# TISSUE DISTRIBUTION OF UNUSUAL RAT ESTROGEN-BINDING PROTEIN STUDIED BY IMMUNOBLOTTING

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The unusual estrogen-binding protein (UEBP) and the pancreatic estrogen-binding protein of rats and other mammals belong to the class of intracellular steromodulin proteins, which regulate the biodynamics (reception and metabolism) of steroid hormones through the formation of reversible complexes with ligands [4, 12]. Considering the essential role of steromodulins in the regional control of biological efficacy of hormonal steroids, the study of the tissue distribution of such proteins is an urgent problem both in connection with general problems in the organization of endocrine function and also on the practical plane, for knowledge of the degree of distribution of steromodulins would allow tissue differences in response to a particular hormone to be predicted in principle. Hormone-binding activity of UEBP, revealed differentially in the form of the labile specific action of the protein with $^3$H-estradiol, sensitive to androgens and insensitive to stilbene estrogens, is found in the liver of male rats but not in the liver of females, and not in other organs which have studied from both male and female rats [1, 2, 10]. The range of organs studied in this respect is very limited. Moreover, the possibility cannot be ruled out that the conditions of detection of hormone-binding activity of UEBP, used in the study of UEBP in the male rat liver, are not optimal for investigations with other tissues. It was accordingly decided to use immunoblotting as a variant of the immunochemical method which is much less sensitive to the conditions of protein isolation than a functional test.

## EXPERIMENTAL METHOD

Experiments were carried out on mature male and female rats of a mixed population, with a body weight of 150-250 g. Tissues and organs for study were homogenized at 0-4°C in 10 mM Tris-HCl buffer (pH 7.5), containing 10 mM KCl, 1 mM EDTA, 10 mM $Na_2MoO_4$, 0.5 mM phenylmethylsulfonyl fluoride, and 6 mM dithiothreitol, with tissue and buffer in the ratio of 1:1. The supernatant fraction (cytosol) obtained by centrifugation of the homogenate at 50,000 g was used immediately or was kept at —20°C. Aliquots of cytosol (15 $\mu$l), after boiling in the presence of SDS and mercaptoethanol, were subjected to vertical electrophoresis in slabs (1 × 100 × 110 mm) of 10% polyacrylamide gel with 6% concentrating gel in the presence of SDS [12] for 3-4 h, with a current of 11 mA. Electrophoretic transfer and processing of the nitrocellulose filters were carried out according to [9] in a modification. The separated protein zones were transferred from the slab of gel to a nitrocellulose filter (BA-85, 0.45 $\mu$, "Schleicher und Schüll," West Germany) in a solution of 20 mM Tris—150 mM glycine—2% methanol (pH 8.3) under a voltage of 50 V for 16-20 h during cooling. Completeness of transfer was verified by staining the gels with Coomassie R-250 [11]. The nitrocellulose was treated with 10 mM Tris-HCl buffer (pH 7.4), containing 300 mM NaCl, 1% gelatin, 1% bovine serum albumin, 1% human serum albumin, and 10% nonimmune rabbit serum, at 37°C for 30 min. The nonimmune serum in the solution was then replaced by monospecific rabbit antiserum to UEBP, obtained by the method described previously [5] of immunization with highly purified UEBP, and incubation was then continued at 20°C for 1.5 h. To verify the specificity of the reaction, incubation with nonimmune serum was used. The filters were repeatedly washed with a solution of 300 mM NaCl in 10 mM Tris-HCl buffer (pH 7.5) for 1.5 h, after which horseradish peroxidase-labeled sheep immunoglobulins, specific for rabbit $\gamma$-globulins (N. F. Gamaleya Institute of Epidemiology and Microbiology) were added. The concentration of secondary antibodies was chosen experimentally for each batch. After 1.5 h the filter was thoroughly washed and treated with a 0.15% solution of $\alpha$-naphthol for 15 min, after which $H_2O_2$ was added up to a final

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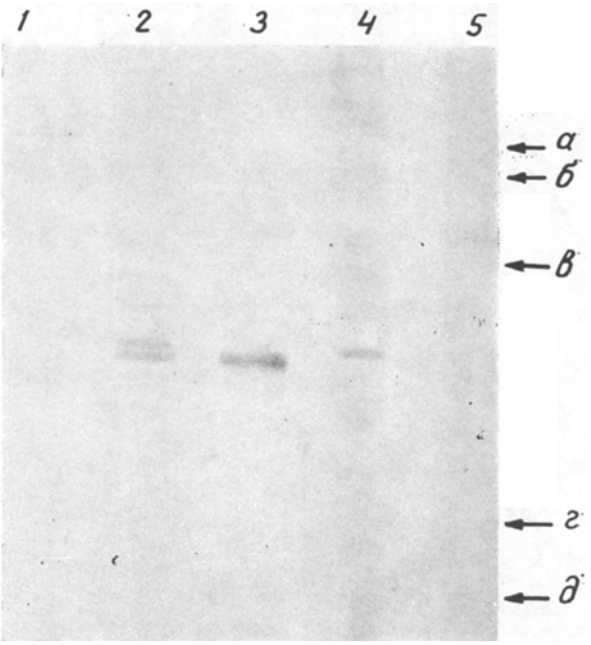


Fig. 1. Immunoblotting of cytosols of liver and pancreas of male and female rats with UEBP-specific antiserum. 1) Male pancreas; 2) male liver; 3) UEBP; 4) female liver; 5) female pancreas; a-e) standards: a) transferrin, b) albumin, c) ovalbumin, d) soy trypsin inhibitor, e) myoglobin.

concentration of 0.06%. The filters with the developed color were dried and photographed. A highly purified preparation of UEBP, obtained by the method described previously [7], was used as the standard for the whole immunoblotting procedure. Human transferrin (apparent Mr 77,000), bovine serum albumin (Mr 67,000), ovalbumin (Mr 45,000), soy trypsin inhibitor (Mr 21,000), and myoglobin (Mr 17,000) were used as markers of the size of the immunoreactive polypeptides. The track with the markers was stained with Coomassie R-250 and the relative mobility of the polypeptides was measured. The experiments were repeated at least 3 times for several specimens of each tissue, obtained from different animals.

## EXPERIMENTAL RESULTS

The photograph shows the results of one typical experiment (Fig. 1). Immunoreactive material with molecular mass similar (Mr $\approx$ 31,000) to that of the UEBP preparation isolated from male rat liver was present in the liver cytosol from male and female rats. Staining was specific for UEBP, for it did not develop after replacement of the specific antiserum to UEBP by preimmune rabbit serum. Besides the zone noted above, in this experiment very intense staining also was obtained in the region of liver proteins. It evidently was due to degradation products of UEBP, although this region did not stain in most other experiments. The sensitivity of the method, estimated by the reaction with different amounts of a highly purified UEBP preparation was of the order of 10-20 ng protein. The zone stained, corresponding to UEBP, in the liver cytosol of males was demonstrated in dilutions of cytosol of 32-64 times, in agreement with data on the content of hormone-binding sites for this protein in liver cytosol, namely 5-10 pmoles/mg protein [6].

Immunoreactive material corresponding to UEBP was found in cytosol of the liver, but not of the other organs and tissues tested on male and female rats (Table 1). The results as a whole thus confirm those of experiments using functional determination of UEBP, and demonstrate the marked organ-specificity of this protein. Meanwhole there was significant divergence of the results obtained by the two methods, as regards sexual differentiation of the presence of UEBP in the liver. The absence of hormone-binding activity of UEBP in the female liver was demonstrated by different methods and can evidently be regarded as a firmly established fact. The similarity of size of the immunoreactive polypeptides of male and female liver suggests that the absence of hormone-binding activity of UEBP in the female liver is not due to proteolysis in vivo or in vitro. The question of the nature of the immunoreactive material, specific for UEBP, in the female liver may be of great interest from the point of view of endocrine regulation

TABLE 1. Detection of Immunoreactor Material Specific for UEBP in Various Rat Organs and Tissues

| Organ, tissue | Males | Females |
|---|---|---|
| Liver | + | + |
| Pancreas | — | — |
| Stomach | — | — |
| Duodenum | — | — |
| Small intestine | — | — |
| Spleen | — | — |
| Kidneys | — | — |
| Lungs | — | — |
| Heart | — | — |
| Diaphragm | — | — |
| Gastrocnemius muscle | — | — |
| Adrenals | — | — |
| Thyroid gland | — | — |
| Pituitary gland | — | — |
| Median eminence of hypothalamus | — | — |
| Cerebral cortex | — | — |
| Submandibular gland | — | — |
| Lacrimal gland | — | — |
| Eyeball | — | — |
| Abdominal adipose tissue | — | — |
| Blood serum | — | — |
| Testes | — | |
| Prostate | — | |
| Seminal vesicles | — | |
| Epididymis | — | |
| Scrotal skin | — | |
| Ovaries | | — |
| Uterus | | — |

of pre- or post-translation modification of proteins. The fact discovered can help to explain the phenomenon, which we observed previously, of inhibition of the negative action of antibodies to UEBP on hormone-binding activity of estrogen receptors, by a protein fraction of female liver cytosol [4].

Data on the absence of immunoreactive material specific for UEBP in the pancreas (Fig. 1, Table 1) can be regarded as evidence of structural and functional differences between UEBP and the hormone-binding protein specific for the pancreas. The latter, unlike UEBP, does not bind androgens but does bind stilbine estrogens [8]. This protein, like UEBP, evidently performs a steromodulin function in the pancreas [12]. Its concentration in other tissues, including the liver, is at least 500-1000 times lower than in the pancreas [14]. It can thus be postulated that the regulatory action of steromodulins is narrowly oriented and organ-specific in character, being finely adapted to the particular functional features of this organ.

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# ACTION OF HUMORAL FACTORS OF MASTOCYTOMA P815 CELLS ON FORMATION OF ALLOSPECIFIC KILLER CELLS IN MIXED LYMPHOCYTE CULTURE AND ON THEIR CYTOTOXIC ACTIVITY

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During tumor growth functional activity of the principal populations of effector cells, namely natural killer cells, macrophages [11], and cytotoxic T lymphocytes (CTL) [10], is depressed. Tumor cells (TC) can exert a similar immunosuppressor action through the secretion of humoral factors (HF), which can influence the activation and differentiation of immunocytes. Reports of the inhibitory action of HF of various TC lines on lymphocyte proliferation induced by mitogens and alloantigens [12], and on functional activity of natural killer cells [8] and macrophages [4], have been published. We could find only one communication dealing with a study of the inhibitory action of P815 cells and their HF on the generation of allo-CTL in mixed lymphocyte culture (MLC) [5]. However, in that study, syngeneity of the responding lymphocytes and TC was not observed. The authors cited also limited themselves to testing the biological activity of tumor cultural supernatants and did not investigate the growth medium of the tumor (ascites fluid) in vivo.

The aim of this investigation was to study the effect of mastocytoma P815 cells and their HF on allo-CTL formation in MLC and on the cytotoxic activity of preformed allo-CTL.

## EXPERIMENTAL METHOD

Experiments were carried out on male mice of inbred lines DBA/2 (H-$2^d$) and C57BL/6 (B6, H-$2^b$), aged 2-4 months, and obtained from the "Rappolova" and "Stolbovaya" nurseries, Academy of Medical Sciences of the USSR. Mastocytoma P815 and leukemia EL4 cells were maintained in vitro and by passage in vivo through syngeneic mice. The animals were killed by cervical dislocation, the spleens were removed under aseptic conditions, cells were obtained fom them in glass homogenizers, and they were allowed to stand for 3-5 min in centrifuged tubes in medium 199 with 10% inactivated bovine serum. The thrice-washed splenocytes were suspended in medium RPMI-1640 ("Flow Laboratories"), with the addition of 5% embryonic calf serum ("Flow"), 2 mM glutamine, $5 \times 10^{-5}$ M 2-mercaptoethanol ("Serva"), 25 mM HEPES buffer ("Flow"), and 50 μg/ml of gentamicin. Allospecific killer cells were generated in 24-well panels ("Flow") with responding cells (splenocytes of DBA/2 mice; $5 \times 10^6$ cells per well) and stimulators (B6 splenocytes) in the ratio of 5:1. The stimulators and cells added as the third component were treated with mitomycin C ("Sigma," USA) in a dose of 50 μg/ml at 37°C for 30 min, followed by washing 3 times. The cells were cultured at 37°C in air with 5% $CO_2$ for 5 days. At the end of the reaction the cells were centrifuged, counted, and their cytotoxic activity (CTA) was determined in the microcytotoxic test, by measuring release of $^{51}Cr$ from labeled target cells (TaC) [3]. TaC in a concentration of $2 \times 10^6$ cells/ml were incubated with 100 μCi $Na_2CrO_4$ (All-Union "Izotop" Combine) for 1 h at 37°C, washed 3 times, and transferred to wells of 96-well micropanels ($10^4$ TAC/well). Effector cells (EC) were added in a volume of 0.1 ml; the ratio EC:TaC was 30:1. The maximal yield of radioactivity was determined with the aid of 2% sodium dodecylsulfate. The spontaneous yield of radioactive label did not exceed 30% of the maximal. Cytotoxic activity (CTA) was expressed as a percentage of specific lysis, calculated by the known formula [3]. Activity of HF of P815 cells was tested in 3-day cultural supernatants and in 7-day ascites fluids. For this purpose they were added as the third component either to the test system of allo-CTL generation or to the microcytotoxic test sample. The

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