EFFECTS OF 17β-ESTRADIOL TREATMENT *IN VIVO* ON THE AMINO ACID INCORPORATING CAPACITY OF RECONSTITUTED RIBOSOMES FROM GUINEA PIG UTERUS

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Summary: The effect of 17β-estradiol treatment *in vivo* on the subunits of guinea pig uterine ribosomes was studied in a cell free protein synthesizing system. The increased synthesizing capacity of ribosomes from treated animals appears to reside principally in the 60S subunit. Gel electrophoresis of protein derived from the subunits revealed the presence of an additional band among proteins associated with the 60S subunits of treated animals.

Introduction: *In vivo* estrogens stimulate uterine growth and protein biosynthesis. Several investigators have shown that this altered biosynthetic capacity is associated with an increase in the quantity of ribosomes within the tissue and an increase in the amino acid incorporating activity of these ribosomes (1-4). Factors such as the rate of peptide chain initiation on the ribosome have been cited to explain this increase (4,5). To date investigations designed to detect quantitative alterations in the synthesizing capacities of the ribosomal subunits have not been reported, nor have steroid induced alterations in the components of these subunits been noted. The association of accessory proteins with ribosomal subunits of eukaryotic cells has been demonstrated by Hirsch *et al* (6) suggests a means by which the capacity of the subunits to alter the rate of protein synthesis might be affected. With the demonstration by Martin and Wool (7) that isolated 40S and 60S ribosomal subunits were capable of reassociation to form 80S ribosomes that were active in polyuridylic acid directed peptide synthesis, a means by which the relative contribution of the estrogen treated animals subunits to the increase in protein synthesis could be

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evaluated became available. We now report a study of the effect of 17β-estradiol treatment in vivo on the cell free protein synthesizing capacity of guinea pig reassociated ribosomal subunits and their protein constituents.

Materials and Methods: Hartley strain nine week old guinea pigs were administered 17β-estradiol 3 weeks post ovariectomy, killed and their uteri frozen as previously described (4). Uteri were then pulverized and homogenized (6 ml/g tissue) in a medium containing 0.05 M-tris, 0.025 M-potassium chloride, 0.005 M-magnesium chloride, 0.25 M-sucrose, 0.2 mg/l of polyvinyl sulfate and 0.005 M-2-mercaptoethanol at pH 7.6. All procedures were done at 0-4°C unless otherwise specified. The homogenate was centrifuged for 10 min at 900 × g followed by 15 min at 27,000 × g. The supernatant fluid was mixed with Triton X-100 to a final concentration of 1% (7). Six ml fractions of Triton treated suspension were layered on discontinuous gradients of 2.5 ml of 0.5 M-sucrose over 2.5 ml of 1.0 M-sucrose (containing 0.05 M-tris, 0.025 M-potassium chloride and 0.005 M-magnesium chloride at pH 7.6) and were centrifuged at 105,000 × g for 11 h. The pellets were stirred in Medium A (0.05 M-tris, 0.08 M-potassium chloride, 0.0125 M-magnesium chloride and 0.02 M-2-mercaptoethanol at pH 7.6) for 12 h.

Polyribosomes from 17β-estradiol treated animals were preincubated for 30 min in a protein synthesizing system to terminate and release nascent polypeptide chains along with the release of free 80S particles from polyribosomes, a technique developed by Staehelin and Falvey (8). Dissociation and reassociation of ribosomes were based on the procedure by Martin and Wool (7) with minor modifications. The supernatant factors were prepared by centrifugation of the post-mitochondrial supernatant fluid at 105,000 × g for 6 h in a 50 Ti rotor. The pellets were discarded and the supernatant factors solution from untreated or estradiol treated uteri was stored in aliquots of 0.5 ml at -90°C. Protein concentrations were determined by the method of Warburg and Christian (9). [^{14}C]phenylalanine-tRNA was prepared by the method of Hawtrey (10) from rat liver homogenates. The specific activity of the preparation used was 50 × 10^3 dpm per mg of tRNA.

The assay for protein synthesis was carried out in a total volume of 1.0 ml containing 50 μmol of tris (pH 7.8), 80 μmol of phosphenol pyruvic acid, 0.1 mg of pyruvate kinase, 200 μg of tRNA charged with [^{14}C]phenylalanine and 19 other [^{12}C]amino acids, and supernatant factors preparation containing 0.75 mg of protein. The reaction was initiated by the addition of 90 μg of the ribosome suspension. The incubation period was 30 min at 37°C in a shaker water bath. The reaction was stopped by adding 4 ml of cold 10% trichloracetic acid. Then the samples were heated at 90°C for 15 min. The samples were cooled and passed through a Millipore filter (Type HA with pore size of 0.45 μm) and washed with 50 ml of 5% trichloracetic acid containing 1 mg/ml of [^{12}C]phenylalanine. The protein on the Millipore filters was dissolved in 0.7 ml of 88% formic acid and 15 ml dioxane containing scintillation fluors was added (11). The radioactivity was measured in a Packard Tricarb liquid scintillation counter Model 3380. The efficiency of counting was about 50% and sufficient counts were always collected to reduce the statistical error in counting to less than 5% (S.E.M.).

Preparation of Subunit Proteins: Uterine ribosomes were prepared from estradiol treated and untreated animals as described by Martin et al (13) using low potassium medium. They were stored at -20°C for no longer than 72 h before use. Preparation of subunit proteins was accomplished by a modification of the method of Sherton and Wool (12) with a simple preparative

centrifugation on 7 to 30% sucrose gradients in a SW 25.1 rotor at 22,500 rpm at 22°C for 6 h. After dialysis and lyophilization the protein was taken up in water to a concentration of 2 mg per ml. The protein solutions were mixed (1:3) with electrophoresis sample buffer (10% glycerol, 5% M-2-mercaptoethanol, 2.3% SDS and 0.0625 M-tris at pH 6.8) and heated for 2 min at 90°C.

Polyacrylamine Gel Electrophoresis: Seven to twenty percent linear polyacrylamide slab gels were prepared from a stock solution of 30 percent by weight of acrylamide and 0.8% by weight of N,N'-methylenebisacrylamide. The final concentrations in the separation gel were 0.375 M-tris (pH 8.8) and 0.1% SDS. Gels were polymerized chemically by the addition of 0.025% by volume, TEMED and ammonium persulfate. Separating gels were 15 cm long and 1 millimeter thick. Samples containing 10 to 50 µg of protein in sample buffer were placed in sample wells formed in the stacking gel and overlaid with running buffer (0.025 M-tris, 0.192 M-glycine, 0.1% SDS at pH 8.3). Electrophoresis was carried out for 6 h using an Ortec pulsed constant current power source (Model 4100) set at 360 pulses per second with a current of 35 mA. The proteins were fixed and stained by submersion of the gel slab in 50% trichloracetic acid containing 0.1% Coomassie Brilliant Blue fro 40 min at 23°C. Destaining was accomplished by immersion in 7% acetic acid with 6 bath changes over 48 h. Individual lanes were then cut out of the slab and scanned at 620 mµ with a Gilford 240 spectrophotometer equipped with a Model 2410 linear transporter.

Results: Using a selected standard set of in vitro assay conditions (14), native ribosomes from 17β-estradiol treated animals were found to incorporate label from aminoacyl-tRNA into protein more than 3 times faster than ribosomes from untreated animals (Table I). Experiments in which the amount of ribosomes, supernatant factor or charged tRNA were varied failed to abolish this effect. Therefore, all subsequent experiments were conducted using

TABLE I

EFFECT OF 17β-ESTRADIOL ON THE AMINO ACID INCORPORATING ABILITY OF NATIVE RIBOSOMES

		SOURCE OF SUPERNATANT FACTORS	
		Untreated Animals	Estrogen Treated Animals
Source of Ribosomes	Untreated Animals	1085	1583
	Estrogen Treated Animals	4784	4821

The capacity of ribosomal particles for incorporation of [^{14}C]phenylalanine-tRNA is expressed as dpm/mg ribosomal RNA. Each result represents an incubation of the ribosomes from a pool of 5 uteri.

standard assay conditions selected so as to saturate the system for preparations from the untreated animals.

The effect of 17β-estradiol on the protein synthesizing capacity of ribosomal subunits. The native ribosomes were dissociated and reassociated as described, and the success of these procedures was always confirmed by sucrose density gradient analysis of the products. The reconstituted ribosomes were evaluated for their ability to incorporate radioactivity from [^{14}C]phenylalanyl-tRNA into protein (Table II). These data show clearly that hybrid 80S particles which contain a 60S subunit derived from uteri of estrogen treated animals, when incubated with supernatant factors derived from treated animals, incorporated radioactivity from charged-tRNA more rapidly than the comparable hybrids containing large subunits from untreated animals. The data for recombination experiments using supernatant factors from untreated animals suggest a similar result which is presumably partially masked by a deficiency of some supernatant factor component. The activity contributed by the small subunit does not appear

TABLE II

AMINO ACID INCORPORATION BY HYBRID 80S PARTICLES RECONSTITUTED FROM RIBOSOME SUBUNITS FROM UNTREATED AND 17β-ESTRADIOL TREATED GUINEA PIG UTERI

		SOURCE OF 40S SUBUNITS	
		Control	Treated
Source of 60S Subunits	Control	(100)	65* 92**
	Treated	111* 216**	143* 212**

*Supernatant factors from untreated animals.
**Supernatant factors from estrogen treated animals.

Each result represents a pool of 5 uteri assayed for incorporation of radioactivity from [^{14}C]phenylalanine-tRNA as described in the text, expressed as a percent of the control. Duplicate determinations which agreed with an average variation of ± 13% were done in the experiments with supernatant factors from untreated animals; the other values are the result of single determinations.

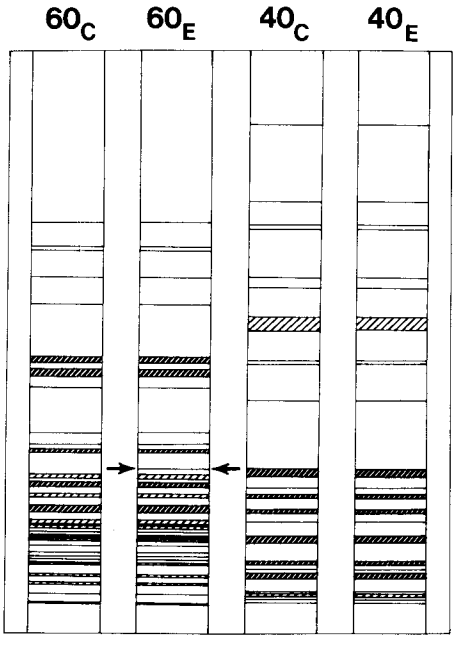

Fig. 1. Diagram of a slab gel electrophoresis of protein preparation from ribosome subunits. An arrow indicates the position of a band present in the estrogen treated preparation that was not present in the untreated preparation from 60S subunits. Migration of proteins was from top to bottom through an increasing acrylamide gradient. The total amount of protein placed in each well varied from 10 to 50 µg on different slabs. All wells on a single slab contained the same amount of protein.

to be stimulated by estrogen treatment.

Ribosomal subunit proteins. Slab rather than disc gels were employed for analysis of subunit proteins so as to facilitate comparison of adjacent lanes and thereby eliminate the problem of matching bands on separate gels. Electrophoretic separation of the proteins obtained from subunit preparations consistently demonstrated the presence of an additional, faint band in the lanes containing 60S subunit protein samples from treated animals (Fig. 1). This band was never present in comparable proteins from untreated animals or in the 40S subunit proteins from either source.

Discussion: Experimentally demonstrable actions of estrogen on uterine protein synthesis include an increase in the relative and absolute amount of ribosomes in the tissue and an increased amino acid incorporating activity of these ribosomes (2-4). The present study offers evidence that a portion

of the increased synthesizing capacity of the treated uterus is inherent in the 80S ribosomal unit as isolated, and that the 60S subunit accounts for much of this altered capacity. Moreover, a qualitative difference in the protein components of the 60S subunit of estrogen and nonestrogen treated animals has been demonstrated. The present study does not, of course, demonstrate that this difference accounts for the change in ribosomal synthesizing capacity. Further study will be necessary to examine this possibility and to establish whether this protein is a previously extant cytoplasmic component (6) or a post-treatment product of protein synthesis. The possibility that this protein is directly related to the estrogen-receptor complex (15) must also be investigated.

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