Detection of Growth Hormone and Growth Hormone-Releasing Hormone-Related Messenger RNA in Rat Leukocytes by the Polymerase Chain Reaction (43302)

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Abstract. **To validate that growth hormone (GH) and growth hormone-releasing hormone (GHRH) can be produced by leukocytes, we have assessed the presence of GH and GHRH-related mRNA in leukocyte cultures by reverse transcription and the polymerase chain reaction. A sample of the polymerase chain reactions were size-fractionated by electrophoresis in a 0.8% agarose gel and examined with ultraviolet light after ethidium bromide staining. Single major DNA bands corresponding in length to the distance between the 5′ ends of the two GH and GHRH specific primers, 603 base pairs and 260 base pairs, respectively, were obtained. The DNA bands hybridized specifically to GH- and GHRH-specific probes after Southern transfer to nitrocellulose. The identity of the GH polymerase chain reaction material was confirmed by restriction enzyme analysis. The results showed that GH and GHRH gene expression occurs in mononuclear leukocytes and support the idea that these neuroendocrine hormones may be common signal molecules between the immune and neuroendocrine systems.** [P.S.E.B.M. 1991, Vol 198]

Lymphocytes are currently under extensive investigation because of their ability to produce and secrete neuroendocrine hormones (1). This is supported in part by findings showing the synthesis and secretion by immune cells of corticotropin and endorphins (2), thyrotropin (3), follicle-stimulating hormone, luteinizing hormone (4), growth hormone (GH; 5), luteinizing hormone-releasing hormone (6), and growth hormone-releasing hormone (GHRH; 7). The leukocyte-derived neuroendocrine hormones have been observed to be identical to their pituitary and hypothalamic counterparts in terms of bioactivity, antigenicity, and molecular weight. More recently, lymphocyte-derived pro-opiomelanocortin mRNA has been cloned and sequenced (8, 9). The nucleotide and amino acid sequence of corticotropin in murine lymphocytes was shown to be identical to that of mouse pituitary corticotropin. Interestingly, the results show that lymphocytes basally transcribe at least two pro-opiomelanocortin transcripts that are up-regulated by corticotropin-releasing factor. These two transcripts lack Exons 1 and 2, but contain either part or all of Exon 3 (10). The overall evidence indicates that neuroendocrine-immune interactions may occur via chemical messengers and their receptors that are common to both neuroendocrine and immune cells (11).

In our previous work, we have reported that leukocytes spontaneously produce and secrete immunoreactive GH and GHRH molecules that are similar to their pituitary and hypothalamic counterparts, respectively. Similarity was evidenced by both immunologic and biological techniques (5, 7). In the present report, the objective was to study leukocyte GH and GHRH mRNA expression to determine whether leukocyte processing of these transcripts was identical to pituitary and hypothalamus processing of these transcripts. We used reverse transcription and polymerase chain reaction (PCR) to obtain GH and GHRH leukocyte cDNA. These cDNA molecules were then characterized by Southern analysis and restriction endonuclease digestion. The results of these experiments showed that the transcripts for GH and GHRH in leukocytes are structurally similar to their pituitary and hypothalamic counterparts, respectively.

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Materials and Methods

Cell Preparations. Adult (150–200 g) male Sprague-Dawley rats were obtained from Harlan Sprague Dawley (Prattville, AL). Following sacrifice, lymphoid tissues, including the spleen and thymus, were prepared as single cell suspensions. The cells were suspended at a final concentration of 10^7 cells per ml in RPMI supplemented with 2% fetal bovine serum plus penicillin, streptomycin, garamycin, and mycostatin (100 units/ml). The cells were cultured for 24 hr *in vitro* at 37°C before they were harvested by centrifugation and frozen for RNA isolation. Rat pituitaries and hypothalamuses were carefully removed and washed in phosphate-buffered saline and quickly frozen, and the RNA was isolated, as described below.

RNA Isolation. Total RNA was prepared by homogenizing leukocytes in 5 *M* guanidine thiocyanate, 1% sarkosyl, 20 m*M* EDTA, 1% 2-mercaptoethanol, and 50 m*M* Tris-HCl (pH 7.5), with subsequent protease K digestion and extraction with phenol and chloroform. Polyadenylated mRNA was isolated on oligo(dT)-cellulose, as described previously (12). Southern gel analysis (12) was performed on 0.8% agarose gels and followed by transfer to nitrocellulose paper. Nitrocellulose paper was hybridized with GH and GHRH probes, to be described.

GH and GHRH cDNA and Hybridization. A rat pituitary GH cDNA was kindly provided by Drs. John Baxter and Fran Denoto (Neurochemistry Laboratories, Veterans Administration Medical Center, Sepulveda, CA; 13). An 800-base pair *Hin*dIII GH cDNA insert was obtained from this plasmid by standard techniques (10, 12), and it was labeled with [^{32}P]dCTP by nick translation (Bethesda Research Laboratories, Rockville, MD) to a sp act of 1–2 × 10^8 cpm/μg. A GHRH oligodeoxynucleotide probe corresponding to amino acids 13–27 (48 bases; 5′-CCTGTTCATGATTTCGTGCAGCAGTTTGCGGGCATATAATTGGCCCAG-3′) was designed and synthesized in our laboratory. The GHRH probe was end-labeled with T4 polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN) using standard procedures (10). Nitrocellulose filters from Southern analysis were prehybridized for 4 hr at 42°C and hybridized for 18 hr at 42°C, according to the procedure of Maniatis *et al.* (12). After hybridization, the membranes were washed by standard techniques until the radioactivity in the final wash was close to background. The nitrocellulose papers were exposed to Kodak AR film at −70°C with Dupont Cronex Lightning-Plus intensifying screens for 2 to 3 days.

Reverse Transcription and Amplification by PCR. First-strand cDNA synthesis was performed using a commercially available reagent kit (Amersham) that is based on the procedure of Okayama and Berg (14). Target sequences from this cDNA were amplified in a PCR using the antisense primer (corresponding to amino acids 180–187) (1 μ*M*) and a sense primer (corresponding to amino acids 4–11) (1 μ*M*) by standard procedures, as described (15). The primers were prepared and purified in our laboratory. PCR reactions were performed in a Perkin Elmer DNA thermal cycler. Generally, reactions were performed in a total volume of 0.1 ml containing 200 μ*M* of each dNTP, 1 μ*M* of each primer, 1–10 ng of template DNA, and 2.5 units of Taq polymerase. The final reaction mixture was overlaid with 0.1 ml of mineral oil (Perkin Elmer) to prevent evaporation. A usual cycle consists of 1 min at 94°C (denaturation), 2 min at 52°C (annealing of primer), and 3 min at 72°C (extension). Twenty-five to 40 cycles (7 min total/cycle) were usually run over a 3- to 5-hr period. Excess primers were separated from amplified DNA on a Sephadex G50 spin column (DuPont). Amplified samples were run on a 2% agarose gel and stained with ethidium bromide to determine efficiency and size. A control template and primers derived from bacteriophage-λ which define a 500-base pair target were always run to ensure reliability of the procedure. To confirm that the GH DNA was specifically amplified, a Southern transfer was performed, as described previously (12). The membrane was hybridized to the radiolabeled GH-specific cDNA probe, as described above.

Restriction Endonuclease Digestion. Restriction enzymes and appropriate 10× buffers were obtained from Boehringer Mannheim. A sample of the GH and GHRH cDNA molecules obtained by reverse transcriptase and PCR was ethanol-precipitated, redissolved in 5 μl of water, and incubated for 1 hr at 37°C with *Hpa*II (5 units) or *Hha*II (5 units) (10 μl final volume). Restriction endonuclease digestion reactions, along with an untreated sample and size standards, were then fractionated by analytical agarose gel electrophoresis, stained, and examined, as described above.

Results

Our previous investigations have shown the presence of GH- and GHRH-related protein and mRNA molecules expressed in rat spleen and thymus cells. To confirm these findings and more completely characterize the RNA molecules, we selectively enriched the concentration of GH- and GHRH-specific nucleotide sequences in leukocytes by reverse transcription and PCR. In the case of GH, the mRNA was reverse transcribed and then amplified by PCR using two oligodeoxynucleotide primers specific for rat pituitary GH (Fig. 1). RNA was used as a template for selective first-strand cDNA synthesis with an antisense oligonucleotide primer complementary to the 3′ end of Exon 5 of GH mRNA. When this purified cDNA was introduced as a template into a PCR with a second sense oligonu-

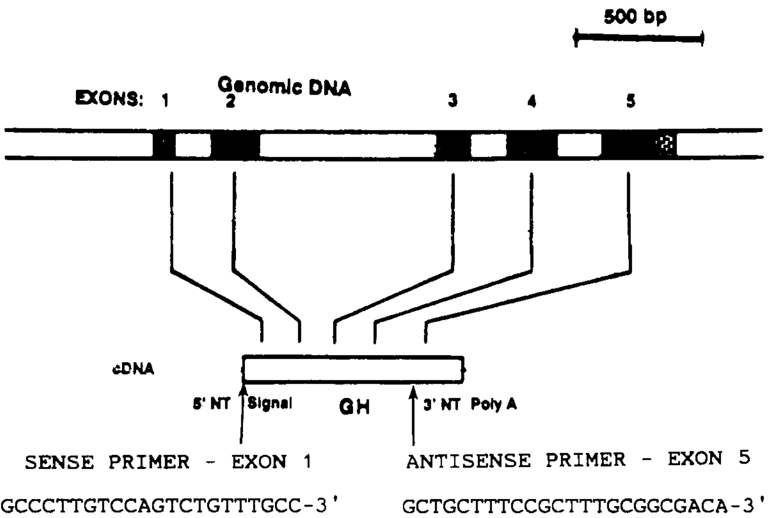

Figure 1. Diagram of rat GH with sequences that are the basis for oligodeoxynucleotide primers used for first-strand cDNA and PCR.

cleotide primer representing the GH sequence, 10 bases 5′ to the GH-containing region of Exon 1, there was amplification of the appropriate-size (603 base pair) target cDNA (Fig. 2). Confirmation that the amplification product was related to pituitary GH was provided by Southern analysis (Fig. 3). The potential problem of amplifying genomic DNA is considered unlikely, since we have used oligothymidine-purified RNA and primers from two different exons for the first-strand synthesis and PCR, respectively.

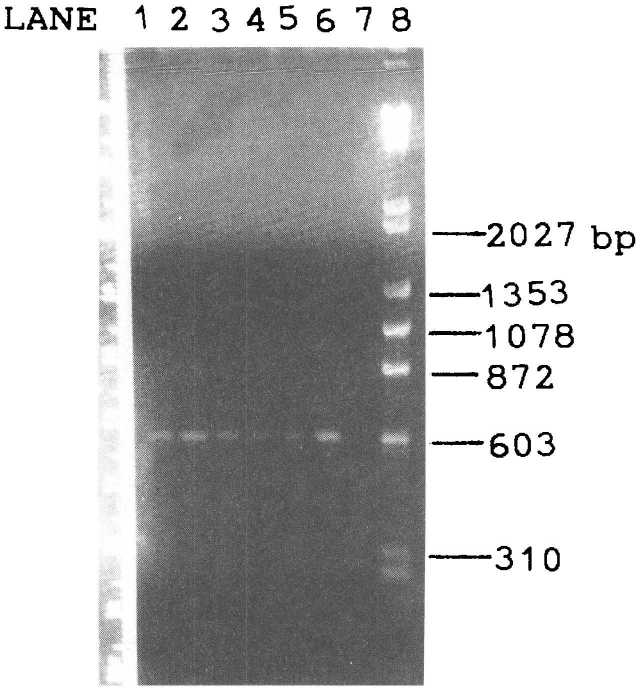

Figure 2. Detection of rat GH RNA in leukocytes by reverse transcription and PCR. PCR and electrophoresis were carried out as described in Materials and Methods. Lane 1, spleen (10 μl); Lane 2, spleen (5 μl); Lane 3, thymus (10 μl); Lane 4, thymus (5 μl); Lane 5, GH_3 pituitary tumor cells (10 μl); Lane 6, rat pituitary (10 μl); Lane 7, blank; and Lane 8, molecular weight standards.

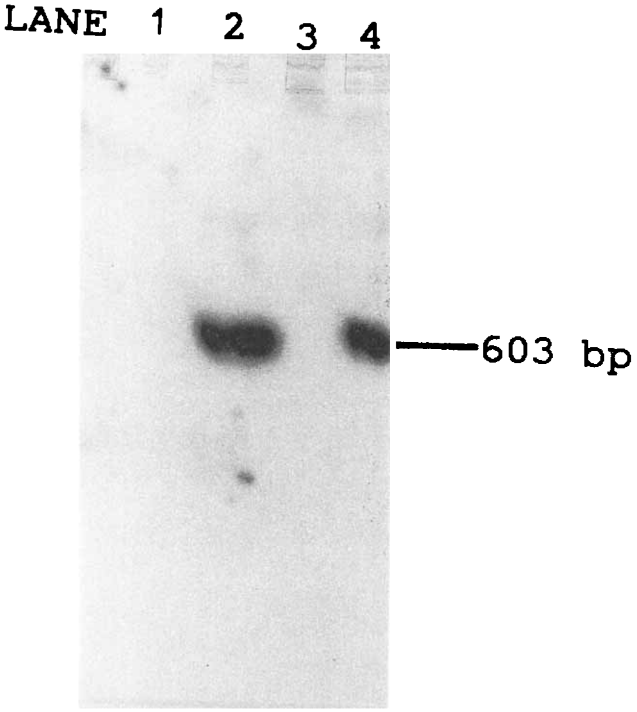

Figure 3. Detection of rat GH RNA in leukocytes by Southern gel transfer. The transfer and probing methods are described in Materials and Methods. Lane 1, molecular weight standards; Lane 2, pituitary; Lane 3, hypothalamus; and Lane 4, spleen.

In a similar series of experiments, we have been able to selectively amplify from leukocytes a predicted-size cDNA fragment of 262 base pairs for GHRH that is similar to the material amplified from the hypothalamus (Figs. 4 and 5). The presence of minor bands could possibly arise as a result of a low stringency-annealing temperature. Taken together, Southern

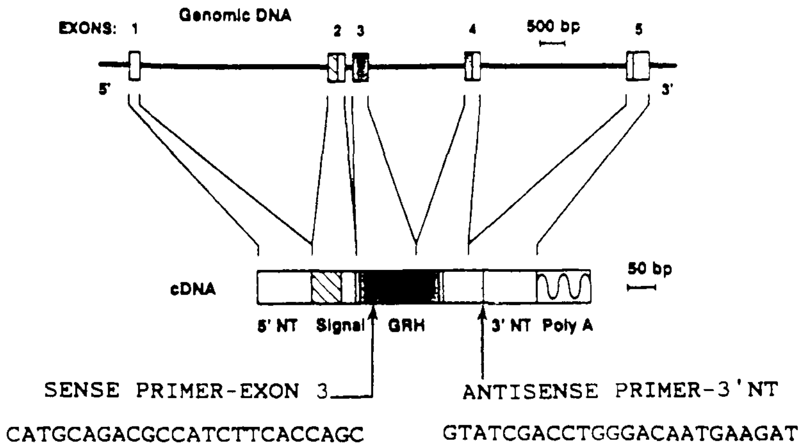

Figure 4. Diagram of rat GHRH with sequences that are the basis for oligonucleotide primers used for first-strand cDNA and PCR.

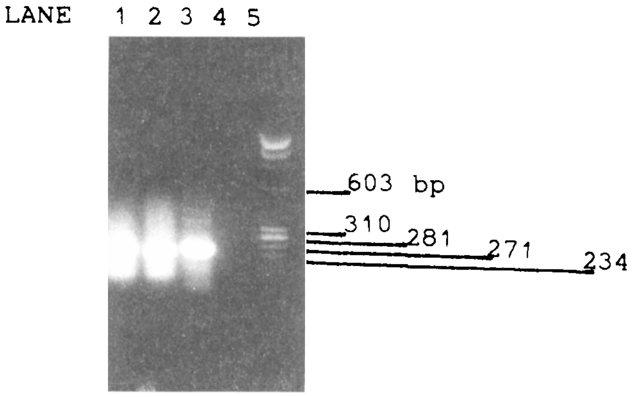

Figure 5. Detection of rat GHRH RNA in leukocytes by reverse transcription and PCR. PCR and electrophoresis were carried out as described in Materials and Methods. Lane 1, spleen (10 μl); Lane 2, thymus (10 μl); Lane 3, rat hypothalamus (10 μl); Lane 4, blank; and Lane 5, molecular weight standards.

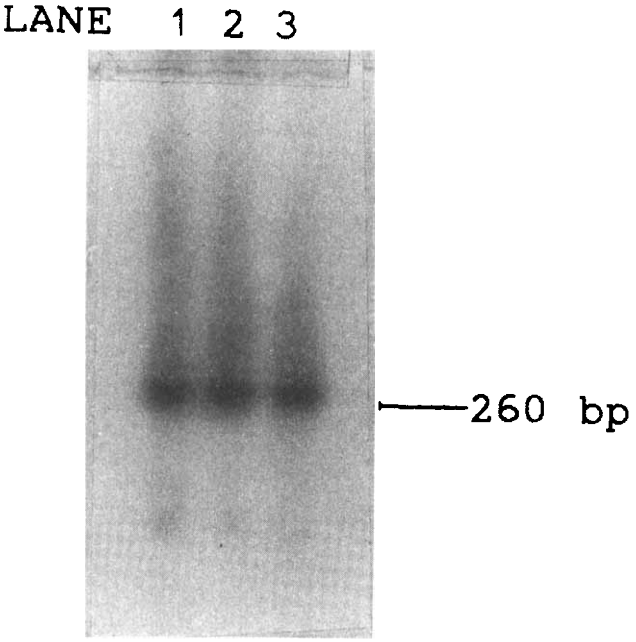

Figure 6. Detection of rat GHRH RNA in leukocytes by Southern gel transfer. The transfer and probing methods are described in Materials and Methods. Lane 1, spleen; Lane 2, thymus; and Lane 3, hypothalamus.

analysis of this amplified cDNA showed that it is related to GHRH, since it hybridized to the ^{32}P-labeled oligodeoxynucleotide GHRH probe (Fig. 6).

Restriction enzyme analysis of reverse transcriptase and PCR products obtained using GH primers. If the leukocyte cDNA generated using GH primers originated from GH mRNA, it should contain one *Hpa*II restriction site, cleavage from which would yield two fragments of approximately 200 and 350 base pairs (13). Similarly, restriction endonuclease digestion of GH cDNA with *Hha*I should yield three fragments of approximately 150, 200, and 300 base pairs in length (13). The results (Fig. 7) show that when *HPa*II and *Hha*I digests of cDNA from leukocytes and pituitary cells were compared, the patterns of bands appeared to be remarkably similar. In particular, *Hpa*II digestion of leukocyte and pituitary cDNA molecules yielded two major fragments of 380 and 245 base pairs and a minor fragment of 225 base pairs. *Hha*I digestion yielded two major fragments of 315 and 200 base pairs and two minor ethidium bromide-staining fragments of 250 and 220 base pairs. The presence of minor bands may be a result of altered restriction enzyme activity due to the presence of contaminants or less-than-optimal restriction reaction conditions, resulting in less specific recognition sequence specificity. Although several minor bands can be observed, the leukocyte cDNA restriction pattern is similar to the pituitary PCR restriction pattern. The results are in close agreement and indicate that the transcripts of leukocyte GH and GHRH are essentially similar to those expressed in the pituitary and hypothalamus, respectively.

Discussion

Previously, we have detected the presence of GH and GHRH-like molecules in leukocytes with immu-

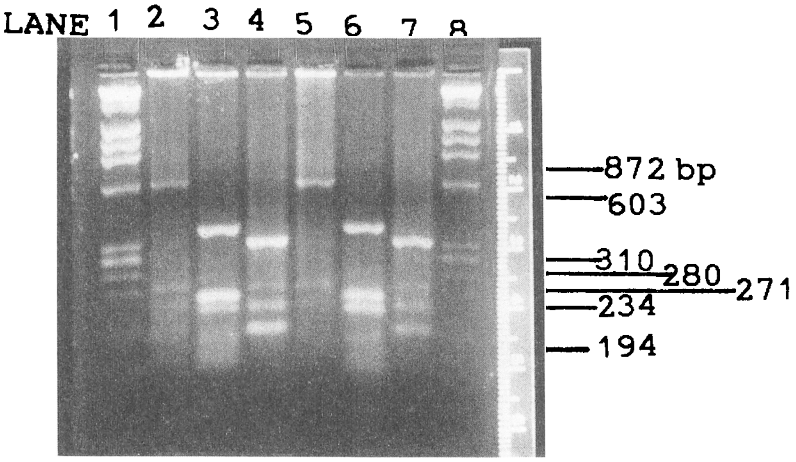

Figure 7. Restriction enzyme analysis of PCR products obtained from rat spleen and pituitary cells. Lanes 1 and 8, DNA size standards; Lane 2, uncut pituitary PCR products; Lane 5, uncut spleen PCR product; Lanes 3 and 6, *Hpa*II (5 units) digest of pituitary and spleen, respectively; Lanes 4 and 7, *Hha*I (5 units) digest of pituitary and spleen, respectively.

nological and biological characteristics similar to GH and GHRH molecules found in the neuroendocrine system (5, 7). In these studies, mRNA was detectable by Northern analysis; however, the chromatograms showed rather diffuse bands. The use of reverse transcriptase PCR has high sensitivity and is capable of detecting low numbers of transcripts from cells. This sensitivity makes it particularly suitable to studies of neuroendocrine hormones in leukocytes in which the amount of message appears to be low. The data reported here clearly demonstrate the expression of GH and GHRH- related RNA in the rat spleen and thymus. In other studies, we have obtained transcripts of similar size for GH and GHRH in purified T cell, B cell, and macrophage populations, as well as in the rat YB2 established cell line (16). The idea that genomic DNA was amplified rather than RNA is considered to be highly unlikely. This is due to the fact that oligothymidine-purified RNA was used in the synthesis of first-strand cDNA and the primers for PCR were designed from two different exons. Although the data we have obtained strongly support the idea that the neuroendocrine hormones derived from leukocytes are the same as their neuroendocrine counterparts, the exact proof awaits the cloning and sequencing of the appropriate cDNA molecules.

The effect of GH and GHRH on the immune system is being extensively investigated (17). Proliferative responses of both transformed (18) and normal (19, 20) lymphoid cells are greater when treated with GH *in vitro*. GH affects the functional activity of cytolytic cells (21) and natural killer cells (22) and has been shown to be as potent as γ-interferon in priming macrophages for the production of superoxide anion (23). Recently, we have shown that leukocytes can be induced to produce GH RNA *in vivo* in rats 6 hr after injection of bacterial lipopolysaccharide (24). In another study, we examined the role of GH in leukocyte proliferation by examining the effect of an antisense oligodeoxynucleotide complementary to GH mRNA. The results of these studies showed that antisense GH oligodeoxynucleotide treatment inhibits leukocyte production of immunoreactive GH and that antisense GH oligodeoxynucleotide-mediated inhibition of immunoreactive GH production resulted in a decrease in leukocyte proliferation. The data indicate that immunoreactive GH produced by leukocytes can stimulate proliferation, suggesting that GH may play an autocrine-paracrine role in leukocyte replication (25). All of these reports considered together strongly support a physiological role for GH in immunoregulation. Several reports indicate that GHRH may also be involved in immunomodulation. Thus, GHRH has been shown to stimulate lymphocyte proliferation (26), inhibit natural killer cell activity (27), and inhibit the chemotactic response (28). Our own studies have identified a specific GHRH receptor on immune cells, as well as measured an increase in Ca^{2+} uptake, thymidine incorporation, and the levels of GH RNA after treatment of leukocytes with GHRH (29, 30). In view of the low concentrations of GHRH found in the hypothalamus, it is highly debatable whether GHRH secreted from the hypothalamus is functional in peripheral plasma. Therefore, for GHRH to function in the periphery at leukocyte GHRH receptors (K_d ~2 nM), it must be produced at alternate sites. Our data (7), along with that of others (31–36), show the extra hypothalamic production of GHRH. Since we know that leukocytes can function as a source of GH (5), this suggests the possibility that GHRH synthesis by leukocytes may function as a signal for the synthesis of leukocyte-derived GH. The finding that leukocyte-derived GHRH is active on pituitary cells (7) and that hypothalamic GHRH is active on leukocytes (30) strongly supports a functional basis for bidirectional communication.

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