Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 4853–4857, August 1982
Biochemistry

Isolation and structure of the gene for the progesterone-inducible protein uteroglobin

(DNA sequence/gene structure/steroid hormone action/uteroglobin cDNA/blastokinin gene)

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Communicated by Erwin Bünning, April 29, 1982

ABSTRACT **Uteroglobin is a small steroid-binding protein that is differentially regulated by steroid hormones in several tissues of the rabbit. In endometrium, the levels of uteroglobin mRNA increase after progesterone administration due to an enhanced rate of transcription of the uteroglobin gene. As a prerequisite for understanding the molecular mechanisms that modulate uteroglobin gene expression, we have isolated and characterized the uteroglobin gene. We first synthesized, cloned, and sequenced a uteroglobin cDNA that was used to screen a rabbit gene library and to show that the uteroglobin gene is not reiterated in the rabbit genome. We obtained three recombinant phages containing uteroglobin gene sequences and covering 35 kilobases of the rabbit genome. The uteroglobin gene is 3 kilobases long and is composed of three short exons separated by a long and a short intron. The complete coding sequence, the short intron, part of the large intron, and the flanking sequences have been subjected to sequence analysis. The salient features of the nucleotide sequence, including the absence of a canonical "T-A-T-A box," are discussed. A possible relationship is considered between the exon–intron structure of the gene and the known structure and function of uteroglobin.**

The induction of uteroglobin synthesis by steroid hormones in the rabbit offers a suitable system for studying the modulation of gene expression in mammals (for a review, see ref. 1). In the endometrium progesterone is the main steroid inducer, whereas in the oviduct estrogens are effective inducers, and in the lung glucocorticoids cause a moderate induction of uteroglobin synthesis. We have shown in a cell-free transcription system that the accumulation of uteroglobin mRNA in endometrial cells after administration of ovarian hormones is mainly due to an increased rate of transcription of the uteroglobin gene (2).

A more detailed understanding of the molecular mechanisms underlying the hormonal control of uteroglobin gene expression depends on our knowledge of the structural organization of the gene and its putative regulatory sequences. An elucidation of the interaction between the hormone receptor and the regulatory sequences in chromatin may eventually provide answers to long-standing questions on the mechanism of action of steroid hormones.

In this paper we report the synthesis, cloning, and sequence analysis of a uteroglobin cDNA and its use for the titration and isolation of the uteroglobin gene. The primary structure of the gene-coding sequences and the flanking regions is reported and discussed.

MATERIALS AND METHODS

Synthesis and Cloning of Double-Stranded Uteroglobin cDNA. The first cDNA strand was synthesized as previously described by using partially purified preuteroglobin mRNA as template (3). Actinomycin D was omitted and [^{3}H]dCTP (specific activity 50 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was used as the radioactive label. After alkaline hydrolysis the cDNA was isolated and a second strand was synthesized with reverse transcriptase and nonradioactive deoxyribonucleotide triphosphates at 1 mM each. The double-stranded cDNA was isolated and the 3′ ends were elongated with short tracts of dCMP by incubation with [α-^{32}P]dCTP and terminal deoxyribonucleotidyl transferase from calf thymus (4). Approximately 15–30 dCMP residues were added to each end. The tailed cDNA was centrifuged through a neutral sucrose density gradient, and the material that sedimented at around 7 S was pooled for further experiments.

Circular plasmid pBR322 was linearized with *Pst* I and elongated with terminal transferase and [^{3}H]dGTP until 10–20 dGMP residues were added to each end. Twenty nanograms of elongated cDNA was annealed with 250 ng of tailed pBR322 in 0.1 M NaCl/10 mM Tris·HCl, pH 7.5/1 mM Na_2EDTA at 63°C for 10 min followed by 3 hr at 43°C. The samples were slowly cooled to room temperature and used to transform competent *Escherichia coli* K-12 χ1776 cells (5). Transformants were selected by antibiotic sensitivity and *in situ* colony hybridization by using [^{32}P]cDNA as probe (3, 6). Clones that appeared positive after colony hybridization were grown in a small volume and plasmid DNA was isolated by a rapid lysate procedure (7). After digestion with *Pst* I the size of the inserts was analyzed on polyacrylamide gels. Preparative isolation of plasmid DNA was performed according to published procedures (8).

Screening of the Rabbit Gene Library. The rabbit gene library (9) was screened by the *in situ* plaque-hybridization technique (10). Sixteen square plates (22 × 22 cm), each containing 7×10^4 phage plaques, were screened with 0.7% agarose as top layer (9) and clone pcUG-G8 labeled with ^{32}P by nick-translation (11) as hybridization probe. Positive plaques were purified by replating three times and the phage DNA was isolated (9).

Analysis of Uteroglobin Gene Sequences in Genomic DNA and Recombinant Phages. High molecular weight DNA was prepared from different tissues of female rabbits (12). Genomic DNA and DNA from recombinant phages were digested with the appropriate restriction enzymes, and the restriction fragments were separated on 1% agarose gels. After transfer of the DNA fragments to nitrocellulose filters by a modification of the Southern technique (13, 14), the filters were hybridized to either pcUG-G8 or uteroglobin mRNA labeled with ^{32}P (11, 15).

DNA Sequence Analysis. The sequence strategy is shown in the corresponding figures. In general, the 5′ ends of purified restriction fragments were labeled with [γ-^{32}P]ATP and phage T4 polynucleotide kinase (16). After secondary restriction the

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Abbreviations: bp, base pair(s); kb, kilobase(s).

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radioactive fragments were isolated on polyacrylamide gels and subjected to sequence analysis (16). In a few cases the 3′ ends were labeled with the Klenow fragment *E. coli* DNA polymerase I and the corresponding [α-^{32}P]dNTP. The *Bam*HI fragment that contains the 5′ end of the mRNA and the putative promoter sequences was labeled both at the 5′ ends and at the 3′ ends (in two separate experiments), and the two strands were separated in a 5% polyacrylamide gel before sequence analysis (16).

Containments. Initial work with cDNA cloning and the screening of the rabbit gene library were carried out under L3 level of physical containment and B2 level of biological containment as specified by the "Richtlinien zum Schutz vor Gefahren durch *in vitro* rekombinierte Nukleinsäuren" of the Bundesministerium für Forschung und Technologie of the Federal Republic of Germany.

RESULTS

Synthesis and Cloning of a Uteroglobin cDNA Probe. Uteroglobin is composed of two identical subunits—each 70 amino acids long—that are synthesized as a precursor containing a signal peptide of 21 amino acids at the NH_2 terminus (1). Preuteroglobin mRNA prepared from membrane-bound endometrial polysomes of pseudopregnant rabbits is >70% pure (3). This material was used for the synthesis of double-stranded cDNA that was introduced into the *Pst* I site of pBR322 by the oligo(G)·oligo(C) tailing procedure and cloned in *E. coli* (see *Materials and Methods*). Thirty clones gave positive hybridization signals with uteroglobin cDNA as probe. After digestion with *Pst* I, 10 of them showed inserts of around 400 base pairs (bp) and a similar restriction map with *Alu* I, *Hpa* II, *Hin*fI, and *Hae* III. One of these clones, designated pcUG-G8, was subjected to sequence analysis (16) and its structure is shown in Fig. 1.

The cDNA insert of pcUG-G8 is composed of 375 nucleotides, which include a track of 30 adenylyl residues corresponding to the 3′ end of the mRNA, and the oligo(G)·oligo(C) tails added during cloning. The internal sequence includes the codons for the amino acids 13–70 of mature uteroglobin (17) and the 3′ untranslated region of the mRNA. The previously published amino acid sequence (17) is confirmed by the nucleotide sequence of the cDNA, with the exception of position 61 where we reported Gln and the nucleotide sequence indicates Glu (17, 18).

Determination of Uteroglobin Gene Copy Number. To determine the number of copies of the uteroglobin gene per haploid rabbit genome, we hybridized trace amounts of pcUG-G8 insert, labeled with ^{32}P, to rabbit genomic DNA and to salmon sperm DNA to which different amounts of pcUG-G8 had been added (19). In this type of experiment a standard straight line is obtained relating the rate of cDNA hybridization to the number of gene copies added (Fig. 2). From these results it is clear that the uteroglobin gene belongs to the class of unique genes and is only present in one to three copies per haploid genome. Experiments performed under less stringent hybridization conditions gave no indication for the existence in the rabbit genome of sequences related to the uteroglobin gene (data not shown). Results similar to those depicted in Fig. 2 were obtained with liver DNA and with DNA prepared from the endometrium of control estrous rabbits, thus demonstrating that gene amplification is not responsible for the increased levels of uteroglobin mRNA observed in endometrial cells of pseudopregnant animals (20).

Isolation and Structural Analysis of the Uteroglobin Gene. For the isolation of the uteroglobin gene(s) a rabbit gene library was screened with labeled pcUG-G8 (9, 10). Of one million phage plaques tested, three independent isolates were found after three plaque purification steps. The inserts in these three recombinant Charon 4A phages overlap partially and cover a region extending over 35 kilobases (kb) of genomic DNA that contains uteroglobin gene sequences at its center. A partial restriction map of these three inserts is shown in Fig. 3*a*. The fragments hybridizing to pcUG-G8 or to uteroglobin mRNA probes in blotting experiments are indicated. Because the cDNA does not contain the 5′ half of the mRNA, a comparison of the results obtained with both hybridization probes (Fig. 4) allowed us to establish the orientation of the gene. The 1.7-kb

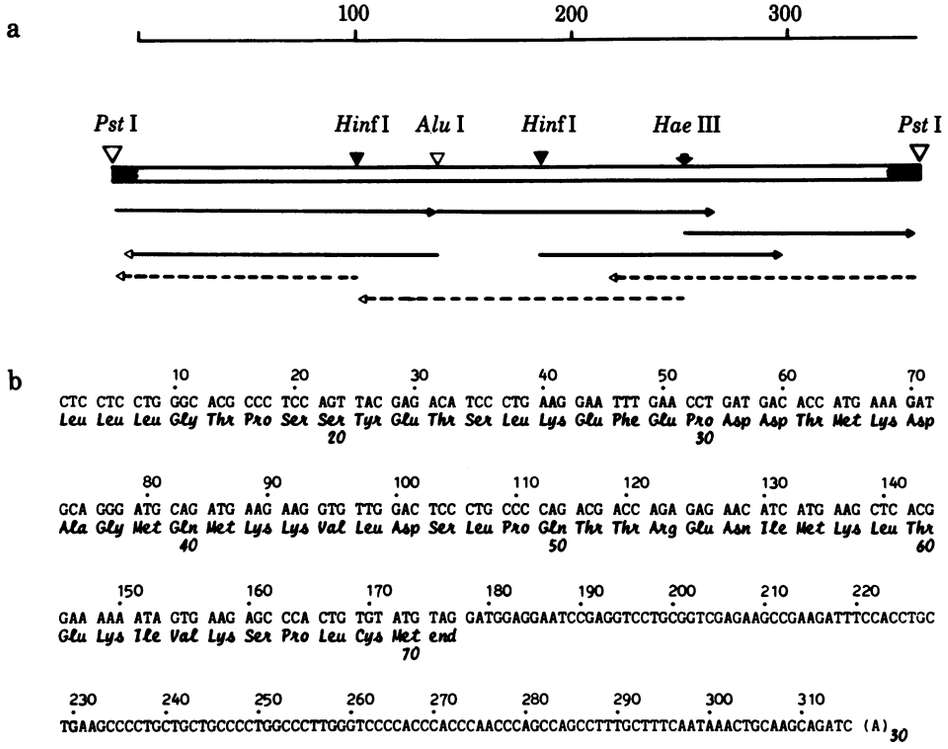

FIG. 1. Structure and sequence of pcUG-G8. (*a*) Partial restriction map of the cDNA insert of pcUG-G8. Only sites for the enzymes used during sequence analysis are shown. The sequence strategy is also shown. The solid lines refer to the sense strand and the broken lines to the antisense strand. The scale on the top is in bp. (*b*) Nucleotide sequence of the pcUG-G8 insert. The corresponding amino acid sequence is shown and numbered according to the known sequence of mature uteroglobin (17). The oligo(G)·oligo(C) tails are not shown.

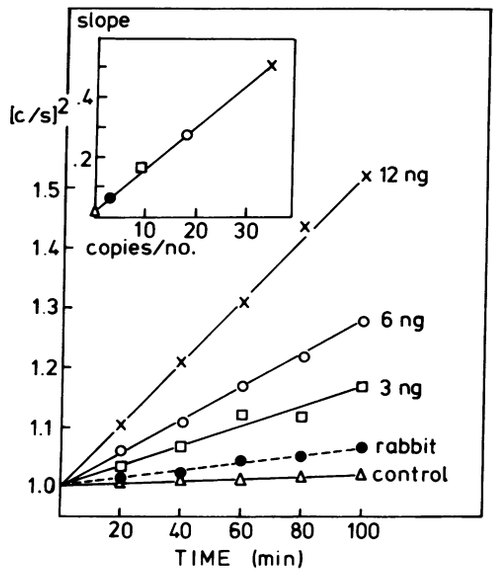

FIG. 2. Determination of the uteroglobin gene copy number. Two hundred micrograms of high-molecular-weight salmon sperm DNA or DNA prepared from the endometrium of pseudopregnant rabbits (●---●) was mixed with 200 pg of the cDNA insert of pcUG-G8 labeled with ^{32}P to a specific activity of 1.5×10^8 dpm/μg (11). The indicated amounts of nonradioactive pcUG-G8 were added to the samples containing salmon sperm DNA. The control sample (△——△) contains salmon sperm DNA and no added pcUG-G8. After treatment with 0.3 M NaOH at 100°C to reduce the size of the DNA to about 600 bp, the DNA was ethanol-precipitated and redissolved in hybridization buffer (19). Poly(A) and poly(C) were added to a final concentration of 50 μg/ml each, and the samples were boiled for 3 min and incubated further at 70°C. At the indicated times, aliquots were taken for measuring the amount of radioactivity resistant to digestion with S1 nuclease (3). The graphical representation of the data has been described (19); c, total radioactive cDNA; s, single-stranded radioactive cDNA. (*Inset*) Plot of the slope of the individual lines versus the calculated number of copies of pcUG-G8 added per haploid genomic equivalent (3.1 pg of DNA).

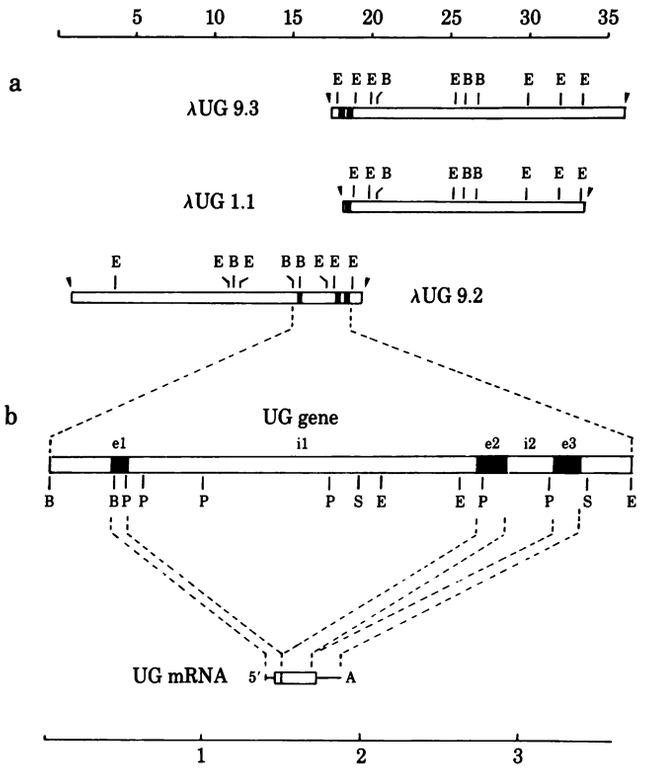

FIG. 3. Structure of recombinant phages that contain uteroglobin (UG) gene sequences and physical map of the uteroglobin gene. (*a*) Restriction sites for *Eco*RI (E) and *Bam*HI (B) in the three recombinant phages λUG9.3, λUG1.1, and λUG9.2. The arrowheads indicate the synthetic *Eco*RI sites introduced during the construction of the gene library (9) and mark the limits of the inserts. The dark regions denote the presence of uteroglobin coding sequences. The 35-kb scale on the top is the reference scale for this part of the figure. (*b*) Ten-fold magnified map of the region that contains uteroglobin gene sequences. The dark regions represent the three exons, labeled e1, e2, and e3. The introns are labeled i1 and i2. The uteroglobin mRNA is shown below for comparison. Abbreviations for restriction sites are: *Eco*RI, E; *Bam*HI, B; *Pst* I, P; and *Sst* I, S. The 3.5-kb scale on the bottom applies to this part of the figure.

*Bam*HI/*Eco*RI fragment that hybridizes to uteroglobin mRNA but not to pUG-G8 should contain the 5′ end of the mRNA, which is missing in the cDNA clone. These data and the results of single- and double-restriction endonuclease digests with enzymes that have known sites on Charon 4A were used to construct the detailed physical map of the uteroglobin gene shown in Fig. 3*b*.

The regions that contain uteroglobin gene sequences and the flanking regions have been subcloned in pBR322 and their sequences partially determined (16). The results are shown in Fig. 5. A comparison of this sequence with the nucleotide sequence of the cDNA (Fig. 1 and ref. 21) allows the following conclusions to be made.

(*i*) The uteroglobin gene extends over 3 kb of genomic DNA and is composed of three exons and two introns. The whole gene and some 15 kb of 5′ flanking sequences are included in the recombinant phage λUG9.2. The 5′ end of the mRNA has been located by S1 nuclease mapping (unpublished observation) and confirms data obtained with cDNA (21).

(*ii*) The first exon contains 47 nucleotides of the 5′ nontranslated region of the mRNA and the codons for the first 18 amino acids of the signal peptide of preuteroglobin. The nucleotide sequence of this region helps to clarify contradictions in the reported amino acid sequence of preuteroglobin. At position −16 there is Thr (22) and not Phe (23), and at position −6 there is Gly (23) and not Cys (22).

(*iii*) The second exon comprises 187 nucleotides including the codons for the last 3 amino acids of the signal peptide of preuteroglobin and for the first 60 amino acids of the mature protein. The nucleotide sequence coincides with that determined for the cDNA (Fig. 1).

(*iv*) The third exon comprises 171 nucleotides and encodes the last 10 amino acids of uteroglobin and 141 nucleotides of the nontranslated region at the 3′ end of the mRNA. The polyadenylation signal A-A-T-A-A-A, which is conserved in eukar-

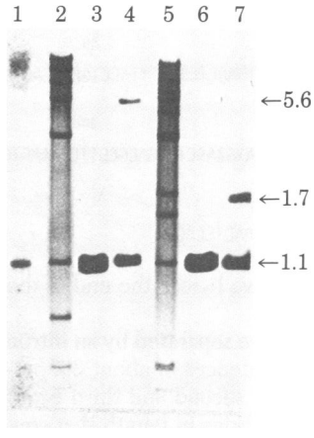

FIG. 4. Blots of restriction fragments of genomic DNA and λUG9.2. Rabbit genomic DNA (lane 1) and DNA from λUG9.2 (lanes 2–7) were digested with *Eco*RI (lanes 1–4) or with *Eco*RI and *Bam*HI (lanes 5–7), and the resulting restriction fragments were separated on a 1% agarose gel. After staining with ethidium bromide (lanes 2 and 5), the DNA fragments were transferred to nitrocellulose filters (13, 14) and hybridized either to pcUG-G8 (lanes 1, 3, and 6) or to uteroglobin mRNA (lanes 4 and 7), both labeled with ^{32}P (11, 15). The numbers on the right represent the size (kb) of the hybridizing fragments as determined by using an *Eco*RI/*Hin*dIII digest of λDNA as standard.

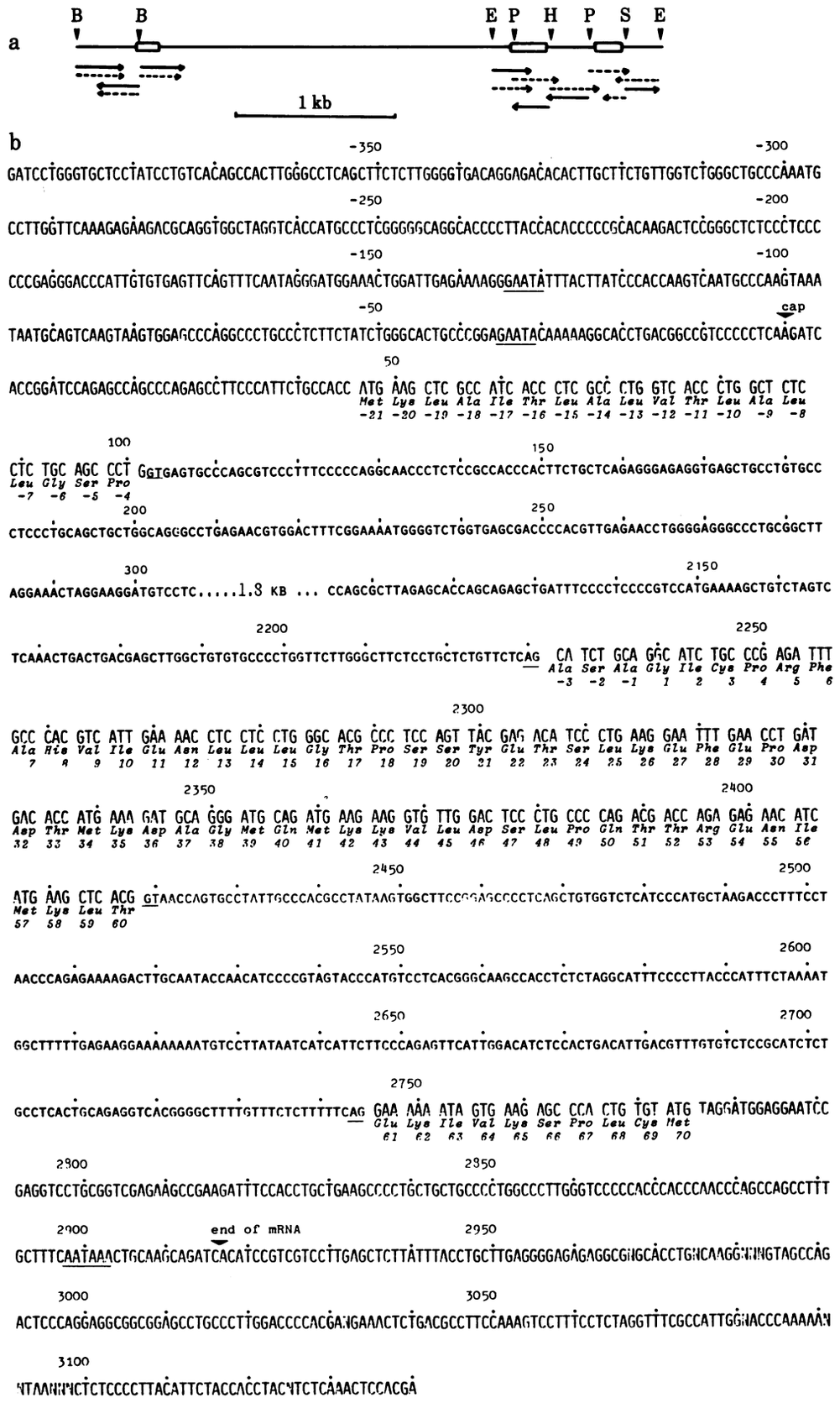

FIG. 5. Partial nucleotide sequence of the uteroglobin gene and its flanking region. (*a*) Sequence strategy. Abbreviations for restriction sites are: *Bam*HI, B; *Eco*RI, E; *Pst* I, P; *Hpa* II, H; and *Sst* I, S. The exons are shown as open boxes. The solid lines refer to the sense strand and the broken lines to the antisense strand. (*b*) Partial nucleotide sequence of the uteroglobin gene and its flanking sequences. The sequence starts at the first *Bam*HI site shown at the left in *a*, and the nucleotides are numbered starting with the 5′ end of the mRNA (21). The putative regulatory sequences preceding the initiation of transcription are underlined and numerated with negative numbers. The exon–intron transitions and the polyadenylylation signal are also underlined. The end of the mRNA is marked by an arrowhead.

yotic genes (24), is found 19 nucleotides before the end of the mRNA (position 2,898 in Fig. 5*b*).

(*v*) The first and the second exons are separated by an intron 2.3 kb in length, from which the sequences of about 300 nucleotides have been determined. The second and third exons are separated by an intron 320 nucleotides in length that contains several tracks of As and Ts. The four exon–intron transitions agree with the canonical sequence 5′ G/GT... intron... AG/$^{G}_{C}$3′ that has been found in most other eukaryotic spliced genes (25).

(*vi*) A comparison of our gene sequence with the cDNA sequence of Chandra *et al.* (21) locates the 5′ end of the mRNA

47 nucleotides upstream from the A-T-G initiation codon. No canonical "T-A-T-A box" (26) is found in the region 25–35 nucleotides upstream from the mRNA initiation site, but, in analogy to the chicken lysozyme gene (27), we find an A+T-rich region at position −35 (G-A-A-T-A) that is repeated at position −134. Other A+T-rich regions, T-A-C-A-A-A-A-A and T-A-A-A-T-A-A-T, are found at positions −32 and −99, respectively. At position −72 there is a sequence, C-C-A-G-G, that has also been found in the human insulin gene (28). Other noncanonical C-C-A-A-T boxes (29) are found at positions −104, −111, and −165.

DISCUSSION

The nucleotide sequence of the cDNA insert of pcUG-G8 confirms a published partial sequence of uteroglobin cDNA (30) but shows differences in five positions when compared to other published sequences (31). Whether this represents genetic polymorphism of the rabbit populations remains to be established. The sequence of the uteroglobin gene reported here coincides with the cDNA sequence (ref. 21 and Fig. 1), thus demonstrating that no artifacts were generated during reverse transcription nor during the construction of the gene library (9).

All the data presented here, as well as previously published restriction maps (32, 33), are compatible with the existence of a single uteroglobin gene in the haploid genome of the rabbit. The general mosaic organization of the uteroglobin gene into three exons and two introns could reflect the existence of structural or functional domains in the protein, as suggested for other eukaryotic genes (34). As already reported (21), the 47 nucleotides preceding the initiation codon exhibit sequences complementary to 18S rRNA.

Thus, the first exon could be considered as a functional unit that contains the information required for the initiation of transcription and translation as well as most of the codons for the signal peptide of preuteroglobin. The second exon contains the information for all but 10 amino acids of the mature protein. It is conceivable that this is the region of the protein that harbors the progesterone binding site, as it contains amino acids such as tyrosine and histidine, which have been implicated in steroid binding (1, 35, 36). Unfortunately, only the three-dimensional structure of the oxidized uteroglobin has been established (35), and this form does not bind progesterone (37). The third exon encodes the last 10 amino acids of mature uteroglobin, including cysteine 69, which is implicated in the interaction between the two subunits that build the native protein (17, 35). Little is known about the functional significance of this region of the protein, but it could be responsible for the extensive conformational changes that accompany reduction of the disulfide bonds (1, 37).

An interesting finding from our sequence work is the lack of a canonical T-A-T-A sequence motif in the expected position with respect to the 5′ end of the mRNA (26). Instead, at positions −35 and −134 we find the sequence G-A-A-T-A that has also been found in human fetal globin genes (38) and other A+T-rich sequences at positions −32 and −99.

The 200–300 bp preceding the putative initiation site show a complex pattern of alternating A·T and G·C clusters, as well as short direct and inverted repeats, that could form extensive cruciform structures. Also of potential interest may be the finding of an open reading frame extending from position −298 to position −82 that is able to encode 72 amino acids (Fig. 5*b*). Whether these structures are relevant to the observed hormonal regulation of transcription of the uteroglobin gene remains to be established. Binding studies with the purified progesterone receptor of rabbit uterus and subcloned fragments of the gene could help to elucidate this intriguing question.

We thank John Collins, Gesellschaft für Biotechnologische Forschung, Stockheim, Federal Republic of Germany, for making L3 facilities available to us during initial cloning, and Tom Maniatis, California Institute of Technology, for the rabbit gene library. This work was supported by grants of the Deutsche Forschungsgemeinschaft (SFB 103 B1 and Br 738/2-2).

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