Translation of Rat Ovarian mRNA to Two 20 α -hydroxysteroid Dehydrogenase Isozymes in *Xenopus* Oocytes

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Abstract. 20 α -Hydroxysteroid dehydrogenase (20 α -HSD) in rat luteal tissue catalyzes the conversion of progesterone into a biologically inactive steroid, 20 α -hydroxypregn-4-en-3-one (20 α -OHP) and depletes the output of progesterone into the circulation. An increase in 20 α -HSD activity in luteal tissue is therefore a prerequisite for the regression of functional corpora lutea in rats. We have reported that ovarian 20 α -HSD is composed of two isoforms (HSD1 and HSD2). In this study, among batches of ovaries collected randomly during the estrous cycle, we selected two batches (batches A and B): the cytosol preparation from batch A contained both HSD1 and HSD2 activities, whereas that from batch B contained only HSD1 activity. From these 2 batches, we extracted mRNA, and each mRNA preparation was subjected to translation in *Xenopus* oocytes. The translation products of batch A exhibited both HSD1 and HSD2 activities, and those of batch B only HSD1 activity in accordance with the enzymatic activities observed in the respective cytosolic preparations. The results are compatible with the presence of two distinct mRNAs coding HSD1 and HSD2, and if so their transcription will be regulated separately according to the functional state of the ovary.

Key words: 20 α -Hydroxysteroid dehydrogenase, mRNA, ovary, *Xenopus* oocyte.

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20 α -HYDROXYSTEROID dehydrogenase (20 α -HSD) (E.C1.1.1.149) is an enzyme widely distributed in a variety of mammalian organs, such as the ovary [1, 2], testis [3, 4], placenta [5, 6], and adrenal [7, 8], and also in spontaneous neoplasms [9]. This enzyme catalyzes the conversion of progesterone to a biologically inactive steroid, 20 α -hydroxypregn-4-en-3-one (20 α -OHP) [1, 2]. An increase in the activity of 20 α -HSD in luteal tissue is always associated with the process of functional luteolysis in rats, and thus plays a central role in the regulation of the recurrence of

the rat estrous cycle [10, 11]. Since progesterone can be a precursor of various C18- and C19-steroid hormones, this enzyme affects the production of those hormones.

We recently carried out complete purification of 20 α -HSD from rat ovarian cytosol. In the process of purification, we found two species of molecule showing 20 α -HSD activity and designated them HSD1 and HSD2. They have similar molecular masses (33kDa) but differ in the electric charge and substrate affinity [12]. And the activities of both isoforms in ovarian or luteal tissues change during pseudopregnancy; ovarian HSD1 activity is low in early and mid- pseudopregnancy and increases abruptly at the end of pseudopregnancy, while medium levels of HSD2 activity are observed almost constantly throughout a pseudopregnant

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period [10, 12].

The objective of this study was to investigate mRNA(s) corresponding to the HSD1 and HSD2 molecules in the ovarian tissue, and to correlate these enzymatic activities expressed in *Xenopus* oocytes with those determined in the original cytosolic preparations.

Materials and Methods

Ovarian cytosol

Female Wistar-Imamichi rats, 8 weeks old, were purchased from Imamichi Institute for Animal Reproduction (Saitama, Japan) and housed under controlled lighting conditions of 14 h light and 10 h darkness (lights on at 0500 h). Food and water were available *ad libitum*.

To make batches, lots of 10 rats at the age of 10–16 weeks were decapitated regardless of the stage of their estrous cycle, and one of the bilateral ovaries was collected for measurement of enzymatic activity, and the other for RNA extraction.

For measurement of enzymatic activity, the ovaries were homogenized with 10 volumes of buffer A [50 mM potassium phosphate buffer (pH 6.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 5 $\mu\text{g}/\text{ml}$ leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)], and the homogenates were centrifuged at $105,000 \times g$ for 60 min at 4°C. The supernatant thus obtained was used for the assay of cytosolic enzymatic activity.

Reagents

PMSF, leupeptin, Bradford dye reagent, β -NADPH, dithiothreitol (DTT), progesterone and 20 α -OHP were purchased from Sigma (St. Louis, USA). Cesium chloride was from Boehringer-Mannheim (Germany), Oligotex-dT30 was from Takara Shuzo (Tokyo, Japan), acetonitrile was from Nakarai Tesque (Osaka, Japan). Guanidinium isothiocyanate, EDTA, sodium dodecyl sulfate (SDS) and dimethylsulfoxide (DMSO) were from Wako Pure Chemical Industries (Osaka, Japan).

Extraction and size-fractionation of mRNA by sucrose gradient centrifugation

Two batches of ovaries of which the cytosolic 20 α -HSD activities had been proven to consist of either HSD1 and HSD2 (batch A), or of HSD1 only (batch B) were selected. RNA was extracted by the guanidinium isothiocyanate/CsCl procedure [13]. Poly A-tailed RNA (mRNA) was isolated using Oligotex-dT30 [14].

A portion of the mRNA isolated from batch A was dissolved in 60 μl DMSO, incubated for 20 min at 37°C and then water was added to make a final volume of 100 μl . This mRNA preparation was loaded on a linear gradient of 5–25% sucrose in 10 mM Tris-HCl buffer (pH 7.0) containing 1 mM EDTA and 0.5% SDS, and centrifuged at $110,000 \times g$ for 15 h at 20°C [15]. This centrifuged preparation was divided into 15 fractions (0.5 ml each). The concentration of RNA was determined by optical density at 260 nm. The RNA was then precipitated with 75% ethanol and 0.3 M sodium acetate at –20°C and the precipitates were stored at –20°C.

mRNA translation system using Xenopus oocytes

Adult female *Xenopus laevis* obtained from Saitama Experimental Animal Supply Co. (Saitama, Japan) were anesthetized by cooling on ice. The ovarian lobes were removed, and separated into individual oocytes with scissors and fine forceps. mRNA was translated using a *Xenopus* oocyte system according to the method reported previously [16, 17]. In brief, the mRNA preparations from batches A and B were dissolved in water to a final concentration of 1 mg/ml. A 50 nl aliquot of the mRNA solution was injected into an oocyte. The oocytes thus treated were incubated for 40 h at 20°C in Barths' medium containing 0.1 mM PMSF and 5 $\mu\text{g}/\text{ml}$ leupeptin. One hundred oocytes were collected for each batch and homogenized with 1 ml of buffer A, followed by centrifugation. The supernatant was used for the assay of 20 α -HSD activity.

Assay of 20 α -HSD activity

The enzymatic activity of 20 α -HSD was assessed in terms of the efficiency of conversion of progesterone to 20 α -OHP in the presence of a

cofactor, β -NADPH. The amounts of progesterone and 20 α -OHP were measured by high performance liquid chromatography (HPLC system-600E, Waters, USA). Each sample (50 μ l) was added to 1 ml of the substrate mixture (0.1 mM Tris-HCl buffer pH 6.0, 1 mM EDTA, 300 μ M β -NADPH, 60 μ M progesterone in 3% ethanol, and 5 mM DTT), and incubated for 60 min at 37°C. The reaction was stopped by adding 2 ml of diethylether, followed by vigorous shaking. The diethylether phase was collected and evaporated at 40°C under nitrogen gas. The residue was dissolved in 500 μ l of 50% ethanol in water, 100 μ l of which was subjected to reverse-phase chromatography (Wakosil-5C18-200, Wako Pure Chemical Industries, Japan) connected to HPLC and eluted with a linear gradient of 50–100% acetonitrile. Steroid peaks were quantified by monitoring at a wavelength of 240 nm.

Separation of HSD1 and HSD2 by DEAE-chromatography

The ovarian cytosolic fraction or the cytosol of *Xenopus* oocytes was subjected to DEAE column chromatography (DEAE Toyopearl-650S, Toyosoda, Japan) connected to a Waters 650S HPLC system (Waters, USA). Samples (1 ml) were developed with a linear gradient of 0–0.25 M KCl in buffer A at a flow rate of 1 ml/min, and fractionated into 0.5 ml aliquots. The protein content of each fraction was determined with the dye reagent according to the method of Bradford [18].

Results

The ovarian cytosol of batch A developed by anion-exchange chromatography exhibited two peaks of 20 α -HSD activity (Fig. 1-A). The activity corresponding to HSD1 appeared in fractions 3–7 and that corresponding to HSD2 in fractions 9–17. The locations of HSD1 and HSD2 coincided with those reported previously [12]. The amount of HSD1 activity is 1498.5 nmol/h ml and that of HSD2 is 683.7 nmol/h ml. The HSD1/HSD2 activity ratio was 2.19.

The translation products of mRNA from batch A contained 2 peaks of 20 α -HSD activity (Fig. 1-B). The first peak corresponded to HSD1 and the second peak to HSD2. The amount of HSD1

Fig. 1. Anion-exchange chromatography on DEAE-cellulose of ovarian cytosol and mRNA translation products in batch A. Ovarian cytosol (1 ml) (A) or *Xenopus* oocyte cytosol (1 ml) (B) was applied to a DEAE-cellulose column and elution was carried out with a linear gradient of 0–0.25 mM KCl in buffer A. Twenty 0.5 ml fractions were collected, and then 20 α -HSD enzymatic activity was measured.

activity is 5731.6 pmol/h ml and that of HSD2 is 2424.1 pmol/h ml. The HSD1/HSD2 ratio was 2.36.

Using batch B, HSD1 and HSD2 activities of either the rat ovarian cytosol or the translation products of *Xenopus* oocytes injected with mRNA were measured in a similar manner to batch A. In rat ovarian cytosol, only HSD1 activity was detected, but there was no detectable activity due to HSD2 (Fig. 2-A). The translation products of the mRNA preparation originating from batch B also exhibited only HSD1 activity (Fig. 2-B).

To separate the mRNAs coding isozymes and determine their size, the mRNA preparation from batch A was fractionated by sucrose gradient centrifugation and each fraction was subjected to translation in *Xenopus* oocytes. The mRNA fractions between 4 and 7, corresponding to 14 S and 8 S, were translated into products possessing 20 α -HSD activity. Among them, the mRNA between 14 S and 12 S showed the highest activity (Fig. 3). Thus, the size of the mRNA was calculated to be

Fig. 2. Anion-exchange chromatography on DEAE-cellulose of ovarian cytosol and mRNA translation products in batch B. Ovarian cytosol (1 ml) (A) or *Xenopus* oocyte cytosol (1 ml) (B) was applied to a DEAE-cellulose column and elution was carried out with a linear gradient of buffer A from 0 mM to 2.5 mM KCl. Twenty 0.5 ml fractions were collected, and then 20 α -HSD enzymatic activity was measured.

approximately 1.0–1.5 kbp.

Discussion

We found that the *Xenopus* oocyte system for the translation of mRNA into protein is thoroughly valid for the translation of rat ovarian 20 α -HSD mRNA; the mRNA from rat ovaries was translated into two separate products showing HSD1 and HSD2 enzymatic activity. Since the molecular mass of both HSD1 and HSD2 proteins is estimated to be 33 kDa [12], the region of mRNA coding for each enzyme, if any, should be longer

Fig. 3. Sucrose gradient centrifugation of ovarian mRNA. Total RNA was denatured and then size-fractionated by sucrose gradient centrifugation. Fractionated RNA was injected into *Xenopus* oocytes, and enzymatic activity of 20 α -HSD was measured. Arrowheads indicate 18s (12,000 bp) and 5s (120 bp).

than 1.0 kbp. In the present experiment, the size of mRNA encoding 20 α -HSD was estimated to be 1.0–1.5 kbp. This result indicates that the noncoding region of 20 α -HSD is very small.

The ovarian cytosol from batch A contained both HSD1 and HSD2 activities, and the HSD1/HSD2 ratio was 2.19. This ratio coincided well with the ratio (2.36) of the translation products from *Xenopus* oocytes which were injected with the mRNA prepared from the same batch. As for the translation products of batch B, a similar relation was observed; the translation product had only HSD1 activity, as in the original cytosol preparation from batch B from which the injected mRNA preparation originated. These results are compatible with the presence of 2 different mRNAs coding HSD1 and HSD2.

Since we could not separate 2 different mRNAs corresponding to HSD1 and HSD2 by sucrose gradient centrifugation, still there is the theoretical possibility that one mRNA codes both HSD isomers. However, this might not be the case, because a "pro-enzyme" should not be processed into HSD1 and HSD2 in *Xenopus* oocytes in a manner completely identical to that in the cytosol of rat ovary.

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