromatin or a gene rearrangement, this seems improbable. Binding of steroid hormone-receptor complexes to chromatin in other systems is a reversible process. Since the RBP genes exhibit a significant basal level of transcription (5), they are likely to be accessible to the cell’s transcription apparatus even in the absence of estrogen. The observation that hydroxytamoxifen reduces the elevated level of RBP mRNA in withdrawn male liver (Fig. 1, panel B (C)) to approximately the basal level observed in control male liver (Fig. 1, panel A (D)), suggests that the persistent estrogen induction of RBP mRNA may be dependent on estradiol-17ß-estrogen receptor complex. Since the serum level of estrogen returns to the basal level (approximately 0.2 nM), which is characteristic of control male Xenopus, within 24 h (14), the residual level of estrogen found in male animals may now be sufficient to maintain the elevated transcription of the RBP gene. A plausible model for this process is based on our observation that estradiol-17ß induces a long-term increase in the level of Xenopus estrogen receptor protein (13, 16). Long-term induction has also recently been reported for the type II estrogen receptor of avian liver (21). When this elevated level of hormone receptor is present, the residual level of hormone receptor complex might be sufficient to maintain transcription of the RBP gene at an elevated level. This hypothesis requires that RBP gene transcription is maintained at an induced level by the small proportion of the estrogen receptor loaded with estrogen in withdrawn animals, while vitellogenin transcription requires a higher level of loaded receptor, and is not detectable in these animals (6, 7, 12). Studies designed to test the several possible models for persistent induction of RBP gene transcription are under way in our laboratory.

The process of cellular differentiation involves successive “choices” about mutually exclusive patterns of gene expression which can be thought of in oversimplified terms as binary switches. The persistent induction of RBP mRNA shares some properties of such irreversible switch systems. We are intrigued by the possibility that elucidation of the properties of the RBP gene which allow persistent estrogen induction of RBP gene transcription, may shed light on the mechanism of long-term gene activation in development.

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REFERENCES