Growth Hormone Induces Two mRNA Species of the Serine Protease Inhibitor Gene Family in Rat Liver*

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In order to study the molecular actions of growth hormone on gene expression, we have cloned and characterized two unique, but related, cDNA sequences from rat liver, λSpi-1 and λSpi-2. These two cDNA sequences are complementary to rat hepatic mRNA species previously designated as Spots 3 and 20 when assayed by in vitro translation and two-dimensional gel electrophoresis. By Northern blot, the two mRNAs are both 1900 bases in length and growth hormone administered to hypophysectomized rats increases the levels of both of these mRNAs. In contrast, the combined administration of thyroxine, corticosterone, and dihydrotestosterone to hypophysectomized rats did not augment these mRNAs. The simultaneous administration of all four hormones resulted in a level greater than that observed for animals treated with growth hormone alone. Analysis of genomic DNA suggests the presence of two similar, but not identical, genes. DNA sequencing of λSpi-1 and λSpi-2 revealed that they were 90% homologous at the nucleotide level and 87% homologous at the amino acid sequence level. λSpi-2 has 78% homology with mouse contrapsin, 60% with human α1-antichymotrypsin, and 51–55% with α1-antitrypsins, all members of the serine protease inhibitor gene family. The nucleotide and deduced amino acid sequences of λSpi-1 and λSpi-2 which align with the reactive centers of known members of this family differ substantially from each other and from other members of the family. The difference in the reactive center suggests that the specificity or function of these proteins may differ from other members of serine protease inhibitor gene family.

A major mechanism of growth hormone action in the liver occurs through modulation of the mass of specific mRNA species. This mechanism of growth hormone action was initially identified for the regulation of the rat urinary protein, α2-globulin (1, 2). The observations on α2-globulin were confirmed and extended with the observation that ovine growth hormone injected into thyroidecimized rats caused significant changes in seven hepatic mRNAs (3). Subsequent work provided evidence that three of these products (arbi-

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The abbreviations used are: GH, growth hormone; Pipes, 1,4-piperazinediethanesulfonic acid; bp, base pairs; Spi, serine protease inhibitor.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EMBL Data Bank with accession numbers J02692.

The mechanism by which growth hormone regulates specific gene expression is unknown. In the case of α2-globulin mRNA, recent evidence suggests that regulation of transcription is an important mechanism by which growth hormone augments the expression of this product (7). It is a matter of speculation whether this example is representative of all of the gene products regulated by growth hormone. Furthermore, there is no evidence which demonstrates that induction of α2-globulin mRNA is the result of a direct and primary action of growth hormone on the liver. To extend our knowledge about growth hormone action, we sought cDNA clones for growth hormone-responsive hepatic gene products which could be used as tools to investigate growth hormone action. We describe the cloning of two cDNAs complementary to the growth hormone-responsive hepatic mRNA species which code for Spots 3 and 20. The nucleotide and deduced amino acid sequences have remarkable homology to the serine protease inhibitor gene family.

MATERIALS AND METHODS

Male rats were used in all experiments. Hypophysectomy was performed by the supplier (Taconic Farms) between 100 and 120 g (approximate age of 30 days). These animals were observed for 3 weeks to confirm growth failure. The dose of hormones injected per 100 g of body weight were methionyl-human growth hormone (GH)1 (supplied by Genentech Inc.), 100 μg; thyroxine, 1.5 μg; corticosterone, 1 mg; and dihydrotestosterone, 200 μg. All hormones were given once daily for 10 days. Bacteriostatic water was the vehicle for growth hormone, 0.1 N NaOH for thyroxine and for corticosterone and dihydrotestosterone as previously described (8). A rat hepatic cDNA library (9) constructed in pBR322 was screened by differential hybridization. Plasmid DNA was isolated from transformants by the alkaline extraction procedure (10). Equivalent amounts of DNA were fixed to duplicate nitrocellulose filters by the method of Bresser and Gillespie (11). One filter was hybridized to 32P-labeled, single strand cDNA synthesized from poly(A)+ RNA isolated from a hypophysectomized rat treated with thyroxine, corticosterone, and dihydrotestosterone (three hormones) and the other filter was hybridized with 32P-labeled cDNA synthesized from poly(A)+ RNA obtained from a three hormone plus GH-treated hypophysectomized rat. At the same time both filters were hybridized to equal amounts of nick-translated [3H]-labeled pBR322 DNA to

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determine the relative quantity of plasmid DNA bound to the filters. Following hybridization and washing of the papers, the radioactivity was determined by autoradiography, followed by liquid scintillation counting. Two clones of interest, pGH1 and pGH4, yielded differential $^{32}P/H$ values from the duplicate filters, suggesting that they contained cDNA sequences for growth hormone-responsive mRNA species. To obtain longer cDNAs, two additional rat liver cDNA libraries were screened. p407 was obtained by screening a cDNA library (12) with nick-translated pGH1 and pGH4 inserts using in situ colony hybridization (13). LP1 and LP2 were obtained by screening a λgt11 cDNA library (14) with nick-translated p407 insert using in situ plaque hybridization (15).

Total RNA and poly(A)$^+$ RNA were isolated from liver as previously described (16, 17). For Northern analysis, RNA samples (10 μg) were treated with formaldehyde and subjected to electrophoresis on a 1.5% agarose, 2.2 M formaldehyde gel (18). RNA was transferred to nitrocellulose paper (19) and hybridized to nick-translated $^{32}P$-labeled plasmid DNA (20) or pGH1 or pGH4 insert (20).

For hybrid select translation, plasmid DNA was prepared by the alkaline extraction procedure and equilibrium centrifugation in an ethidium bromide-cesium chloride gradient (21). Plasmid DNA was linearized with a suitable restriction enzyme and 10 μg was fixed onto a 25-mm$^2$ piece of nitrocellulose (22). Hybrid selection was carried out by a modification of the method of Rickenard et al. (23). Poly(A)$^+$ RNA (15 μg) was hybridized to the filters in a buffer containing 50% deionized formamide, 20 mM Pipes (pH 6.4), 0.4 M NaCl, 0.1% sodium dodecyl sulfate, 1 mM EDTA, and 1 mg/ml yeast tRNA for 20 h at 42 °C. Washing was performed by resuspending the filter in 0.8 ml of hybridization buffer at 60 °C for 5 min and repeated 5 times. Hybridized RNA was eluted in 0.1 ml of 10 mM Tris-HCl (pH 7.6), 0.1 mM EDTA at 95 °C for 1 min and precipitated with ethanol in the presence of 20 μg of calf liver tRNA/mL. RNA samples were translated in vitro and analyzed by two-dimensional gel electrophoresis (5).

A series of overlapping clones was produced for use in DNA sequencing as described by Dale et al. (24). Sequencing was carried out by the Sanger chain termination method (25) and α-[35S]-thio-dATP was used as label. Sequence data was analyzed using software from Intelligenetics.

**RESULTS AND DISCUSSION**

The differential screening of a rat hepatic cDNA library resulted in the identification of several clones which contained inserts for growth hormone-responsive mRNA. Of these, two cDNAs, designated pGH1 (300 bp) and pGH4 (160 bp), were selected for further study on the basis of a rapid induction of the complementary mRNA species with growth hormone (see below). The polypeptides encoded by the mRNA complementary to pGH1 and pGH4 were determined by hybrid select translation (Fig. 1). Two polypeptide products (Spots 3 and 20), both previously demonstrated to be growth hormone responsive, were selected by pGH1, whereas pGH4 selected only Spot 3. The two products selected by pGH1 suggested that two mRNA species which had regions of sequence homology were expressed. The fact that pGH4 only selected one of the mRNA species suggested that it hybridized to a region of less homology between the two mRNA species.

Further analysis of the expressed mRNA by Northern blot using either pGH1 or pGH4 revealed a single hybridizing species of 1900 bases (Fig. 2), suggesting that the mRNA species were the same size. This band was essentially undetectable in hepatic RNA isolated from hypophysectomized rats and from hypophysectomized rats treated with thyroxine, corticosterone, and dihydrotestosterone. In contrast, growth hormone-treated hypophysectomized animals revealed an increased quantity of these RNAs, and animals treated with all four hormones demonstrated an even greater signal. The synergistic interaction of the four hormones in the expression of the product(s) was similar to the hormonal requirements observed when Spot 3 and Spot 20 mRNAs were measured by an in vitro translational assay (26).

The time course of induction of these two mRNA was relatively rapid with a 5-fold increase observed 4 h following growth hormone administration (Table I). These changes parallel or precede changes in hepatic or serum insulin-like growth factor I (27), suggesting that their induction is not mediated through insulin-like growth factor I.

**FIG. 1. In vitro translational products of mRNA selected by pGH1 or pGH4.** Autoradiograms are shown of two-dimensional electrophoretograms of in vitro synthesized labeled translational products obtained from the rabbit reticulocyte lysate translational assay when directed by total poly(A)$^+$ RNA (Panel A), by mRNA selected by pGH1 (Panel B), by mRNA selected by pGH4 (Panel C), and by mRNA nonspecifically selected by the cloning vector pBR322 (Panel D). Hybrid selected translation was performed as described under "Materials and Methods." The location of Spots 3 and 20 are indicated. The location of the reticulocyte lysate protein (RLP), a mRNA-independent labeled product, is shown and permitted the precise alignment of the radiograms. This experiment was repeated once with identical results. SDS, sodium dodecyl sulfate.
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Fig. 2. Size and hormonal regulation of mRNA complementary to pGH1 and pGH4. Northern blot of 10 μg of total RNA obtained from normal animals (N), hypophysectomized animals (H), or hypophysectomized animals treated with methionyl-hGH (+H), hypophysectomized animals treated with a three hormone combination of thyroxine, corticosterone and 5α-dihydrotestosterone (+3), and hypophysectomized animals treated with the three hormone combination plus methionyl-hGH (+4) is shown. Doses of hormones and schedule of injections are described under “Materials and Methods.” Northern blot was performed as described under “Materials and Methods” and the filter hybridized to nick-translated 32P-labeled markers of 28 S and 18 S are shown and were determined by ethidium bromide staining of parallel lanes of the same gel.

To further our understanding of the interrelationship between these two cDNA, a λgt11 cDNA library was screened. This resulted in the identification of two additional cDNA, λSpi-1 and λSpi-2, of 1377 and 1661 bp, respectively. Both pGH1 and pGH4 were capable of hybridizing to these two longer cDNA clones. Endonuclease restriction mapping indicated that these two cDNA were not identical, although they shared several restriction sites (data not shown). To obtain more detailed information on the structure of these cDNA, the nucleotide sequences of λSpi-1 and λSpi-2 were determined. Comparison of the sequence of these two cDNA is shown in Fig. 3. Approximately 90% of the nucleotides matched between the two sequences with extensive homology for the first 920 overlapping nucleotides at the 5‘ end and the last 340 nucleotides at the 3‘ end. Spliced between these two regions of homology was a 45-nucleotide sequence (1175–1220 of λSpi-2) with only 53% homology. The two similar, but not identical cDNA, confirmed the hypothesis that the products, Spot 3 and Spot 20, arise from two distinct mRNA, both of which are regulated by growth hormone. Based on the common restriction enzyme sites of pGH4 and λSpi-1 and the hybrid selection of Spot 3 by pGH4, we surmise that λSpi-1 insert is complementary to the mRNA which codes for Spot 3, whereas λSpi-2 insert is complementary to the mRNA which codes for Spot 20. pGH4 aligns approximately with nucleotides 920–1080 of λSpi-1. The homology between λSpi-1 and λSpi-2 is only 74% in this region and this may explain the hybrid selection of only one of the mRNA species with this cDNA at high stringency. Limited sequencing of pGH1 indicates that it is also a fragment of λSpi-1 and aligns with nucleotides 655–955, a region of 91% homology with λSpi-2.

Table I. Time course of mRNA induction by growth hormone

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Relative mass of mRNA (OD/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.22 ± 0.52 (n = 4)</td>
</tr>
<tr>
<td>30</td>
<td>4.40 ± 0.05 (n = 5)</td>
</tr>
<tr>
<td>90</td>
<td>3.71 ± 0.1 (n = 3)</td>
</tr>
<tr>
<td>120</td>
<td>5.64 ± 0.52 (n = 4)</td>
</tr>
<tr>
<td>240</td>
<td>14.87 ± 1.25 (n = 3)</td>
</tr>
<tr>
<td>240 control</td>
<td>3.25 ± 0.78 (n = 4)</td>
</tr>
</tbody>
</table>

*The change at 120 min was significantly different from zero time values at p < 0.025 as determined by Student t testing.

3 The change at 240 min was significantly different from zero time values at p < 0.005 as determined by Student t testing.
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**Fig. 3. Comparison of nucleotide sequences of XSpi-1 and XSpi-2.** Both strands of both cDNA species were sequenced for 95% of the sequence. In the small regions of single stranded data, at least one additional template was sequenced through the region. Each gel was read by at least two independent readers and differences were resolved either by a third independent reading or by re-sequencing the region in question. The sequences were aligned using the IFind software of Intelligenetics. (The nucleotide sequences for XSpi-1 and XSpi-2 will be deposited in GenBank®, Los Alamos National Laboratory.) Three AUG sites (underlined) in the protein reading frame occur within the first 330 nucleotides of XSpi-2. The middle AUG appears suboptimal as a start site for translational product on two-dimensional electrophoretograms whereas the third AUG resulted in a substantially smaller product. The other two reading frames contained multiple stop sites and failed to predict a polypeptide of appropriate length.

Protease inhibitors, the nucleotide sequences of these two cDNA differed most extensively with the nucleotide sequence coding for the known reactive center of other inhibitors (Fig. 4, Panel A). The amino acid sequence in and around the reactive center of several serine protease inhibitors was also compared with the amino acid sequence of Spi-1 and Spi-2 (Fig. 4, Panel B). The deduced amino acid sequence of \( \text{XSpi-1} \) and \( \text{XSpi-2} \) in this region differed from each other as well as from several known serine protease inhibitors. This difference in the reactive center of protease inhibitors has been previously reported by Hill et al. (32) in their studies comparing the structure of mouse and human inhibitors. Our data indicate that this divergence extends to mouse and rat and more importantly, within a given species. Since the predicted amino acid sequence was similar to the serine protease inhibitor gene family, whereas the reactive centers differed, we have elected to name the products, Spots 3 and 20, with the more descriptive designation, serine protease inhibitor 1 and 2, respectively.

The significance of these alterations within the reactive center is not known. On one hand, these differences may represent differing specificities for proteases. Alternatively, these new members of the serine protease inhibitor gene family may not have antiprotease activity, such as ovalbumin and angiotensinogen. In the case of angiotensinogen, the cleaved C terminus eventually becomes angiotensin II, a potent peptide which causes arteriolar vasoconstriction and aldosterone secretion. An analogous mechanism for \( \text{XSpi-2} \) in vitro translational product on two-dimensional electrophoretograms whereas the third AUG resulted in a substantially smaller product. The other two reading frames contained multiple stop sites and failed to predict a polypeptide of appropriate length.
To estimate the number of genes homologous to \( \text{A} \text{Spi}-1 \) and \( \text{A} \text{Spi}-2 \) in the rat genome, genomic DNA was digested with a variety of restriction endonucleases and probed with either a 5' or 3' probe. The 5' probe was a 204-bp fragment consisting of nucleotides 502-706 (EcoRI-KpnI) of \( \text{A} \text{Spi}-2 \). The 3' probe was a 281 bp fragment consisting of nucleotides 706-987 (KpnI-SphI) of \( \text{A} \text{Spi}-2 \). The homology between \( \text{A} \text{Spi}-1 \) and \( \text{A} \text{Spi}-2 \) for these two probes was 92-94% in these regions. For the 5' probe each enzyme with the exception of BglII resulted in two bands (Fig. 5). The pattern observed with the 3' probe was more complex; however, no endonuclease restriction enzyme resulted in more than two bands in common between the 5' and the 3' probes. This strongly suggests that the mRNA coding for Spots 3 (Spi-1) and 20 (Spi-2) arise from two distinct genes and there are no pseudogene copies present. Furthermore, the difference in complexity of banding between the two probes suggests that the region of the genes bounded by the 3' probe may contain two or more introns. This interpretation of the more complex banding pattern of the 3' probe is consistent with the location of the introns in the 3' region of the \( \alpha \)-antitrypsin and angiotensinogen genes (31, 33).

Progress in our understanding of the mechanism of growth hormone action, one of the most important growth promoting hormones, at a molecular level has been limited by the lack of an adequate model gene. The two cDNA and their mRNA reported in this article have important physiological and biochemical features for a model growth hormone-responsive gene. First, growth hormone acts directly on hepatocytes to augment these two mRNAs (6). Second, Spi-1 and/or Spi-2 are induced 5-fold by 4 h after growth hormone administration to hypophysectomized rats, suggesting that their induction represents a primary event. Third, 5 days of starvation in the euthyroid rat and a 12-day high carbohydrate-low fat diet had only a minimal effect on either Spot 3 or Spot 20 (3, 34). These observations indicate that nutrition, an important co-regulator of insulin-like growth factor I (35) and \( \alpha \text{a}_{\text{r}} \)-globulin (34, 36), does not contribute to the expression of Spots 3 and 20. Streptozotocin-induced diabetes reduces Spot 3 by approximately 30%, suggesting a relatively minor role of insulin (37). Sex steroids also appear unimportant to their expression, since both products are expressed at qualitatively similar levels in female and male animals. Fourth, these two products

\[ \text{FIG. 4. Comparison of the nucleotide and amino acid sequences in the reactive center regions of known serine protease inhibitors to those of \( \text{A} \text{Spi}-1 \) and \( \text{A} \text{Spi}-2 \). The nucleotide sequences in the reactive center of human \( \alpha \)-antitrypsin (HUMAlATM (40)), trypsin (MUSCNT (32)), mouse \( \alpha \alpha \)-antitrypsin (MUSAlPI (32)), and human \( \alpha \alpha \)-antitrypsin (HUMAlATM (41-43)), and human antithrombin III (HUMATIII (44)) are compared with the nucleotide sequences which code for the presumptive reactive centers of \( \text{A} \text{Spi}-1 \) and \( \text{A} \text{Spi}-2 \) (Panel A). The underlined sequence of human \( \alpha \)-antitrypsin, \( \alpha \)-antichymotrypsin and antithrombin III, and mouse \( \alpha \alpha \)-antitrypsin are the nucleotides which code for the reactive center. The asterisk indicates that the nucleotide is identical to that observed in \( \text{A} \text{Spi}-2 \) and "-" indicate gaps introduced into the DNA sequences so that they could be aligned. In Panel B the amino acid sequence in the region of the reactive center of the same serine protease inhibitors, as well as more limited amino acid sequence data of bovine pancreatic trypsin inhibitor (BPTI) (45), \( \alpha \)-PI Pittsburgh (47), soybean and lima bean trypsin inhibitor, are compared to the presumptive reactive center region of \( \text{A} \text{Spi}-1 \) and \( \text{A} \text{Spi}-2 \). The reactive center of several well characterized protease inhibitors are underlined with the P, through P, positions indicated. The strong amino acid sequence homology of \( \text{A} \text{Spi}-1 \) and \( \text{A} \text{Spi}-2 \) with other serine protease inhibitors in the region leading up to the reactive centers is the basis for the designation of the P, through P, positions of \( \text{A} \text{Spi}-1 \) and \( \text{A} \text{Spi}-2 \).]
demonstrate a marked ontogenesis with low levels observed until day 15 which increase to a peak at 35 days (26). This pattern parallels, qualitatively, the ability of the animal to respond to growth hormone with increased growth. Of the physiological stimuli investigated, age of the animal, thyroid hormone and/or glucocorticoids, and growth hormone participate in the regulation of these products. These physiological observations indicate that Spi-1 and Spi-2 are relatively specific markers of growth hormone action. Finally, our studies on the genomic organization of the genes related to these two products suggests the presence of only two similar but not identical genes. The characterization of these two unique genes, one of which presumably codes for Spot 3 while the other codes for Spot 20, should be considerably easier than the characterization of the 18–20 closely related genes for α2-globulin (38). The identification and cloning of these cDNA affords a unique opportunity to investigate the relative specific effects of growth hormone on the liver at a molecular level.

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Note Added in Proof—The extent of homology within the 3’- untranslated regions of the Spi-1 and Spi-2 cDNAs was 86.6%; a value remarkably close to that for the translated portion (87.3%). Likewise, homology in the 3rd positions of codons (89.1%) was similar to that for the 1st two codon positions (85.0%). These findings imply that the two Spi genes diverged quite recently from a common ancestral gene. The surprising observation that sequences within the reactive center diverged to a greater extent (66.7% homology) than other regions of the two genes (including those which are presumed to be evolutionarily neutral) suggests that there must be mechanisms to direct the rapid adaptation of the reactive center. Remarkable divergence in the reactive center of 100 different avian ovomucoid 3rd domains has been reported by Laskowski et al. (1987) Biochemistry 26, 202–221, a result which may be due to a similar evolutionary pathway.

REFERENCES