

The Complementary Deoxyribonucleic Acid Sequence of Guinea Pig Endometrial Prorelaxin*

Y. A. LEE, GILLIAN D. BRYANT-GREENWOOD, M. MANDEL, AND
F. C. GREENWOOD

Department of Anatomy and Reproductive Biology (Y.A.L, G.D.B.-G.), the Pacific Biomedical Research Center (F.C.G), and the Department of Biochemistry and Biophysics (M.M), University of Hawaii, Honolulu, Hawaii 96822

ABSTRACT. The nucleotide sequence of the relaxin gene transcript in the endometrium of the late pregnant guinea pig has been determined. The strategy used was a combination of polymerase chain reaction (PCR) with primers designed from the mRNA sequence of porcine preprorelaxin, rapid amplification of cDNA ends-PCR, and blunt end cloning in M13 mp18. With heterologous primers, a 226-basepair (bp) segment of the guinea pig relaxin gene sequence was obtained and was used to design a guinea pig-specific primer for use with the rapid amplification of cDNA ends-PCR method. The latter allowed completion of the sequence of 336 bp, with a 96-bp overlap. The

sequence obtained shows greater homology at both the nucleotide and amino acid levels with porcine and human relaxins H1 and H2 than with rat relaxin, supporting the thesis that the guinea pig is not a rodent. The transcription of the guinea pig endometrial relaxin gene during pregnancy was confirmed by Northern analysis of guinea pig endometrial tissues with a species-specific cDNA probe. The endometrial relaxin gene is transcribed during pregnancy, but not in lactation, consistent with the observed immunostaining for relaxin. (*Endocrinology* 130: 1165-1172, 1992)

RELAXIN is of particular importance to the guinea pig, since it bears mature young and allows separation of the pelvic bones, thereby facilitating their passage through the pelvic canal. Hisaw (1) used the guinea pig to show that the separation of the pelvis in late pregnancy was related to the secretion of relaxin and exploited this finding as a biological assay (2). It is now 65 yr since this discovery, and we report here the putative amino acid sequence of this hormone.

Natural guinea pig relaxin has not been isolated nor its gene sequenced, but it may be assumed to consist of A- and B-peptide chains linked by two interchain disulfides and one intrachain disulfide derived from a linear peptide with a C-peptide connecting the A- and B-chains. Heterologous antisera to purified porcine relaxin have been used to localize endometrial relaxin and show its release into plasma (3-7), suggesting a significant similarity in structure between pig and guinea pig relaxins at the amino acid level. More recent studies using heterol-

ogous porcine oligoprobes and antisera have confirmed the synthesis of relaxin in both the endometrium and mammary gland (8, 9). The primary source of relaxin and its mRNA is the endometrial gland cells of the late pregnant animal. The finding of both mRNA for relaxin and relaxin peptide in the mammary gland of the lactating guinea pig was unexpected (9) and pointed to the need for homologous antisera and probes. These are particularly needed to further study an apparent signaling of the endometrium by the mammary gland (10).

Accordingly, we have used poly(A)⁺ RNA from the endometrium of late pregnant guinea pig together with reverse transcription and the polymerase chain reaction (PCR) and rapid amplification of cDNA ends (RACE) procedure, dideoxy sequencing, and cloning to obtain the nucleotide sequence of guinea pig prorelaxin.

Materials and Methods

Animals and tissues

Pregnant guinea pigs of the Dunkin Hartley strain were purchased from Simonsen Laboratories, Inc. (Gilroy, CA). These were kept under a 12-h light, 12-h dark photoperiod and had free access to laboratory guinea pig chow, green vegetables, and tap water. The animals were killed in a CO₂ chamber at midpregnancy (approximately day 35), late pregnancy (approximately day 63), and postpartum on day 6 of lactation. The uterus was rapidly removed, and the endometrium was scraped

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Address all correspondence and requests for reprints to: Dr. G. D. Bryant-Greenwood, Department of Anatomy and Reproductive Biology, University of Hawaii, 1960 East-West Road, Honolulu, Hawaii 96822.

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from the myometrium and frozen in liquid nitrogen. All tissues were stored at -80°C until they were used.

Experimental strategy

The strategy used to elucidate the nucleotide sequence of the guinea pig relaxin gene transcribed in the endometrium of the guinea pig in late pregnancy is shown in Fig. 1. This entailed the use of PCR and two heterologous primers corresponding to the partial sequences of mRNA of porcine preprorelaxin (11) as the first step (part I). From the direct sequencing of the PCR products, the sequence of the B-chain plus part of the C-

peptide [226 basepairs (bp)] of guinea pig endometrial relaxin gene were obtained. Part II of this study used the RACE-PCR method (12) and a homologous primer designed from the sequence obtained in part I. Blunt end cloning in M13mp18 and dideoxy sequencing of the RACE-PCR products gave the remaining sequence (336 bp) of this relaxin gene.

Poly(A)⁺ RNA extraction and reverse transcription

Frozen tissues (2–8 g) were used to prepare total RNA by the acid guanidium thiocyanate-phenol-chloroform method (13). Poly(A)⁺ RNA was isolated from the total RNA by affinity

Part I. PCR: Heterologous primers (porcine relaxin)

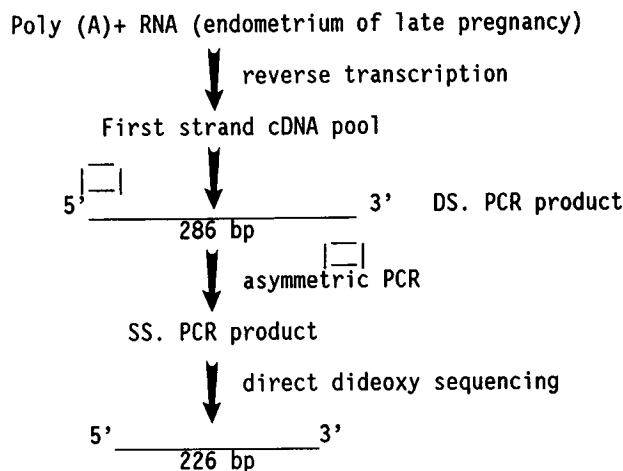
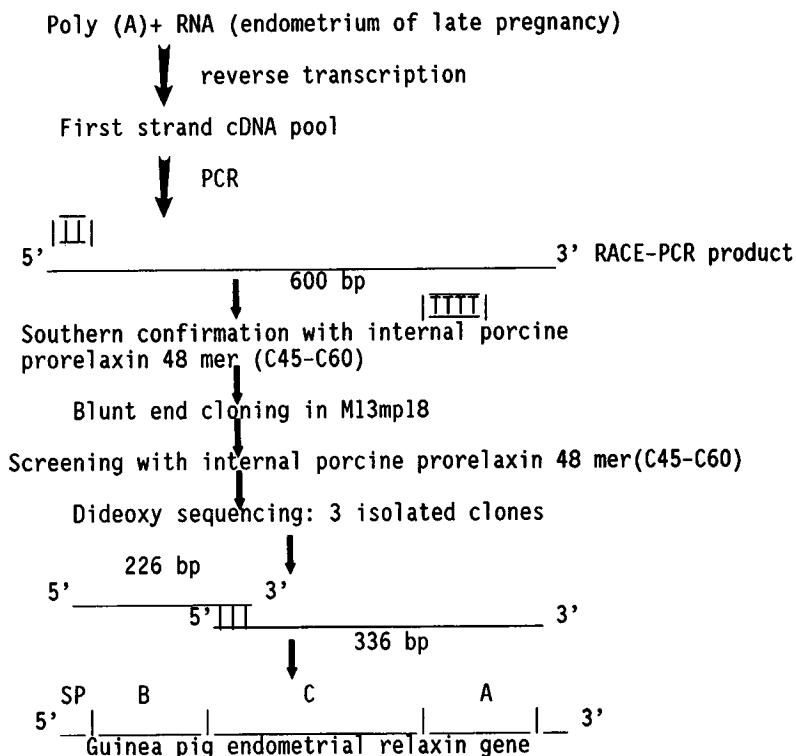


FIG. 1. Strategy for obtaining the cDNA sequence of guinea pig endometrial prorelaxin. Part I, The cDNA sequence of the B-chain plus the 5'-end of the C-peptide of this hormone were elucidated by PCR and direct dideoxy sequencing using heterologous primers (source: porcine preprorelaxin). Part II, The remaining sequence of the guinea pig endometrial prorelaxin was obtained by RACE-PCR and subcloning using homologous primers. These two fragments of guinea pig relaxin gene have a sequence overlap (96 bp), as shown at the bottom of the diagram.

Part II. RACE-PCR and blunt end cloning in M13mp18: Guinea pig relaxin specific primer and poly dT₁₇ adapter primer



chromatography on oligo(dT)-cellulose (14). The poly(A)⁺ RNA samples were stored in 75% ethanol at -80 C for Northern analyses or reverse transcriptions.

A first strand cDNA pool was prepared by the method of Kawasaki (15). Poly(A)⁺ RNA (1 μ l of 1 μ g/ μ l in water) from the endometrium of a late pregnant guinea pig was denatured at 92 C for 3 min and quenched on ice. The following additions were then made: 0.5 μ l RNasin (20 U; Promega Corp., Madison, WI), 2 μ l 10 \times PCR buffer [670 mM Tris-HCl (pH 8.8), 30 mM MgCl₂, and 166 mM (NH₄)₂SO₄], 1 μ l random hexamers (100 pmol; Pharmacia, Piscataway, NJ), 8 μ l 10 mM deoxynucleotide triphosphates (2.5 mM each of dATP, dCTP, dGTP, and dTTP; U.S. Biochemicals, Cleveland, OH), and 2 μ l avian myeloblastosis virus (AMV) reverse transcriptase (32 U; U.S. Biochemicals). The mixture was then made to a final volume of 20 μ l with 0.1% diethylpyrocarbonate (Sigma Chemical Co., St. Louis, MO)-treated double distilled water, mixed gently, and incubated at 42 C for 60 min. The reaction was terminated by heating at 95 C for 5 min, chilled on ice, and diluted to a final volume of 500 μ l with double distilled water. This cDNA pool was frozen until used for PCR.

For the RACE-PCR procedure, a first strand cDNA was prepared from poly(A)⁺ RNA isolated from the endometrium of a guinea pig in late pregnancy, using the method of Frohman (12). The poly(A)⁺ RNA (1 μ g) was mixed with 16.5 μ l RNase-free distilled water and denatured at 65 C for 3 min. The heated RNA was quenched on ice, and the following reagents were added: 2 μ l 10 \times RTC buffer [500 mM Tris-HCl (pH 8.15 at 41 C), 60 mM MgCl₂, 400 mM KCl, 10 mM dithiothreitol (DTT), each dNTP at 10 mM], 0.3 μ l RNasin (10 U; Promega Corp.), 0.5 μ l oligo(dT)₁₇-adapter primer 0.5 μ g; and 0.7 μ l AMV reverse transcriptase (10 U; U.S. Biochemicals). The reaction was carried out at 42 C for 1 h, and then at 52 C for 30 min. The reaction mixture was diluted with TE buffer [10 mM Tris-HCl (pH 7.6) and 1 mM EDTA] to a final volume of 1 ml and stored at 4 C.

Oligonucleotide primers

The mRNA sequence of porcine preprorelaxin was used for PCR primer design (11). Four sets of the synthetic oligonucleotide primers corresponding to different regions of the mRNA

TABLE 1. Oligonucleotide primers (derived from mRNA of porcine preprorelaxin) used in the amplification and sequencing of the guinea pig endometrial relaxin gene by PCR

No.	Flank	Location of amino acids in sequence	Primer size (mer)	Distance between two primers (bp)
I	5'-End	-16...-10	20	241
	3'-End	72...79	23	
II	5'-End	-11...-6	17	225
	3'-End	70...76	17	
III	5'-End	13...19	19	363
	3'-End	142...147	18	
IV	5'-End	49...52	18	225
	3'-End	142...147	18	

sequence of porcine preprorelaxin were designed using a PCR primer selection computer program (Epicenter Software, Inc., Pasadena, CA); their positions in porcine preprorelaxin are shown in Table 1. For the RACE-PCR, the (dT) adapter 35-mer primer was used for the first strand reverse transcription, with the sequence: 5' GACTCGAGTCGACATCGATTTTTTTT-TTTTTTTTTTTT 3'. The guinea pig gene-specific primer used for RACE-PCR was designed from the C-peptide (C6-C12) elucidated in the first stage of this study. The sequences of primers used in the PCR were as follows: 1) 5' TCTGGG-ATCTGGACAATC 3' (guinea pig relaxin C-peptide 18-mer), and 2) 5' GACTCGAGTTCGACATCG 3' (adapter 17-mer primer). All oligonucleotide primers were synthesized by the Biotechnology-Molecular Biology Instrumentation Facility, University of Hawaii, using the phosphoramidite method on an Applied Biosystems DNA Synthesizer (model 380B, Foster City, CA).

PCR

Four sets of PCR primers were used in attempts to amplify relaxin-specific fragments from the cDNA pool. Each PCR reaction mixture was assembled in a PCR tube with the following reagents: 10 μ l 10 \times PCR buffer [670 mM Tris-HCl (pH 8.8), 30 mM MgCl₂, and 166 mM (NH₄)₂SO₄], 10 μ l 10 mM dNTPs (2.5 mM each of dATP, dCTP, dGTP, and dTTP; U.S. Biochemicals), 5 μ l of each 10 μ M PCR primer (25 pmol each), 0.125 μ l cloned *Thermus aquaticus* (*Taq*) DNA polymerase (Amplitaq, Perkin-Elmer Cetus, Norwalk, CT; 5 U/ μ l), 10 μ l cDNA pool, and double distilled water to a final volume of 100 μ l. Template cDNA was replaced by 10 μ l water in a blank control. The reaction mixture was pulse centrifuged, and the mixture was overlaid with two drops of light mineral oil (Sigma). PCR reactions were carried out for 40 cycles in a DNA thermal cycler (Perkin-Elmer Cetus) with a cycle profile of 94 C for 1-min denaturation, 45-55 C for 1-min annealing, and 72 C for 3-min extension.

Single stranded DNA templates for direct sequencing were made by asymmetric PCR. The following reagents were assembled in a PCR tube on ice: 10 μ l 10 \times PCR buffer [670 mM Tris-HCl (pH 8.8), 30 mM MgCl₂, and 166 mM (NH₄)₂SO₄], 10 μ l 10 mM dNTPs (2.5 mM each of dATP, dCTP, dGTP, and dTTP; U.S. Biochemicals), 50 pmol of one PCR primer (5 μ l of a 10- μ M stock solution), 1 pmol of the limiting primer (5 μ l of a 1:50 diluted 10- μ M stock solution), 0.125 μ l Amplitaq DNA polymerase (Perkin-Elmer Cetus; 5 U/ μ l), 2-5 μ l double stranded PCR products (purified by a 2% Nusieve agarose gel; 1:10 or 1:100 diluted), and double distilled water to a final volume of 100 μ l. This was pulse centrifuged and overlaid with 2 drops of mineral oil (Sigma). The PCR reactions were carried out for 40 cycles with the profile of 94 C for 1 min, 55 C for 1 min, and 72 C for 3 min.

The 3'-end of the guinea pig endometrial relaxin gene was amplified by the RACE-PCR method of Frohman (12). The reaction mixture consisted of the following reagents: 10 μ l 10 \times PCR buffer [670 mM Tris-HCl (pH 8.8), 67 mM MgCl₂, 166 mM (NH₄)₂SO₄, and 1.7 mg/ml BSA], 10 μ l 60 mM dNTPs (15 mM each), 10 μ l dimethylsulfoxide (DMSO; Sigma), 5 μ l adapter 17-mer primer (25 pmol), 5 μ l guinea pig relaxin gene-specific primer (25 pmol), 10 μ l template cDNA, and water to a final

volume of 100 μ l. The reaction mixture was heated in a DNA thermal cycler (Perkin-Elmer Cetus) at 95 C for 5 min and cooled to 72 C, and then 5 U Amplitaq DNA polymerase (Perkin-Elmer Cetus) were added. The mixture was overlaid with 30 μ l prewarmed mineral oil (72 C), and then incubated at 55 C for 5 min, followed by a 40-min extension at 72 C. The PCR reaction was then continued for 40 cycles with a profile of 95 C for 40 sec, 55 C for 1 min, and 72 C for 3 min. A final enzymatic extension was carried out at 72 C for 15 min.

PCR and RACE-PCR products were monitored by electrophoresis on a 1.7% agarose gel. The gels were stained with ethidium bromide (0.5 μ g/ml; 30 min), visualized by UV transillumination, and photographed using a Polaroid MP-4 camera (Polaroid Corp., Cambridge, MA).

The gene specificity of PCR products was judged by their size compared with the predicted size based on porcine relaxin and Southern analysis with a radioactive internal porcine oligonucleotide probe (48-mer).

Direct sequencing of PCR product

The PCR products were purified by passage through Centricon-30 (Amicon, Danvers, MA) and sequenced by the dideoxy chain termination method (16), using the Sequenase version 2.0 sequencing kit (U.S. Biochemicals). The annealing mixture was assembled in a microcentrifuge tube on ice: 1 μ l 10 μ M oligonucleotide primer (the limiting primer used in the asymmetric PCR), 2 μ l 5 \times sequencing buffer, and 7 μ l Centriconed single stranded DNA template. The mixture was heated at 95 C for 5 min, immediately cooled at -20 C for 3 min, pulse centrifuged, and then placed into a dry bath block prechilled to 0 C. The block was left on a bench and allowed to warm to room temperature. The labeling mixture was prepared once as follows: 1 μ l 0.1 M DTT, 1.7 μ l 1 \times TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.6), 2 μ l diluted (1:100) labeling mixture (7.5 μ M dCTP, 7.5 μ M dGTP, and 7.5 μ M dTTP), 0.5 μ l [³⁵S] dATP (1000-1500 Ci/mmol; New England Nuclear, Boston, MA), and 0.3 μ l Sequenase (version 2.0). The annealing mixture was mixed with 5.5 μ l radioactive labeling mixture and then incubated in a 20 C water bath for 5 min. The reaction mixture (3.5 μ l) was then distributed into each of the four tubes containing 2.5 μ l prewarmed ddNTPs termination mixtures. The termination reaction was carried out at 37 C for 5 min, and then stopped by adding 4 μ l stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). The sequencing reaction samples were loaded on a 6% denaturing polyacrylamide gel (3 μ l/lane). The electrophoresis gel was run at 1700 V and 75 watt for 6 h, 12 h, or longer, depending on the region of the DNA of interest. The gel was dried with a gel dryer (Bio-Rad Laboratories, Richmond, CA) and exposed to x-ray film (Fuji Photo Co., USA Inc., Stamford, CT) for 48 h.

Blunt end cloning in M13 mp18 and sequencing of the RACE-PCR product

The RACE-PCR product was purified using the method of double gene cleaning (Bio 101, Inc., La Jolla, CA). The ends of the RACE-PCR product were modified with DNA polymerase-I and T4 polynucleotide kinase (U.S. Biochemicals) and cloned into M13 mp18 vector using the M13 cloning system (New

England Biolabs, Beverly, MA). The relaxin gene-specific plaques were identified using a method of colony lifts (17) and Southern hybridization (18), employing a ³²P-labeled 48-mer oligonucleotide corresponding to the mRNA sequence of the C-peptide region (C45-C60) of porcine preprorelaxin. Single stranded DNA templates were prepared from the isolated relaxin gene-specific plaques and sequenced using the Sequenase system (U.S. Biochemicals).

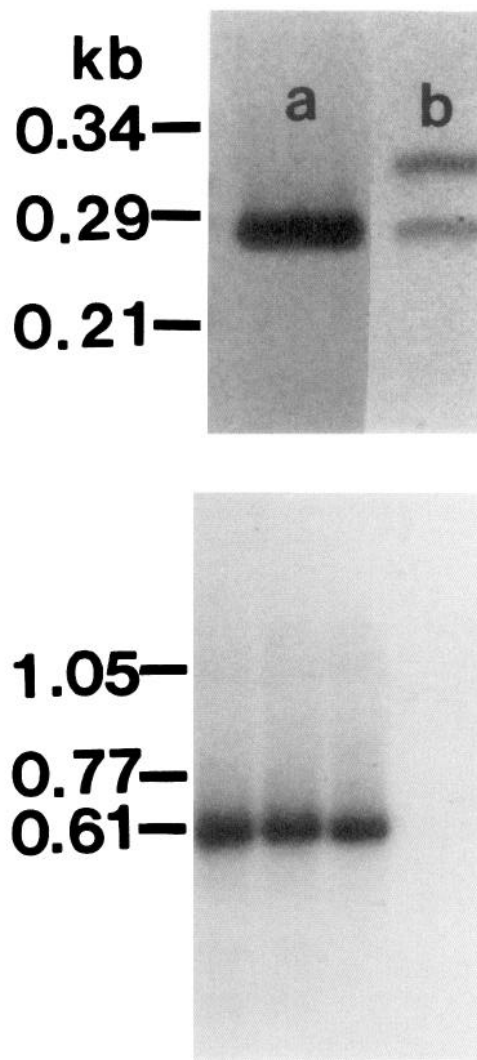


FIG. 2. PCR products from a late pregnant guinea pig endometrial cDNA using standard PCR, asymmetric PCR, and RACE-PCR. The top panel shows the double stranded PCR products (lane a) and single stranded PCR products (lane b) electrophoresed on an agarose gel (1.7%). The double stranded PCR products were amplified using porcine preprorelaxin primers (set 1, Table 1). The single stranded PCR products were obtained by reamplification of the double stranded PCR products using the 5'-terminal primer of set 1 (Table 1) as a limiting primer. The bottom panel shows an autoradiography by Southern blotting of RACE-PCR products hybridized with a ³²P-labeled 48-mer oligonucleotide probe. The homologous primer used in the RACE-PCR corresponds to the C-peptide region of guinea pig endometrial preprorelaxin (C38-C44), and the probe used in hybridization is down-stream of this (C45-C60). Exposure time was 10 min.

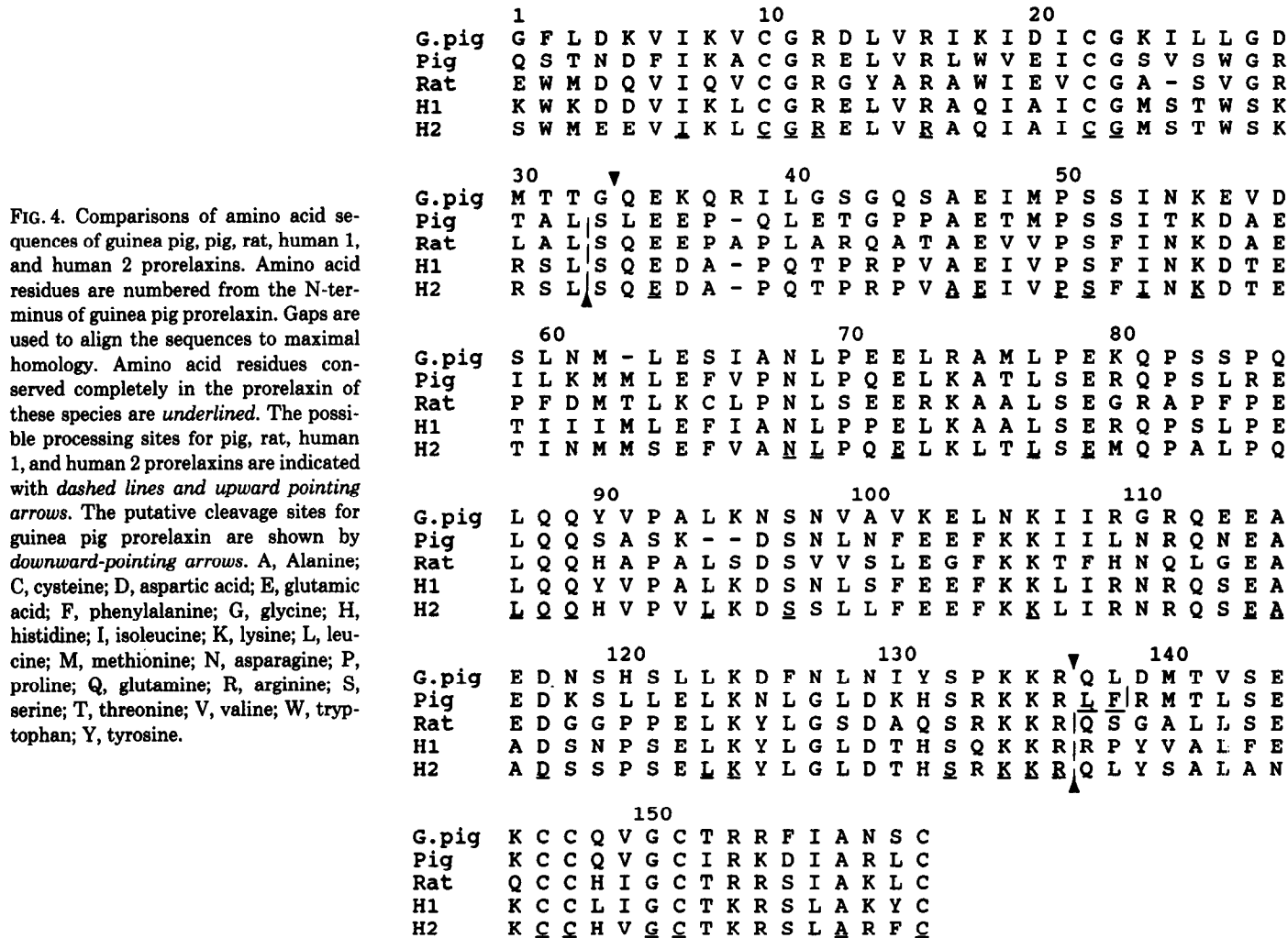


TABLE 2. The percent homologies between the B-chain (B), connecting peptide (C), and A-chain (A) of guinea pig endometrial relaxin and those of porcine relaxin, the two human relaxins H1 and H2, and rat relaxin, at the nucleotide and amino acid levels

	Nucleotide				Amino acid				
	B	C	A	Total	B	C	A	Total	
Porcine	57.3	71.4	74.6	69.6	59.4	65.4	76.2	66.9	
Human H1	57.3	71.3	66.2	68.4	59.4	65.1	47.8	64.8	
Human H2	58.3	72.0	58.3	69.4	67.7	62.1	69.6	65.4	
Rat	57.0	59.0	62.2	59.2	64.5	60.2	73.9	62.9	

resultant cDNA pool with four sets of synthetic oligonucleotide primers in different combinations, corresponding to different regions of the mRNA sequence of porcine prorelaxin. One set (set 1, Table 1) of these primers amplified a fragment from the cDNA pool (Fig. 2). Single stranded template DNA was prepared by amplification of this double stranded putative relaxin-specific fragment using asymmetric PCR and was sequenced by the Sequenase system (Fig. 2). A total of 226 bp was read from three sequencing x-ray films. Computer-aided sequence data analysis showed that this was a relaxin-specific fragment (226 bp) encoding the intact B-chain

of guinea pig prorelaxin plus partial regions of the signal peptide (8 amino acid residues) and C-peptide (33 amino acid residues) of this hormone. The remainder of the guinea pig endometrial prorelaxin (the intact A-chain plus part of the C-peptide) was elucidated using RACE-PCR, M13 cloning, and sequencing (Fig. 1). The nucleotide sequence and its derived amino acid sequence of guinea pig endometrial prorelaxin are shown in Fig. 3. Comparisons of the guinea pig endometrial relaxin with porcine, rat, and human H1 and H2 relaxins at the amino acid level are shown in Fig. 4. Table 2 presents a computer-aided analysis of the homologies of prorelaxins in

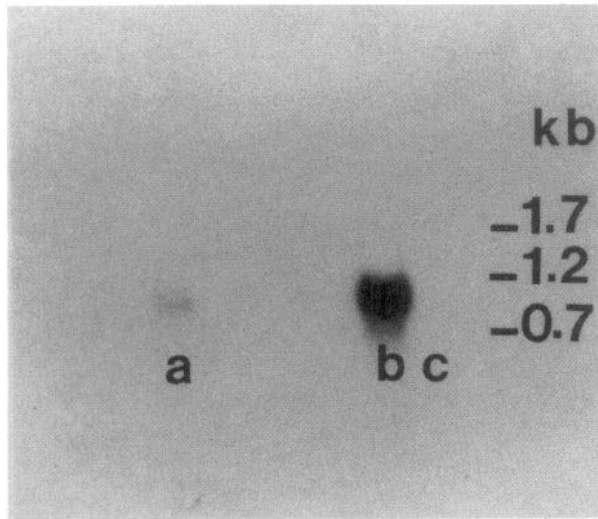


FIG. 5. Northern blot analysis of relaxin gene transcription in the endometrium of the guinea pig during pregnancy and lactation. Poly(A)⁺ RNA was extracted from the guinea pig endometrium collected in midpregnancy (day 35; lane a), late pregnancy (day 63; lane b), and lactation (day 6; lane c). Aliquots of poly(A)⁺ RNA (30 μ g) were electrophoresed, transferred to a Nytran membrane, and hybridized as described in *Materials and Methods*. Exposure time was 48 h. Mol wt markers (in kilobases) are shown on the *right*.

different species and shows that guinea pig endometrial relaxin has higher homologies with porcine relaxin (69.6%) and human relaxin (68.4% H1, 69.4% H2) than with rat relaxin (59.2%) at the nucleotide level. There is also greater homology between guinea pig relaxin and porcine (66.9%), human H1 (64.8%), and human H2 (65.4%) relaxins than with rat relaxin (62.9%) at the amino acid level.

Northern analysis of guinea pig relaxin in the endometrium of pregnancy with a homologous relaxin cDNA probe (286 bp) clearly demonstrated transcription of the relaxin gene in this tissue (Fig. 5) and its increased level of transcription during gestation. The mRNA transcripts had a molecular size of 1.0 kb, consistent with the previously reported data from studies using heterologous porcine relaxin oligonucleotide probes (8). There was no hybridization with poly(A)⁺ RNA obtained from endometrium on day 6 of lactation.

Discussion

Relaxin belongs to the insulin/insulin-like growth factor family of hormones (19). However, unlike the other members of this family, which tend to be conserved in primary structure, relaxin has changed considerably during evolution (20). There is a 52–60% sequence difference in the primary structure of the mammalian relaxins that are currently known: pig, rat, human, *etc.* (20). However, the conservation of some of the amino acid residues suggests that they are needed for the biological activities

of this hormone [the six cystines (residues 10, 22, 146, 147, 151, and 160), two glycines (residues 11 and 23), three arginines (residues 12, 16, and 153), and two lysines (residues 8 and 145)]. Cystines form the insulin-like disulfide bridge structure, glycines may provide unique torsion angles for chain folding, and arginines and lysines may be essential for binding to the receptor of relaxin (20–24). The complete synthesis of human relaxin (H2) and its derivatives carried out by Bullesbach *et al.* (24) provided unequivocal evidence that the amino acid residues in the midregion of the B-chain (Cys-Gly-Arg-Glu-Leu-Val-Arg) are essential for the biological activities of this hormone. Replacement of two arginines in this region by the uncharged isosteric amino acid citrulline rendered the synthetic human relaxin biologically inactive. Guinea pig endometrial prorelaxin contains almost all of these important amino acid residues in the same positions. The critical bioactive sequence identified by Bullesbach *et al.* (24) is present in guinea pig relaxin with a single substitution (Glu by Asp) of little significance according to the GAP program of the GCG sequence analysis software package (Wisconsin University). This change is also present in skate relaxin (25). The structural similarity of this sequence in different relaxins with that of the guinea pig endometrial relaxin suggests that the latter is a potent relaxin. Comparisons at the amino acid level show that the A-chain of guinea pig endometrial relaxin is more similar to that of porcine relaxin, whereas the B-chain of this hormone is more like that of human relaxin H2. However, the overall nucleotide sequence of guinea pig prorelaxin has 69.4% homology with human H2 relaxin and 59.2% homology with rat relaxin. When comparing the structures of guinea pig, porcine, human, and rat relaxins at either the nucleotide or amino acid level, the phylogenetic hypothesis of Graur *et al.* (26) is supported, that “the guinea pig represents a separate evolutionary lineage from the myomorph rodents (rats and mice), and should not be classified in the same order.” Hence, our data on the structure of relaxin in the guinea pig support their view that the guinea pig is not a rodent (26).

It has been known for over 40 yr that the uterus of the guinea pig is the primary source of relaxin in this species (27, 28). Relaxin immunostaining was detected in the endometrial gland cells at different stages of pregnancy: on days 30, 35, and 63 of gestation (3, 5, 6) and, more recently, on days 9 and 14 in the cyclic animal (10). After parturition, endometrial relaxin staining declined rapidly, with only some cells in the glands showing relaxin immunoactivity by midlactation (4, 10). Transcripts of relaxin mRNA have been identified in guinea pig endometrium collected from late pregnancy and shown to be 1.0 kb in size (8). All of the previous work is based upon heterologous antisera or probes (antisera against porcine

relaxin or oligonucleotides complementary to the mRNA sequence of porcine preprorelaxin), and sensitivity may have been limited. The guinea pig relaxin cDNA probe prepared here has been used for Northern analyses of guinea pig endometrial mRNA collected from different stages of pregnancy and lactation. The transcription pattern obtained from the guinea pig relaxin gene in pregnancy is consistent with the immunostaining pattern reported in the literature. The loss of the transcription activity of the guinea pig endometrial relaxin gene in lactation (day 6) in spite of immunostaining for relaxin at this time (6) suggests that relaxin is produced only by the endometrium during pregnancy, and the peptide persists into lactation, as shown for the pig and rat corpus luteum in lactation (29). Plasma relaxin immunoactivity detected in the lactating guinea pig and the uniform and intense relaxin immunostaining in the mammary gland of this species in lactation suggest an extrauterine source for relaxin (7, 9). Homologous guinea pig probes and antisera can now be used or developed to allow more sensitive and specific studies on relaxin to be undertaken in this species.

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