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(54) Title: HUMAN 3 RELAXIN

(57) Abstract: Human H3 preprorelaxin, human H3 prorelaxin, human H3 relaxin, human relaxin analogues having a modified A chain and/or a modified B chain are described. Also described are nucleic acid sequences encoded human H3 preprorelaxin, human H3 prorelaxin, human H3 relaxin, human relaxin analogues. Also described are methods for the treatment of conditions responsive to administration of H3 relaxin or analogues thereof.



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## HUMAN 3 RELAXIN

### Field of the Invention

This invention relates to human 3 relaxin (hereafter referred to as "H3 relaxin"). More specifically, the invention relates to H3 relaxin, pro- and prepro- H3 relaxin, the individual peptide chains which comprise these sequences, analogues of H3 relaxin, compositions including pharmaceutical compositions, as well as therapeutic uses and methods of treatment. Further, the invention relates to nucleic acids encoding H3 relaxin, H3 pro- and prepro- relaxin, H3 relaxin analogues, and individual peptide chains which comprise these sequences.

### Background of the Invention

Pioneering work by Hisaw 1926 first suggested an important role for the peptide hormone relaxin in animals through its effect in dilating the pubic symphysis, thus facilitating the birth process. Relaxin is synthesised in the corpora lutea of ovaries during pregnancy, and is released into the blood stream prior to parturition. The availability of ovarian tissue has enabled the isolation and amino acid sequence determination of relaxin from the pig (James et al (1977), *Nature*, 267, 554-546), the rat (John et al (1981) *Endocrinology* 108, 726-729), and the shark (Schwabe et al (1982) *Ann. N.Y. Acad. Sci.* 380, 6-12).

Relaxin genes and the encoded relaxin polypeptides have been identified in many species including man, pig, rat, sheep and shark. In all these species only one relaxin gene has been characterised in mammals, with the exception of the human and higher primates where two separate genes have been described. The separate human genes were identified by the present applicant and designated H1 (Hudson et al (1983) *Nature* 301, 628-631) and H2 (Hudson et al (1984) *Embo. J.* 3, 2333-2339).

The peptide encoded by the H2 gene is the major stored and circulating form in the human (Winslow et al (1992) *Endocrinology* 130, 2660-2668). H1 relaxin expression is restricted to the decidua, placenta and prostate (Hansell et al (1991) *J. Clin. Endocrinol. Metab.* 72,

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899-904), however, the H1 peptide has similar biological activity to that of H2 relaxin in a rat atrial bioassay (Tan et al (1998) *Br. J. Pharmacol.* 123, 762-770).

The actions of relaxin include an ability to inhibit myometrial contractions, to stimulate  
5 remodelling of connective tissue and to induce softening of the tissues of the birth canal. Additionally, relaxin increases growth and differentiation of the mammary gland and nipple and induces the breakdown of collagen, one of the main components of connective tissue. Relaxin decreases collagen synthesis and increases the release of collagenases (Unemori et al (1990) *J. Biol. Chem.* 265, 10682-10685). These findings were recently  
10 confirmed by the establishment of the relaxin gene-knockout mouse (Zhao et al (1999) *Endocrinology* 140, 445-453), which exhibited a number of phenotypic properties associated with pregnancy. Female mice lacking a functionally active relaxin gene failed to relax and elongate the interpubic ligament of the pubic symphysis and could not suckle their pups, which in turn, died within 24 hours unless cross-fostered to relaxin wildtype or  
15 relaxin heterozygous foster mothers.

Evidence has accumulated to suggest that relaxin is more than a hormone of pregnancy and acts on cells and tissues other than those of the female reproductive system. Relaxin causes a widening of blood vessels (vasodilatation) in the kidney, mesocaecum, lung and  
20 peripheral vasculature, which leads to increased blood flow or perfusion rates in these tissues (Bani et al (1997) *Gen. Pharmacol.* 28, 13-22). It also stimulates an increase in heart rate and coronary blood flow, and increases both glomerular filtration rate and renal plasma flow (Bani et al (1997) *Gen. Pharmacol.* 28, 13-22). The brain is another target tissue for relaxin where the peptide has been shown to bind to receptors (Osherooff et al  
25 (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88, 6413-6417; Tan et al (1999) *Br. J. Pharmacol* 127, 91-98) in the circumventricular organs to affect blood pressure and drinking (Parry et al (1990) *J Neuroendocrinol.* 2, 53-58; Summerlee et al (1998) *Endocrinology* 139, 2322-2328; Sinnahay et al (1999) *Endocrinology* 140, 5082-5086).

30 Important clinical uses arise for relaxin in various diseases responding to vasodilation, such as coronary artery disease, peripheral vascular disease, kidney disease associated with

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arteriosclerosis or other narrowing of kidney capillaries, or other capillaries narrowing in the body, such as in the eyes or in the peripheral digits, the mesocaecum, lung and peripheral vasculature.

- 5 The finding of two human relaxin genes, and encoded human relaxin peptide products nearly 20 years ago was of itself most surprising.

Even more surprisingly with the benefit of nearly 20 years of further research and development in relaxin biology internationally, the applicant has identified, isolated and  
10 characterised nucleic acid sequences encoding a third human relaxin gene (H3), the encoded H3 relaxin peptide and the constituent peptide chains thereof. The production of H3 relaxin and analogues thereof has been made possible, as have uses and therapeutic treatment methods.

### 15 **Summary of the Invention**

In a first aspect the invention relates to the peptides human H3 relaxin, H3 prorelaxin and H3 preprorelaxin, to the individual peptide chains which comprise these sequences and to analogues thereof, particularly truncated and/or amino acid substituted modifications. Preferably the peptides are provided as pharmaceutically acceptable compositions for  
20 human or animal administration, by various therapeutic routes. Peptides are preferably isolated in purified or homogenous form free of contaminating peptides and proteins, or in a form of about 90-99% purity.

In a second aspect of the invention there is provided a composition comprising human H3  
25 relaxin or a human H3 relaxin analogue having an A chain and a B chain,

the A chain having the amino acid sequence:

- 4 -

Asp Val Leu Ala Gly Leu Ser Ser Ser Cys Cys Lys Trp Gly Cys Ser  
 1 5 10 15

5 Lys Ser Glu Ile Ser Ser Leu Cys  
 20

(SEQ ID NO: 4)

or an amino acid sequence truncated by up to about 9 amino acids from N-terminus,

10 the B chain having the amino sequence:

Arg Ala Ala Pro Tyr Gly Val Arg Leu Cys Gly Arg Glu Phe Ile Arg  
 1 5 10 15

15 Ala Val Ile Phe Thr Cys Gly Gly Ser Arg Trp  
 20 25

(SEQ ID NO: 2)

or an amino acid sequence truncated by up to 9 amino acids from the amino-terminus  
 and/or up to about 5 amino acids from the carboxyl-terminus,

20

the A and B chains being linked by interchain disulphide bonds at A11-B10, and A24-B22,  
 and wherein the human H3 relaxin or analogue thereof has relaxin bioactivity.

In a third aspect of the invention there is provided a composition comprising a human H3  
 25 relaxin analogue having a modified A chain and/or a modified B chain,

the H3 relaxin A chain having the amino acid sequence:

30 Asp Val Leu Ala Gly Leu Ser Ser Ser Cys Cys Lys Trp Gly Cys Ser  
 1 5 10 15

Lys Ser Glu Ile Ser Ser Leu Cys  
 20

(SEQ ID NO: 4)

- 5 -

wherein the carboxyl-terminus is an amide derivative and/or Lys at position 12 is replaced with Glu, and/or Glu at position 19 is replaced with Gln,

the H3 relaxin B chain having the amino acid sequence:

5

Arg Ala Ala Pro Tyr Gly Val Arg Leu Cys Gly Arg Glu Phe Ile Arg  
1 5 10 15

Ala Val Ile Phe Thr Cys Gly Gly Ser Arg Trp  
10 20 25 (SEQ ID NO: 2)

wherein the carboxyl-terminus is an amide derivative, and/or Ala at position 2 is replaced with Pro, and/or Arg at position 8 is replaced with Lys,

15 the A and B chains being linked by disulphide bonds between A11-B10 and A24-B22 and wherein the human H3 relaxin analogue has relaxin bioactivity.

In accordance with a fourth aspect of the invention there is provided a composition comprising human H3 preprorelaxin or human H3 prorelaxin, having a signal, A chain, B  
20 chain and C chain in respect of human H3 preprorelaxin, and an A chain, B chain and C chain in respect of human H3 prorelaxin, the said amino acid chains having the amino acid sequences:

the A chain comprising:

25

Asp Val Leu Ala Gly Leu Ser Ser Ser Cys Cys Lys Trp Gly Cys Ser  
1 5 10 15

Lys Ser Glu Ile Ser Ser Leu Cys  
30 20 (SEQ ID NO: 4)

the B chain comprising:

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Arg Ala Ala Pro Tyr Gly Val Arg Leu Cys Gly Arg Glu Phe Ile Arg

1 5 10 15

5 Ala Val Ile Phe Thr Cys Gly Gly Ser Arg Trp

20 25 (SEQ ID NO: 2)

the signal sequence comprising:

10 Met Ala Arg Tyr Met Leu Leu Leu Leu Leu Ala Val Trp Val Leu Thr

1 5 10 15

Gly Glu Leu Trp Pro Gly Ala Glu Ala

20 25 (SEQ ID NO: 1)

15

and the C chain comprising:

Arg Arg Ser Asp Ile Leu Ala His Glu Ala Met Gly Asp Thr Phe Pro

1 5 10 15

20

Asp Ala Asp Ala Asp Glu Asp Ser Leu Ala Gly Glu Leu Asp Glu Ala

20 25 30

Met Gly Ser Ser Glu Trp Leu Ala Leu Thr Lys Ser Pro Gln Ala Phe

25 35 40 45

Tyr Arg Gly Arg Pro Ser Trp Gln Gly Thr Pro Gly Val Leu Arg Gly

50 55 60

30 Ser Arg

65 (SEQ ID NO: 3)

In accordance with a fifth aspect of the invention, there is provided a composition comprising the C chain of human H3 relaxin, the C chain having the amino acid sequence:

35

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Arg Arg Ser Asp Ile Leu Ala His Glu Ala Met Gly Asp Thr Phe Pro  
 1 5 10 15  
 Asp Ala Asp Ala Asp Glu Asp Ser Leu Ala Gly Glu Leu Asp Glu Ala  
 5 20 25 30  
 Met Gly Ser Ser Glu Trp Leu Ala Leu Thr Lys Ser Pro Gln Ala Phe  
 35 40 45  
 Tyr Arg Gly Arg Pro Ser Trp Gln Gly Thr Pro Gly Val Leu Arg Gly  
 10 50 55 60  
 Ser Arg  
 65 (SEQ ID NO: 3)

- 15 In accordance with a sixth aspect of the invention there is provided a nucleic acid sequence encoding human prepro-H3 relaxin comprising the nucleic acid sequence:

tataaatggg gggccaagag gcagcagaga cactggccca ctctcacgtt caaagcgtct 60  
 ccgtccagca tggccaggta catgctgctg ctgctcctgg cggtatgggt gctgaccggg 120  
 20 gagctgtggc cgggagctga ggcccgggca gcgccttacg gggtcaggct ttgcggccga 180  
 gaattcatcc gagcagtcac cttcacctgc gggggctccc ggtggagacg atcagacatc 240  
 ctggcccacg aggctatggg tgaggctggg gagagagtgg atgtagaagg ggaacagggtg 300  
 gctggatggg tcccaggagc taaggacaga gataagagga ggttgctgga ggaggagggg 360  
 ccctgtcctg ccacattcag ccagggacac ctgcccagcc ttgaaacaag ggctcaggag 420  
 25 ttagcagagc tgcagagctg ggatgggggtg ttgcaagcca tccatggggg ctggaagtct 480  
 gaggacaggt gggggcgggg agcgtgccat ttgcaaagac aacaccgaag tgttttccaa 540  
 ccctttccag caggtaatgt gaagggtgtg gtatacacat agctgggttt gtcacctaat 600  
 gcatgacctc tcccagcaa gttggttttt cttccgtctc tgagtgtctt ttttttgag 660  
 atgtggtctc actccattgc ccaggcttga atgcagtggc ccaatcactg ctcatcag 720  
 30 cctcgacctc ccaggctcaa gtgattctcc tgcctccgcc tccagagtag ttgagaccac 780  
 aggcacctga caccatgcct ggctagtttt aaattttttt tttgtagaaa caggggtctc 840  
 actatgttgc ctaggctggg ctggaactcc tgggctcaag tgatcctccc acctcgacct 900  
 ccctaagtgc tgagattaga gtctctgagt gtctttatct tcaaattggga gacacagttc 960  
 ctgaatcttg caggattaag tggtatgatt aaatcaaaac agattagggc agagtctcag 1020  
 35 cagggcagcg gcacaatctg ggatccatca ggagagtcag agggaacaga agacctagct 1080  
 tcatgagggg caggggacctg gcaaatagat attcatgatg gtgagaagga ggataggtat 1140  
 gagcgtggac atagaagaca caccacttgg attcagatag tagctctaca atgtaatagt 1200



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	tgtgtgttca	tgtgctacta	tttttttttt	ttttgagaca	gaatctcatt	ctggttgcca	1260
	ggctggagtg	cagtggtgca	atcttggctc	actgtaacct	ccatcacctg	ggttcaagcg	1320
	attctcgtgc	ctccagcctc	ccaagtagct	gggattacag	atgtgtgcca	ccatacctcg	1380
	ctaattctttt	tatttttagt	agagacagtt	tcaccatggt	ggccaggctg	gtctccaact	1440
5	cctgacctca	ggtgatcctc	ccacctcagc	ctcccaaagt	gctgggatta	caggcatgag	1500
	ccaccgcgcc	cagccatgca	aattctttac	tgagtcctgc	ctcagtgggc	tcctctggaa	1560
	aatacgggtg	ataactgcac	ccacctcaac	tggttatcac	tgagaagaat	aaagaagtta	1620
	acctgctaaa	gcacttaaaa	cgttggttga	cacaaagtaa	gtgatcaata	aattattatt	1680
	attattatta	ttattattat	tattattatt	tttgagacag	ggctcttgctc	tgttgcccag	1740
10	actggagtgc	agtgggtgta	tcacagctca	ctgcagcttc	aacctcttgg	gctcaagcaa	1800
	ttctcctgcc	tcagcctcct	gagtagctgg	gactacaggc	ttgtgccaac	atgtctaact	1860
	ttttattatt	tgtagagaca	gggtagtgtc	gtgttgcca	ggctgttctt	gaactcctgg	1920
	ttctggtgat	cctccagcat	gtgcccctgg	aagtgtctgg	attacagggtg	tgagacaccg	1980
	tgcccgact	caatagtc	ttttgagtgc	tcacatggt	ccagacattg	ttctaagttt	2040
15	ttttttttaa	tgaatattaa	ctccttataa	aacttgagaa	ggttgaggta	attatttttt	2100
	tccactttgc	agaaaagaac	attgaggctc	caagaagtaa	atttacttgc	tcacgattag	2160
	agaagctgga	ttcatgtctc	gtcagcccag	ctcccaaagt	taccaggctc	tcaattaata	2220
	aagagtaagg	agaaataaat	gacagggtcg	ggtgcggtgg	ctcacgcctg	taatcccagc	2280
	actttgggtg	gctgaggtgg	gcacatcact	tgaggtcagg	agtttgcgac	cagcctgaac	2340
20	aacatggtga	accccatctc	tataacaata	caaaaatcag	ccaggcctgc	tggcagacac	2400
	ctgtaatccc	acctactctg	gcagagccag	aatttgaacc	caggactggg	tgggaataaaa	2460
	actctgaact	atgtctatga	ctgttggtcac	aagatcagag	ctagactggc	caggagccat	2520
	gactgtgggt	gcagcagcag	ctgagccctg	atcactaact	ctgttcacat	tttgaggag	2580
	ataccttccc	ggatgcagat	gctgatgaag	acagtctggc	aggcgagctg	gatgaggcca	2640
25	tgggggtccag	cgagtggctg	gccctgacca	agtcacccca	ggccttttac	agggggcgac	2700
	ccagctggca	aggaacccct	ggggttcttc	ggggcagccg	agatgtcctg	gctggccttt	2760
	ccagcagctg	ctgcaagtgg	gggtgtagca	aaagtgaat	cagtagcctt	tgctagtgtg	2820
	agggtggggc	agccgtgggc	accaggacca	atgccccagt	cctgccatcc	actcaactag	2880
	tgtctggctg	ggcacctgtc	tttcgagcct	cacacattca	ttcattcatc	tacaagtcac	2940
30	agaggcactg	tgggctcagg	cacagtctcc	cgacaccacc	tatccaaccc	tgccctttga	3000
	ccagcctatc	atgaccctgg	cccctaagga	agctgtgccc	ctgcctgggc	aagtggggac	3060
	ccccccatcc	tgacccttga	cctctcccca	gccctaacca	tgcgtttgcc	tggcctacac	3120
	actccactgc	cacaactggg	tccctactct	acctaggctg	gccacacaga	gaccctgccc	3180
	cccttcccag	tccaaactgt	ggccattgtc	ccctgaccag	ctaaaatcaa	gcctctgtct	3240
35	cagtccagcc	tttgcaagca	cgttcccttt	gccctgcttt	ccatcccctc	tccttccaac	3300
	tcccctgcca	gagttccaag	gctgtggacc	ccagagaagg	tggcagggtg	ccccctagg	3360
	agagctctgg	gcacattcga	atcttcccaa	actccaataa	taaaaattcg	aagactttgg	3420
	cagagagtgt	gtgtgtgtgt	gtatgggtg				3449

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(SEQ ID NO: 6)

In accordance with a seventh aspect of the invention there is provided a nucleic acid sequence encoding human pro-H3 relaxin including an A chain, B chain and C chain  
 5 sequence,

the A chain sequence comprising:

```

gatgtcctgg ctggcctttc cagcagctgc tgcaagtggg ggtgtagcaa aagtgaaatc      60
10 agtagccttt gc                                          72
  
```

(SEQ ID NO: 7)

the B chain sequence comprising:

```

15 cgggcagcgc cttacggggt caggctttgc ggccgagaat tcatccgagc agtcatcttc      60
   acctgcgggg gctcccgggtg g                                          81
  
```

(SEQ ID NO: 8)

the C chain sequence comprising:

```

20 agacgatcag acatcctggc ccacgaggct atgggagata ccttcccggg tgcagatgct      60
   gatgaagaca gtctggcagg cgagctggat gaggccatgg ggtccagcga gtggctggcc      120
   ctgaccaagt caccacaggc cttttacagg gggcgaccca gctggcaagg aaccctggg      180
   gttcttcggg gcagccga                                          198
25
  
```

(SEQ ID NO: 9)

In an eighth aspect of the invention there is provided a nucleic acid sequence encoding human H3 relaxin having an A and B chain,

30 the A chain sequence comprising:

```

gatgtcctgg ctggcctttc cagcagctgc tgcaagtggg ggtgtagcaa aagtgaaatc      60
agtagccttt gc                                          72
  
```

(SEQ ID NO: 7)

- 10 -

and the B chain sequence comprising:

```
cgggcagcgc cttacgggggt caggctttgc ggccgagaat tcatccgagc agtcatcttc      60
5  acctgcgggg gctcccgggtg g                                          81
                                                                    (SEQ ID NO: 8)
```

In a ninth aspect of the invention there is provided a nucleic acid sequence encoding the A, B or C peptide chains of human H3 relaxin, the said chains comprising the nucleic acid sequences:

A chain:

```
gatgtcctgg ctggcctttc cagcagctgc tgcaagtggg ggtgtagcaa aagtgaaatc      60
15 agtagccttt gc                                          72
                                                                    (SEQ ID NO: 7)
```

B chain:

```
cgggcagcgc cttacgggggt caggctttgc ggccgagaat tcatccgagc agtcatcttc      60
20 acctgcgggg gctcccgggtg g                                          81
                                                                    (SEQ ID NO: 8)
```

and C chain:

```
agacgatcag acatcctggc ccacgaggct atgggagata ccttcccgga tgcagatgct      60
gatgaagaca gtctggcagg cgagctggat gaggccatgg ggtccagcga gtggctggcc      120
ctgaccaagt caccocaggc cttttacagg gggcgaccca gctggcaagg aaccocctggg      180
gttcttcggg gcagccga                                          198
30                                                                    (SEQ ID NO: 9)
```

The nucleic acid sequences are isolated and purified nucleic acids, and may be contained within a vector, such as a plasmid, bacteriophage or virus DNA or RNA, and may be in single or double stranded form, and may include promoters or enhancers or other

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sequences which confer elevated, enhanced or other effects on expression in a host system such as a bacterial cell.

The triplet codons of nucleic acids encode specific amino acids. More than one codon may  
5 encode the same amino acid, as is well and established in the art. Moreover, methods of  
modifying or altering the sequence of nucleic acids are well known in the art. Insofar as  
this invention pertains in its various aspects to nucleic acids encoding human H3 relaxin,  
pro- H3 relaxin, prepro- H3 relaxin, and constituent peptide chains thereof, the invention  
includes nucleic acid variants which encode the same protein products, or a protein product  
10 having relaxin activity.

Nucleotide sequence aspects of this invention also include closely related nucleic acid  
sequences as defined by stringent hybridization, this being annealing of complimentary  
sequences under conditions of 0.25M H<sub>2</sub>PO<sub>4</sub>, pH 7.2, 1mmol EDTA, 20% SDS at 65°C  
15 overnight; followed by 3 washes for 5min in 2xSSC, 0.1% SDS at room temperature; and  
finally a 30 min wash at 65°C in 0.1% SSC; where 6xSSC is 0.9M NaCl, 0.3M Na<sub>3</sub>CO<sub>2</sub>  
H<sub>2</sub>O at pH 7.0. Such sequences will encode H3 relaxin polypeptides having biological or  
immunological or other activity corresponding to those of H3 relaxin.

20 In another aspect of the invention there is provided a method for the treatment of one or  
more of: vascular disease including coronary artery disease, peripheral vascular disease,  
vasospasm including Raynaud's phenomenon, microvascular disease involving the central  
and peripheral nervous system, kidney, eye and other organs; treatment of arterial  
hypertension; diseases related to uncontrolled or abnormal collagen or fibronectin  
25 formation such as fibrotic disorders (including fibrosis of lung, heart and cardiovascular  
system, kidney and genitourinary tract, gastrointestinal system, cutaneous, rheumatologic  
and hepatobiliary systems); kidney disease associated with vascular disease, interstitial  
fibrosis, glomerulosclerosis, or other kidney disorders; psychiatric disorders including  
anxiety states including panic attack, agoraphobia, global anxiety, phobic states;  
30 depression or depressive disorders including major depression, dysthymia, bipolar and  
unipolar depression; neurologic or neurodegenerative diseases (including memory loss or

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- other memory disorders, dementias, Alzheimer's disease); disorders of learning, attention and motivation (including Attention Deficit Hyperactivity Disorder, Tourette's disease, impulsivity, antisocial and personality disorders, negative symptoms of psychoses including those due to schizophrenia, acquired brain damage and frontal lobe lesions);
- 5     addictive disorders (including drug, alcohol and nicotine addiction); movement and locomotor disorders (including Parkinson's disease, Huntington's disease, and motor deficits after stroke, head injury, surgery, tumour or spinal cord injury); immunological disorders( including immune deficiency states, haematological and reticuloendothelial malignancy; breast disorders (including fibrocystic disease, impaired lactation, and
- 10    cancer); endometrial disorders including infertility due to impaired implantation; endocrine disorders (including adrenal, ovarian and testicular disorders related to steroid or peptide hormone production) ; delayed onset of labour, impaired cervical ripening, and prevention of prolonged labour due to fetal dystocia; sinus bradycardia; hair loss, alopecia; disorders of water balance including impaired or inappropriate secretion of vasopressin;
- 15    placental insufficiency; which comprises administering to a subject in need of any such treatments a therapeutically effective amount of human H3 relaxin, or an analogue thereof as herein defined, optionally in association with one or more pharmaceutically acceptable carriers and/ diluents and/or excipients.
- 20    In another aspect of the invention there is provided use of human H3 relaxin or an analogue thereof in the manufacture of medicaments for the treatment of one or more of: vascular disease including coronary artery disease, peripheral vascular disease, vasospasm including Raynaud's phenomenon, microvascular disease involving the central and peripheral nervous system, kidney, eye and other organs; treatment of arterial
- 25    hypertension; diseases related to uncontrolled or abnormal collagen or fibronectin formation such as fibrotic disorders (including fibrosis of lung, heart and cardiovascular system, kidney and genitourinary tract, gastrointestinal system, cutaneous, rheumatologic and hepatobiliary systems); kidney disease associated with vascular disease, interstitial fibrosis, glomerulosclerosis, or other kidney disorders; psychiatric disorders including
- 30    anxiety states including panic attack, agoraphobia, global anxiety, phobic states; depression or depressive disorders including major depression, dysthymia, bipolar and

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unipolar depression; neurologic or neurodegenerative diseases (including memory loss or other memory disorders, dementias, Alzheimer's disease); disorders of learning, attention and motivation (including Attention Deficit Hyperactivity Disorder, Tourette's disease, impulsivity, antisocial and personality disorders, negative symptoms of psychoses

5 including those due to schizophrenia, acquired brain damage and frontal lobe lesions); addictive disorders (including drug, alcohol and nicotine addiction); movement and locomotor disorders (including Parkinson's disease, Huntington's disease, and motor deficits after stroke, head injury, surgery, tumour or spinal cord injury); immunological disorders( including immune deficiency states, haematological and reticuloendothelial

10 malignancy; breast disorders (including fibrocystic disease, impaired lactation, and cancer); endometrial disorders including infertility due to impaired implantation; endocrine disorders (including adrenal, ovarian and testicular disorders related to steroid or peptide hormone production) ; delayed onset of labour, impaired cervical ripening, and prevention of prolonged labour due to fetal dystocia; sinus bradycardia; hair loss, alopecia;

15 disorders of water balance including impaired or inappropriate secretion of vasopressin; placental insufficiency; which comprises administering to a subject in need of any such treatments a therapeutically effective amount of human H3 relaxin, or an analogue thereof as herein defined, optionally in association with one or more pharmaceutically acceptable carriers and/ diluents and/or excipients.

20

### Sequence Listing Table

SEQ ID NO: 1	Signal peptide sequence
SEQ ID NO: 2	B chain peptide sequence
SEQ ID NO: 3	C chain peptide sequence
25 SEQ ID NO: 4	A chain peptide sequence
SEQ ID NO: 6	Genomic DNA sequence
SEQ ID NO: 7	A chain nucleic acid sequence
SEQ ID NO: 8	B chain nucleic acid sequence
SEQ ID NO: 9	C chain nucleic acid sequence

30

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**Description of the Figures****Fig. 1. Assembled DNA sequence of the H3 (A) and M3 (B) genes.**

Start and Stop codons as well as predicted TATA boxes and polyadenylation sequences are underlined. The positions of the putative signal peptide, and B-, C- and A- chain peptide sequences are indicated by arrows. A- and B-chain sequences are boxed and the residues implicated in relaxin receptor binding are shaded. The intron sequence, which is at an identical position in the C-chain in both the human (A) and mouse (B) sequences, is indicated by lower case letters and the exact size of the intron is marked.

10 **Fig. 2. Sequence comparisons of H3 and M3 relaxin with other relaxin and insulin family members.**

Alignments of A- and B-chain sequences from H3 and M3 relaxin with other human and mouse relaxin sequences (A). The consensus sequences are boxed; *Cons 1,2,3*: Consensus sequence between human relaxins 1, 2 and 3. *Cons 3*: Consensus sequence between H3 and M3 relaxin for the B-chain and H3, R3 and M3 relaxin for the A-chain. *Cons Mouse*: Consensus sequence between M1 and M3 relaxin. The rat sequence is derived from an EST clone (see results for details). "+" Denotes a conservative substitution, "." denotes no homology. Phylogenetic tree of evolution of H3 and M3 relaxin full-length sequences with human sequences of the relaxin/insulin/IGF superfamily (B).

20

**Fig. 3. Bioactivity of H3 compared to H1 and H2 relaxin in a human relaxin receptor expressing cell line.**

cAMP accumulation in THP-1 cells upon stimulation with peptides (A). Data are expressed as mean  $\pm$  SEM of the maximum response (%) to H2 relaxin (n = 3). The response to bovine insulin (bINSL) and human INSL3 (hINSL3) are also shown to highlight the specificity of the assay. H1, H2, H3; Human 1, 2 and 3 relaxin respectively. The ability of H1 (n = 7), H2 (n = 11) and H3 (n = 3) relaxin peptides to compete for <sup>33</sup>P-labeled H2 relaxin (B33) binding to THP-1 cells (B). Data are expressed as mean  $\pm$  SEM of the specific binding (%).

30

- 15 -

**Fig. 4. Ability of a well characterized H2 relaxin antibody to recognize H3 relaxin.**

The H2 relaxin antibody was immobilized onto ELISA plates and a competition experiment was performed using H1, H2 and H3 relaxin against  $^{125}\text{I}$ -labeled H2 relaxin. Results are mean  $\pm$  SEM of the specific binding (%) of triplicate determinations from a representative assay.

Unexpectedly, some 20 years after the identification of human relaxin, and the surprising identification at that time of two human relaxin genes, a further relaxin gene has been identified. This invention in its various aspects provides: the characterisation of nucleotide sequences encoding human H3 relaxin; the isolation of purified nucleic acid material; amplification of nucleotide sequences encoding H3 relaxin (mRNA, cDNA and DNA); nucleic acid cloning of H3 relaxin sequences; nucleic acid sequence identification, and peptide sequences encoding human H3 preprorelaxin, H3 prorelaxin and H3 relaxin.

The human H3 relaxin polypeptide comprises disulphide bridged A and B chains. The amino acid sequence of human H3 relaxin is set out in SEQ ID NO: 4. The amino acid sequence of the B chain of human relaxin is set out in SEQ ID NO: 2.

The A and B chains of human H3 relaxin are linked through cysteine residues, A11-B10, A24-B22 disulphide bond formation taking place between these cysteine linkages.

Hence, the amino acid sequence of human H3 relaxin A and B chains are as follows:

**A Chain**

25

Asp Val Leu Ala Gly Leu Ser Ser Ser Cys Cys Lys Trp Gly Cys Ser  
1 5 10 15

Lys Ser Glu Ile Ser Ser Leu Cys

30

20

(SEQ ID NO: 4)



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## B Chain

Arg Ala Ala Pro Tyr Gly Val Arg Leu Cys Gly Arg Glu Phe Ile Arg  
 1 5 10 15  
 5  
 Ala Val Ile Phe Thr Cys Gly Gly Ser Arg Trp  
 20 25 (SEQ ID NO: 2)

the A and B chains being linked by disulphide bonds between A11-B10, A24-B22.

10

Human H3 relaxin possesses classical relaxin bioactivity. Human relaxins, H1 and H2 relaxin, bind to cells expressing relaxin receptors, such as THP-1 cells (Parsell et al (1996) *J. Biol. Chem.* 271, 27936-27941). H2 relaxin produces a dose dependent increase in cAMP production from these cells. Synthetic H3 relaxin produced according to this  
 15 invention stimulated a dose dependent increase in cAMP in keeping with human H2 relaxin. The specificity of response in target cells bearing the human relaxin receptor as exhibited by H3 relaxin is demonstrated by the inability of bovine insulin (bINSL) or human insulin (hINSL3) to stimulate cAMP responses at doses up to 1  $\mu$ m in THP-1 cells.

20 The elicitation of a second messenger response (cAMP) by stimulating human relaxin receptors with human H3 relaxin, provides definitive evidence that human H3 relaxin has classic relaxin biological activity. Such assays in cells containing relaxin receptors, for example THP-1 cells as referred to above provides, a ready way to determine relaxin activity. In addition, the ability of human H3 relaxin to compete with P<sup>32</sup>-labelled H2  
 25 relaxin in binding to relaxin binding sites in cells expressing relaxin receptors, again provides definitive confirmation of relaxin activity.

Other biological activities/assays for determining relaxin activity are known in the art. For example, bioassays used for the measurement of active relaxin during pregnancy and non-  
 30 pregnancy, such as the guinea pig interpubic ligament assay may be used (Steinetz et al (1960) *Endocrinology* 67, 102-115, and Sirosi et al (1983) *American Journal of Obstetrics and Gynaecology* 145: 402-405) may be used. Other bioassays include cAMP production

- 17 -

in THP-1 cells (Parsell *et al* (1996) J. Biol. Chem 271, 27936-27941).

Applicant has found that H3 relaxin analogues may be prepared where up to 9 amino acids are truncated from the N-terminus of the A chain, and up to 9 amino acids are truncated from the N-terminus of the B chain, and up to 5 amino acids are truncated from the C-terminus of the B chain.

The resulting relaxin analogues comprise a H3 relaxin A and B chain, the A chain having the amino acid sequence

10

Asp Val Leu Ala Gly Leu Ser Ser Ser Cys Cys Lys Trp Gly Cys Ser  
1 5 10 15

15

Lys Ser Glu Ile Ser Ser Leu Cys

20

(SEQ ID NO: 4)

truncated by up to about 9 amino acids from amino-terminus,

and the B chain having the amino acid sequence:

20

Arg Ala Ala Pro Tyr Gly Val Arg Leu Cys Gly Arg Glu Phe Ile Arg  
1 5 10 15

25

Ala Val Ile Phe Thr Cys Gly Gly Ser Arg Trp

20

25

(SEQ ID NO: 2)

truncated by up to 9 amino acids from the amino-terminus and/or up to about 5 amino acids from the carboxyl-terminus,

the A and B chains being linked by disulphide bonds between A11-B10 and A24-B22, and wherein the human H3 relaxin or analogue thereof has relaxin bioactivity. The A chain of human H3 relaxin contains an intrachain disulphide bond between Cys residues 10 and 15.

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In standard assays looking at second messenger elicitation in cells bearing human relaxin receptors, the H3 relaxin analogues referred to above all elicited cyclic AMP production in a manner which was characteristic of full length, non-truncated human H3 relaxin, and indeed human H2 relaxin. Hence, such truncated H3 relaxin analogues possess relaxin  
5 bioactivity.

Another aspect of the present invention relates to compositions comprising a human H3 relaxin analogue having a modified A chain and/or a modified B chain. The carboxyl-terminus of the A chain, and/or the B chain, may be an amide derivative. Lys at position  
10 12 in the A chain may be replaced with Glu, and/or Glu at position 19 may be replaced with Gln. In the B chain, the Ala at position 2 may be replaced with Pro, and/or Arg at position 8 may be replaced with Lys. The resulting H3 relaxin analogues having modified amino acids comprise an amino acid sequence which may be depicted as follows:

15 In accordance with another aspect of the invention, there is provided a human H3 relaxin analogue having a modified A chain and/or a modified B chain,

the H3 relaxin A chain having the amino acid sequence:

20 Asp Val Leu Ala Gly Leu Ser Ser Ser Cys Cys Lys Trp Gly Cys Ser  
1 5 10 15

Lys Ser Glu Ile Ser Ser Leu Cys  
20

(SEQ ID NO: 4)

25

wherein the carboxyl-terminus is an amide derivative and/or Lys at position 12 is replaced with Glu, and/or Glu at position 19 is replaced with Gln,

the modified B chain having the amino acid sequence:

30

- 19 -

Arg Ala Ala Pro Tyr Gly Val Arg Leu Cys Gly Arg Glu Phe Ile Arg  
 1 5 10 15

Ala Val Ile Phe Thr Cys Gly Gly Ser Arg Trp  
 5 20 25 (SEQ ID NO: 2)

wherein the carboxyl-terminus is an amide derivative, and/or Ala at position 2 is replaced with Pro, and/or Arg at position 8 is replaced with Lys,

- 10 the A and B chains being linked by disulphide bonds between A11-B10 and A24-B22, and wherein the human H3 relaxin analogue has relaxin bioactivity.

The isolation, purification and characterisation of nucleic acid sequences encoding human H3 relaxin has allowed the characterisation and production of the signal sequence of  
 15 human H3 relaxin, and the pro-sequence of human H3 relaxin.

The identification, purification and characterisation of the signal sequence and C chain of human H3 relaxin enables the prepro- and pro-human H3 relaxin to be produced.

- 20 In accordance with another aspect of the invention there is provided a composition comprising human H3 preprorelaxin or human H3 prorelaxin, having a signal, A chain, B chain and C chain in respect of human H3 preprorelaxin, and an A chain, B chain and C chain in respect of human H3 prorelaxin, the said amino acid chains having the amino acid sequences:

25

the A chain comprising:

Asp Val Leu Ala Gly Leu Ser Ser Ser Cys Cys Lys Trp Gly Cys Ser  
 1 5 10 15

30

Lys Ser Glu Ile Ser Ser Leu Cys  
 20

(SEQ ID NO: 4)

- 20 -

the B chain comprising:

Arg Ala Ala Pro Tyr Gly Val Arg Leu Cys Gly Arg Glu Phe Ile Arg  
 1 5 10 15  
 5  
 Ala Val Ile Phe Thr Cys Gly Gly Ser Arg Trp  
 20 25 (SEQ ID NO: 2)

the signal sequence comprising:

10  
 Met Ala Arg Tyr Met Leu Leu Leu Leu Leu Ala Val Trp Val Leu Thr  
 1 5 10 15  
 Gly Glu Leu Trp Pro Gly Ala Glu Ala  
 15 20 25 (SEQ ID NO: 1)

and the C chain comprising:

Arg Arg Ser Asp Ile Leu Ala His Glu Ala Met Gly Asp Thr Phe Pro  
 20 1 5 10 15  
 Asp Ala Asp Ala Asp Glu Asp Ser Leu Ala Gly Glu Leu Asp Glu Ala  
 20 25 30  
 25 Met Gly Ser Ser Glu Trp Leu Ala Leu Thr Lys Ser Pro Gln Ala Phe  
 35 40 45  
 Tyr Arg Gly Arg Pro Ser Trp Gln Gly Thr Pro Gly Val Leu Arg Gly  
 51 55 60  
 30  
 Ser Arg  
 65 (SEQ ID NO: 3)

In accordance with a further aspect of the invention there is provided the C chain of human  
 35 H3 relaxin, said C chain having the amino acid sequence:

- 21 -

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Arg Arg Ser Asp Ile Leu Ala His Glu Ala Met Gly Asp Thr Phe Pro
1           5           10           15

5  Asp Ala Asp Ala Asp Glu Asp Ser Leu Ala Gly Glu Leu Asp Glu Ala
           20           25           30

Met Gly Ser Ser Glu Trp Leu Ala Leu Thr Lys Ser Pro Gln Ala Phe
           35           40           45
10
Tyr Arg Gly Arg Pro Ser Trp Gln Gly Thr Pro Gly Val Leu Arg Gly
           50           55           60

Ser Arg
15 65
(SAQ ID NO: 3)

```

Human H3 prorelaxin possesses characteristic relaxin bioactivity.

Human H3 relaxin, prorelaxin, preprorelaxin and constitutive peptide chains may be  
 20 products using techniques previously described as useful in the production of relaxin such  
 as US Patent No. 5,991,997, US Patent No. 4,758,516, US Patent No. 4,871,670, US  
 Patent No. 4,835,251, PCT/US90/02085, and PCT/US94/0699.

Relaxin analogues and derivatives where amino acids are substituted as indicated above  
 25 may be produced recombinantly using, for example, site directed mutagenesis techniques  
 as set forth, for example, in Tsurushita et al (1988) *Gene Tissue*: 135-139.

The disclosed sequence information for human H3 relaxin, analogues thereof wherein one  
 or more amino acids are truncated from the N- and/or C-terminus of the A and/or B chains,  
 30 or human H3 relaxin analogues having amino acid substitutions as referred to above, may  
 be synthesised according to the methods of Büllesbach (1991) *J. Biol. Chem.* 266, 10754-  
 10761, for synthesising relaxin. Additionally, well known methods of peptide synthesis  
 may be utilised to produce the various H3 relaxin forms referred to herein.

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Relaxin has been implicated consequently in the treatment and diagnosis of various diseases and disorders. For example, studies provide evidence that relaxin is effective in the treatment of scleroderma, sinus bradycardia, cardiovascular disease, neurodegenerative and neurologic disorders, hair loss, depression. See e.g., U.S. Patent No. 5,166,191 and  
5 International Patent Application No. PCT/US92/069). Evidence also suggests the use of relaxin in diseases and disorders related to the abnormal expression of collagen or fibronectin, such as rheumatoid arthritis.

Human H3 relaxin, human H3 relaxin truncated analogues, amino acid modified H3  
10 relaxin analogues, and human prorelaxin provided by the instant invention bind to the relaxin receptor and possess relaxin biological activity. It directly follows that these human H3 relaxin forms possessing relaxin biological activity may be used for the treatment of the above-identified diseases and other diseases.

15 In accordance with another aspect of the present invention there is provided a method for the treatment of one or more of: vascular disease including coronary artery disease, peripheral vascular disease, vasospasm including Raynaud's phenomenon, microvascular disease involving the central and peripheral nervous system, kidney, eye and other organs; treatment of arterial hypertension; diseases related to uncontrolled or abnormal collagen or  
20 fibronectin formation such as fibrotic disorders (including fibrosis of lung, heart and cardiovascular system, kidney and genitourinary tract, gastrointestinal system, cutaneous, rheumatologic and hepatobiliary systems); kidney disease associated with vascular disease, interstitial fibrosis, glomerulosclerosis, or other kidney disorders; psychiatric disorders including anxiety states including panic attack, agoraphobia, global anxiety, phobic states;  
25 depression or depressive disorders including major depression, dysthymia, bipolar and unipolar depression; neurologic or neurodegenerative diseases (including memory loss or other memory disorders, dementias, Alzheimer's disease); disorders of learning, attention and motivation (including Attention Deficit Hyperactivity Disorder, Tourette's disease, impulsivity, antisocial and personality disorders, negative symptoms of psychoses  
30 including those due to schizophrenia, acquired brain damage and frontal lobe lesions); addictive disorders (including drug, alcohol and nicotine addiction); movement and

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- locomotor disorders (including Parkinson's disease, Huntington's disease, and motor deficits after stroke, head injury, surgery, tumour or spinal cord injury); immunological disorders( including immune deficiency states, haematological and reticuloendothelial malignancy; breast disorders (including fibrocystic disease, impaired lactation, and cancer); endometrial disorders including infertility due to impaired implantation; endocrine disorders (including adrenal, ovarian and testicular disorders related to steroid or peptide hormone production) ; delayed onset of labour, impaired cervical ripening, and prevention of prolonged labour due to fetal dystocia; sinus bradycardia; hair loss, alopecia; disorders of water balance including impaired or inappropriate secretion of vasopressin; placental insufficiency; which comprises administering to a subject in need of any such treatments a therapeutically effective amount of human H3 relaxin, or an analogue thereof as herein defined, optionally in association with one or more pharmaceutically acceptable carriers and/ diluents and/or excipients.
- 15 In accordance with another aspect of the present invention there is provided use of human H3 relaxin or an analogue thereof in the manufacture of medicaments for the treatment of one or more of: vascular disease including coronary artery disease, peripheral vascular disease, vasospasm including Raynaud's phenomenon, microvascular disease involving the central and peripheral nervous system, kidney, eye and other organs; treatment of arterial hypertension; diseases related to uncontrolled or abnormal collagen or fibronectin formation such as fibrotic disorders (including fibrosis of lung, heart and cardiovascular system, kidney and genitourinary tract, gastrointestinal system, cutaneous, rheumatologic and hepatobiliary systems); kidney disease associated with vascular disease, interstitial fibrosis, glomerulosclerosis, or other kidney disorders; psychiatric disorders including anxiety states including panic attack, agoraphobia, global anxiety, phobic states; depression or depressive disorders including major depression, dysthymia, bipolar and unipolar depression; neurologic or neurodegenerative diseases (including memory loss or other memory disorders, dementias, Alzheimer's disease); disorders of learning, attention and motivation (including Attention Deficit Hyperactivity Disorder, Tourette's disease, impulsivity, antisocial and personality disorders, negative symptoms of psychoses including those due to schizophrenia, acquired brain damage and frontal lobe lesions);



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addictive disorders (including drug, alcohol and nicotine addiction); movement and locomotor disorders (including Parkinson's disease, Huntington's disease, and motor deficits after stroke, head injury, surgery, tumour or spinal cord injury); immunological disorders (including immune deficiency states, haematological and reticuloendothelial malignancy; breast disorders (including fibrocystic disease, impaired lactation, and cancer); endometrial disorders including infertility due to impaired implantation; endocrine disorders (including adrenal, ovarian and testicular disorders related to steroid or peptide hormone production) ; delayed onset of labour, impaired cervical ripening, and prevention of prolonged labour due to fetal dystocia; sinus bradycardia; hair loss, alopecia; disorders of water balance including impaired or inappropriate secretion of vasopressin; placental insufficiency; which comprises administering to a subject in need of any such treatments a therapeutically effective amount of human H3 relaxin, or an analogue thereof as herein defined, optionally in association with one or more pharmaceutically acceptable carriers and/ diluents and/or excipients.

15

Without wishing to be bound on mechanism of action, applicant believes that H3 relaxin may act as a neurotransmitter or neuroregulator in the brain, and other parts of the body including nerves, for example through inducing eAMP production in cells. H3 relaxin may also allow nutrient uptake by cells, or may be involved in autoregulatory presynaptic and/or conventional postsynaptic actions. Applicant further believes that H3 relaxin may also be axonally transported by nerve projections.

20

As defined hereinafter, H3 relaxin has surprisingly been found to be expressed in neuroanatomical region of the pars ventromedialis of the dorsal tegmental nucleus (vmDTg), which may otherwise be referred to as the *nucleus incertus* (Goto *et al* (2001) *Journal of Comparative Neurology* 438: 86-122). With the extensive pattern of efferents and afferents to and from key forebrain areas from the *nucleus incertus*, this region has been proposed as part of a brain stem network that may regulate behavioural activation via influences on attention, motivation, locomotion and learning (Goto *et al*) and may give rise to the therapeutic treatment modalities herein described. This is consistent with the abundant distribution of relaxin binding sites in cerebral cortex and other relevant brain

25

30

- 25 -

areas (Osheroff and Phillips (1991) *Proc. Natl. Acad. Sci. USA* 88, 6413-6417; and Tan *et al* (1999) *Br. J. Pharmacol.* 127, 91-98).

5 H3 relaxin may cross the blood brain barrier, or may be treated to facilitate crossing of the blood brain barrier, by methods known in the art including use of one or more sugars or amino acids, or other substances which open the blood brain barrier or make it leaky allowing coadministered/timed administration with H3 relaxin (see for example Naito US Patent 6,294,520), by intranasal administration according to the methods of Frey (US Patent 6,313,093), for example using a lipophilic vehicle, and by methods described in  
10 PCT/WO89/10134.

H3 relaxin and analogues as herein described may be effective in the treatment of a wide range of what may broadly be described as neurologic diseases including psychiatric disorders, disorders of learning, attention and memory, addictive disorders and movement  
15 and locomotor disorders.

H3 relaxin binds to the relaxin receptor as described hereinafter.

For convenience, human H3 relaxin, analogues of human H3 relaxin where one or more  
20 amino acids are truncated from the N- and/or C-terminus of the A and B chains of human H3 relaxin, analogues of human H3 relaxin where one or more amino acids are modified or substituted with another amino acid as described herein, and human H3 preprorelaxin shall collectively be referred to as human H3 relaxin, unless otherwise specifically indicated.

25 Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. More specifically, a therapeutically effective amount means an amount effective to prevent development of or to alleviate the existing symptoms of the subject being treated. Determination of the effective amounts is well within the capability of those  
30 skilled in the art, especially in light of the detailed disclosure provided herein.

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For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC<sub>50</sub> as determined in cell culture. Such information can be used to more accurately determine  
5 useful doses in humans.

A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms or a prolongation of survival in a patient. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical  
10 procedures in cell cultures or experimental animals, e.g., for determining the LD<sub>50</sub> (the dose lethal to 50% of the population) and the ED<sub>50</sub> (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD<sub>50</sub> and ED<sub>50</sub>. Compounds which exhibit high therapeutic indices are preferred. The data  
15 obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED<sub>50</sub> with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage  
20 can be chosen by the individual physician in view of the patient's condition. (See e.g. Fingl et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p1).

Dosage amount and interval may be adjusted individually to provide serum levels of the active moiety which are sufficient to maintain the relaxin activity and effects.

25 Administration of H3 relaxin can be via any of the accepted modes of administration for agents that serve similar utilities, preferably by systemic administration.

While human dosage levels for treating many of the above-identified relaxin related  
30 diseases or disorders have yet to be optimized for H3 relaxin generally, a daily dose is from about 0.05 to 500.0  $\mu$ g/kg of body weight per day, preferably about 5.0 to 200.0

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.mu.g/kg, and most preferably about 10.0 to 100.0 .mu.g/kg. Generally it is sought to obtain a serum concentration of the H3 relaxin approximating or greater than normal circulating levels of relaxin in pregnancy, i.e., 1.0 ng/ml, such as 1.0 to 20 ng/ml, preferably 1.0 to 20 ng/ml.

5

For administration to a 70 kg person, the dosage range would be about 7.0 .mu.g to 3.5 mg per day, preferably about 42.0 .mu.g to 2.1 mg per day, and most preferably about 84.0 to 700.0 .mu.g per day. The amount of the H3 relaxin administered will, of course, be dependent on the subject and the severity of the affliction, the manner and schedule of administration and the judgment of the prescribing physician and the biological activity of such analog or derivative. One treatment regimen can employ a higher initial dosage level (e.g., 100 to 200 .mu.g/kg/day) followed by decreasing dosages to achieve steady H3 relaxin serum concentration of about 1.0 ng/ml. Another treatment regimen, particularly postpartum depression, entails administration of an amount of H3 relaxin sufficient to attain normal pregnancy levels of relaxin (about 1.0 ng/ml) followed by gradual decreasing dosages until H3 relaxin serum levels are no longer detectable (e.g. less than about 20 picograms/ml), optionally discontinuing treatment upon reaching that dosage level.

Any pharmaceutically acceptable mode of administration can be used. H3 relaxin can be administered either alone or in combination with other pharmaceutically acceptable excipients, including solid, semi-solid, liquid or aerosol dosage forms, such as, for example, tablets, capsules, powders, liquids, gels, suspensions, suppositories, aerosols or the like. Such proteins can also be administered in sustained or controlled release dosage forms (e.g., employing a slow release bioerodable delivery system), including depot injections, osmotic pumps (such as the Alzet implant made by Alza), pills, transdermal (including electrotransport) patches, and the like, for prolonged administration at a predetermined rate, preferably in unit dosage forms suitable for single administration of precise dosages. The compositions will typically include a conventional pharmaceutical carrier or excipient and/or H3 relaxin, H3 prorelaxin, and H3 preprorelaxin or derivatives thereof. In addition, these compositions may include other active agents, carriers, adjuvants, etc.

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In a preferred aspect of the invention, a sustained/controlled release H3 relaxin formulation was a selectively permeable outer barrier with a drug dispensing opening, and an inner H3 relaxin containing portion designed to deliver dosage of the H3 relaxin progressively  
5 diminishing at a predetermined rate (e.g. containing about 30 mg of H3 relaxin in a matrix for delivery of initially about 500 .mu.g per day diminishing as a rate of 10 .mu.g per day.

In another preferred aspect of the invention, a sustained/controlled release of H3 relaxin has a selectively permeable outer barrier with a drug dispensing opening, a first inner H3  
10 containing portion designed for steady state release of H3 relaxin at a therapeutically effective daily dosage (e.g. containing about 50 mg of H3 relaxin in a matrix for continuous delivery of about 500 .mu.g per day), and a second inner H3 relaxin a portion designed to deliver a dosage of H3 relaxin progressively diminishing at a predetermined rate (e.g. containing about 3 mg of H3 relaxin in a matrix for delivery of initially about 500  
15 .mu.g per day diminishing at a rate of 50.mu.g per day) commencing upon exhaustion of the H3 relaxin from the first inner portion.

Generally, depending on the intended mode of administration, the pharmaceutically acceptable composition will contain about 0.1% to 90%, preferably about 0.5% to 50%, by  
20 weight of H3 relaxin, either alone or in combination with H3 relaxin, the remainder being suitable pharmaceutical excipients, carriers, etc. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., 15th Edition, 1975.

25

In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

The amount of composition administered will, of course, be dependent on the subject being  
30 treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

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Suitable routes of administration may, for example, include oral, rectal, transmucosal, or intestinal administration. Parenteral administration is generally characterized by injection, either subcutaneously, intradermally, intramuscularly or intravenously, preferably  
5 subcutaneously. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like. In addition, if desired, the pharmaceutical compositions to be administered may also contain minor amounts of non-toxic auxiliary substances such as  
10 wetting or emulsifying agents, pH buffering agents, solubility enhancers, and the like, such as for example, sodium acetate, sorbitan monolaurate, triethanolamine oleate, cyclodextrins, and the like.

A more recently devised approach for parenteral administration employs the implantation  
15 of a slow-release or sustained-release system, such that a constant level of dosage is maintained. See, e.g., U.S. Pat. No. 3,710,795.

Alternately, one may administer the H3 in a local rather than systemic manner, for example, via injection of the compound directly into a solid tumor, often in a depot or  
20 sustained release formulation.

Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with tumor-specific antibody. The liposomes will be targeted to and taken up selectively by the tumor.

25

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

30

- 30 -

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

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In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

A pharmaceutical carrier for the hydrophobic compounds of the invention is a cosolvent system comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. The cosolvent system may be the VPD co-solvent system. VPD is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant polysorbate 80, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. The VPD co-solvent system (VPD:5W) consists of VPD diluted 1:1 with a 5% dextrose in water solution. This co-solvent system dissolves hydrophobic compounds well, and itself produces low toxicity upon systemic administration. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied: for example, other low-toxicity nonpolar surfactants may be used instead of polysorbate 80; the fraction size of polyethylene glycol may be varied; other biocompatible polymers may replace polyethylene glycol, e.g. polyvinyl pyrrolidone; and other sugars or polysaccharides may substitute for dextrose.

Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various of sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and



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the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

5 The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

10 Formulations comprising human H3 relaxin may also be administered to the respiratory tract as a nasal or pulmonary inhalation aerosol or solution for a nebulizer, or as a microfine powder for insufflation, alone or in combination with an inert carrier such as lactose, or with other pharmaceutically acceptable excipients. In such a case, the particles of the formulation may advantageously have diameters of less than 50 microns, preferably less than 10 microns. See, e.g., U.S. Pat. No. 5,364,838, which discloses a method of  
15 administration for insulin that can be adapted for the administration of H3 relaxin.

H3 relaxin for treatment of such disorders such as alopecia, may also be administered topically in a formulation adapted for application to the scalp, such as a shampoo (e.g., as disclosed in U.S. Pat. No. 4,938,953, adapted according to methods known by those skilled  
20 in the art, as necessary for the inclusion of protein ingredients) or a gel (e.g., as disclosed in allowed U.S. Ser. No. 08/050,745) optionally with increased H3 relaxin concentrations to facilitate absorption.

For oral administration, the compounds can be formulated readily by combining the active  
25 compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable  
30 auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose

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preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid  
5 or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions,  
10 and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of  
15 gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid  
20 polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized  
25 packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable  
30 powder base such as lactose or starch.

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Various aspects of the invention will be described with reference to the following non-limiting examples.

In the examples which follow rats are used as an experimental model to map H3 relaxin  
5 expression at the mRNA and protein level in the rat brain, this allowing human brain mapping. In this regard, the rat brain is a standard comparative anatomical model of the human brain (Goto *et al* (2001) *The Journal of Comparative Neurology* 438: 86-122).

### Example 1

#### 10 Nucleotide sequence identification, characterisation, purification and manipulation

*Tissue RNA/DNA Extraction and RT-PCR*-Human genomic DNA was extracted from human CL using standard protocols (Sambrook *et al* 1989) In *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbour Laboratory Press, NY). Human CL and mouse tissues were finely diced in the presence of liquid nitrogen and immediately  
15 homogenized with RNAWiz reagent (Ambion Inc., Austin, TX), and the RNA extracted according to the manufacturer's instructions. Total RNA (5 µg) from each sample was used for the reverse transcription (RT) reaction, which was performed using the Superscript II RT-PCR kit (Gibco-BRL, Rockville, MD) in a 20 µl volume according to the manufacturer's instructions. A 50 µl reaction containing 100 ng of primers and 150 ng  
20 of the cDNA template was used for all PCR reactions. Mouse tissues were screened for M3 relaxin expression using specific forward [5' TGCGGAGGCTCACGATGGCGC 3'] and reverse [5' GACAGCAGCTTGCAGGCACGG 3'] primers, which generated a 319-bp product. Mouse relaxin (M1) expression was determined using a specific forward [5' GTGAATATGCCCCGTAATTGATC 3'] and reverse [5'  
25 AGCGTCGTATCGAAAGGCTCT 3'] primer based on the published sequence (Evans *et al* (1993) *J. Mol. Endocrinol.* 10, 15-23), generating a 150-bp product.

Human CL cDNA was used in RT-PCR reactions with specific primers for H3 relaxin, forward 1 [5' ACGTTCAAAGCGTCTCCGTCC 3'], forward 2 [5'  
30 CGGTGGAGACGATCAGACATC 3'] and reverse [5' ATGGCAGGACTGGGGCATTGG 3'], generating products of 504- and 310-bp for

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forward 1/reverse and forward 2/reverse, respectively. All primer combinations we subsequently show to cross the single introns in the mouse and human relaxin sequences, respectively, so as to control for genomic DNA contamination. In all experiments GAPDH forward [5' TGATGACATCAAGAAGGTGG 3'] and reverse [5' TTTCTTACTCCTTGGAGGCC 3'] primers generating a product of 246-bp were used in separate PCR reactions to control for quality, and equivalent loading of the cDNA. M3 relaxin expression by RT-PCR was performed on cDNA samples extracted from at least two animals, although the results from only one representative experiment are shown. The mouse PCR reactions were completed in a Perkin Elmer Gene Amplifier using the following (touch-down) annealing temperatures: 64°C (2 cycles), 63°C (2 cycles), 62°C (2 cycles), 61°C (2 cycles), 60°C (32 cycles). H3 relaxin expression in human CL cDNA was performed by RT-PCR at the following annealing temperatures: 60°C (2 cycles), 59°C (2 cycles), 58°C (2 cycles), 57°C (2 cycles), 56°C (32 cycles). Aliquots of the PCR products were electrophoresed on 2% (w/v) agarose gels stained with ethidium bromide and photographed. Mouse tissue samples were transferred to Hybond NX membranes (Amersham International, Aylesbury, UK) for Southern blot analysis.

An additional PCR reaction was performed using mouse brain and ovarian cDNA using the reverse M3 primer (above) and a forward primer from in front of the ATG start codon (5' GGG TCGCAGGCATCTCAACTG 3'). The resulting product contained the full H3 relaxin coding sequence. PCR was performed as above, but with the following annealing temperatures: 60°C (2 cycles), 59°C (2 cycles), 58°C (2 cycles), 57°C (2 cycles), 56°C (32 cycles). To generate a specific H3 relaxin cDNA probe for <sup>32</sup>P-labeling and to utilize it for subsequent probing of a human multi tissue array, RT-PCR was performed on human genomic DNA (50 ng). Specific forward (5' CGGATGCAGATGCTGATGAAG 3') and reverse (5' GTGCCTGAGCCCACAGTGCCT 3') primers from the exon II sequence of the H3 relaxin gene were used at the following annealing temperatures: 60°C (2 cycles), 59°C (2 cycles), 58°C (2 cycles), 56°C (2 cycles), 54°C (32 cycles). These products as well as the mouse PCR product described above, were separated on 2% agarose gels. Bands were detected of the expected size under UV light (mouse 319-bp, 478-bp; human 374-bp), excised and eluted from the gel using the Ultraclean™ 15 DNA purification kit

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(Geneworks Pty Ltd, Adelaide, Australia). The bands were subsequently subcloned into the pGEM-T vector (Promega, Madison, WI) and multiple subclones were then sequenced on both strands using the ABI PRISM 377 automatic DNA sequencer, according to the manufacturers instructions (Applied Biosystems, Melbourne, Australia).

5 *Southern Blot Analysis*-PCR products on membranes were hybridized against specific internal oligonucleotide primers for the M1 relaxin (5' CAAGCAGAGCTGGCTCCTCCTGGCT CAAAGCCAATCTTC 3') and M3 relaxin (5' AATTGGCTCTTGCTACAGCCCCACTCG CAGCAACTGCT 3') cDNA sequences, which had been labeled using T4 polynucleotide kinase and [ $\gamma$ - $^{32}$ P] ATP. Hybridization  
10 was performed at 55°C overnight in 5 x SSC (1 x SSC; 0.15 M NaCl, 15 mM sodium citrate, pH 7), 5 x Denhardtts, 1% SDS and 100  $\mu$ g/ml sonicated herring sperm. Membranes were washed three times for 5 min in 2 x SSC, 0.1% SDS at room temperature followed by a 30 min wash at 55°C in 0.1 x SSC, 0.1% SDS, before being exposed to BioMAX MR  
15 film (Eastman Kodak Co., Rochester, NY) for 24 h at room temperature.

*Northern Blot Analysis*-To further examine the expression of M3 relaxin mRNA, total RNA (5-25  $\mu$ g) from the heart, brain, lung, thymus and spleen of male mice, and ovary, endometrium, myometrium, cervix and vagina of female mice pooled from day 7.5, 10.5  
20 and 18.5 of pregnancy, were run on standard MOPS/formaldehyde gels. RNA was then transferred to optimized Hybond-NX membranes and probed for M3 relaxin mRNA with a  $^{32}$ P-labeled probe that corresponded to the 319-bp PCR product, generated by specific primers to M3 relaxin (see above). This product was labeled with [ $\alpha$ - $^{32}$ P]dCTP using the specific reverse primer (above) and T7 polymerase as previously described (31). The  
25 membrane was hybridized at 65°C overnight in 0.25M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.2, 1 mM EDTA, 20% SDS, followed by three washes for 5 min in 2 x SSC, 0.1% SDS at room temperature, and finally a 30 min wash at 65°C in 0.1 x SSC, 0.1% SDS. Membranes were first exposed to a phosphoimager plate for 48h at room temperature before being analysed in a FujiX 2000 Phosphoimager (Fuji Photo Company, Japan), and then exposed to BioMAX MS  
30 film (Integrated Sciences, Melbourne, Australia) together with a Hyperscreen (Amersham Pharmacia, Sydney, Australia) at -80°C. In separate experiments, total RNA (200  $\mu$ g) from

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the brain, spleen, liver and testes was purified to poly-A RNA using an mRNA purification kit (Amersham Pharmacia), and Northern blotting performed as described above. A human multiple tissue expression array (CLONTECH laboratories, Palo Alto, CA) was hybridized with a  $^{32}\text{P}$ -labeled H3 relaxin specific probe according to the manufacturers  
 5 recommendations. The 374-bp fragment of the H3 relaxin sequence isolated from genomic DNA was labeled with  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$  using the H3 relaxin specific reverse primer (described above), and T7 polymerase (Bathgate et al (1999) *Biol. Reprod.* 61, 1090-1098). The membrane was exposed to a phosphorimager plate and BioMAX film as described above.

10

*In Situ Hybridization Histochemistry*-Coronal sections (14  $\mu\text{m}$ ) were cut on a cryostat at  $-16^\circ\text{C}$  and mounted on silane-coated slides. Sections were delipidated in chloroform for 10 min, rinsed and stored in 100% ethanol at  $4^\circ\text{C}$ . Three oligonucleotides (39 mers) [5' GGTGGTCTGTATTG GCTTCTCCATCAGCGAAGAAGTCCC 3']; [5'  
 15 AATTTGGCTCTTGCTACAGCCCCACTC GCACGAACTGCT 3'] and [5' TAAGGAGACAGTGGACCCCTTGGTGCCTCGCCTGT AGGA 3'], of the M3 relaxin mRNA sequence, and three oligonucleotides to [5' GCACATCCGAATGAATCCGTCCTCCACTCCTCCGAGAC 3'], [5' CAAGCAGAGCT GGCTCCTCCTGGCTCAAAGCCAATCTTC 3'] and [5'  
 20 GTTGTAGCTCTGGGAGCGAGGC CTGAGCCTCAGACAGTA 3'] of the previously known M1 relaxin sequence (Evans et al (1993) *J. Mol. Endocrinol.* 10, 15-23) were prepared commercially (Geneworks Pty Ltd). Probes were labeled with  $[\alpha\text{-}^{35}\text{S}]\text{dATP}$  (1200 Ci/mmol; NEN, AMRAD-Biotech, Melbourne, Australia) to a specific activity of  $1 \times 10^9$  d.p.m./ $\mu\text{g}$  using terminal deoxynucleotidyl transferase (Roche Diagnostics; Wisden  
 25 et al (1994) In *In Situ Hybridization Protocols for the Brain* (Wisden, W. and Morris, B.J. eds), pp 9-34, Academic Press, London). Screening of the sequences used against gene sequence databases (Celera, EMBL and Genbank; NCBI/NIH Blast Service) revealed homology only with the appropriate M1 and M3 relaxin mRNAs.

30 Sections were incubated overnight at  $42^\circ\text{C}$  with multiple  $^{35}\text{S}$ -labeled probes (30 fmol each probe/slide) in hybridization buffer containing 50% formamide, 4 x SSC, 10% dextran

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sulphate and 0.2 M dithiothreitol. Slides were washed in 1 x SSC at 55°C for 1 h, rinsed in 0.1 x SSC, then dehydrated before being apposed to Kodak BioMAX MR for 10 d.

The authenticity of the hybridization was confirmed by the demonstration that the signal could be successfully blocked in all areas by the addition of a 100-fold excess of unlabeled probes to the hybridization buffer, except those that corresponded to non-specific or background hybridization (data not shown). In addition, three oligonucleotide probes were used that were complementary to different, non-overlapping regions of the M3 relaxin gene sequence.

10

*Human relaxin (H3) studies:*

*Solid Phase Synthesis*-A putative peptide sequence encoded by the H3 gene was assembled by solid phase synthesis procedures based on the predicted signal peptide and proteolytic enzyme cleavage sites between the signal peptide and the B-chain, and the B/C and C/A chain junctions of the H3 relaxin prohormone (see Results for details). For ease of synthesis we chose to prepare the A- and B-peptides as their carboxyl-terminal amide derivatives. Selectively S-protected A- and B-chains were synthesized on a 0.1 mmol scale by the continuous flow Fmoc solid-phase method as previously described Dawson et al (1999) *J. Pept. Res.* 53, 542-547. Selective S-protection was afforded for the following cysteine residues: trityl (Trt) for A<sup>10,15</sup> and B<sup>22</sup>, tert-butyl for A<sup>24</sup>, and acetamidomethyl (Acm) for A<sup>11</sup> and B<sup>10</sup> (see Fig. 2A for numbering of amino acid residues).

20

On completion of the syntheses, the S-protected A- and B-chains were cleaved from the solid supports and simultaneously sidechain deprotected by treatment with TFA in the presence of scavengers. Selective disulfide bond formation was achieved essentially as described for the synthesis of bombyxin Maruyama et al (1992) *J. Prot. Chem.* 11, 1-12.

25

*Peptide Characterization*-Peptides were quantitated by duplicate amino acid analysis of 24 h acid hydrolyzates on a GBC automatic analyser (Melbourne, Australia). MALDITOF mass spectrometry (MS) was performed in the linear mode at 19.5kv on a Bruker Biflex instrument (Bremen, Germany) equipped with delayed ion extraction.

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*Other Relaxin and Insulin Peptides*-Human INSL3 was synthesized using the same methodology used for ovine INSL3 (Dawson et al (1999) *J. Pept. Res.* 53, 542-547), and was characterized by MS and amino acid analysis as outlined above. H1 relaxin was  
5 synthesized previously (Wade et al (1996) *Biomed. Pept. Prot. Nucl. Acids* 2, 27-32), recombinant H2 relaxin was a gift from the Connetics Corporation (Palo Alto, CA) and bovine insulin was purchased from Roche Diagnostics (Sydney, Australia).

*THP-1 Cell Bioassay*-The ability of H3 relaxin to induce cAMP production in the human  
10 monocytic cell line (THP-1) was compared to H1 and H2 relaxin following the procedure of Parsell and colleagues (Parsell et al (1996) *J. Biol. Chem.* 271, 27936-27941), with the following modifications; THP-1 cells which had been viability tested using Trypan Blue were resuspended in media, and transferred to a 96 well plate at a density of 60,000 cells/well. Peptides (H1, H2, H3 relaxin, human INSL3 and bovine insulin) were added to  
15 the wells together with 1  $\mu$ M forskolin and 50  $\mu$ M isobutylmethylxanthine (IBMX) in RPMI media, and incubated at 37°C for 30 min. The plate was then briefly centrifuged, the media removed and the cells resuspended in lysis buffer. cAMP levels were measured in the lysates using the cAMP Biotrak EIA system (Amersham International, Aylesbury, UK). The results are expressed as the maximum relaxin response (%) in comparison to the  
20 maximum stimulation of cAMP achieved with H2 relaxin. Data represent the mean  $\pm$  SEM of three experiments performed in quadruplicate, and are plotted using PRISM (Graphpad Inc., San Diego, CA).

*THP-1 Cell Binding Assay*- THP-1 cells were spun down and resuspended in binding  
25 buffer (20 mM HEPES, 50 mM NaCl, 1.5 mM CaCl<sub>2</sub>, 1% BSA, 0.1 mg/ml lysine, 0.01% NaN<sub>4</sub>, pH 7.5) (Parsell et al (1996) *J. Biol. Chem.* 271, 27936-27941) to give  $2 \times 10^6$  cells/well in a 96-well plate. The cells were incubated in binding buffer with <sup>33</sup>P-labeled H2 (B33) relaxin (100 pM: labeled as previously described (Tan et al (1999) *Br. J. Pharmacol.* 127, 91-98) at 25°C for 90 min in the absence or presence of increasing  
30 concentrations of unlabeled H1, H2 and H3 relaxin (100 pM to 30 nM). Non-specific binding was defined with H2 relaxin (1  $\mu$ M). Cells were harvested using a Packard 96-



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well plate cell harvester and Whatman GF/C glass fibre filters treated with 0.5% polyethylenimine. The filters were washed three times with modified binding buffer (20 mM HEPES, 50mM NaCl, 1.5mM CaCl<sub>2</sub>), dried in a 37°C oven, and the radioactivity counted by liquid scintillation spectrometry (TopCount™, Canberra Packard, Australia).

5

*Antibody Crossreactivity*-The ability of well characterized human relaxin antibodies to recognize H3 relaxin was tested in comparison to H1 and H2 relaxins by radioimmunoassay. Briefly, goat anti-H2 relaxin (Lucas et al (1989) *J. Endocrinol.* 120, 449-57) was coated onto 96 well ELISA plates (Disposable Products, Adelaide, Australia) at a dilution of 1:1000 with 0.05M sodium carbonate buffer at 4°C overnight. After washing twice with PBS-T (phosphate buffered saline; 0.05% Tween 20, pH 7.4) dilutions of human relaxin peptides dissolved in 50 µl of assay buffer (1% BSA in PBS-T) were added together with 50,000 cpm <sup>125</sup>I-labeled relaxin, in 50 µl of assay buffer. H2 relaxin was <sup>125</sup>I-labeled and purified by HPLC (Palejwala et al (1998) *Endocrinology* 139, 1208-1212). After an overnight incubation at 4°C the plates were washed twice with PBS-T. The antibody-bound-<sup>125</sup>I-labeled H2 relaxin was collected by the addition of 1M NaOH and decanted into tubes for counting on a Packard 5010 gamma counter (Canberra Packard). Experiments were performed at least twice and similar results obtained. Data was plotted as the mean ± SEM from one representative experiment performed in triplicate and plotted using PRISM.

20

*Mouse relaxin (M3) studies:*

*Animals*-All male and female mice used in these studies were age-matched and had the same background (C57BLK6J). Animals were housed in a controlled environment and maintained on a 14 h light, 10 h dark schedule with access to rodent lab chow (Barastock Stockfeeds, Melbourne, Australia) and water. Female mice (3.5 months old) were mated and pregnancy timed from the identification of the vaginal plug. At day 7.5, 10.5 and 18.5 of pregnancy, mice were sacrificed for tissue collection. Tissues were also collected from non-pregnant female and male mice (4 months old). These experiments were approved by the Howard Florey Institute's Animal Experimental Ethics Committee, which adheres to

30

the Australian Code of Practice for the care and use of laboratory animals for scientific purposes.

*Tissue Collection*-Animals were killed with an overdose of Isoflurane (Abbott Australasia Pty Ltd, Sydney, Australia). The brain, heart, thymus, spleen, lung, liver, kidneys, skin and gut were collected along with the reproductive organs from female (ovary, endometrium, myometrium, cervix, vagina; n = 2 for each pregnancy stage) and male (testes, epididymis, prostate; n = 3) mice. From additional animals, male brains (n = 3) were dissected into specific regions including the hypothalamus, cortex, hippocampus, thalamus, medulla and cerebellum, and immediately placed in liquid nitrogen and stored at -80°C until used for RNA preparation. Female brains (n = 3) were collected and immediately frozen over dry ice for in situ hybridization histochemistry (Burazin et al (2001) *J. Neuroendocrinol.* 13, 358-370). Human CL from women in early pregnancy undergoing surgery for ectopic pregnancies were utilized with the approval of the Howard Florey Institute Human Ethics Committee and the written consent of the patients.

## **Example 2**

### Human H3 relaxin genes in the human and mouse

Both H3 relaxin sequences in the human and mouse contain features representative of functional genes (Fig. 1A human; 1B mouse). Each contain a putative TATA box for initiation of transcription 65, and 59 bp, upstream of putative ATG start codons for human and mouse, respectively. A polyadenylation signal is present in the 3' untranslated region of both genes, in a position 582 and 448 bp downstream from an inframe TAG stop codon for the human, and mouse genes respectively. A single intron interrupts the coding region in an identical position in the sequence of both genes, corresponding to a similar position to that of other relaxin and insulin family members (Hudson et al (1983) *Nature* 301, 628-631; Evans et al (1993) *J. Mol. Endocrinol.* 10, 15-23; Ivell, R (1997) *Rev. Reprod.* 2, 133-138). The H3 relaxin gene is localized on chromosome 19 at 19p13.3, whereas the mouse gene is located on chromosome 8 at 8C2. The derived coding regions of the H3 and M3 relaxin genes were 142, and 141, amino acids, respectively.

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The cysteine residues necessary for disulphide bond formation are retained in the correct positions, together with conserved glycine residues necessary for flexibility around the cysteine linkages (Büllesbach et al (2000) *Int. J. Pept. Prot. Res.* 46, 238-243). Most importantly, the residues demonstrated to be essential for relaxin receptor binding in the core of the B-chain (R-X-X-X-R-X-X-I) (Büllesbach et al (2000) *J. Biol. Chem.* 275, 35276-35280), have been retained in both the human and mouse sequences. Therefore, although the human sequence most closely resembles the hINSL5 peptide sequence on direct amino acid homology, the presence of this binding motif indicates that the peptide is more like a relaxin peptide. Interestingly, the M3 relaxin A-chain conforms to the cysteine pattern of family members, whereas the previously characterized M1 relaxin sequence contains an extra tyrosine residue before the final cysteine residue (Fig. 2A).

The H3 (human H3) and M3 (mouse "3" relaxin) sequences share greater than 70% homology in the coding region at the nucleotide level. However, the homology is most striking in the derived amino acid sequence. Both derived pro-hormone sequences contain a typical signal sequence after the ATG start codon which is likely to be cleaved at an identical position between alanine and arginine in both the human and mouse peptides (Nielsen et al (1997) *Prot. Engineer.* 10, 1-6). The arginine-arginine pair of basic amino acids at the B/C junction found with other members of the relaxin family strongly suggests cleavage between tryptophan and arginine. Similarly, cleavage at the C/A junction is most likely to occur between the arginine and aspartic acid as indicated in Figs. 1A and 1B, as this corresponds to a weak furin (proprotein convertase) cleavage site (Nakayama, K. (1997) *Biochem. J.* 327, 625-635). Therefore, it is believed that both H3 and M3 relaxins comprise a B-chain of 27 amino acids, a C-peptide of 66 amino acids and an A-chain of 24 amino acids.

A comparison of the A- and B-chain sequences of H3 and M3 relaxin with H1, H2 and M1 relaxin is outlined in Fig. 2A. There are only two amino acid differences in both the A- and B-chains between the M3 and H3 sequences, of which three of these changes are conserved. In contrast, the homology between M1 and H2 relaxin is only 42% and 45% in the A-, and B-, chains respectively. Furthermore, other than the key core elements in the B-

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chain and the key structural elements in the A-chain, there is very little homology between H2 and H3 relaxin, and between M1 and M3 relaxin. Interestingly, H3 and M3 relaxin show high homology of the C-peptide domain (73%), compared with less than 20% homology in this region of other insulin/relaxin family members. The C-peptide lengths of H3 and M3 relaxin are 65, and 66 amino acids, respectively, and are much shorter than that of other relaxins (102 amino acids for H1 and H2 and 99 amino acids for M1 relaxin). The C-peptide chain length and sequence homology is most similar to INSL5 (24%).

The full length amino acid sequences of the two genes were aligned to other members of the insulin/relaxin family and a phylogenetic tree generated (Fig. 2B). Additionally, the H3 and M3 relaxin sequences are grouped under a separate branch, indicating that the evolution of these particular relaxins diverged from other relaxins early in evolution. This was also the case for INSL5 within this analysis which interestingly shares closest primary structural similarity to H3 relaxin.

### Example 3

#### Peptide synthesis

H3 relaxin was prepared by solid phase synthesis in low overall yield (0.7%). MALDITOF MS showed a single product with an  $MH^+$  of 5,494.7 (theoretical value: 5,497.5). Amino acid analysis also confirmed its correct composition.

#### Chemical Synthesis of Human Relaxin H3 [hRIx-3 A(1-24) amide-B(1-27) amide

Selectively S-protected A- and B-chains representing the amino acid sequence of the separate H3 relaxin peptide chains, were synthesized by the continuous flow 9-fluorenyl methoxycarbonyl (Fmoc) solid-phase method using the general procedures described in Atherton, E and Sheppard, RC. (*Solid Phase Peptide Synthesis*. IRL Press at Oxford University Press, Oxford, United Kingdom, 1989). Both peptides were prepared on a 0.1 mmol scale as peptide-carboxyl terminal amides using Fmoc peptide amide linker polyethylene glycol polystyrene (Fmoc-PAL-PEG-PS) supports (Applied Biosystems). For the A-chain assembly, four-fold excesses of Fmoc-amino acids (Auspep, Melbourne, Australia) were activated by 1,3-diisopropylcarbodiimide (DIC) and 1-

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hydroxybenzotriazole (HOBt) in dimethylformamide (DMF), whereas during the B-chain synthesis each residue was activated by 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and diisopropylethylamine (DIEA) in DMF. N<sup>α</sup>-Fmoc deprotection for both chain assemblies was with 20% piperidine in DMF.

5 Couplings were generally of 30 minutes duration, with the exception of double couplings and extended times for A-chain residues Ser<sup>7,8,21</sup> and all cysteines, and double couplings of B-chain residues Arg<sup>1,12,16</sup>, Ala<sup>2,3,17</sup> and Cys<sup>10</sup>. Side chain protection was afforded by *tert*-butyl esters and ethers for Asp, Glu, Thr and Ser, butoxycarbonyl (Boc) for Lys and Trp, 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl (Pbf) for Arg and the amide bond

10 protection N<sup>α</sup>-(2-Fmoc-oxy-4-methoxybenzyl) [FmocHmb] for B-chain Gly<sup>11</sup>. Selective S-protection was afforded for the following cysteine residues: trityl (Trt) for Cys<sup>10,15</sup> in the A-chain and Cys<sup>22</sup> in the B-chain, *tert*-butyl (tBu) for Cys<sup>24</sup> in the A-chain, and acetamidomethyl (Acm) for A-chain Cys<sup>11</sup> and B-chain Cys<sup>10</sup>.

15 (i) Synthesis of Human Relaxin H3, A-chain [Cys<sup>11</sup>(Acm), Cys<sup>24</sup>(tBu)](1-24) amide [1]

On completion of the synthesis, the protected A-chain resin was treated at room temperature for 2.5 hours with 95% trifluoroacetic acid (TFA)/2.5% ethanedithiol (EDT)/2.5% H<sub>2</sub>O plus 4 drops triethylsilane, to aid the quenching of thiols. TFA was removed to a minimum volume under a stream of nitrogen and precipitated twice from

20 chilled diethyl ether. The precipitate was then dissolved in 0.1% aq. TFA and lyophilized. The crude S-reduced [thiol-Cys<sup>10,15</sup>, Cys<sup>11</sup>(Acm), Cys<sup>24</sup>(t-Bu)] A-chain was directly subjected to air oxidation in 0.1M Gly-NaOH, pH 8.3, for 4 hours at room temperature. Analytical reverse-phase high performance liquid chromatography (RP-HPLC) monitoring confirmed the completeness of the intramolecular disulfide bond formation, after which

25 several drops of neat TFA were added and the crude oxidized material directly lyophilized.

(ii) Synthesis of Human Relaxin H3, B-chain [Cys<sup>10</sup>(Acm)] (1-27) amide [2]

On completion of the synthesis, the protected B-chain resin was treated at room temperature for 2.5 hours with 82.5%TFA/5% phenol/5% H<sub>2</sub>O/5% thioanisole/2.5%

30 ethanedithiol plus 4 drops of triethylsilane, to aid the quenching of thiols. TFA was removed to a minimum volume under a stream of nitrogen and precipitated twice from

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chilled diethyl ether. The precipitate was then dissolved in 0.1% aq. TFA and lyophilized. The crude B-chain was then purified by RP-HPLC as described below.

(iii) Synthesis of Human Relaxin H3, A-chain [Cys<sup>11</sup>(Acm), Cys<sup>24</sup>(Pyr)](1-24) amide [3]

5 25 mg of peptide **1** (9.65  $\mu$ mol) and 35 mg (158.86  $\mu$ mol) 2,2'-dipyridyl disulfide (DPDS) were dissolved together in 4.5 ml TFA and 0.5 ml thioanisole and the resulting solution then chilled. To this was added 5 ml trifluoromethanesulfonic acid (TFMSA)/TFA (1:5 v/v) and the whole mixture allowed to stir at  $\leq 0^{\circ}\text{C}$  for 30 mins. The [Cys<sup>11</sup>(Acm), Cys<sup>24</sup>(Pyr)] A-chain amide peptide was precipitated from cold ether and the pellet obtained  
10 on centrifugation then suspended in 6M guanidine hydrochloride (GdHCl), pH 8.0, and purified by RP-HPLC. (Yield peptide **3**: 4%). (Alternative to RP-HPLC purification, peptide **3** was desalted on a Sephadex G-25 gel filtration column in 20% aq acetic acid).

(iv) Synthesis of Human Relaxin H3, A[Cys<sup>11</sup>(Acm)] (1-24) amide-B[Cys<sup>10</sup>(Acm)] (1-27) amide [4]

15 Purified A-chain peptide **3** (1.0 mg, 0.38  $\mu$ mol) and purified B-chain peptide **2** (1.2 mg, 0.38  $\mu$ mol) were dissolved separately in 1.0 ml and 0.5 ml 0.1M NH<sub>4</sub>HCO<sub>3</sub> respectively. The B-chain solution was then slowly added to A-chain and the reaction mixture was stirred vigorously at room temperature for 30 min. The solution was acidified with 0.5 ml  
20 glacial acetic acid and then subjected to RP-HPLC, as detailed below, to isolate the *bis*-disulfide bonded chain combined product. (Alternative to RP-HPLC purification, the resulting A/B product, peptide **4**, was desalted on a Sephadex G-25 gel filtration column in 20% aq acetic acid).

25 (An alternative method for chain combination which improves B-chain solubility, is as follows: peptide **3** and purified [Cys<sup>10</sup>(Acm)] B-chain were dissolved separately, at a concentration of 1.0 ml/mg, in 8M GdHCl, pH 4.5 buffer. The B-chain solution was then slowly added to A-chain and the reaction mixture was stirred vigorously at 37°C for 24 hours).

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(v) Synthesis of Human Relaxin H3 [hRlx-3A(1-24) amide-B(1-27) amide

All of the purified 4 peptide was used to form the third and final disulfide bond (assuming 100% recovery, estimated at 0.39  $\mu$ mol). The peptide was dissolved in a solution of 80mM HCl and acetic acid. 20mM iodine in 95% aqueous acetic acid was then added dropwise  
5 (25 equivs of iodine per AcM group). The reaction was performed for 1 hour in the dark at room temperature after which excess oxidant was quenched with 20mM aqueous ascorbic acid. Purification of the relaxin was by RP-HPLC, with a final yield, relative to peptide 3 starting material, of 0.74%.

10 *Purification*

The separate crude chains and intermediate peptides were purified by RP-HPLC, using a Waters 600 multisolvent delivery system connected to a model 996 photodiode array detector. A 10 $\times$ 250mm Vydac 218 TP column packed with C<sub>4</sub> silica gel (330A pore size, 10 $\mu$ m particle size) was used. The peptides were eluted with a solvent system of (A) 0.1%  
15 aq. TFA (v/v) and (B) 0.1% TFA in acetonitrile (v/v) in a linear gradient mode (25-50% B over 30 minutes). The target fractions were collected and identified by matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF MS) and lyophilized.

*Peptide characterisation*

20 Peptide quantitation was by duplicate amino acid analysis of 24 hr acid hydrolyzates on a GBC automatic analyser (Melbourne, Aust). MALDITOF MS was performed in the linear mode at 19.5kv on a Bruker Biflex instrument (Bremen, Germany) equipped with delayed ion extraction.

25 **Example 4**

Relaxin biological activity

*Demonstration of Relaxin Activity of Synthetic H3 Relaxin*-Synthetic H3 relaxin C-terminal amide derivatives were tested for relaxin activity in a relaxin receptor expressing cell line, THP-1 (Parsell et al (1996) *J. Biol. Chem.* 271, 27936-27941). H2 relaxin produces a dose  
30 dependent increase in cAMP production from these cells (Fig. 3A). Synthetic H3 relaxin also stimulated a dose dependent increase in cAMP ( $pEC_{50} = 8.68 \pm 0.08$  [2.11nM]; n=3),

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albeit with slightly lower activity than H1 ( $pEC_{50} = 9.10 \pm 0.05$  [0.794 nM];  $n=3$ ) and H2 ( $pEC_{50} = 9.67 \pm 0.11$  [0.214 nM];  $n=3$ ) relaxin. The specificity of this response was demonstrated by the inability of bovine insulin (bINSL), or human insulin 3 (hINSL3), to stimulate cAMP responses at doses up to 1  $\mu$ M.

5

Synthetic H3 relaxin was also tested for its ability to compete for  $^{33}\text{P}$ -labeled H2 relaxin binding to relaxin binding sites in THP-1 cells (Fig. 3B), with an affinity ( $pK_i = 7.5 \pm 0.16$ ;  $n = 3$ ) lower than that of H2 ( $pK_i = 8.74 \pm 0.11$ ;  $n = 11$ ) and H1 ( $pK_i = 8.9 \pm 0.11$ ;  $n = 7$ ) relaxin. Nevertheless, these data provide definitive evidence that the synthetic H3 relaxin peptide binds to, and elicits a second messenger response by stimulating human relaxin receptors.

*Ability of a Well Characterized H2 Relaxin Antibody to Recognize H3 Relaxin*-The ability of a well characterized anti-H2 relaxin antibody to recognize H1 and H3 relaxin was tested by radioimmunoassay. As shown in Fig. 4, H2 relaxin was able to displace  $^{125}\text{I}$ -labeled H2 relaxin binding to the anti-H2 relaxin antibody with high specificity. In contrast, H1 and H3 relaxin showed poor cross reactivity with the antisera as determined by their poor ability to displace  $^{125}\text{I}$ -labeled H2 relaxin binding. Furthermore, the non-parallelism of the displacement curves indicates that not all the antibody epitopes are recognized by the two peptides.

20

## Example 5

### H3 relaxin expression

*Relaxin Gene Expression in the Mouse*-The expression of M3 relaxin mRNA was compared to M1 relaxin mRNA expression using southern blotting of RT-PCR products. Although this technique is only semi-quantitative, it enabled us to determine the potential sites of expression of M3 relaxin compared to M1 relaxin. The results of a representative experiment and duplicate experiments gave identical results. M3 relaxin mRNA was expressed in a number of tissues in C57BLK6J mice where M1 relaxin was found, but the pattern of expression, between the two mouse relaxins was different. In male non-reproductive tissues, highest levels of M1 relaxin expression were seen in the brain,

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moderate levels in the thymus, heart and kidney, lower levels in the lung, spleen and skin, with no expression seen in the gut. Interestingly, M3 relaxin expression was detected at highest levels in brain, however, it was expressed at moderate levels in the thymus, lung and spleen, only at very low levels in the heart and liver, and not at all in the kidney, skin and gut. Female mice showed an almost identical pattern of expression for both genes in these tissues. In male reproductive tissues M3 relaxin mRNA was significantly expressed only in the testis whereas, M1 relaxin mRNA was detected in the testis, epididymis and prostate. Both relaxins were also detected in female reproductive organs in the mammary gland, ovaries of non-pregnant, pregnant and lactating mice, and the endometrium and myometrium of pregnant mice. Significant expression of M3 relaxin mRNA was observed in all ovarian stages, while M1 relaxin expression was higher in ovaries of late gestation compared to ovaries from non-pregnant and lactating mice. High levels of M3 relaxin mRNA were detected in the brain and further analysis of this tissue revealed that both relaxins were expressed in several distinct regions. While M1 relaxin mRNA was consistently expressed in the hypothalamus, hippocampus, cortex, thalamus, pons/medulla and cerebellum, M3 relaxin mRNA was found to be highly expressed in the thalamus and pons/medulla, thus suggesting, that the two relaxins may play distinct roles in the mouse.

*Northern Analysis*-Tissues in which M3 relaxin mRNA was positively identified by RT-PCR and Southern blot analysis, were further examined by Northern blotting. Total RNA (5-25 µg) from the heart, brain, lung, thymus, spleen, ovary, endometrium, myometrium, cervix and vagina were initially probed with a <sup>32</sup>P-labeled M3 relaxin specific probe, but no specific hybridizing bands were found in any tissue. Poly-A RNA from the brain (15 µg), spleen (5 µg), liver (5 µg) and testis (25 µg) were then analyzed and a specific ~1.2-kb hybridizing band was identified in the brain, consistent with M3 relaxin expression detected by RT-PCR and Southern blot analysis. The obtained transcript size was consistent with the predicted size based on the M3 relaxin transcript sequence (~1kb) plus a poly-A tail (~200-bp).

*Expression of H3 Relaxin in Human Tissues*-A Clontech Multi Tissue Expression Array was used to examine sites of expression of H3 relaxin in human tissues. The array

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contained normalized poly-A RNA (50-750 ng) from 76 different human tissues including 8 different control RNAs and DNAs, spotted onto a nylon membrane. The array was probed with a <sup>32</sup>P-labeled 374-bp H3 relaxin specific gene fragment from the 3' end of the H3 relaxin transcript, generated from genomic DNA. This DNA fragment was sequenced  
5 on both strands. Very weak hybridizing signals were observed in spleen, thymus, peripheral blood leukocytes, lymph node and testis however, these signals were barely discernable above background and hence, the data is not shown. RT-PCR was also performed on human CL from early pregnancy using two different primer combinations based on the H3 relaxin sequence. No specific bands were observed in any PCR reaction  
10 even after changing the PCR conditions, whereas transcripts for H2 relaxin and GAPDH were easily amplified (data not shown), confirming the integrity of the cDNA.

*Distribution of Relaxin mRNA in the Mouse Brain*-Given the high levels of M3 relaxin mRNA expression detected by RT-PCR and Northern blotting in the brain, its distribution  
15 was further examined using *in situ* hybridization histochemistry (Burazin et al (2001) *J. Neuroendocrinol.* 13, 358-370. Multiple specific <sup>35</sup>S-labeled oligonucleotide probes were utilized to determine the cellular distribution of M3 relaxin mRNA throughout the rostro-caudal extent of the female C57BLK6J mouse brain. M3 relaxin mRNA was not widely detected throughout brain nuclei, but was most strongly detected in the pons/medulla (Fig.  
20 7). The strongest level of M3 relaxin mRNA was present in the pars ventromedialis of the dorsal tegmental nucleus. In addition, M3 relaxin mRNA was also detected, albeit at far lower levels, in the hippocampus and olfactory regions. Brain regions containing low levels of mRNA encoding M3 relaxin may not have been detected in the current study due to sensitivity limitations associated with *in situ* hybridization histochemistry. The  
25 distribution of M3 relaxin mRNA in the brain differs from that of M1 relaxin mRNA, as no M1 relaxin mRNA was detected in the pars ventromedialis of the dorsal tegmental nucleus (data not shown).

### Example 6

30 Pror relaxin H3 cDNA sequences from human, mouse and rat are expressed in both prokaryotic and eukaryotic cell systems using appropriate expression transfer vectors.

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These systems include appropriate mammalian host cells, other higher eukaryotic cells including insect cells, plant cells and avian cells as well as bacterial and yeast expression systems. Additionally, fusion protein products of these three sequences are produced by linking a portion of a prokaryotic or eukaryotic protein characteristic of the host cell. The  
5 fusion products facilitate the purification of the protein product such that the fusion product may be subsequently removed. All transfer vectors may also be modified by codon substitutions/deletions/additions with the modifications giving rise to shortened C peptide prorelaxins with B/C and C/A junction modifications to facilitate the removal of the modified C peptide sequence.

10

Relaxin synthesis using shortened C peptide substitutions and B/C and C/A junction modifications are described in US patent 5,759,807, and such methods may be used for the production of H3 relaxin.

15 Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

20 The reference to any prior art in this specification is not, and should not be taken as an acknowledgment or any form of suggestion that that prior-art forms part of the common general knowledge in Australia.

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**The claims defining the invention are as follows:**

1. A method for the treatment of one or more of: vascular disease; treatment of  
arterial hypertension; diseases related to uncontrolled or abnormal collagen or fibronectin  
5 formation; kidney disease; psychiatric disorders; depression or depressive disorders;  
neurologic or neurodegenerative diseases; disorders of learning, attention and motivation;  
addictive disorders; movement and locomotor disorders; immunological disorders; breast  
disorders; endometrial disorders; endocrine disorders; delayed onset of labour, impaired  
cervical ripening, and prevention of prolonged labour due to fetal dystocia; sinus  
10 bradycardia; hair loss; alopecia; disorders of water balance including impaired or  
inappropriate secretion of vasopressin; or placental insufficiency; which comprises  
administering to a subject in need of any such treatments a therapeutically effective  
amount of human H3 relaxin, or an analogue thereof as herein defined, optionally in  
association with one or more pharmaceutically acceptable carriers and/ diluents and/or  
15 excipients.

2. A method according to claim 1 wherein the H3 relaxin or analogue thereof is  
human H3 relaxin, human H3 prorelaxin, human H3 preprorelaxin, or the constitutive A, B  
or C peptide chains thereof.

20

3. A method according to claim 2 wherein the human H3 relaxin or a human H3  
relaxin analogue thereof comprises an A chain and a B chain,

the A chain having the amino acid sequence:

25

Asp Val Leu Ala Gly Leu Ser Ser Ser Cys Cys Lys Trp Gly Cys Ser

1

5

10

15

Lys Ser Glu Ile Ser Ser Leu Cys

30

20

(SEQ ID NO:4)

or an amino acid sequence truncated by up to about 9 amino acids from N-terminus,

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the B chain having the amino sequence:

Arg Ala Ala Pro Tyr Gly Val Arg Leu Cys Gly Arg Glu Phe Ile Arg  
 5 1 5 10 15

Ala Val Ile Phe Thr Cys Gly Gly Ser Arg Trp  
 20 25

or an amino acid sequence truncated by up to 9 amino acids from the amino-terminus  
 10 and/or up to about 5 amino acids from the carboxyl-terminus,

the A and B chains being linked by disulphide bonds between A11-B10 and A24-B22, and  
 wherein the human H3 relaxin or analogue thereof has relaxin bioactivity.

15 4. A method according to claim 1 wherein a human H3 relaxin analogue comprises a  
 modified A chain and/or a modified B chain,

the H3 relaxin A chain having the amino acid sequence:

20 Asp Val Leu Ala Gly Leu Ser Ser Ser Cys Cys Lys Trp Gly Cys Ser  
 1 5 10 15

Lys Ser Glu Ile Ser Ser Leu Cys  
 20

(SEQ ID NO: 4)

25

wherein the carboxyl-terminus is an amide derivative and/or Lys at position 12 is replaced  
 with Glu, and/or Glu at position 19 is replaced with Gln,

the H3 relaxin B chain having the amino acid sequence:

30

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Arg	Ala	Ala	Pro	Tyr	Gly	Val	Arg	Leu	Cys	Gly	Arg	Glu	Phe	Ile	Arg
1				5					10					15	
Ala	Val	Ile	Phe	Thr	Cys	Gly	Gly	Ser	Arg	Trp					
5				20				25							(SEQ ID NO: 2)

wherein the carboxyl-terminus is an amide derivative, and/or Ala at position 2 is replaced with Pro, and/or Arg at position 8 is replaced with Lys,

10 the A and B chains being linked by disulphide bonds between A11-B10 and A24-B22, and wherein the human H3 relaxin analogue has relaxin bioactivity.

5. A method according to claim 1 which is a method for the treatment of arterial hypertension.

15

6. A method according to claim 1 which is a method for the treatment of peripheral vascular disease including coronary artery disease, peripheral vascular disease, vasospasm including Raynaud's phenomenon, microvascular disease involving the central and peripheral nervous system, kidney, eye and other organs.

20

7. A method according to claim 1 which is a method for the treatment of kidney disease including vascular disease, interstitial fibrosis, glomerulosclerosis, or other kidney disorders.

25 8. A method according to claim 1 which is a method for the treatment of fibrotic disorders including psychiatric disorders including anxiety states including panic attack, agoraphobia, global anxiety, phobic states.

9. A method according to claim 1 which is a method for the treatment of depression or depressive disorders including major depression, dysthymia, bipolar an dunipolar depression; neurologic or neurodegenerative diseases (including memory loss or other memory disorders, dementias, Alzheimer's disease).

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10. A method according to claim 1 which is a method for the treatment of disorders of learning, attention and motivation including Attention Deficit Hyperactivity Disorder, Tourette's disease, impulsivity, antisocial and personality disorders, negative symptoms of  
5 psychoses including those due to schizophrenia, acquired brain damage and frontal lobe lesions).
11. A method according to claim 1 which is a method for the treatment of hair loss including drug, alcohol and nicotine addiction.
- 10 12. A method according to claim 1 which is a method for the treatment of neurologic or neurodegenerative diseases including memory loss or other memory disorders, dementias, Alzheimer's disease.
- 15 13. A method according to claim 1 which is a method for the treatment of movement and locomotor disorders including Parkinson's disease, Huntington's disease, and motor deficits after stroke, head injury, surgery, tumour or spinal cord injury.
14. A method according to claim 1 which is a method for the treatment of diseases  
20 related to uncontrolled or abnormal collagen or fibronectin formation including fibrosis of lung, heart and cardiovascular system, kidney and genitourinary tract, gastrointestinal system, cutaneous, rheumatologic and hepatobiliary systems.
15. A method according to claim 1 which is a method for the treatment of delayed  
25 onset of labour, impaired cervical ripening, and prevention of prolonged labour due to fetal dystocia.
16. A method according to claim 1 which is a method for the treatment of endocrine disorders including adrenal, ovarian and testicular disorders related to steroid or peptide  
30 hormone production.

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17. A method according to claim 1 which is a method for the treatment of breast disorders including fibrocystic disease, impaired lactation, and cancer.
18. A method according to claim 1 which is a method for the treatment of  
5 immunological disorders including immune deficiency states, haematological and reticuloendothelial malignancy.
19. A method according to claim 1 which is a method for the treatment of endometrial disorders including infertility due to impaired implantation.
- 10 20. A method according to claim 1 which is a method for the treatment of endocrine disorders including adrenal disorders, ovarian disorders, and testicular disorders related to steroid or peptide hormone production.
- 15 21. A method according to claim 1 which is a method for the treatment of diseases associated with water balance including impaired or inappropriate secretion of vasopressin.
22. A method according to claim 1 which is a method for the treatment of placental insufficiency.
- 20 23. Use of H3 relaxin or an analogue thereof and the manufacture of a medicament for the treatment of one or more of: vascular disease; treatment of arterial hypertension; diseases related to uncontrolled or abnormal collagen or fibronectin formation; kidney disease; psychiatric disorders; depression or depressive disorders; neurologic or  
25 neurodegenerative diseases; disorders of learning, attention and motivation; addictive disorders; movement and locomotor disorders; immunological disorders; breast disorders; endometrial disorders; endocrine disorders; delayed onset of labour, impaired cervical ripening, and prevention of prolonged labour due to fetal dystocia; sinus bradycardia; hair loss; alopecia; disorders of water balance including impaired or  
30 inappropriate secretion of vasopressin; or placental insufficiency; which comprises administering to a subject in need of any such treatments a therapeutically effective



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amount of human H3 relaxin, or an analogue thereof as herein defined, optionally in association with one or more pharmaceutically acceptable carriers and/ diluents and/or excipients.

5 24. Use according to claim 23 wherein the H3 relaxin or analogue thereof is human H3 relaxin, human H3 prorelaxin, human H3 preprorelaxin, or the constitutive A, B or C peptide chains thereof.

25. Use according to claim 25 wherein the human H3 relaxin or a human H3 relaxin  
10 analogue thereof comprises an A chain and a B chain,

the A chain having the amino acid sequence:

Asp	Val	Leu	Ala	Gly	Leu	Ser	Ser	Ser	Cys	Cys	Lys	Trp	Gly	Cys	Ser
15	1			5					10					15	
Lys Ser Glu Ile Ser Ser Leu Cys															
	20	(SEQ ID NO:4)													

20 or an amino acid sequence truncated by up to about 9 amino acids from N-terminus,

the B chain having the amino sequence:

Arg	Ala	Ala	Pro	Tyr	Gly	Val	Arg	Leu	Cys	Gly	Arg	Glu	Phe	Ile	Arg
25	1			5					10					15	
Ala Val Ile Phe Thr Cys Gly Gly Ser Arg Trp															
				20										25	

or an amino acid sequence truncated by up to 9 amino acids from the amino-terminus  
30 and/or up to about 5 amino acids from the carboxyl-terminus,

the A and B chains being linked by disulphide bonds between A11-B10 and A24-B22, and wherein the human H3 relaxin or analogue thereof has relaxin bioactivity.

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26. Use according to claim 23 wherein a human H3 relaxin analogue comprises a modified A chain and/or a modified B chain,

5 the H3 relaxin A chain having the amino acid sequence:

Asp	Val	Leu	Ala	Gly	Leu	Ser	Ser	Ser	Cys	Cys	Lys	Trp	Gly	Cys	Ser
1				5					10					15	

10 Lys Ser Glu Ile Ser Ser Leu Cys

20

(SEQ ID NO: 4)

wherein the carboxyl-terminus is an amide derivative and/or Lys at position 12 is replaced with Glu, and/or Glu at position 19 is replaced with Gln,

15

the H3 relaxin B chain having the amino acid sequence:

Arg	Ala	Ala	Pro	Tyr	Gly	Val	Arg	Leu	Cys	Gly	Arg	Glu	Phe	Ile	Arg
1				5					10					15	

20

Ala Val Ile Phe Thr Cys Gly Gly Ser Arg Trp

20

25

(SEQ ID NO: 2)

25 wherein the carboxyl-terminus is an amide derivative, and/or Ala at position 2 is replaced with Pro, and/or Arg at position 8 is replaced with Lys,

the A and B chains being linked by disulphide bonds between A11-B10 and A24-B22, and wherein the human H3 relaxin analogue has relaxin bioactivity.

30 27. Use according to claim 23 for the treatment of areterial hypertension.

28. Use according to claim 23 for the treatment of peripheral vascular disease including coronary artery disease, peripheral vascular disease, vasospasm including Raynaud's

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phenomenon, microvascular disease involving the central and peripheral nervous system, kidney, eye and other organs.

29. Use according to claim 23 for the treatment of kidney disease including associated  
5 with vascular disease, interstitial fibrosis, glomerulosclerosis, or other kidney disorders.

30. Use according to claim 23 for the treatment of psychiatric disorders including  
psychiatric disorders including anxiety states including panic attack, agoraphobia, global  
anxiety, phobic states.

10

31. Use according to claim 23 for the treatment of depression or depressive disorders  
including major depression, dysthymia, bipolar and unipolar depression; neurologic or  
neurodegenerative diseases (including memory loss or other memory disorders, dementias,  
Alzheimer's disease).

15

32. Use according to claim 23 for the treatment of disorders of learning, attention, and  
motivation including Attention Deficit Hyperactivity Disorder, Tourette's disease,  
impulsivity, antisocial and personality disorders, negative symptoms of psychoses  
including those due to schizophrenia, acquired brain damage and frontal lobe lesions.

20

33. Use according to claim 23 for the treatment of hair loss including drug, alcohol and  
nicotine addiction.

34. Use according to claim 23 for the treatment of neurologic or neurodegenerative  
25 diseases including memory loss or other memory disorders, dementias, Alzheimer's  
disease.

35. Use according to claim 23 for the treatment of movement and locomotor disorders  
including Parkinson's disease, Huntington's disease, and motor deficits after stroke, head  
30 injury, surgery, tumour or spinal cord injury.

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36. Use according to claim 23 for the treatment of diseases related to uncontrolled or abnormal collagen or fibronectin formation including fibrosis of lung, heart and cardiovascular system, kidney and genitourinary tract, gastrointestinal system, cutaneous, rheumatologic and hepatobiliary systems.
- 5
37. Use according to claim 23 for the treatment of difficult foetal delivery including delayed onset of labour, impaired cervical ripening, and prevention of prolonged labour due to fetal dystocia.
- 10
38. Use according to claim 23 for the treatment of haematological disorders including adrenal, ovarian and testicular disorders related to steroid or peptide hormone production.
39. Use according to claim 23 for the treatment of breast disorders including fibrocystic disease, impaired lactation, and cancer.
- 15
40. Use according to claim 23 for the treatment of immunological disorders including immune deficiency states, haematological and reticuloendothelial malignancy.
41. Use according to claim 23 for the treatment of endometrial disorders including
- 20 infertility due to impaired implantation.
42. Use according to claim 23 for the treatment of endocrine disorders including adrenal disorders, ovarian disorders, and testicular disorders related to steroid or peptide hormone production.
- 25
43. Use according to claim 23 for the treatment of diseases associated with water balance including impaired or inappropriate secretion of vasopressin.
44. Use according to claim 23 for the treatment of placental insufficiency.

Fig. 1A

**A: H3 relaxin assembled gene sequence**

**TATAAATGGGGGGCCAAGAGGCAGAGACACTGGCCCACTCTCACGTTCAAAGCGTCT**  
**CCGTCCAGCATGGCCAGGTACATGCTGCTGCTGCTCCTGGCGGTATGGGTGCTGACCGGG**  
M A R Y M L L L L L A V W V L T G  
← Signal peptide →  
**GAGCTGTGGCCGGGAGCTGAGGCCCGGGCAGCGCCTTACGGGGTCAGGCTTTGCGGCCGA**  
E L W P G A E A R A A P Y G V R L C G R  
←  
**GAATTCATCCGAGCAGTCATCTTCACCTGCGGGGGCTCCCGGTGGAGACGATCAGACATC**  
E F I R A V I F T C G G S R W R R S D I  
← B Chain →  
**CTGGCCACGAGGCTATGG>**gtgaggctggggagagagtggatgtagaaggggaacag-  
L A H E A M  
-----intron 2318bp-----  
-cactaactctgttcacatcttttgcag<<**GAGATACCTTCCCGGATGCAGATGCTGATGAA**  
G D T F P D A D A D E  
**GACAGTCTGGCAGGCGAGCTGGATGAGGCCATGGGGTCCAGCGAGTGGCTGGCCCTGACC**  
D S L A G E L D E A M G S S E W L A L T  
← C Chain →  
**AAGTCACCCCAGGCCTTTTACAGGGGGCGACCCAGCTGGCAAGGAACCCCTGGGGTTCTT**  
K S P Q A F Y R G R P S W Q G T P G V L  
**CGGGGCAGCCGAGATGTCCTGGCTGGCCTTTCCAGCAGCTGCTGCAAGTGGGGGTGTAGC**  
R G S R D V L A G L S S S C C K W G C S  
← A Chain →  
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K S E I S S L C \*  
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CCCAGAGAAGGTGGCAGGTGGCCCCCTAGGAGAGCTCTGGGCACATTCGAATCTTCCCA  
AACTCCAATAATAAAATTGGAAGACTTTGGCAGAGAGTGTGTGTGTGTGTATGGTTG

Fig. 1B

**B: M3 relaxin assembled gene sequence**

TATAAATAGGGGATCGGAGGTGGTGCAGATAGAGCACCTGGGTTCGCAGGCATCTCAACTG

ATCATGGCAATGCTCGGGCTGCTGCTGCTGGCTTCTGGGCTCTCCTCGGGGCTCTGGGG

M A M L G L L L L A S W A L L G A L G  
 ← signal peptide →

CTGCAGGCCGAGGCGAGGCCGGCGCCCTACGGGGTGAAGCTCTGCGGTTCGGGAGTTCATC

L Q A E A R P A P Y G V K L C G R E F I

CGCGCGGTCTATCTTCACTTGCGGAGGCTCACGATGGCGCCGGGCGGACATCTTGGCCAC  
 R A V I F T C G G S R W R R A D I L A H

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E S L  
 -----intron 1446b-----  
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 G D F F A D G E A N T D H L A S

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E L D E A V G S S E W L A L T K S P Q A

TTCTACGGTGGTTCGAGCCAGCTGGCAAGGGTCACCTGGAGTGGTTCGGGGCAGCAGAGAT

F Y G G R A S W Q G S P G V V R G S R D

GTGTTGGCTGGCCTTTCCAGCAGTTGCTGCGAGTGGGGCTGTAGCAAGAGCCAAATTAGC

V L A G L S S S C C E W G C S K S Q I S

AGCTTGTGCTAGGATCAGGGTTGAGCAATGGAGAAGCGGGCCGTGCCTGCAAGCTGCTGT

S L C \*

CAGCTGTGCGATGTTCAAGAGCATTCTTACAGGCGAGGCACCAAGGGGTCCACTGTCTCC

TTACAGACCCCTCTGCCAAGATGCACACACTACGTGCCAACCTTTCCCCACCTTGCTGCCG

GCCCCCTCCTCTATCCAGCCAAACAGAACTTGTTTTTTCATGACTGAGTTCTTCCGTGCCA

CAACCTCACCCCCAGCAGCCAGCAGCAACCAGATGCCATCTTCTTAACTGGCTACAC

TAGAGTCTGCCCCACCTCCACCCTCAGTCCGGCCCTAATTGCCGCCACTGTCCCTGGCTA

ACCTGCCCCCCCCCAAAAAAAAAAAAAACAGAGCACTCTGTTGCAGACCCAGGACTGAG

GGCCCCCTGGTCCTCAGTACTCAGACTTCCTCACCACATAAAATAAAGGTTCAAGTTCTGAG

Fig. 2A

## A.

## B Chain Aligns

	1	5	10	15	20	25
Human 1	KWKDDVIKLCGRELVRAQIAICGMSTWS					
Human 2	DSWMEEVIKLCGRELVRAQIAICGMSTWS					
Cons 1,2,3	.....++LCGRE.+RA.I..CG.S.W.					
Human 3	RAAPYGVRLCGREFIRAVIFTCGGSRW					
Cons 3	R.APYGV+LCGREFIRAVIFTCGGSRW					
Mouse 3	RPAPYGVKLCGREFIRAVIFTCGGSRW					
Cons Mouse	.....+++CGRE+.R.+I..CG.S..					
Mouse 1	RVSEEWMDGFIRMCGREYARELIKICGASVGRLAL					

## A Chain Aligns

	1	5	10	15	20
Human 1	RPYVALFEKCCCLIGCTKRSLAKYC				
Human 2	QLYSALANKCCHVGCTKRSLARFC				
Cons 1,2,3	...+.L...CC..GC+K...+...C				
Human 3	DVLAGLSSSSCCKWGCSKSEISLC				
Cons 3	DVLAGLSSSSCC+WGCSKS+ISSLC				
Rat 3	DVLAGLSSSSCCEWGCSKSQISSLC				
Mouse 3	DVLAGLSSSSCCEWGCSKSQISSLC				
Cons Mouse	+...+S..CC..GCS+..I..L-C				
Mouse 1	ESGGLMSQQCCHVGCSRRSIKLYC				

Fig. 2B

B.

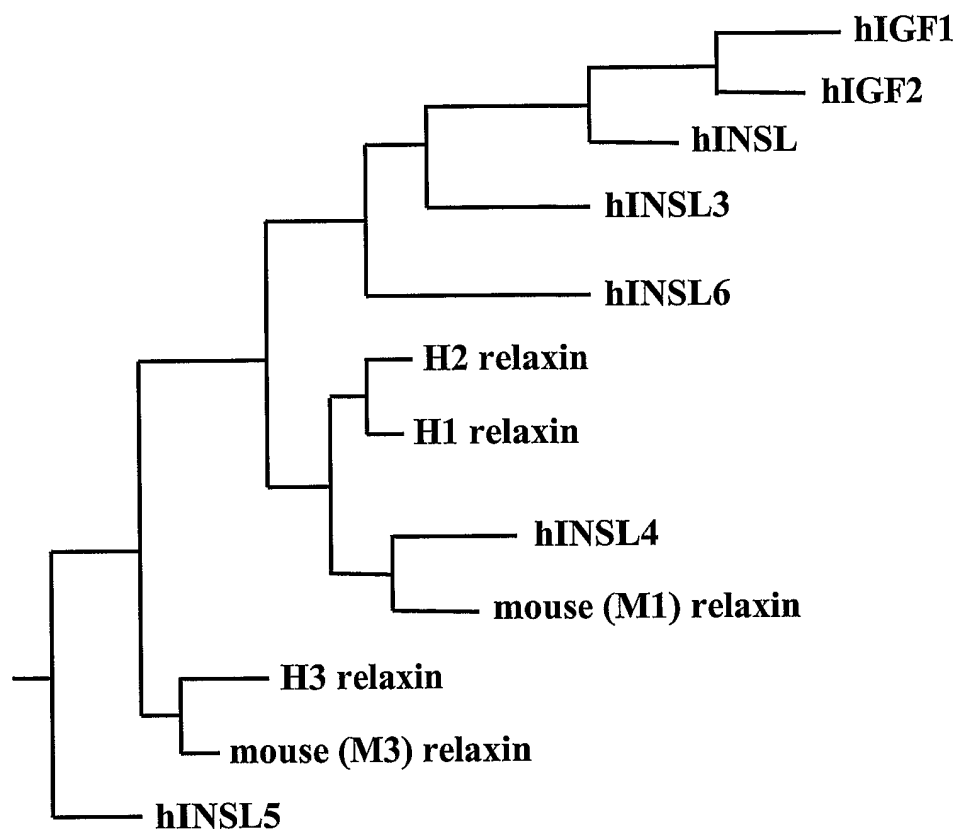
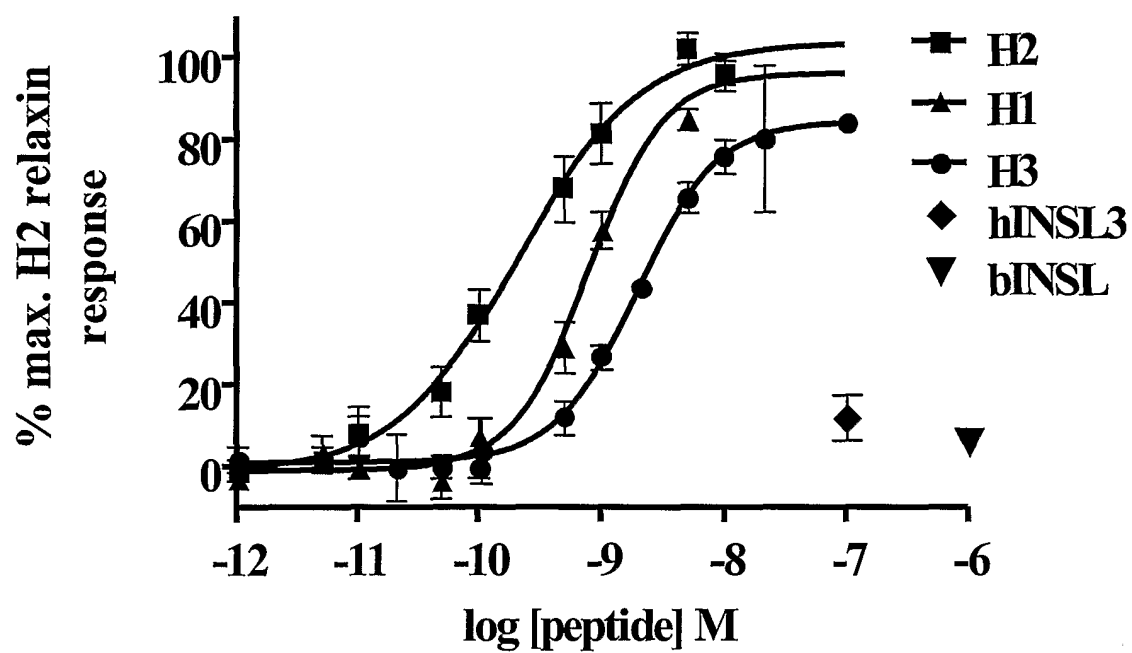




Fig. 3

A.



B.

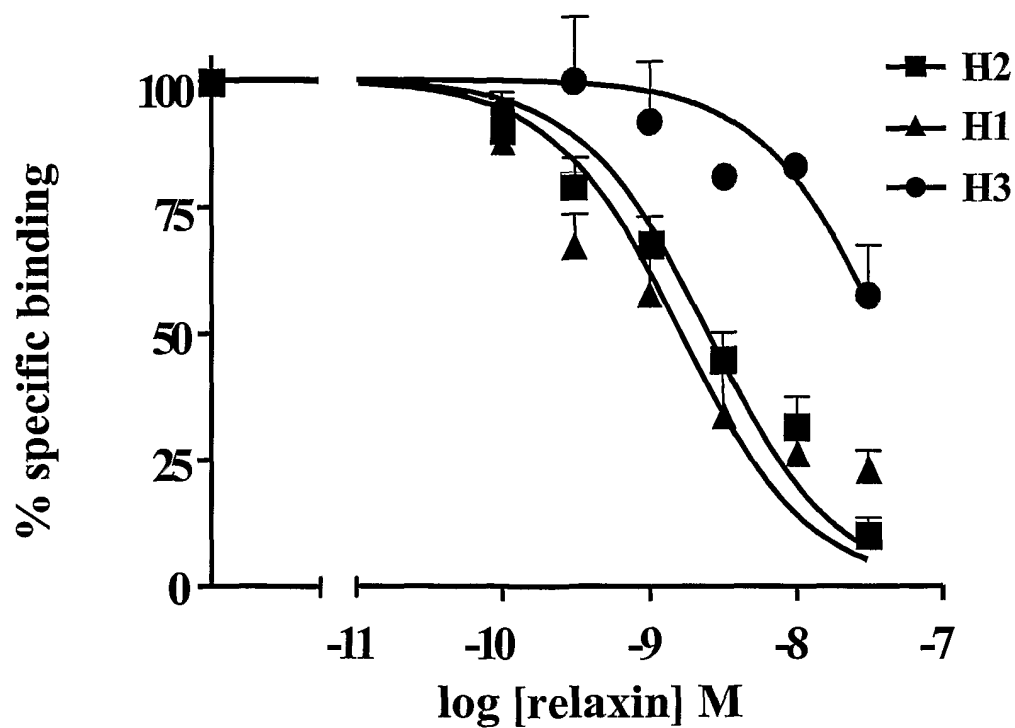
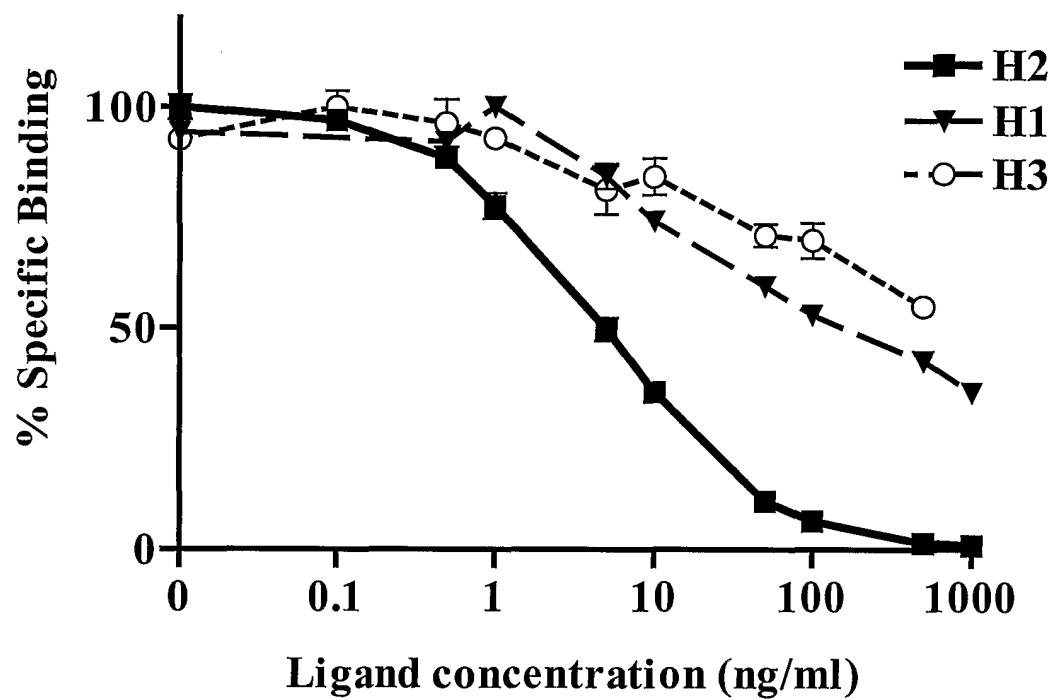


Fig. 4



## SEQUENCE LISTING

<110> Howard Florey Institute of Experimental Physiology  
and Medicine  
5 University of Melbourne

<120> H3 Relaxin

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Ala Val Ile Phe Thr Cys Gly Gly Ser Arg Trp

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5	65	70	75	80
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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU02/01338

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
Int. Cl. 7: A61K 38/22		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols) IPC 7		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched SEE DATABASES BELOW		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPAT, Medline: Keywords used - relaxin, prorelaxin, preprorelaxin, human, H3, disease, disorder, therapy, treatment, administration GenBank, EMBL, PDB Nucleic Acids, GenPept, TREMBL, Swiss-Prot, PIR - SEQ. ID. NOs.:1-10		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	BATHGATE, R.A. et al. Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene. The Journal of Biological Chemistry. 2002, January. Vol. 277, No. 2, pages 1148-1157. See the entire document, in particular Figure 1.	1-44
X	WO 01/68862 A1 (ZYMOGENETICS, INC.) 20 September 2001. See the entire document, in particular SEQ. ID. NOs:1 and 2; and pages 42 to 46.	1-44
A	GAVINO, E.S. and FURST, D.E. Recombinant relaxin: a review of pharmacology and potential therapeutic use. BioDrugs. 2001. Vol. 15, No. 9, pages 609-14. See entire document.	1-44
<input type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex		
<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>		
Date of the actual completion of the international search 10 December 2002		Date of mailing of the international search report 20 DEC 2002
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustalia.gov.au Facsimile No. (02) 6285 3929		Authorized officer  <b>JULIE CAIRNDUFF</b> Telephone No : (02) 6283 2545

## INTERNATIONAL SEARCH REPORT

### Information on patent family members

International application No.

**PCT/AU02/01338**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
WO	01/68862	AU	36817/01

END OF ANNEX

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