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Regulation of Growth Hormone-Releasing Hormone Gene Expression and Biosynthesis

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Growth hormone-releasing hormone (GRH) was initially isolated, characterized, sequenced, and cloned from human tumors and subsequently from the hypothalamus of humans and other animal species. Extensive structure-function studies have indicated the amino terminus to be most important for its biologic action, and the primary mechanism of its bioinactivation occurs by cleavage of an amino terminal dipeptide. The GRH gene is expressed primarily in the hypothalamic arcuate nucleus but also in the placenta. Expression of the GRH gene is regulated by growth hormone in a classical feedback manner, with hypophysectomy leading to increased expression that is reversed by growth hormone treatment. GRH gene overexpression in transgenic mice leads to a syndrome similar to that of ectopic GRH secretion with massive pituitary hyperplasia and markedly enhanced growth. The transgenic mouse has been used for studies of GRH biosynthesis and provides a suitable model for the study of precursor processing to the mature hormone.

ISOLATION AND CHARACTERIZATION OF GRH

The concept of a growth hormone (GH)-releasing hormone (GRH) was first proposed in the early 1960s and evidence for its existence appeared later in the same decade (see [1] for review). Although GH-releasing activity in hypothalamic tissue extracts was demonstrated by several laboratories [2,3], isolation of GRH from the hypothalamus proved to be a formidable task. The isolation and sequencing of GRH first occurred as a result of the clinical recognition that a GH-releasing factor was being secreted by extra-pituitary tumors associated with acromegaly [4,5]. Identification of two such tumors (both of pancreatic islet origin) with high levels of activity led to the definitive characterization of GRH as a 44 amino acid peptide in 1982 [6,7]. Three forms of GRH were isolated from the tumors ($GRH_{1-44}NH_2$, $GRH_{1-40}OH$, and $GRH_{1-37}OH$) with $GRH_{1-40}OH$ actually being the most abundant form. Shortly thereafter, GRH was identified in human hypothalamus [8] and its sequence was shown to be identical to that found in the human tumors [9]. Both the 1–40 and 1–44 forms of the hormone were shown to exist in human hypothalamus. Based on the structure of GRH, oligonucleotide probes were constructed, the cDNA for human GRH was reported by two different groups [10,11], and, shortly thereafter, the entire GRH gene cloned [12].

The human genome contains only a single GRH gene with a length of approximately

Abbreviations: GH: growth hormone GRH: growth hormone-releasing hormone

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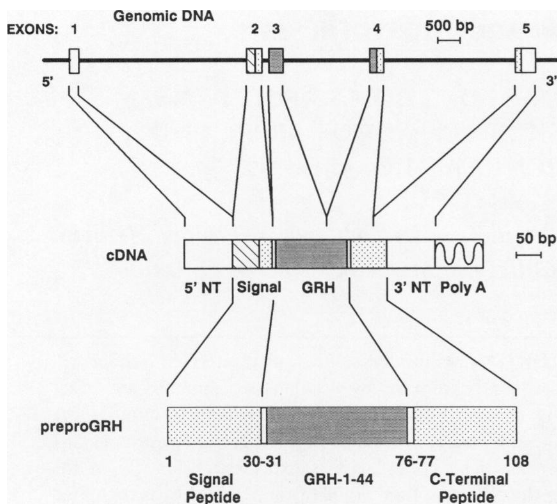

FIG. 1. Schematic representations of the human GRH gene, cDNA, and precursor peptide. The coding sequence for the mature peptide is contained in exons 3 and 4, while the signal peptide and C terminal peptide extension are contained in exons 2 and 4–5, respectively.

10 kilobases (Fig. 1). The gene contains five exons resulting in a mRNA species, including the polyadenylation signal, of about 750 bases. Two nearly identical mRNAs have been reported, which differ only in a slight alteration of the splice site of the fifth exon, resulting in the absence of a single serine near the 3′ end of the coding region. The mRNA codes for a precursor peptide of 107 (or 108) amino acids, consisting of a characteristic signal peptide, the 44 amino acid GRH, and a 30 (or 31) amino acid C terminal peptide. The last two splice sites occur in the N terminal half of GRH and in the middle of the C terminal peptide and explain some of the species variation observed in primary sequence, particularly in rodents.

The structure of GRH has now been reported in several other species [1] and varies only minimally from the human hormone in most of the larger animals studied. In contrast, rat GRH, the second species in which the cDNA sequence has been published, exhibits considerable dissimilarity with human GRH (only 68 percent homology). Even more extenisve differences occur in the C terminal peptide (deduced from the structure of rat GRH mRNA) [13] where the carboxy terminal half of the peptide is completely dissimilar, a change explained by an alteration in the splice site of the fifth exon. It was anticipated that mouse GRH would resemble rat GRH more closely that human GRH, since rat GRH exhibits greater GH-releasing potency (60 percent) than does human GRH in primary cultures of mouse pituitaries [1]. Recent data indicates that the sequence of mouse GRH exhibits comparable homology with human and rat GRH, though comparison of its C terminal peptide suggests a greater evolutionary association with rat than with human GRH [14].

The biologic activity of GRH appears to be centered around the amino terminal region of the peptide, and the presence of tyrosine or histidine in position one is essential for receptor binding [9,15]. In contrast, the C terminal third of the molecule is not essential and $GRH_{1-29}NH_2$ exhibits full biologic potency *in vivo* and *in vitro.* This pattern is thus similar to that of ACTH, where full biologic activity is present in the first 24 of the peptide's 39 amino acid residues. As with ACTH, species variability is greater in the carboxy than the amino terminal region. Current attempts to develop superanalogs are therefore based on using $GRH_{1-29}NH_2$ as the parent compound. In a single study, the biologic activity of the C terminal peptide has been examined and

shown to alter food intake after central nervous system administration in the rat [16]. It is not known, however, whether this observation has physiologic relevance.

GRH METABOLISM

Detailed studies of the metabolism of human GRH *in vivo* and *in vitro* [17–19] have shown that the major mechanism for enzymatic destruction occurs by cleavage of the amino terminal dipeptide by a plasma enzyme, dipeptidylpeptidase, type IV. This enzymatic conversion occurs rapidly; the first and second components of the plasma disappearance rate for GRH are 1.0 and 6.8 minutes, respectively [18]. The product, $GRH_{3-44}NH_2$, is biologically inactive, thus ensuring that GRH action is limited to a single pass phenomenon. This degradation process is also of importance in the metabolism of exogenously injected GRH, and the structural modification of GRH to create dipeptidylpeptidase resistance has been a critical requirement in the development of superactive GRH analogs [19]. GRH contains several sites of potential cleavage by trypsin-like endopeptidases and evidence for a GRH_{12-44} fragment has also been reported [19].

GRH GENE EXPRESSION

The development of sensitive methods for measurement of GRH mRNA in extracts of hypothalamus has been followed by the accumulation of data concerning the regulation of GRH gene expression. The anatomic sites of GRH gene expression were predicted by previous immunocytochemical studies with anti-GRH serum [20,21]. To date, GRH mRNA has been identified only in the arcuate nucleus of the hypothalamus and in the placenta ([22]; [Downs TR, Chomczynski P, Frohman LA: unpublished observations]). A considerably larger (1.9 kb) mRNA species that hybridizes to the rat GRH mRNA probe has been reported in rat testis [23], though the GRH peptide has not been detected in this organ and the significance of this finding remains to be determined.

Target organ hormone (GH) removal by hypophysectomy results in a marked increase in hypothalamic GRH mRNA levels in the rat [22,24]. This change is seen within three days of hypophysectomy and persists for at least six weeks (Fig. 2). There is a concomitant decrease in GRH content in the hypothalamus, a phenomenon that is only partially explained by the slight increase in GRH release observed *in vitro* [24,25]. The possibility of an impairment in post-transcriptional control has been suggested [24], though it remains to be proven. A parallel set of findings has recently been observed with preproTRH [26]. Adrenalectomy and gonadectomy, alone or in combination, do not result in any changes in hypothalamic GRH mRNA levels [24]. Treatment of hypophysectomized animals with GH alone or in combination with other target organ hormones decreases hypothalamic GRH mRNA levels and increases GRH peptide content, supporting the role of GH in the regulation of GRH gene expression. It is not presently known whether this effect of GH occurs directly or is mediated by other factors, such as IGF-1. The extent of pituitary GH depletion that must occur in order to eliminate its inhibitory effect on GRH gene expression is not precisely known, though it is believed to be at least 75 percent.

Thyroid hormone deficiency also increases hypothalamic GRH mRNA levels and decreases GRH peptide content to levels that are as pronounced as after hypophysectomy, though the changes require from one to two weeks post-thyroidectomy to become evident and are maximal only after three to four weeks [27]. The effects are mediated

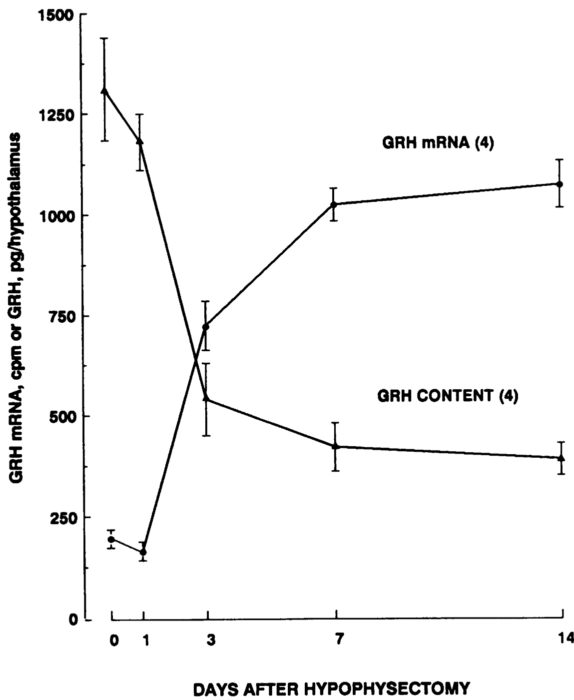

FIG. 2. Changes in hypothalamic GRH mRNA and GRH peptide content at varying times after hypophysectomy in rats. Shown are the mean ± S.E.M. The number of animals is shown in parentheses. Reprinted from Chomczynski et al.[24]

through depletion of pituitary GH rather than by a direct effect on the hypothalamus, since treatment with GH, even in the presence of continued hypothyroidism, markedly decreases the elevated levels of GRH mRNA and increases the levels of GRH peptide content.

TRANSGENIC hGRH MOUSE

A model of GRH overproduction has been developed using the transgenic approach in mice, in which a fusion gene (Fig. 3) consisting of the mouse metallothionein I promoter linked to a human GRH "minigene" containing GRH exons 2–5 as well as the second intron and the 3′ non-coding region [28]. Transgenic mice exhibit markedly elevated GH levels, a growth rate of up to twice normal, and massive pituitary

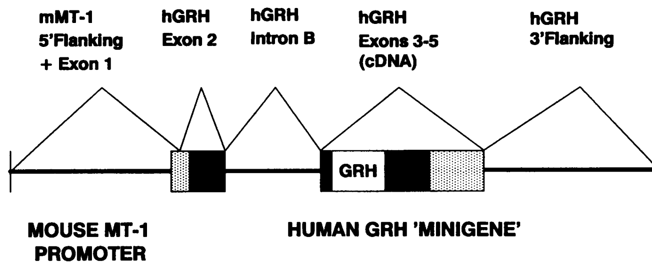

FIG. 3. Schematic representation of the mouse metallothionein I-human GRH fusion gene used for the production of GRH transgenic mice. The mouse promoter was fused to a GRH "minigene" in which a portion of the GRH gene was replaced by a GRH cDNA that eliminated two introns. Modified from Hammer et al.[28]

enlargement due to somatotroph hyperplasia. The mice are fertile and exhibit germline gene expression, resulting in transmission of the gene as a simple Mendelian dominant. Identification of hGRH mRNA was initially reported by Northern blotting in liver, intestine, pancreas, kidney, and spleen. By use of more sensitive radioimmunoassay, however, we have observed GRH immunoreactivity, as well, in pituitary, brain, adrenal, gonads, heart, lung, and skeletal muscle [29]. By means of standard immunohistochemistry and double label immunofluorescence, GRH has been identified in four separate cell types of the anterior pituitary (somatotrophs, lactotrophs, gonadotrophs, and thyrotrophs), two cell types in the pancreatic islets (glucagon-containing A cells and somatostatin-containing D cells), Brunner's glands of small intestine, proximal convoluted tubules of the kidney, adrenal medulla, atrial cardiocytes, peribronchiolar epithelium, testicular Leydig cells, and oocytes [30]. In the brain, hGRH was identified in neuronal perikarya of the arcuate nucleus, where the hormone would normally be expected to occur, extensively concentrated in the outer layer of the median eminence, in the supraoptic nuclei, the paraventricular region, and the amygdala. Although some of the tissues in which GRH is expressed also express the metallothionein gene [31,32], some of the selectivity appears unique to GRH and suggests that tissue-specific regulatory elements are present within the second intron, the 3′ end of the message, or possibly the coding region itself.

The widespread distribution of GRH suggests several possibilities by which the releasing hormone could produce GH hypersecretion and somatotroph hyperplasia: autocrine, paracrine, neuroendocrine, and endocrine. The specific contributions of each possibility remain to be evaluated, though some clarification has been provided by initial studies of the molecular heterogeneity of GRH in specific tissues. Using two separate radioimmunoassay systems to measure GRH, one of which detects the mid-portion of the molecule, and also carboxy terminally extended molecules (including the GRH precursor), and the other of which requires an amino terminus in the 44 position (and thus should not recognize the precursor), we have examined GRH heterogeneity using multiple chromatography systems, including gel filtration and reverse-phase high-performance liquid chromatography. Initial studies indicate that the GRH precursor is present in multiple tissues and that the pituitary, pancreas, and brain all appear capable of processing the precursor to the mature hormone, though with varying degrees of effectiveness. Primary cultures of transgenic mouse pancreas secrete multiple forms of immunoreactive GRH, and preliminary studies of GRH biosynthesis using ^{35}S-methionine incorporation indicate the presence of label in the precursor form and $GRH_{1-40}OH$, and possibly other forms as well. In contrast, the liver is able to process the precursor partially, though incompletely. Nearly all GRH immunoreactivity in plasma is in the form of $GRH_{3-44}NH_2$, indicating that the mouse, like the rat and human, has an active dipeptidylpeptidase system and, despite the extremely high levels of total GRH immunoreactivity, very little of it is in a biologically active form. This finding suggests that the contribution of circulating hormone to the overall development of GH overproduction is small. The source of the hormone in plasma is still unknown and other transgenic models are now being examined to provide more information.

SUMMARY

In the short period of time since the first identification of GRH in human tumors, its structural forms have been identified and its gene has been cloned in several species, its structural-functional relationships have been defined, its metabolism has been charac-

terized, and considerable information has been generated concerning its gene regulation. A model of GRH overproduction has been developed with similarities to the disease that led to the isolation of GRH and which has permitted for the first time studies of GRH biosynthesis. During the next few years, equally exciting data can be expected concerning neuroendocrine mechanisms involved in the regulation of its gene expression, leading to enhanced understanding of its role in the control of growth hormone secretion. In particular, the potential role of GRH in the pathogenesis of dwarfism in both laboratory animals and man and in various states of GH overproduction may be clarified and the development of agonist and antagonist analogs of the hormone may provide new approaches to therapy of disorders of GRH under- and overproduction.

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