Molecular Cloning of Rat Insulin-Like Growth Factor I Complementary Deoxyribonucleic Acids: Differential Messenger Ribonucleic Acid Processing and Regulation by Growth Hormone in Extrahepatic Tissues

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Two classes of insulin-like growth factor I (IGF-I) cDNAs were isolated from an adult rat liver library using a human IGF-I cDNA probe. The two types of rat IGF-I cDNA differed by the presence or absence of a 52-base pair insert which altered the derived C-terminal amino acid sequence of the E peptide, but not the 3′-untranslated region or the sequence coding for the mature IGF-I protein. When probes derived from these cDNA clones were hybridized to Northern blots of rat mRNA, specific bands of 8.6, 2.1, and 1.0–1.4 kilobases were seen. Hybridization to poly(A)$^+$ RNA from various tissues from GH-treated and control rats demonstrated an increase in IGF-I mRNA due to GH treatment in all tissues examined. (Molecular Endocrinology 1: 243–248, 1987)

INTRODUCTION

The somatomedin hypothesis as originally formulated by Daughaday *et al.* (1) proposed that the effects of GH on cartilage are not due to a direct interaction of GH with the target tissue, but are mediated through a class of peptide growth factors termed somatomedins. Subsequent research has indicated that insulin-like growth factor I (IGF-I), or somatomedin C, is the most likely candidate for the role of an intermediary in GH action (2). For example, the levels of IGF-I protein are increased by GH in liver as well as in other tissues (3, 4). Additionally, recent studies using a human IGF-I cDNA probe have shown that hypophysectomy decreases the levels of IGF-I mRNA in rat liver, and that GH treatment partially restores the amount of IGF-I mRNA (5), suggesting that GH can regulate the biosynthesis of IGF-I at the level of IGF-I mRNA synthesis, processing, or stability. In order to extend this work to nonhepatic tissues, we undertook the isolation and characterization of IGF-I cDNAs from adult rat liver. DNA sequence analyses of these clones revealed certain significant differences in the sequence and putative processing of rat IGF-I mRNA as compared to the transcription of the human gene (6–9), and provided homologous probes with which to determine the extent and level of control by GH of IGF-I mRNA synthesis in nonhepatic rat tissues.

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RESULTS AND DISCUSSION

The isolation of rat IGF-I cDNA clones was accomplished by screening approximately 10^6 recombinant phage from an adult rat liver cDNA library cloned into λgt11 (purchased from Clontech, Palo Alto, CA) with the human IGF-Ia cDNA. Fifty phage plaques which gave signals on duplicate filters were rescreened at progressively lower densities three more times, until single purified plaques were obtained. Phage DNA was prepared from 32 individual clones isolated and purified in this manner, digested with *Eco*R1 to cleave out the cDNA inserts, and run on an agarose gel. The DNA was probed with the human IGF-Ia cDNA. The inserts ranged in size from approximately 600 to 1346 base pairs (bp) and all hybridized to the human IGF-Ia probe. Several inserts were subcloned into pUC-18 or pUC-19

vectors and two clones of 1346 bp (prIGF-I-25) and 703 bp (prIGF-I-42) were sequenced. Interestingly, the DNA sequence analysis revealed that these clones differed by the presence of a 52-bp insert in prIGF-I-42. The difference between these two completely characterized clones (described in detail below) was corroborated by partial sequencing of two independently isolated inserts, one similar to prIGF-I-25, and the other to prIGF-I-42. The isolation of these clones from the same cDNA library suggests that both of these forms of rat IGF-I mRNA are present in adult rat liver. Figure 1 shows the sequence of the prIGF-I-25 cDNA clone, the sequence of the 52-bp insert found in clone prIGF-I-42, and the derived amino acid sequences of the prepro-IGF-I peptides. Also indicated are amino acid differences between the rat sequence and the derived amino acid sequences reported for the human IGF-Ia and IGF-Ib cDNAs (6, 8).

The region upstream from nucleotide 794 (met-22) constitutes an open reading frame which includes two met codons in frame with the met codon at position 794. The significant divergence of this sequence from the reported human sequence, in both nucleotide and derived amino acid sequence, and the extensive homology downstream from position 794, suggests that the prepeptide sequence of rat (and human) IGF-I translated *in vivo* begins at met-22.

The amino acid sequence of the mature (pre-B-C-A-D) IGF-I peptide of rat shows a number of differences from the human sequence, specifically at residues B20, C6, A21, D1, D2, and D5. Although these amino acid substitutions range from potentially neutral to potentially drastic (*i.e.* the Pro-Asp change at position B10), none occur at positions which would necessarily alter the predicted overall tertiary structure of the mature IGF-I protein (10). Additionally, none of the residues predicted to occur on the surface of the IGF-I molecule (10) are affected by the amino acid differences between the rat and human proteins, suggesting that interactions with IGF-I carrier proteins are probably unaffected.

In terms of the C-terminal extension (E) peptide, the rat amino acid sequence is identical to the human sequence through residue E16, with the exception of a change at residue E3. At the first base of the codon for residue E17 (nucleotide 1118) the amino acid sequences of the two rat clones characterized here diverge due to the 52-bp insert in prIGF-I-42. This insert changes the derived amino acid sequence as well as the reading frame, resulting in two possible C-terminal E peptide sequences and the use of two different UAG stop codons in the variants (beginning at nucleotides 1193 and 1227).

There are three obvious mechanisms which could account for the 52-bp insert present in prIGF-I-42. In the first case, an alternate splice donor site 52 bp into the 5′-end of a putative intron present at this position in the rat IGF-I genomic sequence could be used to generate the insert. Alternatively, the use of an alternate splice acceptor site could be employed to generate the extra 52 bp of coding sequence from the 3′-end of the pertinent intron. Finally, the 52-bp insert could arise from a completely separate exon, which is spliced out in a certain fraction of mature rat liver IGF-I mRNAs.

A preliminary distinction between these mechanisms is possible based upon a comparison of the sequences surrounding the site of the insert with consensus sequences of splice junctions in other eukaryotic genes (11). Mechanism 1 above would predict the sequence 5′-AG:GUAAGU-3′ at the 5′-end of the insert in prIGF-42. Mechanism 2 would result in the sequence 5′-$(Py)_{10}$NCAG:G-3′ at the 3′-end of this insert. Existence of a separate exon encoding this insert (mechanism 3) would result in the consensus sequence 5′-AG:G-3′ at the 5′- and 3′-junctions of the insert with the flanking common coding regions, as well as at the site in prIGF-I-25 at which the insert occurs in prIGF-I-42. Analysis of the rat cDNA sequence in light of the rationale described above suggests that the presence of the 52-bp insert in prIGF-I-42 results from differential splicing of a separate exon, since the sequences AG:G, AG:G, and AG:U occur at the putative exon-exon boundaries in these cDNAs. Although the exon-exon consensus sequence is AG:G, U is found at the 3′-position in approximately 8% of the pertinent eukaryotic sequences in the Gen Bank data base (10). A more definitive resolution of these alternatives will require a direct comparison of the sequences described in this work with the rat IGF-I genomic sequence.

A comparison of the E peptide and 3′-untranslated regions of the rat IGF-I cDNAs to the reported human IGF-Ia (6, 7) and IGF-Ib (8) cDNA and genomic sequences (9) allows some tentative conclusions to be drawn concerning the evolution of differential processing in the 3′-end of the mammalian IGF-I gene. As discussed above, rat IGF-I cDNAs differ at the 3′-end by the presence or absence of an insert probably coded for by its own distinct exon. Differential splicing produces mature mRNAs coding for IGF-I molecules containing E peptides with differing C-termini, but whose mRNAs have identical 3′-untranslated regions. In human liver, on the other hand, IGF-I mRNAs diverge in the E peptide coding region, but this divergence results from the use of alternate exons, each encoding an entirely separate E peptide C-terminus as well as divergent 3′-untranslated regions. The sequence reported here reveals that the rat cDNAs diverge at the exact nucleotide position where the divergence occurs in human cDNAs. The terminus of the E peptide seen in the prIGF-I-25 clone, as well as the common 3′-untranslated region, are extremely homologous to the human IGF-Ia sequence, whereas the first part of the 52-bp insert in prIGF-I-42 is significantly homologous to the human IGF-Ib sequence (31/45 nucleotides, or 69%, through the codon for residue E31, but only 36/75 nucleotides, or 48%, through the codon for residue E41). These data support the hypothesis that the two divergent 3′-untranslated regions coded for by separate exons in the human genome arose as a result of an alteration involving the 3′-splice junction sequence of the short 52-bp exon presumably present in the rat gene, such that splicing of the altered 3′-end of this exon occurred much further downstream, resulting in a

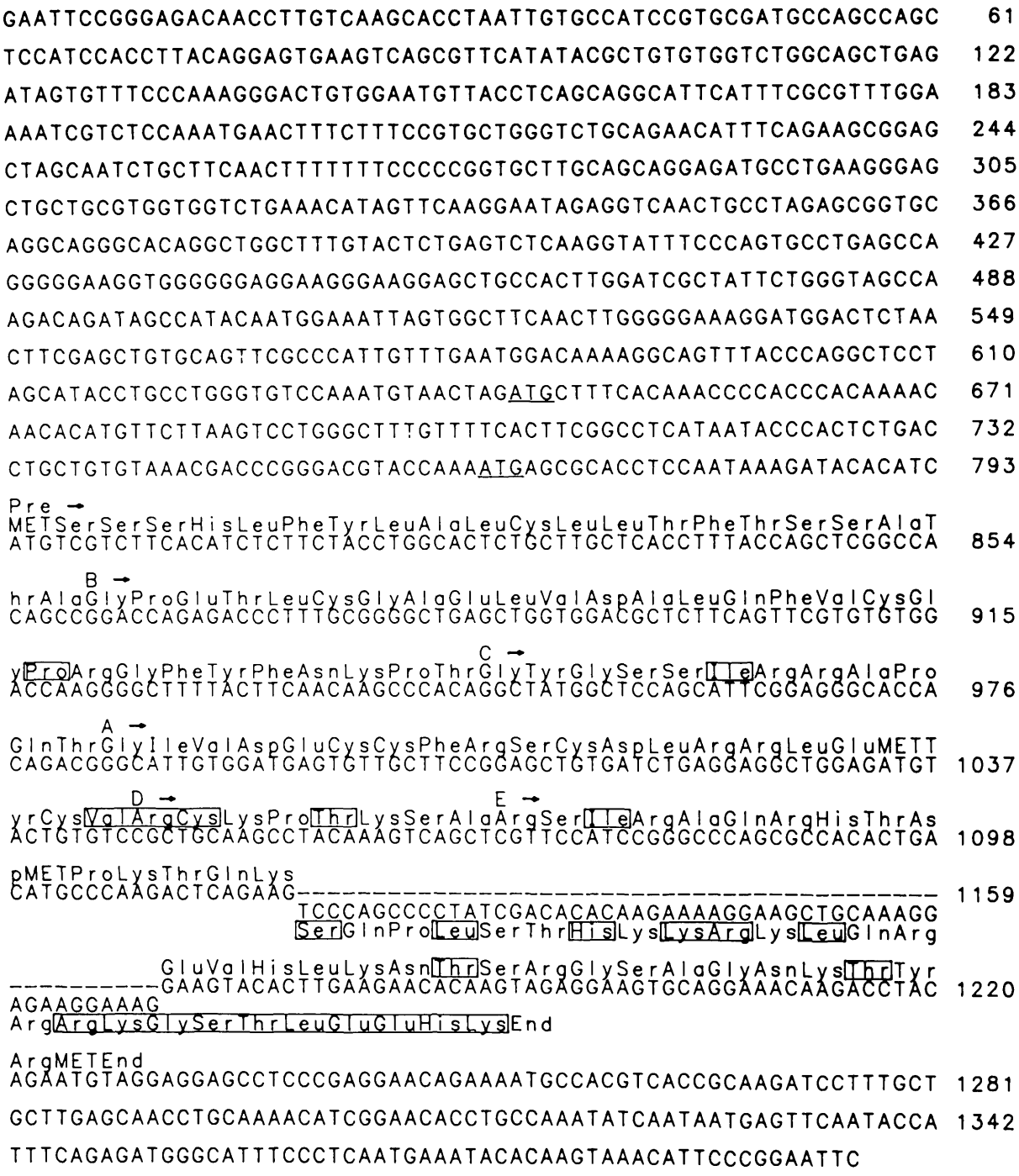

Fig. 1. DNA and Derived Amino Acid Sequences of Rat IGF-I cDNAs
The cDNA inserts in clones prIGF-I-25 and prIGF-I-42 were purified, cleaved with *Hinf*1, *Sau*3A, and *Xma*1, and the resulting subfragments recloned into pUC-18 or -19 using standard procedures. The 5′-end of clone prIGF-I-42 corresponds to position 688 of the prIGF-I-25 sequence. The sequence from position 1118 through position 1170 represents the 52-bp insert found in prIGF-I-42, but absent in prIGF-I-25. The two in-frame initiation codons upstream from the met codon at position 794 are underlined. The *boxed* amino acid residues are those which are different in the human IGF-I peptide.

distinct E peptide C-terminus and 3′-untranslated region, including cleavage and polyadenylation signals.

Additionally, all 32 cDNA inserts isolated from the adult rat liver library hybridized under stringent conditions to the 3′-*Hin*fI-*Eco*R1 fragment of prIGF-I-25 which contains the common 3′-untranslated region, but not the 52-bp insert (data not shown). This suggests that, if there is indeed an alternate 3′-untranslated sequence in the rat similar to the situation in human liver (8), it is not expressed in adult rat liver to any great extent.

No consensus polyadenylation site (5′-AAUAAA-3′) is present in the 3′-untranslated region of the rat IGF-I cDNA sequence. In light of the fact that all rat IGF

mRNA species are present only in the poly(A)$^+$-enriched RNA fraction from liver (Lowe, Jr., W. L., unpublished observations), it is likely that the original synthesis of the rat IGF-I cDNAs analyzed in this work resulted from annealling of the oligo dT primer to an A-rich RNA sequence upstream from the polyadenylation signals present in the full-length IGF-I mRNA. A similar situation apparently occurred in the initial cloning of the human IGF-Ia cDNA (compare Refs. 6 and 7).

As described above, one of the purposes of isolating rat IGF-I cDNA clones was to obtain homologous probes with sufficient sensitivity to allow the study of the effects of GH treatment on IGF-I mRNA levels in nonhepatic tissues of hypophysectomized rats. A previous study using a human IGF-I cDNA probe (5) demonstrated that there are multiple IGF-I mRNA species in adult rat liver, and that the level of IGF-I mRNA can be increased in livers from hypophysectomized rats by GH treatment. The results shown in Fig. 2, obtained with a homologous rat IGF-I cDNA probe (nucleotides 688-1016, corresponding to the prepeptide, B, C, and part of the A domains), demonstrate the presence, in all tissues examined, of the multiple IGF-I mRNA species (8.6, 2.1, and 1.0–1.4. kilobases) previously reported in liver. Of all these tissues, liver contained the highest levels of IGF-I mRNA, suggesting that liver is quantitatively the primary site of IGF-I synthesis. IGF-I mRNA levels (as determined by densitometric scanning of the Northern blot of Fig. 2) in liver were approximately 15-fold higher than in lung, the nonhepatic tissue producing the greatest amount of IGF-I mRNA. Since the actin mRNA levels were similar in lung and liver, the IGF-I mRNA levels in these tissues were not normalized to the actin mRNA levels. Densitometric scanning also revealed that GH increased the overall content of IGF-I mRNA in each tissue; the fold increase was 2.6 in liver, 1.9 in lung, 3.0 in heart, and 1.3 in kidney. Furthermore, preliminary experiments using a solution hybridization-RNase protection assay have confirmed the increase in IGF-I mRNA levels by GH (Lowe, Jr., W. L., unpublished observations). Not all IGF-I mRNA species were affected equally by GH treatment in each tissue. The significance of these diverse effects is unclear at present, due to the fact that the translational capacity, sequence, and function of the various IGF-I mRNA species is not known.

When these blots were reprobed with a 3′-fragment common to the rat IGF-I cDNAs (nucleotides 1170-1393), results identical to those shown in Fig. 2 were obtained (data not shown). These data, in conjunction

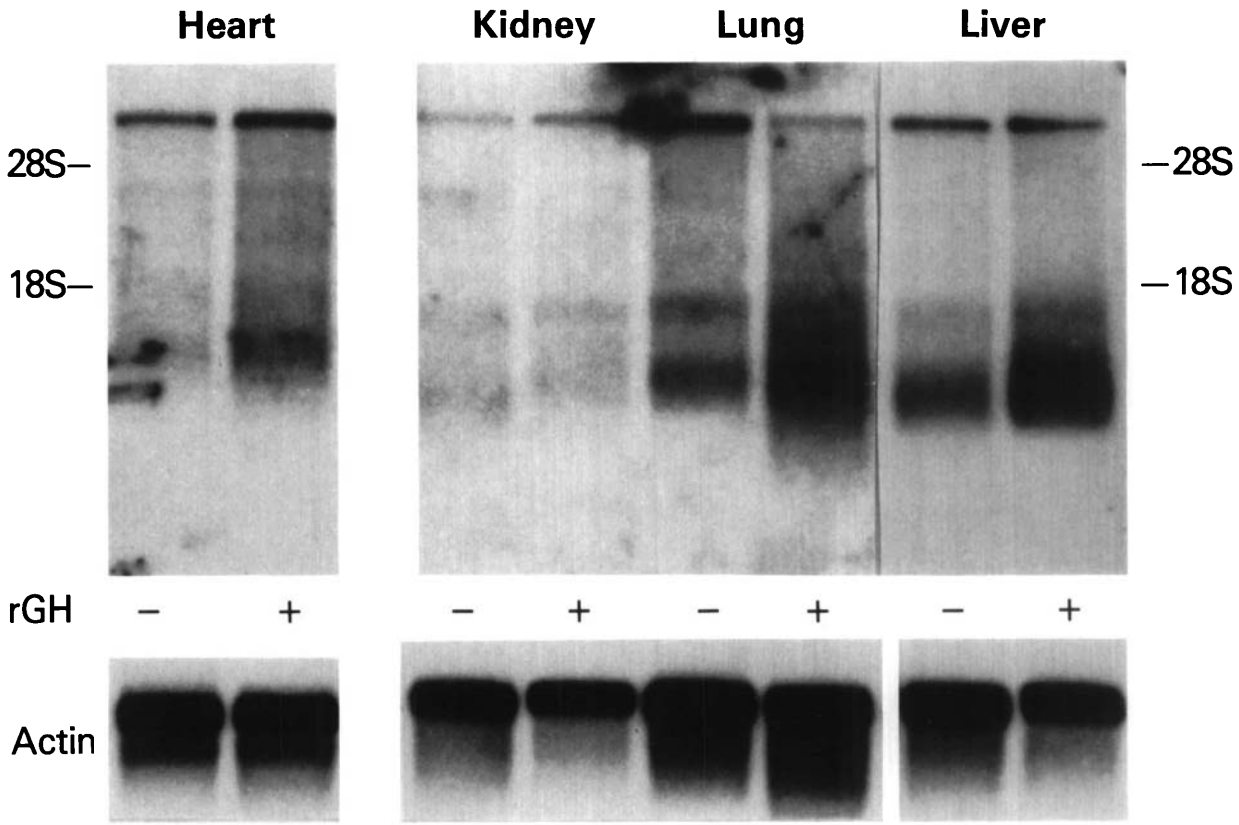

Fig. 2. Effect of GH Treatment on IGF-I mRNA Levels in Various Tissues from Hypophysectomized Rats
Northern blots of poly(A)$^+$ RNA (liver, 3.5 μg; kidney, 4.5 μg; lung, 4.5 μg; heart, 7.0 μg) from GH-treated and control hypophysectomized rats were hybridized with the rat IGF-I cDNA probe as described in *Materials and Methods*. All samples represent RNA from the tissues of three to five animals which were pooled at the time of RNA extraction. The blots were exposed to Kodak X-Omat AR film for varying periods of time (liver, 1 day; kidney and lung, 3 days; heart, 7 days). The 28S and 18S markers correspond to the position of the ribosomal RNA bands on the ethidium bromide stain of the gel. The blots were washed and rehybridized with a rat β-actin cDNA as described above. Hybridization with the labeled β-actin cDNA demonstrated that similar amounts of poly(A)$^+$ RNA were present on the GH (+) and (–) lanes for each tissue.

with the results of the Southern hybridization with this probe (described above), suggest that the possibility of an entirely different 3′ untranslated region being expressed in rat is unlikely.

A comparison of the results of this study with a previous study of IGF-I peptide levels in tissues of GH-treated, hypophysectomized rats (3) reveals interesting similarities and contrasts. Consistent with the results described here, the tissue levels of IGF-I peptide were increased by GH in liver, lung, heart, and kidney. In contrast to the relative effect of GH on IGF-I mRNA levels in the various tissues described above, the most dramatic effect of GH on IGF-I peptide levels was in the kidney. This discrepancy between the IGF-I peptide and mRNA levels in the kidney is most likely due to filtration of IGF-I from the serum as opposed to *de novo* synthesis as the basis for the relatively elevated IGF-I peptide levels in the kidney. Other possible reasons for the different results include the duration of hypophysectomy and the length of GH treatment in the two studies. The increase in tissue IGF-I peptide levels in the liver, lung, and heart in GH-treated as compared to control animals was reported to be approximately 3.5-, 2.0- and 1.8-fold. These values are similar to the increase in IGF-I mRNA levels seen in these tissues in the present study.

In summary, then, the work reported here establishes the presence of two differentially processed forms of IGF-I mRNA in adult rat liver as predicted from DNA sequence analyses of the analogous cDNAs, and demonstrates the regulation of rat IGF-I gene expression by GH in various nonhepatic tissues using homologous rat IGF-I cDNA probes. Future studies will address the potential differential tissue-specific or hormonal regulation of the various processing forms of rat IGF-I mRNA.

MATERIALS AND METHODS

Hybridization Probes

The cDNA probes used in this study were: the 520-bp *Pst*1-*Bam*H1 fragment of the human IGF-Ia cDNA described by Jansen *et al.* (6) which includes the entire coding region for the mature IGF-I peptide but lacks a majority of the 3′-untranslated region; the 326-bp *Eco*R1-*Sau*3A fragment at the 5′-end of the rat IGF-I cDNA insert in prIGF-I-42, which contains the coding region of the pre, B, C, and part of the A domains; the 232-bp *Hin*fI-*Eco*R1 fragment at the 3′-end of prIGF-I-25, which contains the C-terminus of the E peptide as well as the 3′-untranslated region; and a 650-bp *Eco*R1-*Hin*dIII fragment of a rat β-actin cDNA.

All probe fragments were purified free of vector DNA by agarose gel electrophoresis and labelled with $\alpha[^{32}P]$dCTP to specific activities of approximately 5×10^8 cpm/μg DNA using the previously described technique of random priming (12, 13).

Library Screening, Southern Analyses, and DNA Sequencing

Screening of the rat liver cDNA library, phage DNA isolation, and Southern analysis of cDNA inserts followed standard procedures (14). Rat IGF-I cDNA inserts were subcloned into pUC-18 or pUC-19 plasmid vectors (15) and double-stranded DNA was prepared and both strands sequenced as previously described (16).

RNA Isolation, Gel Electrophoresis, and Northern Blot Analyses

Forty-day-old male Sprague-Dawley rats were hypophysectomized by the supplier, Zivic-Miller Laboratories (Allison Park, PA). The adequacy of the hypophysectomy was assessed at the time of killing by visual inspection of the sella turcica. After hypophysectomy, the rats were maintained for 10 days with *ad libitum* feeding of rat chow and fluid (H_2O with 5% dextrose, 8.12 g/l NaCl, 0.33 g/l KC1, 0.14 g/l $CaCl_2$, 0.07 g/l Mg Cl_2.) Beginning at 11 days after hypophysectomy, rats were injected ip with either 150 μg rat GH (lot B-9, a gift of the National Hormone and Pituitary Program) or vehicle daily for 4 days, and killed 1½ h after the final injection. Tissues were removed and immediately frozen in liquid nitrogen and RNA was prepared from the tissues using a modification of the method of Cathala *et al.* (17) as described previously (12). Poly(A)$^+$ RNA was prepared from GH-treated and control animals as described by Aviv and Leder (18) and size-separated by electrophoresis on a 1.5% agarose-17.5% formaldehyde gel (12). RNA was transferred to a nylon membrane (Gene Screen, New England Nuclear, Boston, MA) using a model HBS horizontal blotting system (I.B.I., New Haven, CT). Transfer was effected at 0.25 amps for 12 h and 0.55 amps for 1 h in 0.025 M Na_2HPO_4/NaH_2PO_4 (pH 6.5). RNA was fixed to the membrane by baking *in vacuo* at 80 C for 2 h. Hybridizations were performed in 5× SSPE, 50% formamide, 10% dextran sulphate, 5× Denhardt's solution (19), 1% sodium dodecyl sulfate, and 10 μg/ml salmon sperm DNA at 50 C, and washed as described previously (12).

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Note Added in Proof

Recent cloning of mouse IGF-I cDNAs (Bell, G. I., M. M. Stempien, N. M. Fong, and L. B. Rall, Nucleic Acid Res 14:7873–7882, 1986) has demonstrated a similar 52 base insertion which alters the C-terminal amino acid sequence of the E-peptide.

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