Stimulation of Uridine Phosphorylation in Primary Cultures of *Xenopus laevis* Hepatocytes by Estradiol-17β

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**ABSTRACT.** The effects of estrogen on the uridine uptake into cells were examined in primary cultures of liver parenchymal cells from *Xenopus laevis*. The total uptake of [3H]uridine into the estrogen-treated cells and its incorporation into RNA were about 1.5 times higher than the values for control cells. The uptake of [3H]adenosine and its incorporation into RNA were not affected by estrogen. An experiment in which liver parenchymal cells were double labeled with [3H]uridine and [3H]adenosine showed that estrogen elevated the specific radioactivity of the UTP pool 1.4-fold the value found for the control cells, but that of the ATP pool was not altered by estrogen. Short term labeling revealed that estrogen did not significantly alter the rate of the initial uptake of [3H]uridine into the cells, but it did stimulate [3H]uridine phosphorylation about 1.7-fold. Uridine kinase activity measured in cell-free extracts of hepatocytes treated with estrogen had a value 1.6 times that of the control cells. These data indicate that the stimulation of [3H]uridine uptake and phosphorylation in *Xenopus laevis* hepatocytes in the presence of estrogen is caused by the enhancement of uridine kinase activity.

Estrogen induces the synthesis of the egg yolk protein precursor vitellogenin in the liver of male oviparous vertebrates, and this has provided valuable information about the hormonal regulation of gene expression (24, 26). A primary culture system of liver parenchymal cells from male *Xenopus* (7, 8) was established to study hormonal regulation of the hepatocyte function. In this culture system, liver parenchymal cells could synthesize and secrete vitellogenin for more than one month by responding to estrogen. Another report (27), however, has stated that liver pieces maintained for 1 to 2 weeks prior to the addition of estrogen could not respond to the hormone nor synthesize vitellogenin, an indication that some functions of the parenchymal cells were lost during culture. Our culture system provides valuable information about vitellogenin induction by estrogen.

It has been reported that estrogen did not alter the rate of total RNA synthesis even when vitellogenin was synthesized in large amounts (0.34 µg/h/10⁵ cells; on day 7 of estrogen treatment) in a primary culture of *Xenopus laevis* hepatocytes (7). Tata

Abbreviations used: PCA, perchloric acid; TCA, trichloroacetic acid; DPO, diphenyloxazol PEI, polyethyleneimine.

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and Baker (23) reported that the rate of incorporation of $[^3H]$uridine into hepatic nuclear RNA increased from 4 to 8 times following estrogen administration to male *Xenopus*, but they made no analysis of the effect of estrogen on the rate of $[^3H]$-uridine uptake into the cells nor on the specific radioactivity of the UTP pool after $[^3H]$uridine administration. This stimulation of the incorporation of $[^3H]$uridine into RNA by estrogen *in vivo* may be due to an increase in the specific radioactivity of the UTP pool rather than to a change in the actual rate of RNA synthesis. Recently, Brock and Shapiro (2) reported that estrogen increased $[^3H]$uridine incorporation into the nuclear RNA of cultured *Xenopus* liver fragments. Various factors are known to alter the uridine uptake of cells as well as its incorporation into RNA, apart from the actual rate of RNA synthesis (6, 22, 28).

In this paper, the effects of estrogen on $[^3H]$uridine uptake and its phosphorylation in a primary culture of liver parenchymal cells of *Xenopus laevis* which synthesizes vitellogenin efficiently by responding to estrogen.

MATERIALS AND METHODS

Isolation and culture of liver parenchymal cells. Liver parenchymal cells were isolated by the method described previously (7). The culture medium was 60% Leibovitz's L-15 medium supplemented with 10 mU/ml of insulin, 0.5 mg/ml of glucose, 100 mU/ml of penicillin and 100 µg/ml of streptomycin. The estrogen-treated cultured cells were maintained continuously in the presence of $10^{-6}$ M estradiol-17β starting one day after the inoculation of the cells. Cells were cultured at 25°C under atmospheric pressure. Under these conditions, parenchymal cells could be maintained for at least 8 days without significant loss of the cell number (7). The method used to determine the cell number has been described elsewhere (7).

Chemicals. The sources of the materials used were Leibovitz's L-15 medium from GIBCO, New York, U.S.A.; ACS-II scintillator solution, [2-$^3$H]adenosine (21 Ci/m mole) and [5,6-$^3$H]uridine (40 Ci/m mole) from the Radiochemical Center, Amersham, England; insulin (bovine pancreas) and estradiol-17β from Sigma Chemical Co., St. Louis, U.S.A. and Protosol from New England Nuclear, Boston, U.S.A.

Labeling procedure. Cells labeled with $[^3H]$adenosine, or with $[^3H]$uridine, were washed with cold culture medium containing 1 mM adenosine (or 1 mM uridine) then homogenized in a buffer solution (1% Triton X-100, 0.2 M sucrose, 50 mM Tris-HCl (pH, 7.6), 1% diethylpyrocarbonate). Portions of the cell homogenate were placed on glass fiber filters (Whatman GF/C). Some filters were dried immediately (total cellular uptake); others were washed thoroughly with cold 5% TCA containing 1 mM adenosine, or 1 mM uridine (incorporation into RNA). Radioactivity was counted in DPO-toluene scintillator solution (4 g/l) with an Aloka LSC-700 counter. The counting efficiency was 42%.

To determine the initial uptake of $[^3H]$uridine into the cells and its phosphorylation, $3 \times 10^4$ isolated cells were inoculated into each well of the microtest plates (Falcon, 6 mm in dia.). These cells were labeled with $[^3H]$uridine and immediately washed by five successive dips into beakers containing 500 ml of ice-cold amphibian Ringer solution (113 mM NaCl, 2 mM KCl, 0.7 mM CaCl$_2$, 0.3 g/l NaHCO$_3$). Following this, the Ringer solution was aspirated. To determine the initial uptake of $[^3H]$uridine, the cells were homogenized with 0.4 N PCA and counted the radioactivity of a portion of the cell homogenates in ACS-II scintillator solution after making them soluble with Protosol. The counting efficiency was 32%. To separate the uridine phosphates from uridine, another portion of the cell homogenates was centrifuged; the uridine phosphates in the supernatant fraction were neutralized.
with KOH (1) and separated from the uridine as described below, after which the radioactivity was counted in ACS-II scintillator solution. Counting efficiency was 24%. The acid-insoluble fractions were used to determine the amount of protein.

Protein determination. The amount of protein was determined by the method of Lowry et al. (12) using bovine serum albumin as the standard. Data were normalized with reference to the protein amount and cell number (60 µg protein = 10^5 cells).

Polyethyleneimine (PEI)-cellulose chromatography. To separate ATP and UTP, we used the two dimensional chromatographic method described previously (18). To separate the uridine phosphates from uridine, we applied samples to PEI-cellulose thin layers and developed them with methanol-H_2O (4 : 1, v/v) (9). In this system, uridine moved to the top of the plate, but the uridine phosphates remained in their original positions. The uridine phosphate spots were cut out and eluted (14).

Uridine kinase assay. Uridine kinase activity was measured by a modification of the procedure of Krystal and Webb (10). Parenchymal cells were homogenized with 10 mM Tris-HCl (pH, 7.6)-10 mM MgCl_2-10 mM KCl-20 mM 2-mercaptoethanol then sonicated for 15 sec at 4°C. The enzymatic activity did not change when the sonication time used to disrupt the cells was varied from 5 to 30 sec. This treatment resulted in complete cell lysis. The homogenate was centrifuged at 10,000 × g_{max} at 4°C for 30 min. Appropriate amounts of the supernatant fraction were added to the reaction mixtures, each of whose final volumes contained 200 µl, 10 mM Tris-HCl (pH, 7.6)-10 mM KCl-5 mM MgCl_2-10 mM 2-mercaptoethanol-2 mM ATP-6.5 µM [3H]uridine (0.9 µCi). After 60 min of incubation at 25°C, each sample was boiled for 2 min then centrifuged. A portion of the supernatant fraction was applied to PEI-cellulose as described above, and the radioactivity of the uridine phosphates obtained was counted. Each experiment included a zero time incubation as the control. The enzyme activity increased linearly up to 90 min and was proportional to the amount of the cell extract.

RESULTS

Difference between the cellular uptake of [3H]adenosine and [3H]uridine in the presence of estrogen. Estrogen stimulated the total uptake of [3H]uridine into the cells and its incorporation into RNA 1.5 to 1.6 times the value for the control (Fig. 1A). But the total cellular uptake of [3H]adenosine and its incorporation into RNA were not stimulated by estrogen (Fig. 1B). It was observed that the uptake of both labeled compounds into the cells and their incorporation into RNA increased with the duration of culture whether or not estrogen was present.

The specific radioactivity of the ATP and UTP pools was determined after double labeling the cells with [3H]uridine and [3H]adenosine to confirm the different incorporation patterns of these compounds into RNA under the same experimental conditions. ATP and UTP were separated by two dimensional chromatography on a PEI-cellulose thin layer. Estrogen increased the amount of specific radioactivity in the UTP pool 1.4 times the value found for the control cells (Table 1), but it did not enhance the specific radioactivity of the ATP pool, as reported previously (18). No interconversion of tritium was found between ATP and UTP. These results indicate that estrogen stimulates [3H]uridine incorporation into the cellular UTP pool and enhances its incorporation into RNA.

Initial rate of [3H]uridine uptake into liver parenchymal cells and its phosphorylation. The initial rate of uridine uptake by the cells is limited by the rate of transport,
whereas the subsequent accumulation of uridine is limited by the rate of phosphorylation (13, 16, 17). Therefore, stimulation of $^3$H]uridine uptake by estrogen may be caused by an increase in the rate of $^3$H]uridine transport, $^3$H]uridine phosphorylation, or both. To determine the rates of transport and phosphorylation, cells were labeled with $^3$H]uridine at short intervals of up to 90 sec. Up to 10 sec, the initial rates of uptake were approximately the same, independent of the presence of estrogen (Fig. 2). But following this, the uptake of $^3$H]uridine into the estrogen-treated cells was greater than into the control cells. This means that the transport of $^3$H]uridine into the cells was unaffected by estrogen.

To confirm whether uridine phosphorylation, rather than its transport, was stimulated by estrogen, the acid-soluble materials from the cells were analyzed by chromatography. By this procedure, uridine nucleotides could be separated from uridine to give an indication of the intracellular phosphorylation rates. Although the total uptakes of $^3$H]uridine were approximately the same in the presence or absence of estrogen within 5 to 10 sec, the amount of phosphorylated uridine was increased.

| TABLE 1. SPECIFIC RADIOACTIVITIES OF ATP AND UTP$^a$ |
|-----------------|---------------|--------------|
| estrogen        | UTP ($\times 10^3$ cpm/nmole) | ATP (c.p.m.) |
| ---             |               |              |
| -               | 2.83 (2.62, 3.04) | 4.97 (5.02, 4.92) |
| +               | 3.92 (3.92, 3.92) | 4.93 (5.06, 4.79) |

$^a$ Results are the means of the two determinations shown in parentheses. A culture of $8 \times 10^6$ cells, which contained 15.6 to 23.0 nmole of UTP and 138.4 to 157.2 nmole of ATP, was double labeled with 10 $\mu$Ci each of $^3$H]uridine and $^3$H]adenosine per 40 ml of culture medium for 24 h on day 4 of estrogen treatment. The specific radioactivities of the ATP and UTP pool were determined as described in MATERIALS AND METHODS.
Effect of Estrogen on Uridine Uptake

Estrogen stimulated $[^3H]$uridine phosphorylation about 1.7 times the value found for the control cells. This is evidence that the stimulation of $[^3H]$uridine uptake by estrogen is caused by enhanced phosphorylation of $[^3H]$uridine in the hepatocytes.

Stimulation of uridine kinase activity by estrogen. The phosphorylation of uridine is a relatively simple enzymatic reaction that involves two substrates, uridine and Mg$^{2+}$-ATP. It can be studied in cell-free extracts (21, 25). The effects of Mg$^{2+}$, ATP and uridine on uridine kinase activity were investigated in a high-speed supernatant fraction of hepatocyte homogenates from young adult Xenopus laevis. The reaction rate of uridine phosphorylation for different concentrations of Mg$^{2+}$ is shown in Fig. 3A. The maximum rate was obtained at concentrations exceeding 0.25 mM of Mg$^{2+}$. Enzymatic activity was not inhibited by a high Mg$^{2+}$ concentration, such as 10 mM. The effects of the ATP concentration of the enzyme reaction also were investigated (Fig. 3B). The maximum reaction rate was found at 0.25 to 2 mM of ATP; concentrations of ATP higher than 4 mM inhibited the uridine kinase reaction. Maximum uridine kinase activity took place at concentrations of uridine exceeding 5 $\mu$M (Fig. 3C).

Estrogen increased uridine kinase activity 1.6 times the value found for the control cells (Table 2). Because the enzyme activity was measured in cell-free extracts of hepatocytes, these extracts of the estrogen-treated cells may have contained factors that affected the uridine kinase activity of the control cells. This possibility was investigated by mixing enzyme extracts from the control and estrogen-treated cells in a 1:1 ratio then assaying $[^3H]$uridine phosphorylation. Uridine kinase activity in this mixture was approximately equal to half the sum of the activities of the respective enzyme preparations (Table 3). Thus, the estrogen-treated cells do not appear to contain factors that would affect the uridine kinase activity of the control cells.
Fig. 3. Effect of assay conditions on the uridine kinase activity of Xenopus laevis hepatocytes. A cell-free extract was prepared as described in MATERIALS AND METHODS. (A): the MgCl₂ concentration determined in the presence of 6.5 µM of [³H]uridine (0.9 µCi) and 2 mM of ATP; (B): the ATP concentration determined in the presence of 6.5 µM of [³H]uridine (0.9 µCi) and 5 mM of MgCl₂ and (C): the uridine concentration determined in the presence of [³H]uridine (0.9 µCi), 2 mM of ATP and 5 mM of MgCl₂. Bars show the range of values.

TABLE 2. URIDINE PHOSPHORYLATION

<table>
<thead>
<tr>
<th>estrogen</th>
<th>uridine phosphorylated a (pmol/mg protein/h)</th>
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<tbody>
<tr>
<td>–</td>
<td>48.8 ± 5.6</td>
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<tr>
<td>+</td>
<td>76.2 ± 5.1</td>
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</table>

a Mean of six determinations ± S.E. Each determination was done in duplicate.
DISCUSSION

The experimental results described above demonstrate that estrogen produces an increase in the rate of uridine phosphorylation accompanied by an increase in uridine kinase activity in primary cultures of *Xenopus laevis* hepatocytes. Consequently, estrogen increases the specific radioactivity of the UTP pool and the subsequent incorporation of [3H]uridine into RNA.

Previous reports (7, 18) have shown that estrogen caused no significant stimulation of the initial rate of total RNA synthesis in cultured *Xenopus laevis* liver parenchymal cells, even when vitellogenin synthesis was fully induced. Results obtained in vivo, however, indicate that estrogen enhanced [3H]uridine incorporation into hepatic nuclear RNA (23). [3H]uridine incorporation into RNA does not always reflect the rate of RNA synthesis (6, 22, 28). Cunningham and Pardee (3) reported that the increase in RNA labeling in the presence of [3H]uridine following the addition of fresh serum to a confluent 3T3 cell culture medium could be accounted for by stimulation of the rate of uridine uptake. In estrogen-treated *Xenopus laevis* hepatocytes, stimulation of the rate of incorporation of [3H]uridine into RNA is believed to be the result of stimulation of the uptake of [3H]uridine into the cells. Nucleoside kinase is known to be the key enzyme in nucleoside uptake into the cells (5, 19, 20). Stimulation of uridine uptake into *Xenopus* liver parenchymal cells by estrogen probably is caused by the activation of uridine kinase (Table 2). Our experimental results (Figs. 3A and B) show that the uridine kinase reaction is sensitive to the Mg²⁺ and ATP concentration as in other cell systems (25). Furthermore, the activity of the enzyme is inhibited by a high concentration of ATP. Additional studies are required to identify the mechanism by which estrogen stimulates uridine kinase activity. There are two possibilities. Estrogen may stimulate the synthesis of uridine kinase, or it may change the inactive enzyme into the active form with some modification.

The amount of stimulation of [3H]uridine incorporation into RNA by estrogen under our experimental conditions was about 25 to 30% of that found in an in vivo experiment (23). The possible explanations for this difference include the fact that insulin enhances the [3H]uridine uptake of cells by stimulating uridine phosphorylation (4, 15, 17). In the study reported here, the culture medium was supplemented with 10 mU of insulin per milliliter to maintain the liver parenchymal cells in primary culture. The basal value for uridine uptake may increase during culture; therefore, the true extent of the increase in uridine uptake produced by estrogen may not have been sufficiently evaluated under the present culture conditions, in comparison with that of the in vivo determination. Also, Lewis *et al.* (11) reported that the total RNA

<table>
<thead>
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<th>source of enzyme</th>
<th>uridine phosphorylated <em>a</em> (pmol/mg protein/h)</th>
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<tbody>
<tr>
<td>control cell extracts</td>
<td>40.2 ± 5.4</td>
</tr>
<tr>
<td>estrogen-treated cell extracts</td>
<td>64.3 ± 5.6</td>
</tr>
<tr>
<td>1 : 1 mixture of extracts from control cells and estrogen-treated cells</td>
<td>55.2 ± 2.3</td>
</tr>
</tbody>
</table>

*a* Mean of three determinations ± S.E. Each determination was done in duplicate.
content per liver increased and the maximum RNA content per gram of liver was 1.5 times that of the controls following estrogen injection into male *Xenopus*. Estrogen thus seems to stimulate the rate of RNA synthesis, to stabilize RNA, or both, under *in vivo* conditions. Certain factors, which may be produced by estrogen in other organs *in vivo*, probably act on the hepatocytes in concert with estrogen to bring about the reported *in vivo* responses.

Recently, Brock and Shapiro (2) found that estrogen decreased the specific radioactivity of the UTP pool and stimulated nuclear RNA synthesis 20- to 60-fold the value of the control in cultured *Xenopus* liver fragments. The differences between their findings and ours (18) may be due to differences in the experimental conditions used. Brock and Shapiro studied the short term effects of estrogen on cultured pieces of liver, but in our experiment the long term effects of estrogen on [*H]uridine metabolism in cultured hepatocytes were examined. Brock and Shapiro did indicate that in a cultured *Xenopus* kidney cell line estrogen caused an increase in the specific radioactivity of the UTP pool and in the incorporation rate of [*H]uridine into RNA, but the rate of RNA synthesis was not affected at all by estrogen (2). The results we report here agree well with the results of their *Xenopus* kidney cell system.

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