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Second gene for gonadotropin-releasing hormone in humans

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ABSTRACT Gonadotropin-releasing hormone (GnRH) is a decapeptide widely known for its role in regulating reproduction by serving as a signal from the hypothalamus to pituitary gonadotropes. In addition to hypothalamic GnRH (GnRH-I), a second GnRH form (pGln-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly; GnRH-II) with unknown function has been localized to the midbrain of many vertebrates. We show here that a gene encoding GnRH-II is expressed in humans and is located on chromosome 20p13, distinct from the GnRH-I gene that is on 8p21-p11.2. The GnRH-II genomic and mRNA structures parallel those of GnRH-I. However, in contrast to GnRH-I, GnRH-II is expressed at significantly higher levels outside the brain (up to 30%), particularly in the kidney, bone marrow, and prostate. The widespread expression of GnRH-II suggests it may have multiple functions. Molecular phylogenetic analysis shows that this second gene is likely the result of a duplication before the appearance of vertebrates, and predicts the existence of a third GnRH form in humans and other vertebrates.

MATERIALS AND METHODS

Library Screen. A 270-nt partial cDNA for the putative human GnRH-II was cloned from human thalamus poly(A) RNA (CLONTECH) by using reverse transcription–PCR (RT-PCR) and 3'-RACE (rapid amplification of cDNA ends) as described (1). Oligomers flanking putative intron B were used to screen a human genomic P1 artificial chromosome (PAC) library (Genome Systems, St. Louis) by using PCR, and a single PAC clone of ≈100 kb containing the entire gene for GnRH-II was identified.

Sequence Analysis. Two overlapping PAC subclones spanning 4,498 bp, from 1.3 kb upstream to 1.1 kb downstream of the GnRH-II gene, were sequenced on both strands. Exons were predicted by using GRAIL (Oak Ridge National Laboratory Informatics Group), FGENEH, HSPL (V. Solovyev, Baylor College of Medicine), and NNSSP (M. Reese, Lawrence Berkeley National Laboratory), and by comparison with our cDNA information. Promoter predictions were made with GRAIL and NNPP (M. Reese, Lawrence Berkeley National Laboratory).

Fluorescence in Situ Hybridization Mapping. The PAC clone was labeled with digoxigenin-dUTP by nick translation. Labeled probe was combined with sheared human DNA and hybridized to normal metaphase chromosomes in a solution containing 50% formamide, 10% dextran sulfate and 2× standard saline citrate (SSC; 1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7). A biotin-labeled probe specific for the centromere of chromosome 20 (D20Z1) was cohybridized with the PAC probe, and 71 of 80 metaphase cells analyzed exhibited specific labeling.

RNA Blot Hybridizations. A dot blot of human RNA (MasterBlot, CLONTECH) was used to quantify expression levels of GnRH-II. The blot consisted of poly(A) RNAs from 50 human tissues, with loadings of 80–448 ng per dot, normalized for eight housekeeping genes (7, 8), along with appropriate positive and negative controls. Samples on the blot were taken from whole brain, amygdala, caudate nucleus, cerebellum, cerebral cortex, frontal lobe, hippocampus, medulla oblongata, occipital lob, putamen, substantia nigra, temporal lobe, thalamus, subthalamic nucleus, spinal cord, heart, aorta, skeletal muscle, colon, bladder, uterus, prostate, stomach, testis, ovary, pancreas, pituitary gland, adrenal gland, thyroid gland, salivary gland, mammary gland, kidney, liver, small intestine, spleen, thymus, peripheral leukocyte, lymph node, bone marrow, tonsil, lung, trachea, placenta, fetal brain, fetal heart, fetal kidney, fetal liver, fetal spleen, fetal thymus, fetal lung, yeast total RNA, yeast tRNA, Escherichia coli rRNA, E. coli DNA, poly r(A), human cDNA, and human DNA. Hybridization was overnight at 65°C in ExpressHyb (CLONTECH) using a GnRH-II cDNA probe and final

Abbreviations: GnRH, gonadotropin-releasing hormone; RT-PCR, reverse transcription–PCR; PAC, P1 artificial chromosome; GAP, GnRH-associated peptide.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF036329 and AF036330).

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RESULTS AND DISCUSSION

To discover whether humans have a second GnRH form, we used RT-PCR on human thalamic mRNA and obtained a partial cDNA encoding a putative second GnRH. This cDNA was used to design oligonucleotide primers to screen a human genomic PAC library with PCR. A single clone was isolated and subsequent sequence analysis confirmed that it contained the complete gene encoding GnRH-II. We then used fluorescence in situ hybridization to map GnRH-II unambiguously to the p terminus of chromosome 20, corresponding to band 20p13, different from GnRH-I, which is located at 8p21-p11.2 (14). Additionally, the human GnRH-II gene is remarkably short (2.1 kb) compared with GnRH-I (5.1 kb) (15) (Fig. 1), which is the largest human GnRH gene (16) have four exons, as do GnRH genes in other species (2), and the predicted preprohormone encoded by human GnRH-II is organized identically to that of other GnRHs: all have a signal sequence, followed by a GnRH decapeptide, a conserved proteolytic site (Gly-Lys-Arg), and a GAP. Corresponding preprohormone elements of the two human GnRHs are quite similar in length, with the striking exception that GAP is 50% longer in GnRH-II than in GnRH-I (84 vs. 56 amino acids). A similar disparity in GAP was recently reported (8). Surprisingly, human GnRH-II mRNA expression is highest outside the brain. Of 50 tissues tested, expression levels in the kidney were ~30-fold higher than in any brain region, and

![Diagram of human GnRH-II gene](image)

**Fig. 1.** Organization of the human GnRH-II gene compared with that of human GnRH-I (16). Note that only exonic regions are drawn to scale. Intron sizes in bp are indicated. GnRH-II exon 1 size is based on predicted location of the most proximate promoter.
expression in bone marrow and prostate was ∼4-fold greater than in the brain. GnRH-I expression was not observed at a high level outside the brain. Within the brain, GnRH-II expression is most obvious in the caudate nucleus but can also be seen in the hippocampus and amygdala (Fig. 2a). Hybridization of the same blot with a probe specific for human GnRH-I showed a wholly different pattern (Fig. 2b), confirming that cross-hybridization cannot account for these results. Further confirmation was obtained by using RT-PCR to amplify GnRH-II mRNA in three tissues (thalamus, fetal brain, and kidney). Each yielded a cDNA fragment of the predicted size, and Southern blot analysis established these as human GnRH-II RNA products (Fig. 3). The blot revealed the existence of a GnRH-II cDNA splice variant extended at the 5'-end of exon 3, such that the predicted length of GAP is increased from 77 to 84 amino acids. This longer GnRH-II transcript was found in both fetal brain and adult thalamus, but not in adult kidney, implying tissue-specific processing of GnRH-II transcripts.

Our discovery of a GnRH-II gene in humans, in combination with recent GnRH-II cDNA data for tree shrew (R.B.W., T.L.K., S. White, and R.D.F., unpublished data), led us to examine the evolution of GnRH peptides. Molecular phylogenetic reconstruction was used to infer the evolutionary relationship among GnRH forms (Fig. 4). The methods of maximum parsimony, maximum likelihood, and neighbor-joining were used to test the robustness of our analysis, and each method showed essentially the same branching patterns. The phylogenetic tree shows the existence of three evolutionarily distinct GnRH groups: “releasing” forms localized to the hypothalamus (GnRH-I), forms previously localized solely to midbrain nuclei (GnRH-II), and forms localized to the telencephalon in several fish species (GnRH-III).

Several lines of evidence support the conclusion that the three GnRH groups identified by this phylogenetic comparison comprise evolutionarily distinct forms. First, the groupings correspond to GnRH forms with distinct expression patterns and thus presumed biological roles. This suggests the groupings can be used to predict the activities of yet uncharacterized forms. Second, although only the neighbor-joining tree is shown, the same groupings were found by using other distance-based methods as well as maximum parsimony and maximum likelihood (not shown). Third, high bootstrap values were generated for the neighbor-joining and parsimony trees, strongly supporting identification of these groups. Finally, the branch lengths to each group are relatively long, suggesting an ancient divergence. Furthermore, the branching order within each GnRH group matches the evolutionary branching order of the species (see in particular GnRH-I, for which sequences were available from diverse species).

Although these phylogenetic relationships cannot identify the selective forces that generated multiple GnRH forms, the analysis can suggest when distinct forms arose. The structure of this phylogenetic tree strongly suggests that multiple forms exist in many different species because of ancient duplications of a gene encoding GnRH. Moreover, it seems likely that different forms of GnRH diverged from each other before the divergence of species represented in the tree. Because the tree is unrooted, the exact order of the duplications in unclear. However, GnRH-I and GnRH-II each include representatives from fish and mammals, so the separation of these two groups must have occurred before the separation of mammalian and fish ancestors. The origin of GnRH-III is less clear, because there are only representatives from fish in this group, but two alternative hypotheses are suggested by our data. First, it is possible that the gene duplication leading to GnRH-III occurred only recently, within the fish lineage, and thus is restricted to this taxon. Alternatively, GnRH-III may have resulted from an ancient duplication, and has either been lost in higher vertebrates during evolution or has not yet been discovered. We favor the latter hypothesis because if the duplication were recent, the GnRH-III forms would be expected to cluster with one of the other GnRH forms in fish. Perhaps another form of GnRH remains to be found in humans.

Among the GnRH deceptides, only GnRH-II is identical in all species in which it has been found. This suggests that the
peptide has been subject to extremely stringent selective pressures. Such selection might have arisen if GnRH-II serves different functions at different loci in the body, as supported by several examples. First, GnRH-II has been shown to be present in the sympathetic ganglia of amphibia (17) where it has been shown that GnRH can act as a neuromodulator in spinal cord ganglion neurons in amphibia (3). In fish, neurons shown previously to control sperm duct and oviduct contractility receive input from cells that show GnRH-like immunoreactivity (5). Moreover, in newts, immunoreactivity to GnRH-II shifts from midbrain cell bodies to terminal regions following the initiation of courtship (4). Examples of possible GnRH-II expression outside the midbrain cells include transient GnRH immunoreactivity within mast cells in the habenula of ring doves following courtship (18, 19). Similarly, mast cells in musk shrew pup brains (6) are immunoreactive for GnRH by using an antibody shown to be specific to GnRH-II. These data have led to the suggestion that mast cells represent an alternative delivery system for GnRH (18). Indeed, several types of differentiated lymphocytes including spleenocytes, thymocytes, peripheral T- and B-lymphocytes, and mast cells (20) have been demonstrated to produce GnRH or a GnRH-like peptide. Because kidney, bone marrow, and prostate contain significant numbers of mast cells (21–23), perhaps these cells account for the high GnRH-II expression there. Furthermore, although it is not known whether different forms of GnRH might have different receptor types, GnRH receptors have been found throughout the body, including the kidney (24) and prostate (25). Because previous studies have shown that GnRH receptors bind GnRH-II peptide up to 100 times more effectively than GnRH-I (26), GnRH-II could act through these receptors outside the brain. Perhaps GnRH-II has multiple functions as suggested by its presence in diverse loci.

Has GnRH-II remained unchanged because of selective pressure from multiple functions? Perhaps GnRH-II originated in the immune system and only later acquired a neuromodulatory function in the brain. Additional characterization of GnRH-II should help clarify whether it serves distinct functions in the body and what selective forces are responsible for its remarkable conservation over evolutionary time.

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