Isolation and analysis of the 3'-untranslated regions of the human relaxin H1 and H2 genes

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ABSTRACT

The human has two relaxins, termed H1 and H2, both of which are biologically active and co-expressed in the decidua, placenta and prostate; in the corpus luteum, the main source of circulating relaxin, only the H2 form is expressed. The reasons for this differential expression of the relaxin genes are unknown. The possibility that their 3'-untranslated regions (UTRs) contribute to this differential expression by affecting their mRNA stabilities was investigated. Thus the 3'-UTRs of both relaxin genes were isolated through a combined 3'-rapid amplification of cDNA ends-PCR (RACE-PCR) using poly (A)⁺ RNA from human decidua, placenta, prostate and corpus luteum. The sequences obtained for each 3'-UTR were identical in the tissues examined, were AT-rich (72%) and showed 91% homology between relaxin H1 and H2 when maximally aligned to include several gaps, the significance of which is unknown. Relaxin H1 has two, and relaxin H2 has one, poly (A)⁺ signal,

in addition to one cytoplasmic polyadenylation element 30 nucleotides upstream of this.

The mRNA levels of relaxin H1 and H2 in the prostate adenocarcinoma LNCaP.FGC cell line were determined by quantitative competitive RT-PCR. Relaxin H1 had a 10-fold greater number of molecules ($\sim 2.5 \times 10^7$) per μg of total RNA than relaxin H2 ($\sim 2.5 \times 10^6$). The stability of relaxin H1 and H2 mRNAs were compared in LNCaP cells treated with the transcription inhibitor actinomycin D (10 mM) for 0, 1, 2, 4, 8, 10, 14, or 24 h. Half-lives of 3.17 days for relaxin H1 mRNA and 11.4 h for relaxin H2 mRNA were obtained from semi-logarithmic plots. Thus both mRNAs are relatively stable; however, relaxin H1 mRNA is considerably more stable than relaxin H2, at least in LNCaP cells. This difference in their mRNA stability may partly explain the greater level of expression of relaxin H1 in these cells.

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INTRODUCTION

Relaxin is a 6 kDa polypeptide hormone structurally related to insulin and insulin-like growth factors (Isaacs *et al.* 1978). It is classically an endocrine hormone, produced by the ovary, and targeting the mammalian reproductive system to ripen the cervix, elongate the pubic symphysis and inhibit uterine contraction (Bryant-Greenwood & Schwabe 1994, Sherwood 1994). The identification of additional autocrine/paracrine sources of relaxin such as the prostate (Ivell *et al.* 1989, Hansell *et al.* 1991), endometrium, decidua (Bryant-Greenwood *et al.* 1993), placenta (Sakbun *et al.* 1990), breast

(Tashima *et al.* 1994), and gastrointestinal tract (Stemmerman *et al.* 1994) suggests additional roles for relaxin. Indeed, this hormone was reported to enhance sperm motility (Weiss 1989), regulate blood pressure (Kakouris *et al.* 1992, Han *et al.* 1994), control heart rate (Mumford *et al.* 1989, Ward *et al.* 1992) and release of oxytocin and vasopressin (Summerlee *et al.* 1984, Wilson & Summerlee 1994). The human appears to be unique among the vertebrates in having two functional relaxins, termed H1 and H2 (Hudson *et al.* 1984, Evans *et al.* 1994), and it is of interest to know whether both genes are actively transcribed and translated at the same level in all tissues. This

information will provide insights into whether these hormones have separate or overlapping functions. Of the two human relaxin peptides, only the H2 has been isolated from the corpus luteum (Weiss *et al.* 1976) and prostatic fluid (Winslow *et al.* 1992); the H1 peptide has not been isolated from any tissue to date. However, both genes have been shown by reverse transcription (RT)-PCR to be actively transcribed in all tissues considered as autocrine/paracrine sources (Bryant-Greenwood & Schwabe 1994), albeit at a lower level compared with the relaxin H2 transcribed in the corpus luteum, consistent with a local role. The high degree of sequence similarity between these two genes (Evans *et al.* 1994) and their low levels of expression preclude the use of northern analysis for their separate quantitation. A quantitative competitive RT-PCR method was therefore developed in order to differentiate and quantitate the individual mRNAs for the two relaxin H1 and H2 genes in a model system, the prostate adenocarcinoma cell line, LNCaP.FGC. In addition, their mRNA stabilities and half-lives were determined by RT-PCR in these cells treated with the transcription inhibitor, actinomycin D.

Gene expression in almost all organisms is influenced by the stability of mRNA, and the abundance of a particular mRNA may fluctuate many-fold after a change in the half-life of the mRNA, without any change in the rate of transcription (Ross 1995). The 3'-untranslated region (UTR) contains sequence and structural motifs affecting mRNA stability (Chen & Shyu 1995) and translation efficiency (Jackson & Standart 1990, Ross 1995), thereby affecting overall gene expression (Ross 1995). Thus a comparison of the 3'-UTR of the two human relaxin genes and determination of their relative mRNA stabilities could provide insights into their importance in different tissues.

MATERIALS AND METHODS

Human tissues

Placentas with the fetal membranes and decidua attached were obtained from women undergoing elective cesarean section at term before labour at Kapiolani Medical Center for Women and Children (Honolulu, Hawaii), with Institutional Board approval and approval of the Human Experimentation Committee of the University of Hawaii. These tissues were transported on ice within 1 h of removal, then stored frozen at -80°C before use. Dr A H MacLennan (Queen Victoria Hospital, South Australia) kindly provided the cyclic corpus

luteum, which was frozen in liquid nitrogen and shipped to Hawaii in dry ice. The prostate gland was obtained from the National Disease Research Interchange (Philadelphia, PA, USA). This was a tissue collected at autopsy from a young male who died of a gunshot wound. It was collected and frozen in liquid nitrogen and shipped in dry ice.

Preparation of poly (A)⁺ RNA and 3'-RACE-PCR

Total RNA was isolated from 2–8 g corpus luteum, prostate, placenta or decidua using the acid guanidinium thiocyanate–phenol–chloroform method (Chomczynski & Sacchi 1987). Poly (A)⁺ RNA was isolated by affinity chromatography on oligo(dT) cellulose (Aviv & Leder 1972), and yields were estimated by spectrophotometry at 260 nm. The extracted poly (A)⁺ RNAs (1 µg) were used for the first-strand cDNA synthesis, using primers and reagents from Clontech's Marathon kit (Palo Alto, CA, USA) and following the manufacturer's protocol. The resulting cDNAs were used for the PCR-based 3'-rapid amplification of cDNA ends (RACE-PCR) with the gene-specific primers shown in Fig. 1. These primers were chosen from the C-terminus of the coding region that showed the least homology between the two genes. Amplified products were gel extracted and purified using the Qiagen (Valencia, CA, USA) gel extraction kit, and cloned into the TA-cloning vector pCR2.1 (Invitrogen, San Diego, CA, USA). Selected clones were sequenced in both directions at the University of Hawaii's Biotechnology Molecular Biology Instrumentation Facility, using the double-stranded dideoxynucleotide termination method (Sanger *et al.* 1977).

Sequence analysis of the 3'-UTR and RNA folding

Sequence analysis, comparison and identification of putative regulatory elements were performed using GAP, BESTFIT, LINEUP, REPEATS and FINDPATTERNS programs as implemented in the GCG molecular biology software package (GCG-Wisconsin Package Version 9.1, Madison, WI, USA). RNA secondary structure predictions were performed using Zuker's MFOLD program (SeqWeb Version 1.1, GCG-Wisconsin Package Version 10).

Construction of the H1 and H2 cRNA standard molecules and RT-PCR

The H1 and H2 cRNA standard molecules were constructed using total RNA from LNCaP.FGC

FIGURE 1. Primers for the 3'-RACE-PCR of relaxins H1 and H2. A diagram of the pre-prorelaxin structure is shown above the primer sequences, as a reference point for the location of the primers. S, signal peptide; B, B-chain; C, C-peptide; A, A-chain. The relaxin H1 outer primer (H1-O) is shown at nt 315 within the C-chain, and the nested primer (H1-N) is further downstream at nt 482 between the C-peptide and A-chain. The relaxin H2 primers overlap within the C-chain, with the outer primer (H2-O) shown at nt 296, and the nested primer (H2-N) at nt 316.

cells and corpus luteum, respectively, with the GeneAmp RT-PCR kit (Perkin Elmer, Foster City, CA, USA). The schematic representations of the construction of the relaxin H1 and H2 cRNA standard molecules and RT-PCR assays are shown in Fig. 2. The gene-specific primers used for the construction of the internal standards and quantitative RT-PCR reactions shown in Table 1 were prepared by and obtained from Gibco BRL (Grand Island, NY, USA). These were carefully selected using a computer-based primer selection program (Gene Runner; Hastings Software Inc., Hastings, NY, USA) from the region of least homology, thereby ensuring that the products amplified were specific to each relaxin gene. The very high level of sequence homology between the relaxin H1 and H2 genes made it difficult to obtain gene-specific primers. There were several primer pairs designed and tested, and the best primer pair for each of the relaxin genes shown in Table 1 and Fig. 2 was chosen for its high specificity and amplification efficiency. The gene-specific forward (5'-) primers H1-a or H2-c for relaxin H1 or H2, respectively (Table 1), were used for both the construction of the cRNA standards and the quantitative RT-PCR. In contrast, two different reverse (3'-) primers were designed for each relaxin gene; one set was used only for the construction of the cRNA standards, and another for the RT-PCR (Fig. 2). The 3'-gene-specific primers used for the quantitative

competitive RT-PCR were H1-b or H2-b for relaxin H1 or H2, respectively (Table 1, Fig. 2A). H1-b or H2-b was modified with additional 24 (relaxin H1) or 26 (relaxin H2) oligonucleotides, resulting in the primers H1-e/b and H2-f/d, respectively (Table 1, Fig. 2B) for the construction of the cRNA standards. These additional oligonucleotides were derived from their respective cDNAs at a position 59 (relaxin H1) or 55 (relaxin H2) bp upstream of their respective 3'-gene-specific primers. The use of the H1-e/b or H2-f/d primers, together with their respective 5'-gene-specific primers, amplified 389 bp relaxin H1 or 400 bp relaxin H2 cDNAs (Fig. 2B). These were subsequently cloned and reverse transcribed to produce the internal cRNA standards. In contrast, the use of the 5'- and 3'- (H1-b or H2-b) gene-specific primers for RT-PCR amplified 448 bp relaxin H1 or 455 bp relaxin H2 cDNA products (Fig. 2A). Thus the internal standards were shorter than the endogenous target mRNAs, allowing them to be clearly identified by PCR. The PCR primers were also designed to span the intron (within the C-peptide), thereby eliminating contamination from genomic DNA.

The RT reaction was carried out in a total volume of 20 µl containing 5 mM MgCl₂, 1 × PCR buffer II, 10 mM each of dATP/dCTP/dGTP/dTTP, 20 U RNase inhibitor, 2.5 µM random hexamer primer, 2.5 U MuLV reverse transcriptase and 1 µg

A) RT-PCR amplification of relaxin H1 and H2 mRNA with gene specific primers **B) Construction of the H1 and H2 internal standards**

FIGURE 2. RT-PCR amplification of relaxin H1 and H2 mRNAs and construction of internal cRNA standards. A diagram of the pre-prorelaxin cDNA is shown to illustrate the positions of the primers, which are in turn indicated by solid arrows. S, signal peptide; B, B-chain; C, C-peptide; A, A-chain. The full length cDNA for both relaxins is 558 bp. (A) RT-PCR amplification of relaxin H1 and H2 mRNA with gene-specific primers. The use of the forward or 5'-primer (H1-a) and reverse or 3'-primer (H1-b) specific to relaxin H1 amplified a 448 bp PCR product, and the 5'-primer (H2-c) and the 3'-primer (H2-d) for relaxin H2 amplified a 455 bp PCR product. (B) Construction of the relaxin H1 and H2 cRNA internal standards. The 5'-gene specific primers from above were used directly for the construction of the cRNA standards. The 3'-primers were modified by the addition of 24 oligonucleotides for relaxin H1 or 26 oligonucleotides for relaxin H2. These oligonucleotides were derived from their respective cDNA sequences 59 bp (Rlx H1) or 55 bp (Rlx H2) upstream of the 3'-primers used in the above RT-PCR reactions. The modified primers are shown with tails and are termed H1-e/b for relaxin H1 and H2-f/d for relaxin H2, where e and f represent the oligonucleotides added, and b and d refer to the original 3'-primer sequence. The use of these primers amplified 389 bp relaxin H1 and 400 bp relaxin H2 PCR products, which were subsequently cloned and transcribed *in vitro* for the construction of the cRNA molecules.

total RNA. The reaction mixture was incubated as follows: 23 °C for 10 min, 42 °C for 15 min, 99 °C for 5 min, then 8 °C for 15 min. A 10 µl aliquot from this reaction was used for the PCR with the gene-specific primers H1-a, and H1-e/b (relaxin H1), H2-c and H2-f/d (relaxin H2; Table 1, Fig. 2B). Amplification was performed in a 50 µl

total volume containing 1 mM MgCl₂, 1 × PCR buffer II, 0.1 µM each of the 3'- and the 5'- primers, 1.25 U Amplitaq Gold (polymerase), and the 10 µl aliquot from the RT reaction. The PCR was cycled as follows: 1 × at 95 °C for 10 min, 40 × at 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 45 s and a final extension at 72 °C for 10 min. The

TABLE 1. Oligonucleotides used for the semi-quantitative RT-PCR and construction of internal standards. The name, sequence, nucleotide positions and the use of each oligonucleotide are indicated. Nucleotide positions are as reported by Hudson *et al.* (1984). For details see the text and Materials and Methods

Name	Sequence	Position (nucleotide)	Usage
H1-a	5'-TCGCGGCCAAATGGAAGGAC-3'	68–87	Q-PCR 5'-H1 primer
H1-b	5'-GCAACATTTCTCAAACAGTGCCAC-3'	493–516	Q-PCR (RT) 3'-H1 primer
H1-e/b	5'-GCAACATTTCTCAAACAGTCCAC- ATTGCTGTCTGCGGCTTCACTTTG-3'	b: 493–516 e: 409–432	H1 cRNA Construct
H2-c	5'-CGGACTCATGGATGGAGGAAG-3'	71–91	Q-PCR 5'-H2 primer
H2-d	5'-AACCAACATGGCAACATTTATTAGC-3'	502–526	Q-PCR (RT) 3'-H2 primer
H2-f/d	5'-AACCAACATGGCAACATTTATTAGC- TTTAATTCTGAAGGACTGCTGTCTGC-3'	d: 502–526 f: 421–446	H2 cRNA Construct

RT-PCR products were gel purified (QIAquick PCR purification kit by Qiagen), subcloned into the pGEM-T vector (Promega, Madison, WI, USA) and amplified into the One Shot *E. coli* competent cells (Invitrogen). Recombinant plasmids were selected and sent for sequencing to ensure that the correct DNA templates were made. The selected recombinant plasmids were digested with Sal I endonuclease (New England Biolabs, Beverly, MA, USA) to obtain linear DNA templates which were used for the *in vitro* transcription preparation of the H1 and H2 RNA standards. *In vitro* transcription was carried out using the Riboprobe *in vitro* Transcription Kit (Promega) in a reaction mixture (20 µl) containing 1 × transcription buffer, 10 mM dithiothreitol (DTT), 20 U RNasin, 10 mM each of ATP/CTP/GTP/TTP, 0.4 µg linearized DNA template and 15 U T7 RNA polymerase, and incubated at 37 °C for 90 min. The reaction products were treated with DNase (1 U/µg DNA) to remove the DNA template completely. The resulting cRNA standards were serially diluted to the desired concentrations as determined by spectrophotometric analysis.

Cell culture and incubation with actinomycin D

LNCaP.FGC cells (ATCC; Rockville, MD, USA) were maintained and grown at 37 °C under 5% CO₂ in RPMI 1640 medium, containing 10% fetal calf serum (Gibco BRL), and 100 U/ml penicillin G/streptomycin (Sigma, St Louis, MO, USA). At 85% confluence, cells were washed twice with Hank's balanced salt solution (HBSS) (Gibco BRL), then maintained in growth medium with-

out actinomycin D for control cells, or treated with 10 mM actinomycin D (Sigma) for 1, 2, 4, 8, 10, 14 and 24 h. After incubation, cells were washed completely in HBSS and lysed with Trizol reagent (Gibco BRL) for the isolation of total RNA.

Determination of H1 and H2 mRNA levels by quantitative competitive RT-PCR

The high degree of sequence homology between relaxin H1 and H2 genes makes it difficult to distinguish between relaxin H1 and H2 mRNAs using northern blotting analysis, despite several efforts with specific oligonucleotide probes (Ivell *et al.* 1989, Gunnarsen *et al.* 1995). Therefore a quantitative competitive RT-PCR method for relaxins H1 and H2 was developed and used to determine the relative levels of relaxin H1 and H2 mRNAs in the prostate adenocarcinoma LNCaP.FGC cell line. Exogenous homologous internal standards were prepared for both relaxin genes in order to minimize the variation due to the differences in amplification efficiencies and to determine the amount of target mRNA within the cells. In order to limit the differences in the efficiency of amplification, the sizes of the internal standards prepared were only 59 bp (for relaxin H1) and 55 bp (for relaxins H2) shorter than the target mRNAs.

The specificity of the RT-PCR primers was tested by using total RNAs from the corpus luteum, decidua, and LNCaP.FGC cells. The corpus luteum was selected because it expresses only relaxin H2, whereas both the decidua and

LNCaP.FGC cells express relaxins H1 and H2. Primer specificity was confirmed by demonstrating that only relaxin H2 mRNA was amplified from the corpus luteum, whereas both relaxins H1 and H2 mRNAs were obtained from the decidua and LNCaP.FGC cells.

The relative efficiency of these primers in amplifying the correct cDNA products was also tested, using increasing amounts of the internal cRNA standards only. These primers were able to amplify the same number of molecules of the internal standards and showed a linear increase using a range of 10^2 - 10^8 molecules. Subsequent quantitative competitive RT-PCRs to determine the mRNA levels of relaxin H1 and H2 in LNCaP cells were therefore carried out using internal standards at 10^2 - 10^8 mol/ μ g total RNA. The efficiency of these primers in synthesizing the first-strand DNA was also compared with primers for β -actin, which served as an internal control. RT-PCR assays were performed combining the primers for the β -actin internal control with those of either relaxin H1 or H2 gene-specific primers, to assess their individual efficiencies in the synthesis of the first-strand cDNA from LNCaP RNA. These assays further showed that the gene-specific primers for both relaxins H1 and H2 were equally efficient in the synthesis and amplification of their respective cDNA products. The amount of each product amplified was analyzed and compared using an AMBIS Image Acquisition and Analysis System (San Diego, CA, USA).

The mRNA levels of relaxin H1 and H2 in LNCaP.FGC cells were determined as follows. Total RNAs, along with a known copy number of the H1 or H2 cRNA standard molecules, were reverse transcribed using random hexamers as described above. Increasing amounts of either H1 or H2 cRNA standards were added to 1 μ g total RNA from LNCaP cells in order to determine the equilibration point of the endogenous relaxin H1 or H2 mRNAs. Aliquots (10 μ l) of the RT products were used as templates for the subsequent PCR reactions, using the same conditions as above but with only 35 cycles and using the common 5'-gene-specific primers paired with the 3'-gene-specific primers H1a or H2b (Fig. 2A). The products were analysed on 2% agarose gels. Initially, the amounts of H1 and H2 internal standards used in the RT-PCR ranged between 10^4 and 10^8 molecules. Once the range was narrowed down, the RT-PCRs were repeated using finer gradations to determine a more precise copy number of the endogenous relaxin H1 and H2 mRNAs.

Determination of relaxin mRNAs half-lives

The mRNA half-lives for the relaxin H1 and H2 genes were determined using 1 μ g total RNA from untreated LNCaP cells (control) or cells treated with the transcription inhibitor, actinomycin D (10 mM), for 1, 2, 4, 8, 10, 14, and 24 h for the RT-PCR assays. To test the effectiveness of the transcription inhibition, additional RT-PCRs were performed to amplify the constitutively expressed β -actin transcripts, and the unstable c-myc mRNA using 5'- and 3'-gene-specific amplimer sets from Clontech. Using these primers, a 540 bp and a 479 bp PCR product were obtained for β -actin and c-myc, respectively. PCR products were analysed on 2% agarose gels, bands were visualized with ethidium bromide, and the densities were compared using an AMBIS Image Acquisition and Analysis System. Data (means $\pm$ s.d.) were expressed as percentage change in relation to control values (control=100%). The mRNA half-lives were calculated from the semi-log plot of these data following the methods of Ross (1995) and Loflin *et al.* (1999).

RESULTS

Isolation and analysis of the 3'-UTRs of the human relaxin H1 and H2 genes

The 3'-UTRs of both relaxins H1 and H2 genes were isolated by 3'-RACE using poly (A)⁺ RNA from decidua, placenta, prostate, and corpus luteum. The length of the relaxin H1 3'-UTR is 284 bp, whereas that of relaxin H2 is 234 bp. A sequence comparison and diagram of these, starting from the TGA translation stop codon, is shown in Fig. 3A. The nucleotide sequences for the 3'-UTR of both relaxins were AT-rich (72%), and identical in each of the tissues examined. Using the GCG-BESTFIT program, a maximal alignment was obtained when three gaps (11 nt, 89 nt, 45 nt) were introduced, and resulted in an overall homology of 91% (Fig. 3B). This shows two unique regions (11 nt upstream and 89 nt downstream) within the relaxin H1 sequence that did not correspond to any sequences in the relaxin H2 3'-UTR. A search of the transcription factor database failed to show any sequence motif in the upstream 11 nt region that may act as a regulatory element. The downstream 89 nt region showed three direct repeats (G/TTCTTTT), located at positions 754, 762 and 792. This sequence motif has not been shown to correspond to any known regulatory element. Within the relaxin H2 3'-UTR, a unique sequence of 45 nt showed no homology with any regions of the relaxin H1 sequence. A direct repeat (AGGTCCCATCAATTCTTAGAATATCTAA) of 28 nucleotide residues at position 651 was found in this 45 nt

A

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H1 556 TGAGATGAAGCTAATTGTGCACATCTTGTCTAATTTTCACACATAGTCTTGATGACATTTCACTGATGCT 626
      |||
H2 556 TGAGATGAAGCTAATTGTGCACATCTCGTATAATATTCACACATATTCTTAATGACATTTCACTGATGCT 626
      Stop
H1 627 TCTGTCAGGTCCCACTAATTATTAGAATA..TAAGAA..... 661
      |||
H2 627 TCTATCAGGTCCCATCAATTCTTAGAATATCTAAGAACTCTTGTAGATATTAGGTCCCATCAATTCTTA 696
      a. b.
H1 662 .....ATCTTTATTAATGTTTAGA.TTTTTCATTTGGTGTGTAAGAAATATTCTTTGTATGA 718
      |||
H2 698 GAATATCTAAACATCTTTGTTGATGTTTAGATTTTTTTATTTGATGTGTAAGAAATGTTCTTTGTGTGA 766
      CPE
H1 721 TTGTAGTTTCTGTAAATGACAC..TTTCTATGCTGAAGTCTTTTTTGTCTTTTTTATTAACAGTATAATTGT 786
      1 2
H2 767 T.....TAAATGACACATTTTTTTTGCTG..... 792
      3
H1 787 GTTGATTCTTTTTAATGCTGTAACTTAAATACAATAAAACCTTTGCCACTTTTAAAAAAAAAAAAA 856
      3
H2 793 .....AAAAAAAAAAAAA 806

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B

FIGURE 3. The 3'-untranslated regions of human relaxins H1 and H2. (A) Sequence alignment between the human relaxin H1 (GenBank Accession No. AF057705) and H2 (GenBank Accession No. AF057706) 3'-UTRs were obtained using the GCG-BESTFIT program. The nucleotides are numbered starting at the 'T' residue of the **TGA** stop codon (in bold), following the published cDNA sequence (Hudson *et al.* 1984). The polyadenylation signals are shown in bold, underlined and overlined; the cytoplasmic polyadenylation element (CPE) in relaxin H2 is underlined. The gaps that represent absence of DNA sequence are shown as dotted lines. Direct repeats on the 89 nt relaxin H1 unique region are enclosed by rectangles and numbered 1, 2 and 3, and the 28 nt direct repeats identified at the relaxin H2 unique region are enclosed by dashed rectangles and marked as a and b. (B) A schematic comparison of the relaxin 3'-UTRs shows a 91% homology, and the presence of several gaps. For relaxin H1, the 11 nt and 89 nt segments absent from relaxin H2 are indicated by triangular shaped gaps, and the two polyadenylation signals by stippled ovals. The unique 45 nt segment in relaxin H2 is indicated by a diamond shaped gap, the CPE by a hatched box, and the single polyadenylation signal by a stippled circle. Direct repeats within the unique regions are indicated by solid stars (★).

domain, separated from its upstream counterpart by 18 nucleotides at position 633 (Fig. 3A).

A computer search of the database showed that relaxin H1 has two poly (A)⁺ sites; that located

at nucleotide 652 is atypical (AATATA), whereas the other at nucleotide 822 (AATAAA) follows the consensus sequence (Fig. 3A, B). Relaxin H2, in contrast, showed the presence of a single

FIGURE 4. Relaxin (Rlx) H1 and H2 mRNA levels in LNCaP cells. The basal levels of relaxin H1 and H2 mRNA in LNCaP.FGC cells were measured and compared with their respective internal cRNA standards by quantitative competitive RT-PCR. (A) The basal level of relaxin H1 mRNA was initially determined to lie within the range 10^7 – 10^8 mol/ μ g total RNA, and that for relaxin H2 mRNA was within the range 10^5 – 10^6 mol/ μ g total RNA. (B) A more precise determination of the basal levels of relaxin H1 and H2 mRNAs was performed using finer gradations of the cRNA standards. Results show a 10-fold greater basal level of relaxin H1 mRNA ($\sim 2.5 \times 10^7$ mol/ μ g total RNA) than of relaxin H2 mRNA ($\sim 2.5 \times 10^6$ mol/ μ g total RNA).

non-canonical poly (A)⁺ signal (ATTAAA) located at nucleotide 766. In addition, a putative cytoplasmic polyadenylation element (CPE) that fits the

consensus TTTTTTTTAT motif was found at 30 nucleotides upstream of the relaxin H2 polyadenylation signal (Fig. 3A, B).

Analysis of the secondary structures of the two relaxin 3'-UTRs was performed using Zuker's MFOLD program (GCG-package). The predicted structures for relaxin H1 and H2 3'-UTRs showed similar conformation with seven putative stem loops positioned in the same orientation. The free energy obtained for these secondary structures was -44 kcal/mol for relaxin H1 and -51 kcal/mol for relaxin H2 (data not shown).

Determination of mRNA levels of relaxins H1 and H2 by quantitative competitive RT-PCR

The basal levels of relaxin H1 and H2 mRNAs in LNCaP.FGC cells were measured by quantitative competitive RT-PCR. During the RT-PCR reaction, a known amount of either mRNA standard for relaxin H1 or H2 was added, in order to determine the equilibration point of each standard with the endogenous levels of either relaxin H1 or H2 mRNA. At the equilibration point, the estimated amount of the endogenous relaxin mRNA in 1 μ g total RNA would be equivalent to the concentration of cRNA molecules. Densitometric comparison of the endogenous mRNA levels of relaxin H1 and H2 with each homologous exogenous internal standard gave initial results of approximately 10^7 – 10^8 mol/ μ g total RNA for relaxin H1 and approximately 10^5 – 10^6 mol/ μ g total RNA for relaxin H2 (Fig. 4A). A more detailed comparison using finer gradations of the internal standards showed the endogenous relaxin H1 mRNA at approximately 2.5×10^7 mol/ μ g total RNA, whereas that of relaxin H2 mRNA was 10-fold lower, at 2.5×10^6 mol/ μ g total RNA (Fig. 4B).

FIGURE 5. Determination of relaxin (Rlx) H1 and H2 mRNA half-lives. The decays of relaxin H1 and H2 mRNAs were measured after treatment of LNCaP cells with 10 mM actinomycin D for 0, 1, 2, 4, 8, 10 and 24 h.

(A) Measurements were obtained from four independent experiments and plotted on a semi-logarithmic scale. The amount of remaining mRNA is expressed as percentage change in relation to the control value (control=100%). The amount of β -actin mRNA was used as an internal control and was shown to be unaffected by actinomycin D treatment. In contrast, the unstable c-myc transcript showed a rapid decline in its mRNA level and a calculated half-life of 2.5 h. Actinomycin D initially stimulated relaxin H1 and H2 mRNAs at 1 h treatment; this was followed by a slow decline in relaxin H1 mRNA, and a more rapid degradation of relaxin H2 mRNA. The relaxin H1 mRNA half-life was calculated to be 3.17 days, and that of relaxin H2 was 11.4 h. (B) Representative agarose gels for the RT-PCR assays of mRNA half-lives. The DNA size marker (M) is shown on the left. Amplified PCR products are shown in lanes 1–8 for relaxin H1 genes, lanes 9–16 for relaxin H2 genes, lanes 17–24 for c-myc genes and lanes 25–32 for β -actin genes. The experimental conditions were as follows: lanes 1, 9, 17, 25 untreated or control cells; lanes 2, 10, 18, 26 treated with actinomycin D for 1 h; lanes 3, 11, 19, 27 treated for 2 h; lanes 4, 12, 20, 28 treated for 4 h; lanes 5, 13, 21, 29 treated for 8 h; lanes 6, 14, 22, 30 treated for 10 h; lanes 7, 15, 23, 31 treated for 14 h; and lanes 8, 16, 24, 32 treated for 24 h.

Assessment of the mRNA half-lives and stabilities of relaxins H1 and H2 in actinomycin-D-treated LNCaP.FGC prostate adenocarcinoma cells

The half-lives of relaxin H1 and H2 mRNAs were determined by measuring the decay of pre-existing mRNA molecules by RT-PCR after blockade of new RNA synthesis by 10 mM actinomycin D. To assess the effectiveness of actinomycin D in blocking new RNA synthesis in the LNCaP cells, the amounts of remaining mRNA molecules for two other transcripts expressed in LNCaP cells were also assayed. β -Actin, a highly expressed house-keeping gene, is a commonly used internal control and is also known for its high mRNA stability, unaffected by actinomycin D treatment (Chen *et al.* 1993). The other transcript used as a measure of mRNA decay was c-myc, known to have a short half-life and to be easily affected by actinomycin D (Leygue *et al.* 1995).

The decay of relaxin H1 and H2, β -actin and c-myc mRNAs after treatment with actinomycin D over a period of time is shown in Fig. 5A; agarose gels from a representative RT-PCR assay showing the effect of actinomycin D on the levels of these mRNAs are shown in Fig. 5B. The amount of remaining mRNA is expressed as percentage change in relation to the control (untreated cells) value with control taken as 100%. The data represent the results of four independent RT-PCR experiments. Actinomycin D initially stimulated the levels of both relaxin H1 and H2 mRNAs. This was followed by a gradual decline in the relaxin H1 mRNA, which was finally reduced by only 1.5% of the control over the 24-h period. In contrast, the amount of relaxin H2 mRNA rapidly declined after the 2 h treatment (Fig. 5A). Using a semi-logarithmic extrapolation, we calculated the half-life of relaxin H1 mRNA to be 3.17 days, and that for relaxin H2 mRNA was only 11.4 h. On the basis of these calculations, relaxin H2 mRNA was found to be approximately seven times less stable than relaxin H1.

DISCUSSION

In this study, the 3'-UTRs of the two human relaxin genes have been isolated and analysed, in an effort to determine their contributions to the differential expression of these genes. In the prostate adenocarcinoma LNCaP.FGC cell line used in this study, we have shown that relaxin H2 has a lower level of expression and a less stable mRNA than relaxin H1. Analysis of the 3'-UTR sequences and comparison with nucleic acid

databases showed the presence of regulatory elements that may be involved in the regulation of the relaxin mRNA levels and stabilities.

A single type of mRNA species for each of the relaxins, H1 and H2, was isolated from all the tissues examined. This is consistent with the reported northern blot studies showing a single transcript of 1.0 kb (Sakbun *et al.* 1990, Tashima *et al.* 1994). An additional 2.0 kb relaxin H2 transcript previously reported and detected by northern analysis of the corpus luteum (Hudson *et al.* 1984, Hansell *et al.* 1991), was not identified in the tissues used in this study, suggesting that it may result from an alternative transcription initiation site in the 5'-upstream sequence. We have recently isolated and analysed the 5'-flanking regions of both relaxin H1 and H2 genes, and identified three transcription initiation sites in both genes through primer extension analysis using LNCaP cells (Garibay-Tupas *et al.* 1999). Moreover, Gunnersen *et al.* (1996) reported that the C-peptide of relaxin H2 is alternatively spliced in a transcript isolated from the placenta, resulting in the insertion of a 51 nt sequence within this region. The length contributed by using alternative transcription start sites and insertion at the C-peptide is not enough to account for a 2.0 kb relaxin H2 mRNA transcript. It is possible that another alternatively spliced relaxin H2 mRNA is present in the corpus luteum, but was not detected in this present study.

A comparison of the nucleotide sequences showed that the 3'-UTRs of both genes shared very high homology (91%) when several large gaps were introduced. This high degree of sequence similarity between the two 3'-UTRs may indicate a functional role in directing the expression of the relaxin genes. On the other hand, the regions of no homology, shown as gaps, represent gene-specific segments of unknown significance, as these appeared to contain no known regulatory elements. Although direct repeats were found in the unique regions of both relaxin H1 and H2 3'-UTR, the significance of these repeats in terms of regulating the expression levels of these genes remains unknown. It is possible that these repeats encode regulatory elements that may influence the differential expression of the relaxin genes by a mechanism unrelated to their mRNA stability. The predicted folding patterns of both relaxin 3'-UTRs showed similar configuration and free energy, thus it is unlikely that there are distinct structural features within their 3'-UTRs that could differentially affect mRNA stability.

The analysis of the 3'-UTR of relaxin H1 showed the presence of two polyadenylation signals. The use of the upstream atypical polyadenylation signal would result in a transcript size 100 nucleotides

shorter than the reported 1.0 kb transcript for relaxins (Sakbun *et al.* 1990, Tashima *et al.* 1994). This size difference is probably too small to be detected by northern analysis. The significance of the shorter transcript is not known; however, the isolation of a single clone with a shorter relaxin H1 3'-UTR in the prostate, but not in either decidua or placenta, suggests it is not a common product and that the downstream poly (A)⁺ site is the active site. The relaxin H2 3'-UTR, however, has a single polyadenylation signal (ATTAAA) that differed from the consensus AATAAA motif. A single base change in relaxin H2 poly (A)⁺ signal may affect its mRNA expression level in a manner similar to the finding by Gottschling *et al.* (1993) that a non-canonical polyadenylation signal (ATTAAA) in the 3'-UTR of laminin-A chain was responsible for the down-regulation of its mRNA and protein expression. Conversely, Sheets *et al.* (1990) reported that, among the rare natural variants of the poly (A)⁺ site, the ATTAAA motif is the most commonly found. This type of polyadenylation signal is the mildest mutation in terms of its effect on the polyadenylation and cleavage efficiency – and hence on the translation of the mRNA (Sheets *et al.* 1990). It is likely that the mRNA of relaxin H2, but not its protein concentration, would be affected by this variant poly (A)⁺ site in the LNCaP cells. Further studies will be needed to show that there is, indeed, a difference in the protein concentrations of these relaxins in the LNCaP cells. A system to differentiate the two relaxin peptides is currently being developed, and quantitation of the gene products in cells or tissues may help to resolve this issue.

A putative CPE located at 30 nucleotides upstream of the relaxin H2 polyadenylation signal fits the consensus for CPE reported to be necessary for the developmentally regulated polyadenylation that occurs in the cytoplasm (Sachs & Wahle 1993, Curtis *et al.* 1995). The rate of translation initiation of an mRNA can be regulated by modulating the length of the poly (A)⁺ tail; in the cytoplasm, the CPE may be involved in this regulation (Curtis *et al.* 1995). The CPE in the relaxin H2 3'-UTR may, indeed, enhance the translation of its mRNA particularly in cells and tissues that undergo differentiation and development, such as the placental trophoblast and endometrium, in which relaxin peptides have been demonstrated (Sakbun *et al.* 1990, Bryant-Greenwood *et al.* 1993). The role of the putative CPE and other regulatory elements present in the 3'-UTR of relaxins H1 and H2 is currently being investigated in our laboratory.

We have determined the relative levels of expression of relaxin H1 and H2 mRNA using

LNCaP.FGC cells as a model system in a quantitative competitive RT-PCR. The primers used for these assays were shown to be equally efficient, specific, and amplified only the correct relaxin cDNA, in spite of the very high level of sequence homology between the relaxin H1 and H2 genes, which limited the choice of gene-specific primers. These RT-PCR assays were used to determine the individual levels of the relaxin H1 and H2 mRNAs and assess the relative stabilities of their mRNAs.

A report by Gunnensen *et al.* (1995) showed that the promoter of relaxin H2 was more active than that of relaxin H1 in LNCaP cells, which suggests that, in these cells, the transcription rate for relaxin H2 is greater, implying a greater concentration of relaxin H2 than of relaxin H1. This is contrary to our observation that the basal level of relaxin H2 mRNA was 10-fold lower than that of relaxin H1. It is likely that the mRNA level of relaxin H2 is affected by its half-life without any change in its transcription rate, as its half-life was sevenfold shorter than that of relaxin H1 mRNA. The use of a non-canonical poly (A)⁺ signal in its 3'-UTR may further influence the level of relaxin H2 mRNA. These results suggest that post-transcriptional regulation of relaxin H1 and H2 gene expression through their 3'-UTRs contributes to the observed differential expression of these genes.

This study shows that the differential expression of the relaxin genes in human tissues is regulated at both the transcription and post-transcription levels, involving their 3'-UTRs in addition to their 5'-flanking regions (Gunnensen *et al.* 1995, Garibay-Tupas *et al.* 1999). The gene-specific segments in the 3'-UTRs and the promoter regions involved in regulation are currently being investigated, and the further elucidation of such mechanisms could provide some insight into the relative roles of the two relaxins in both normal and pathological processes.

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