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Two human relaxin genes are on chromosome 9

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We have recently cloned two different human relaxin gene sequences. One of these (H1) was isolated from a human genomic clone bank and the other (H2) from a cDNA library prepared from human pregnant ovarian tissue. Southern gel analysis of the relaxin genes within the genomes of several unrelated individuals showed that all genomes contained both relaxin genes. Hence it is unlikely ($p < 0.001$) that the two relaxin gene sequences are alleles. Rather, it is probable that there are two relaxin genes within the human genome. It is likely that relaxin and insulin genes have evolved from a common ancestral gene by gene duplication, since structural similarities between insulin and relaxin are evident at both the peptide and gene level. To investigate the evolutionary relationship between the two human relaxin genes and the insulin gene, we have determined the chromosomal position of the relaxin genes using mouse/human cell hybrids. We found that the human insulin and relaxin genes are on different chromosomes. Both human relaxin genes are located on the short arm region of chromosome 9.

Key words: relaxin/gene/somatic cell hybrids/hybridisation

Introduction

Recently we isolated two different human relaxin gene sequences that code for peptides resembling rat and porcine relaxins in amino acid sequence. One of these (H1) was isolated from a human genomic clone bank (Hudson *et al.*, 1983) and the other (H2) from a cDNA clone bank prepared from human pregnant ovarian tissue (Hudson *et al.*, preceding paper). Extensive nucleotide homology within the 5'-flanking regions and within the known parts of the intron sequence (unpublished data) suggested that these two genes might be allelic. However, the predicted amino acid sequences of these relaxin peptides contained considerably more variation than might be expected if these two genes represented alleles.

The different positions of some restriction enzyme sites within the genes provided a method for distinguishing between them. Accordingly we analysed the relaxin genes of several unrelated individuals, using the Southern technique (Southern, 1975). The results of these experiments show that the two relaxin genes are indeed separate, non-allelic genes.

The relaxin peptide and its gene share structural features with those of insulin and the insulin-like growth factors suggesting that the genes coding for these peptides have evolved from a common ancestral gene by gene duplication. One of the proposed consequences immediately following gene duplication is the close linkage of two or more related gene sequences within the genome (for example, see Jeffreys *et al.*, 1980). Subsequent events can result in the separation of these linked genes, even to different chromosomes. We have determined the chromosomal location of these relaxin genes and report here that the relaxin genes and the insulin gene are on different chromosomes. Both human relaxin genes are located on the short arm region, just proximal to the centromere, of chromosome 9.

Results

The two human relaxin genes are non-allelic

If the separate relaxin sequences we have isolated were allelic we reasoned that homozygous individuals with only one type of relaxin gene would readily be detected within the population. Conversely if we could not detect individuals having only one type of relaxin gene sequence it is probable that the related relaxin gene sequences are separate genes. Accordingly we determined whether each of 10 unrelated individuals possessed either one or both of the relaxin genes. 10 μg of DNA from each individual was digested with *Eco*RI, and hybridised with a human relaxin cDNA probe encompassing the entire coding region of gene 2, using Southern gel analysis (Southern, 1975). This probe readily hybridises to both human relaxin genes, and the two genes are easily distinguished on the basis of their *Eco*RI restriction sites (Figure 1). The 2.25-kb and 1.95-kb *Eco*RI fragments correspond in size to the relaxin gene *Eco*RI fragments within the H1 gene (Hudson *et al.*, 1983). The other two hybridising fragments (7 kb and 5 kb in length, Figure 1) are consistent with the restriction fragments within the second relaxin gene clone.

It is clear that each of the individuals analysed in Figure 1 contains both relaxin genes – not one of the genomes contained just one of the relaxin genes. If we considered the two relaxin genes to be allelic, then each of the 10 individuals analysed (Figure 1) would be relaxin gene heterozygotes, and each of the relaxin genes would occur within the population with approximately equal frequency. It follows that the probability of randomly selecting only relaxin gene heterozygotes, and no homozygotes, would be less than $(1/2)^{10}$ (i.e., <0.001). This significantly low probability of selecting only relaxin gene heterozygotes, argues strongly against the hypothesis that the two related relaxin genes are allelic. Rather, the data (Figure 1) indicate that the two gene sequences code for separate relaxin related peptides, and that both of the genes are invariably present within the human genome.

The human relaxin genes are on chromosome 9

The chromosomal location of the human relaxin genes was determined by analysing the human relaxin gene content of

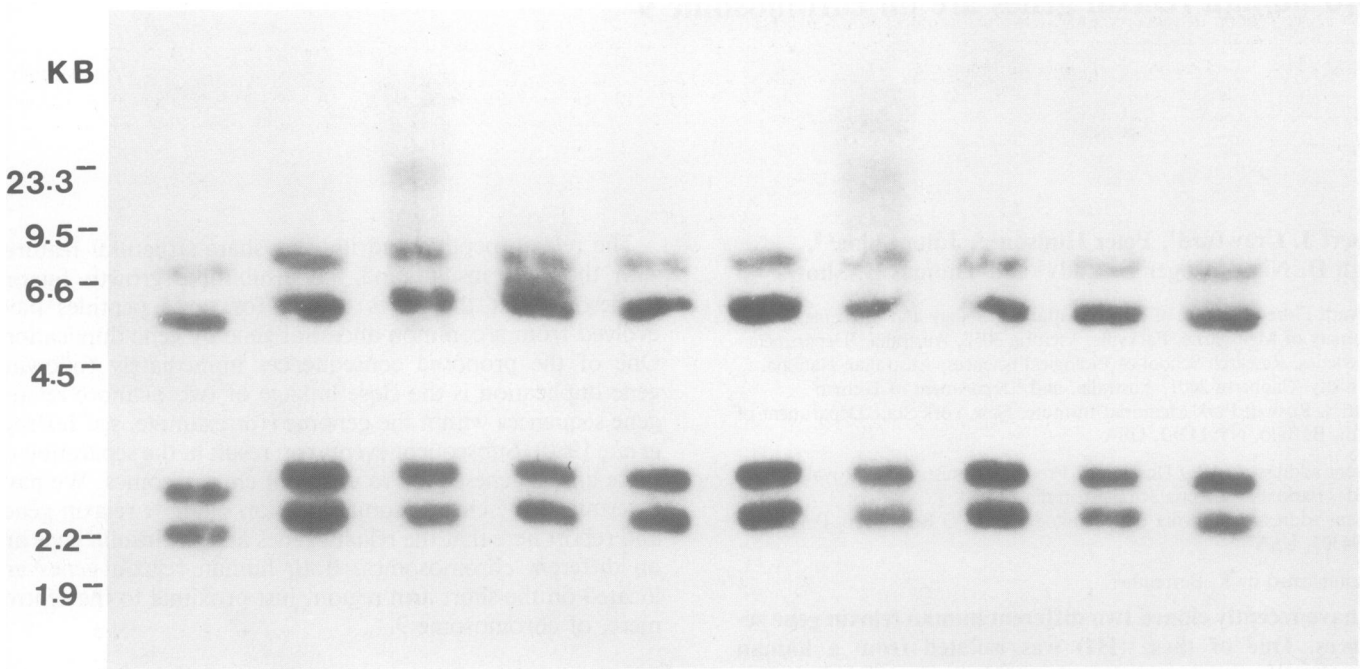

Fig. 1. Hybridisation of human relaxin cDNA probe to human DNA. DNA was isolated from leucocytes from 10 unrelated individuals, digested with *Eco*RI and electrophoresed in separate lanes on a 1% agarose gel. The DNA was transferred to nitrocellulose (Southern, 1975) and hybridised with ^{32}P-labelled human relaxin cDNA probe.

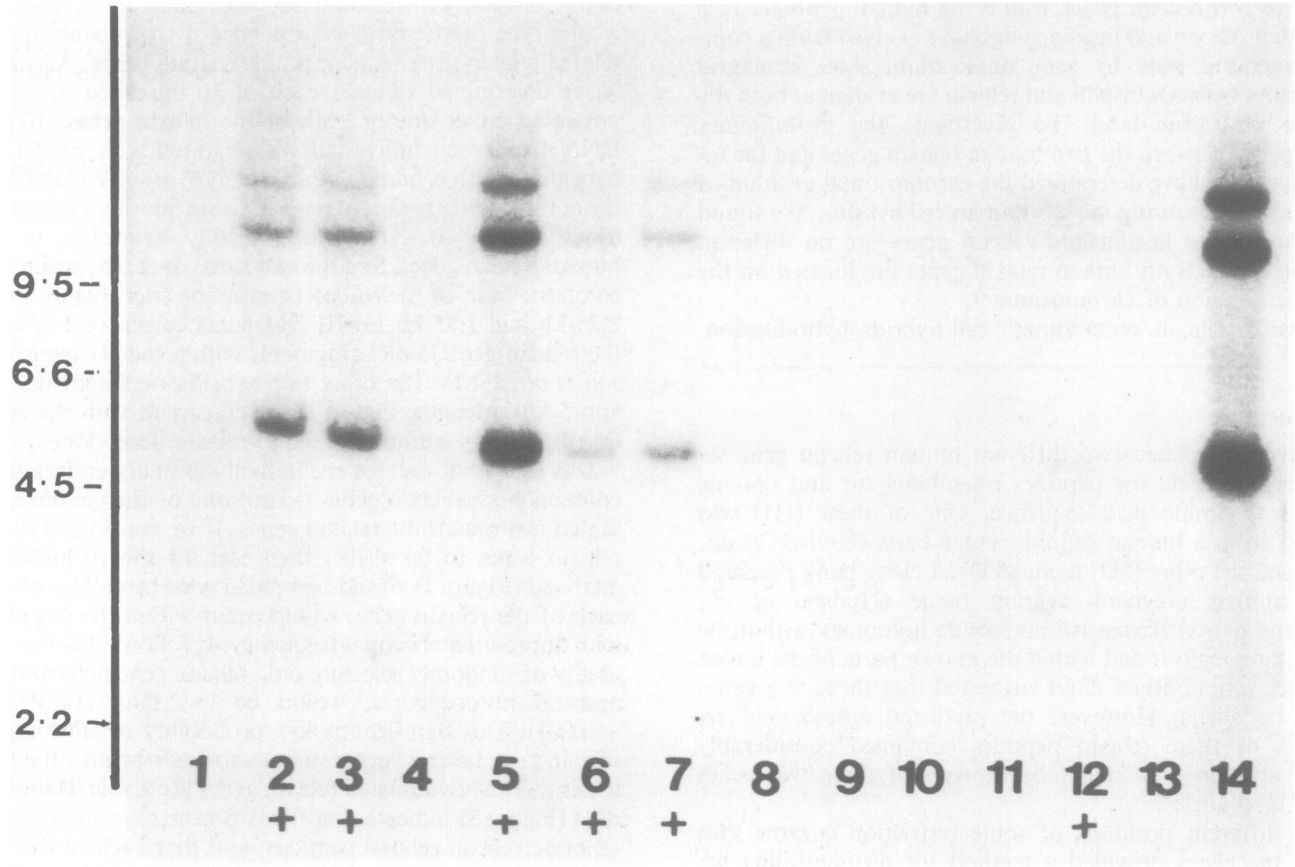

Fig. 2. Segregation of relaxin sequences in human-mouse cell hybrids. Mouse/human hybrid DNA was digested with *Hind*III and electrophoresed on a 0.8% agarose gel. Human relaxin gene sequences were detected by the Southern transfer technique (Southern, 1975). Human (MLD) and mouse (LM/TK$^-$) controls are in **lanes 14** and **13**, respectively. Relaxin-positive hybrids are in **lanes 2, 3, 5, 6, 7** and **12**. Relaxin-negative hybrids are in **lanes 1, 4, 8–11**.

Table I. Segregation of relaxin with human chromosomes in cell hybrids

Hybrid DNA	Relaxin	Human chromosomes																						Translocation chromosomes
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	
WIL-2	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	+	−	+	−	−	−	+	−	+	
WL-5	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	+	+	−	−	+	−	+	
WIL-6	−	−	+	−	+	+	+	+	+	−	+	+	−	−	+	−	−	+	−	+	+	+	−	+	
WIL-7	−	−	+	+	−	+	+	−	+	−	+	+	−	+	+	−	−	+	+	−	−	+	−	+	
WIL-8	−	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−	+	
WIL-8X	−	−	−	+	+	+	−	+	+	−	+	+	+	−	+	−	−	+	+	+	+	+	−	+	
WIL-13	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+	+	−	
WIL-15	−	−	+	+	+	−	−	+	−	−	+	+	+	+	+	−	−	+	+	−	+	+	−	+	
REW-7	−	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	
REW-11	−	−	−	−	+	−	−	−	−	−	−	+	+	+	−	−	+	−	−	−	+	+	−	+	
REW-15	−	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	
TSL-2	−	−	+	−	−	+	+	−	−	−	+	−	+	−	−	−	−	−	+	−	+	+	−	+	17/3
DUM-13	−	+	+	+	−	+	+	−	−	−	+	+	+	−	+	−	+	+	+	+	+	+	+	−	X/15 15/X
ATR-13	−	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+	+	−	−	−	−	5/X
XTR-22	−	−	+	−	+	+	+	−	+	−	+	+	−	−	−	+	−	−	+	+	+	+	+	−	X/3
XTR-3 BSAgB	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	5/X 10q$^-$
XTR-11 BSAgA	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	20p$^+$
REX-11 BSAgB	−	−	−	+	−	−	−	−	−	−	+	−	−	−	+	+	−	−	+	−	−	−	+	−	
JWR-26C	+	−	+	+	+	+	+	+	−	+	+	+	+	−	+	+	+	−	+	−	+	+	−	+	1p$^-$
JWR-22H	+	−	−	−	+	−	+	−	−	−*	+	+	−	−	+	+	−	+	+	−	+	+	−	−	2/1
% Discordancy		25	50	60	50	60	45	40	55	0	70	50	60	50	50	40	25	70	65	50	60	75	40	80	

Plus (+) and minus (−) indicate the presence or absence of relaxin, human chromosomes, and chromosome-specific isozyme markers; all analyses were determined on the same cell passage. The chromosome content of these cell hybrids may appear different in other publications since different cell passages of the cell hybrids were analysed.

*A normal chromosome 9 was not observed in this hybrid; however, the isozyme short arm marker *ACO1* was detected, indicating a rearrangement of this chromosome with the presence of chromosome 9 gene markers.

% Discordancy indicates the presence of relaxin but the absence of a specific chromosome or the reverse. No discordancy indicates the concordant segregation (+/+ or −/−) of relaxin and specific human chromosomes.

Translocation chromosomes are described in the publications cited in Materials and methods.

Table II. Distribution of relaxin in translocation cell hybrids

Hybrid	Relaxin	9	17/9	9/17	17	AK1	ACO1	GALK
NSL-5	−	−	+	−	−	+	−	+
NSL-9	−	−	+	−	+	+	−	+
NSL-15	−	−	−	−	+	−	−	+
NSL-16	+	−	+	+	+	+	+	+

NSL cell hybrids were constructed from the fusion of a human fibroblast line with a [46,XY,t(9;17)(q12;p11) reciprocal translocation and mouse LM/TK$^-$ cells (Shows *et al.*, 1982a). The 17/9 translocation contains the (17qter→17pll::9q12→9qter) rearrangement and the 9/17 translocation contains the (9pter→9q12::17p11→17pter) rearrangement. Adenylate kinase-1 (AK1) and aconitase-1 (ACO1) are chromosome 9 isozyme markers. Galactokinase (GALK) is a chromosome 17 isozyme marker.

human/mouse cell hybrids. Cell hybrids were prepared by fusing human fibroblast cell lines with mouse parental cells as previously described (Shows *et al.*, 1978, 1982b). DNA from each hybrid cell line was prepared and analysed for human relaxin genes by the Southern gel technique (Southern, 1975). The results of one of these experiments is shown in Figure 2. *Hind*III-digested human DNA (lane 14) contains a 13-kb relaxin gene 1 fragment (Hudson *et al.*, 1983) as well as relaxin gene 2 fragments of 9.5 and 4.5 kb. Under the hybridization conditions used, no cross-reaction between mouse genomic DNA and the human relaxin gene probe was detected (lane 13). Both human relaxin genes were always detected in hybrid cell lines retaining human relaxin sequences (lanes 2, 3, 5, 6, 7, Figure 2). Analysis of the results of several Southern gel experiments is shown in Tables I and II. The human chromosome content of each hybrid cell line listed in these tables was identified by both chromosome-specific isozyme markers and karyotyping. It is clear that the human relaxin genes co-segregate without exception, with chromosome 9 (Table I).

An additional 10 cell hybrids whose chromosome content was determined only by chromosome-specific isozyme markers were also analysed for human relaxin gene content. Four of these 10 cell hybrids were positive for human relaxin genes (for example see lanes 5, 6, 7, Figure 2) and for aconitase 1 (ACO1), a chromosome 9 short enzyme marker; the remaining six cell hybrids were negative for relaxin and ACO1. Hence experiments with these 10 cell hybrids further indicate that the human relaxin genes only co-segregate with chromosome 9.

The relaxin genes were regionally localised within chromosome 9 by using cell hybrids prepared from a human fibroblast cell line (GM 2836) with a reciprocal translocation involving chromosomes 9 and 17. This translocation involved a breakage within chromosome 9 at 9q12 and within chromosome 17 at 17p11, resulting in chromosomes 9/17 (9pter→

9q12:17p11→17pter) and 17/9 (17qter→17p11:9q12→9qter) (Shows *et al.*, 1982a). The 9/17 and 17/9 chromosomes were identified in cell hybrids by chromosome and isozyme marker analysis as previously described (Shows *et al.*, 1982a).

DNA from informative cell hybrids was analysed for human relaxin gene content by the Southern gel technique (Southern, 1975), and the results are shown in Table II and Figure 2. The human relaxin genes co-segregate with the translocation chromosome 9/17 – the relaxin genes are contained within the cell hybrid NSL-16 which includes chromosome 9/17, yet were not detected within the cell hybrids lacking chromosome 9/17. Hence, the human relaxin genes are located on the 9pter→9q12 region of chromosome 9.

The results of experiments with the JWR-22H cell hybrid (Figure 2, lane 3 and Table I) are consistent with the localisation of the human relaxin genes on the 9pter→9q12 region of chromosome 9. This cell hybrid contains no normal human chromosome 9, but does contain the human ACO1 gene located in the short arm region 9p22→9p13 (Owerbach *et al.*, 1980). This finding indicates that human chromosome 9 has been altered, and is not recognizable by karyotyping. Hence it is possible that the human relaxin genes are encoded close to ACO1, on the short arm of chromosome 9.

Discussion

One of the conclusions from this work is that the human genome contains two non-allelic relaxin genes. In this sense the human genome differs from the pig, rat and mouse genomes, which have only one relaxin coding gene (unpublished data). Hence the need for two non-allelic relaxin genes does not appear to be universal among mammals.

The discovery of two human relaxin genes introduces several new possibilities about relaxin gene expression, and relaxin activity. Whereas human relaxin gene H2 is clearly transcribed within pregnant ovaries, no ovarian expression of gene H1 has been detected (Hudson *et al.*, preceding paper). We do not yet know whether one or both relaxin genes are specifically expressed in other tissues, including the prostate (Essig *et al.*, 1982) and placenta (Bryant-Greenwood, 1982). It is also possible that relaxin gene H1 is non-functional.

The human relaxin gene locus shares one feature with the human growth hormone locus (Seeburg, 1982) and the β chorionic gonadotropin locus (Talmadge *et al.*, 1984) – each of these human loci contain more genes than expected on the basis of known protein products. It is also worth noting that the additional genes within the relaxin gene locus and the growth hormone locus, have been found only within the human genome. For example, additional genes are not present in these gene systems of the rat (Barta *et al.*, 1981). It is not known why genes additional to those present in other mammals, have evolved in these hormone gene loci in humans.

Relaxin, insulin and the insulin-like growth factors are each derived from a single chain prepropeptide with the same basic organisation: signal peptide/B chain/C chain/A chain (Chan *et al.*, 1976). Furthermore the biologically active peptides each consist of an A and B chain linked by one intra- and two inter-disulphide bonds located at homologous positions (Chan *et al.*, 1976). There is also limited sequence homology between insulin and relaxin, particularly within residues surrounding the disulphide linkages. Similarities between relaxin and insulin are also apparent at the gene level. For example, an intron interrupts the C peptide coding sequence at a similar position within both genes (Hudson *et al.*, 1983; Bell *et al.*, 1980).

These similarities suggest that insulin, the insulin-like growth factors and relaxin evolved from a common ancestral gene by gene duplication. Hence the insulin and relaxin-like gene sequences were presumably closely linked on the same chromosome immediately following the gene duplication event. As we have shown, however, both human relaxin genes are located on the 9pter→9q12 region of chromosome 9. This region of chromosome 9 also contains the interferon gene cluster and the aconitase 1 gene (Shows *et al.*, 1982a), although the significance of these linkages is not clear. The human insulin gene has previously been located on the short arm of chromosome 11 (Owerbach *et al.*, 1980, 1981). Hence the genes coding for insulin and relaxin are unlinked in humans. The separation of these genes may have occurred by a chromosomal translocation, involving a breakpoint between the insulin-like and relaxin-related genes. Subsequently two human relaxin genes evolved, probably by a second gene duplication event. This second duplication apparently occurred after the evolutionary divergence of pigs, rat and mouse genomes from that of human, since only a single relaxin gene is found in all mammals so far investigated, other than humans. It remains to be determined whether other primate genomes contain two relaxin genes.

Materials and methods

Cell hybrids and genetic analysis

Human-mouse cell hybrids were prepared and isolated as described (Shows, 1972). Specific human-mouse cell hybrids making up the chromosome assignment panel (Table I) were previously described and analyzed for their chromosome content (Shows, 1972; Shows and Brown, 1975; Lalley *et al.*, 1976, 1977; Shows *et al.*, 1979; Sakaguchi and Shows, 1982; Naylor *et al.*, 1982). The cell hybrids used for regionally assigning the relaxin genes to chromosome 9 (Table II) segregate reciprocal 9/17 translocation chromosomes as previously described (Shows *et al.*, 1982a). Chromosome analysis of the cell hybrids and testing of chromosome specific isozyme markers were described previously (Shows *et al.*, 1978, 1982b).

DNA preparation

Human genomic DNA (a gift from Dr. Matthews), was prepared from leucocytes collected from 10 unrelated individuals. Cell hybrid DNA was prepared as previously described (Wigler *et al.*, 1979).

Analysis of the relaxin genes

About 10 μg of each DNA sample was digested with *Hind*III or *Eco*RI in buffers recommended by the suppliers, and electrophoresis was carried out in 0.8% agarose gels in Tris-acetate buffer pH 8.1. The DNA was transferred to nitrocellulose by the Southern procedure (Southern, 1975).

Hybridisation and subsequent washing of the filters was carried out as previously described (Owerbach *et al.*, 1980). The relaxin gene probe was isolated from a cDNA library prepared from human pregnant ovarian tissue (Hudson *et al.*, preceding paper). The probe encompassing the entire coding region of the relaxin mRNA, was nick-translated with [α-^{32}P]dATP and [α-^{32}P]dCTP, to a specific activity of $>10^8$ c.p.m./μg (Rigby *et al.*, 1977).

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