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CLONING OF THE RABBIT UTEROGLOBIN STRUCTURAL GENE

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The mRNA coding for uteroglobin, a progesterone-induced uterine protein, has been partially purified from 4-day pregnant rabbit uterus. Double-stranded DNA synthesized from the partially purified mRNA preparation was inserted into the Pst I site of pBR 322. Bacterial transformants containing uteroglobin DNA sequences were identified by their ability to enrich for uteroglobin mRNA on hybridization with total uterine poly A-RNA. The identity of one recombinant was confirmed unambiguously by matching its nucleotide sequence with the amino acid sequence of the uteroglobin polypeptide.

INTRODUCTION

Uteroglobin is a predominant protein in rabbit uterine secretions during early pregnancy or pseudopregnancy (2,3). The protein consists of two identical subunits each of 70 amino acids; its primary sequence has been reported (4,5). Like other secretory proteins, uteroglobin contains a leader sequence at the N-terminus, consisting of 21 amino acids (5,6). The protein has been called blastokinin, based on its alleged involvement in blastocyst development (3,8,9). Such a property could not be demonstrated, however, in the growth of the blastocyst *in vitro* (10). Furthermore, uteroglobin-like proteins have been detected immunologically in other rabbit tissues such as the oviduct (11-13), the male genital tract (14,15) and the lung (16-20).

Despite the uncertain physiological function(s) of uteroglobin, it provides an excellent system in which to study the mechanism of steroid hormone action in mammals. The increased secretion of the protein in the uterine lumen coincides with the

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rise in serum progesterone during early pregnancy (21) and can be induced by injection of virgin rabbits with progesterone (22). The synthesis of uteroglobin is also modulated by estrogen, since pretreatment of pregnant animals with estradiol-17B delays the onset of uteroglobin accumulation (23), and administration of estrogen to progesterone-induced virgin rabbits represses uteroglobin synthesis (24). Uteroglobin mRNA has been isolated from the rabbit uterus and its activity has been shown by us and others to increase during early pregnancy or in response to progesterone (25-27). Arnemann et al. (28) used complementary DNA synthesized from uteroglobin mRNA as a hybridization probe to demonstrate that the steady state level of uteroglobin mRNA per uterine endometrial cell is increased 70-fold after progesterone treatment. In order to determine unequivocally whether the increased steady state level of uteroglobin mRNA results from an enhanced rate of synthesis or a decreased rate of turnover, or both, we have constructed a recombinant plasmid containing double-stranded uteroglobin cDNA synthesized from partially purified uteroglobin mRNA.

MATERIALS AND METHODS

Preparation of Uteroglobin mRNA

Adult female New Zealand white rabbits were anesthetized with pentobarbitol on Day 4 of pregnancy and both uterine horns were frozen in liquid N_2 within 1 min of excision. Nucleic acid was extracted from the frozen tissue with SDS-phenol and poly A-containing RNA was prepared by chromatography on a column of oligo-dT cellulose, as reported previously (25). Cell-free translation of mRNA using the wheat germ system and immunoprecipitation of uteroglobin were as reported (19,25).

Cloning of Uteroglobin cDNA

Double-stranded complementary DNA to partially purified uteroglobin mRNA was synthesized using AMV reverse transcriptase according to previously published procedures (29,30). The double-stranded DNA was treated with 5000 units/ml of S1 nuclease (Miles) at 25° C for 30 min in 0.3 M NaOAc, pH 4.6/0.6 M NaCl/4 mM $ZnCl_2$. Poly dC tracts of 15 nucleotides length were added onto the dsDNA using calf thymus terminal nucleotidyl transferase. The plasmid vector pBR 322 DNA was made linear with Pst I and 14 dGMPs were added per terminus using the same enzyme. Reannealing of the DNA in 0.01 M Tris-HCl, pH 7.6/0.1 M NaCl and transformation of *E. coli* RR1 were carried out as described previously (31). Cloning was performed in a P3 facility in accordance with the NIH guide-

lines for recombinant DNA research. Bacterial transformants grown in L-agar containing 12.5 ug/ml of tetracycline were screened for recombinants by colony hybridization (32) using [^{32}P]cDNA synthesized from uteroglobin mRNA enriched by sucrose density gradient centrifugation.

Affinity Hybridization and Cell-free Translation

Recombinant plasmid DNA was prepared from individual bacterial colonies by the clear lysate procedure (33). The DNA was cleaved by Hha I, denatured thermally and linked covalently to aminobenzyloxymethyl cellulose (34). The immobilized DNA was allowed to hybridize with 500 ug/ml of uterine poly A-RNA for 2 hr at 37° C in the presence of 50% formamide/0.4 M NaCl/0.5% SDS/50 mM Tris-HCl, pH 7.5/1 mM EDTA. The cellulose was washed extensively to remove unhybridized RNA and the bound RNA fraction was released by incubation at 80° C for 3 min in 100% deionized formamide (Fluka). RNA was precipitated from ethanol and was redissolved in water. Translation was performed in a micrococcal nuclease-treated (35) rabbit reticulocyte lysate system (New England Nuclear Corp.) under the manufacturer's recommended conditions, using [^{35}S]-methionine as tracer. The translational products were treated with pancreatic ribonuclease A and were applied to a 15% SDS-polyacrylamide gel (36). The gel was treated with EN^{3}HANCE (New England Nuclear) and a fluorograph of the gel was obtained after 20 hr of exposure.

Direct DNA Sequencing

Restriction endonuclease-cleaved DNA (3 mg/ml) was dephosphorylated by incubation at 60° C for 30 min with 2.5 units/ml of bacterial alkaline phosphatase (Boehringer-Mannheim) in the presence of 0.05 M Tris-HCl, pH 8.0. The reaction was terminated by extraction with phenol saturated with 0.05 M Tris-HCl, pH 8.0. The dephosphorylated DNA was labeled with 4 mM y[^{32}P]ATP (Amersham-Searle, 2000 Ci/mmol) at 37° C for 30 min with 800 units/ml of T4 polynucleotide kinase (Boehringer-Mannheim) in the presence of 50 mM glycine-NaOH, pH 9.5/10 mM $MgCl_2$/5 mM dithiothreitol/ 0.1 mM spermidine/25% glycerol. The labeled DNA fragments were separated from plasmid DNA by preparative polyacrylamide gel electrophoresis and were cleaved with Alu I. The labeled fragments were degraded chemically according to the procedure of Maxam and Gilbert (37) and the products were separated on thin polyacrylamide gels (38).

RESULTS AND DISCUSSION

The sucrose gradient profile of uterine mRNA is shown in Figure 1. The sedimentation distances of 4S, 18S and 28S RNA in a parallel gradient are indicated by the arrows. Individual sucrose gradient fractions were analysed for translational activity using the wheat germ system. The profile of [^{35}S]methionine incorporation into TCA-precipitable peptides demonstrated that the mRNAs in the preparation were heterogenous in size. Immunoprecipitation of radioactive translational products using a specific antibody against uteroglobin indicated that uteroglobin mRNA sed-

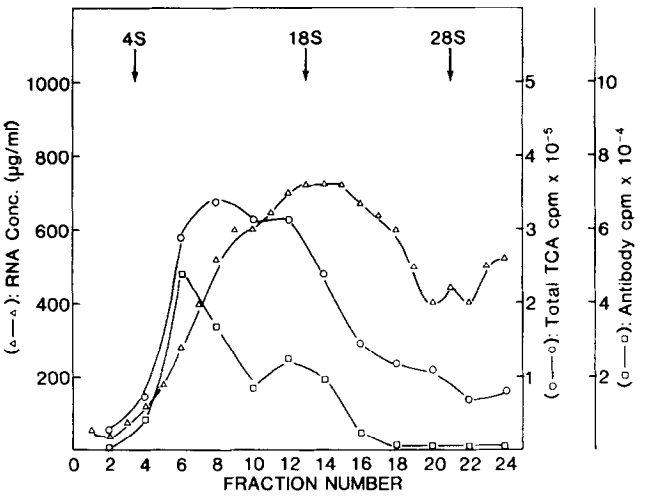

FIGURE 1. Sucrose density gradient centrifugation of total uterine poly ARNA. Six hundred ug of RNA in 0.2 ml water were heated at 70° C for 1 min, quick-chilled in ice-water and applied to a 12.4-ml linear gradient of 5 to 20% sucrose in 0.01 M NaOAc, pH 5.0/0.1 M NaCl/1 mM EDTA. The gradient was centrifuged at 38,000 rpm in a SW 40 rotor for 20 hr at 4° C. Fractions of 0.5 ml were collected while monitoring absorbance at 254 nm (Δ-Δ). RNA was precipitated from ethanol and the pellets were redissolved in 50 ul water. Alternate fractions (2 ul) were used for cell-free translation in a final volume of 100 ul. TCA-precipitation of 10 ul of the translational products was used to determine total mRNA activity (○-○) and 75 ul were taken for immunoprecipitation to assess uteroglobin mRNA activity (□-□). Both sets of values were adjusted to the 100-ul total volume of the assay.

imented at about 8S. The mRNA was enriched about 30-fold from total uterine poly A-RNA and constituted about 20% of the activity in fraction 6 (Fig. 1).

The complexity of the RNA in the fraction most enriched for uteroglobin mRNA was analysed prior to molecular cloning. Complementary DNA of high specific radioactivity was synthesized from RNA in fraction 6 of the sucrose gradient and allowed to hybridize with the same RNA fraction to various R_0t values. Hybrids were scored as S1 nuclease-resistant radioactivity and the R_0t curve is shown in Figure 2. Most sequences hybridized at high R_0t values and there was a low complexity component with a half-R_0t transition of about 2×10^{-3}. This low complexity component constitutes about 25% of the cDNA preparation and should be mostly

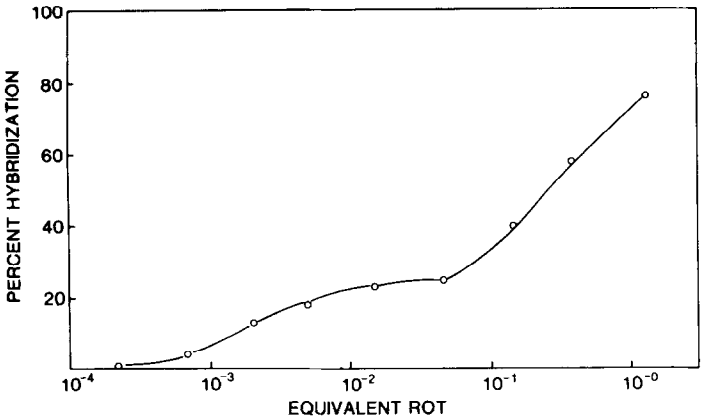

FIGURE 2. Complexity analysis of the partially purified uteroglobin mRNA preparation. Highly labeled [^{3}H]cDNA (10^7 cpm/ug) was synthesized from the RNA in fraction 6 of Fig. 1 and allowed to hybridize with the same RNA preparation (1 ug/ml) to various R_0t values. Hybridization was carried out in 10 mM HEPES-KOH, pH 7.0/0.6 M NaCl/2 mM EDTA with 10^4 cpm per vial of cDNA in a final volume of 50 ul. The mixture was treated with S1 nuclease and S1-resistant hybrids were scored as reported previously (29). The equivalent R_0t values were calculated from the apparent R_0t values and plotted.

uteroglobin cDNA, in agreement with the cell-free translation data indicating that 20% of the translatable mRNA in fraction 6 is uteroglobin mRNA (Fig. 1). Since the low complexity component reached a plateau at 25% hybridization, its half-R_0t transition value should be reduced by a factor of 4. The complexity of this component then becomes 5×10^{-4}, similar to that of globin mRNA.

The partially purified uteroglobin mRNA preparation was used for molecular cloning. Double-stranded DNA synthesized from 5 ug of the RNA population was inserted into the Pst I site of pBR 322. Bacterial transformants were screened by hybridization with [^{32}P]-cDNA synthesized from the partially purified mRNA population. A total of 315 individual colonies containing rabbit DNA was detected and 16 were selected for further analysis. The translational products of RNA recovered after affinity hybridization are shown in Figure 3. Control uterine mRNA directed the synthesis of many polypeptides, one of which was uteroglobin (lane b) which migrated

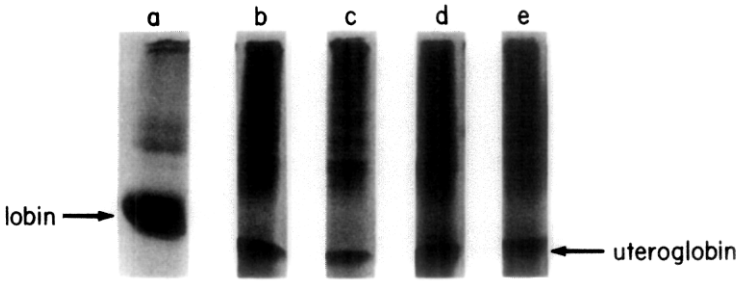

FIGURE 3. Fluorogram of a polyacrylamide gel containing [^{35}S]-labeled translational products synthesized in a reticulocyte lysate assay driven by RNA recovered from immobilized plasmid DNA. Lane a, rabbit globin mRNA control; lane b, total uterine poly A-RNA; lane c, RNA eluted from pBR 322 vector DNA; lane d, RNA eluted from pUG 221 DNA and lane e, RNA eluted from a negative clone.

slightly faster than globin (lane a). The pattern of protein products using RNA recovered from resin containing only pBR 322 vector DNA (lane c) was essentially the same as the control uterine mRNA. The same is true with RNA recovered from a negative clone (lane e). Using DNA of another clone, however, a significant increase in intensity of the uteroglobin band relative to the other bands is apparent (lane d). This clone is designated pUG 221 and was subjected to nucleotide sequence analysis.

The inserted rabbit DNA in pUG 221 is about 400 b.p. in length and both Pst I sites are reconstituted. The inserted DNA was cleaved with Pst I and the fragments were labeled at their 5' ends with y[^{32}P]ATP and were sequenced. The nucleotide sequence (Table 1) matched exactly with the amino acid sequence of uteroglobin at positions 18 to 56 of the native monomer (5). The nucleotide sequence allows us to reconcile some discrepancies in the amino acid sequence proposed by Popp et al. (4).

Since we have demonstrated unambiguously that pUG 221 DNA contains rabbit uteroglobin DNA sequences and that both Pst I sites are reconstituted in this recombinant plasmid, uteroglobin DNA can be excised from the plasmid using Pst I. The excised fragment can

TABLE 1. PARTIAL NUCLEOTIDE SEQUENCE OF CLONE pUC 221

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CCC TCC AGT TAC GAG ACA TCC CTG AAG GAA TTT GAA CCT
Pro Ser Ser Tyr Glu Thr Ser Leu Lys Glu Phe Glu Pro
        20                  25                  30

GAT GAC ACC ATG AAA GAT GCA GGG ATG CAG ATG AAG AAG
Asp Asp Thr Met Lys Asp Ala Gly Met Gln Met Lys Lys
                35                  40

GTG TTG GAC TCC CTG CCC CAG ACG ACC AGA GAG AAC ATC
Val Leu Asp Ser Leu Pro Gln Thr Thr Arg Glu Asn Ile
    45                  50                  55
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The numbers refer to the known amino acid residues of the native uteroglobin monomer (5)

be used as a specific hybridization probe to study hormonal effects on uteroglobin mRNA accumulation as well as the molecular structure and sequence organization of the chromosomal gene in the rabbit genome.

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Note added in proof: While this paper was in preparation, Atger et al. published their results of cloning uteroglobin cDNA (Biochem. Biophys. Res. Comm. 93, 1082-1088, 1980).

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