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Two insulin-like growth factor I messenger RNAs are expressed in human liver

(prohormone)

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ABSTRACT **Through use of a synthetic oligonucleotide probe, human insulin-like growth factor I (IGF-I) cDNA clones were isolated from a liver library. Two types of cDNAs were defined by restriction enzyme analysis and DNA sequencing. Both encode IGF-I precursors of either 195 or 153 amino acids. The two predicted protein precursors are identical from their amino terminus to a lysine residue 16 codons beyond the IGF-I sequence, and then they diverge. Both cDNAs predict additional unique carboxyl-terminal extension peptides. Since there is only one *IGF-I* gene in the human genome, the finding of two different cDNAs suggests that alternative RNA processing plays a role in *IGF-I* gene expression. The functions of the different extension peptides remain to be elucidated.**

The somatomedins or insulin-like growth factors (IGFs) comprise a family of peptides that circulate in plasma and stimulate DNA synthesis in a variety of cultured cells (1, 2). Two human IGFs have been characterized. IGF-I, a 70-amino acid basic protein (3, 4), plays a fundamental role in postnatal mammalian growth as the major mediator through which growth hormone (GH) exerts its biological effects (5, 6). The function of IGF-II, a 67-amino acid neutral peptide (7, 8), is less clear, as IGF-II serum levels do not show GH dependence (5, 9), and unlike IGF-I, IGF-II cannot substitute for GH as a growth-promoting peptide (10). Both IGFs circulate in blood bound to specific carrier proteins (11).

Very little is known about IGF-I biosynthesis, in part because its content in tissues is low (12), and also because, in contrast to IGF-II (8, 10), no cultured cell lines elaborate significant quantities of the peptide (13, 14). Vassilopoulou-Sellin and Phillips (12) have estimated, by molecular sieve chromatography, that IGF-I activity extracted from rat liver has a higher molecular weight (≈30,000) than activity extracted from plasma (M_r, ≈8000) and have asserted that the larger molecular weight material represents an IGF-I precursor. Amino acid sequence derived from a human IGF-I cDNA clone by Jansen *et al.* (15) supports the observation of a larger precursor, but since this cDNA is not full length the precise beginning of translation of IGF-I messenger RNA could not be determined.

Here I describe the characterization of two different IGF-I cDNAs isolated from a human liver library. The nucleotide sequences of these cDNAs predict two different IGF-I protein precursors and define the size of these peptides, 153 and 195 amino acids. The two IGF-I mRNAs have identical 5′ ends and are expressed in human liver. Since current evidence points to the existence of only one *IGF-I* gene in the human genome (16–18), these observations suggest that alternative RNA processing accounts for at least two different IGF-I mRNA species. As in other genes elaborating multiple peptides, tissue-specific regulation of RNA biosynthesis and maturation may play a role in *IGF-I* gene expression (19–23). In addition, processing of two different IGF-I protein precursors provides another potential level for control of IGF-I biosynthesis and raises the possibility that the peptide extensions at the amino and carboxyl ends have biological functions.

MATERIALS AND METHODS

Materials. Enzymes including restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, DNA polymerases, ribonuclease A, and proteinase K were purchased from commercial suppliers (New England Biolabs and Bethesda Research Laboratories). Nitrocellulose was obtained from Schleicher & Schuell and Millipore. Radionuclides were purchased from New England Nuclear and Amersham; deoxynucleoside triphosphates and dideoxynucleoside triphosphates were from Pharmacia P-L.

Methods. *Oligonucleotide synthesis.* A 42-base oligonucleotide corresponding to the DNA sequence encoding amino acids 10–23 of human IGF-I (15, 16) was produced at Monsanto Company, St. Louis, on an Applied Biosystems solid-phase DNA synthesizer. The sequence of the probe is as follows: 5′ AAA GCC CCT GTC TCC ACA CAC GAA CTG AAG AGC ATC CAC CAG 3′. The probe was 5′-end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (24).

cDNA cloning. A human liver cDNA library in λgt11 (kindly provided by S. L. C. Woo and T. Chandra) was plated on *Escherichia coli* K-12 strain Y1088 (25). Duplicate nitrocellulose filters were prepared (26) and hybridized at 42°C in buffer containing 5× SSC (1× SSC = 150 mM NaCl/15 mM Na citrate, pH 7)/50 mM Na phosphate, pH 6.8/40% deionized formamide/denatured salmon sperm DNA (50 μg/ml)/5× Denhardt's solution [0.1% Ficoll/0.1% bovine serum albumin/0.1% polyvinylpyrrolidone (27)]. Following hybridization, the filters were washed for 15 min at 22°C and for 15 min at 40°C in 0.2× SSC/0.1% $NaDodSO_4$, and exposed to Kodak XAR5 film with intensifying screens. Positive plaques were rescreened at lower density and purified to homogeneity. DNA was prepared (28) and mapped by restriction enzyme digestion and gel electrophoresis.

DNA sequence analysis. DNA sequencing was performed by the dideoxy-chain-terminating method (29, 30) after subcloning restriction fragments into M13 mp18 and mp19 bacteriophage (31). The sequences of two cDNA isolates were determined in their entirety. DNA sequence was determined on both strands and across all restriction enzyme sites used as initiation points except for the extreme 3′ end of one clone, which was sequenced three times in only one orientation. Data analysis was simplified by computer programs run on an Apple II microcomputer (32) and on a Digital Equipment Corporation VAX minicomputer. Protein homology searches were conducted by using the National Biomed-

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Abbreviation: IGF, insulin-like growth factor.

ical Research Foundation Protein Sequence Data Bank with the computer programs of Lipman and Pearson (33). Nucleotide alignments were achieved by using the NUCALN program (34).

RNA isolation and analysis. Liver polyadenylylated RNA was isolated from tissue fresh-frozen at −70°C by extraction with guanidinium thiocyanate (35) and one round of chromatography on oligo(dT) cellulose (36). The polyadenylylated RNA was denatured with glyoxal (37), electrophoresed through a 1.25% agarose gel, and transferred to a nitrocellulose filter by blotting (38). IGF-I cDNA probes were labeled with ^{32}P by nick-translation (39) to 8–12 × 10^8 dpm/μg and were hybridized to the filters at 42°C in 50% formamide/5× SSC/50 mM Na phosphate, pH 6.5/denatured salmon sperm DNA (100 μg/ml)/1× Denhardt's solution/10% dextran sulfate. Filters were washed for 15 min at 22°C in 0.2× SSC/0.1% $NaDodSO_4$, for 30 min in two changes of the same buffer at 47°C, and then autoradiographed using intensifying screens for 62 hr at −70°C.

RESULTS

Isolation of IGF-I cDNA Clones. Plaques (5 × 10^5) of the human liver cDNA library were screened with the IGF-I-specific oligonucleotide, leading to the isolation of seven positives with DNA inserts ranging from 800 to 1150 nucleotide pairs. By restriction enzyme mapping, the cDNAs were found to be of two types. Two inserts of 800 and 850 nucleotide pairs containing internal *Bam*HI restriction sites (data not shown) corresponded to the IGF-I cDNA reported by Jansen *et al.* (15) and are designated IGF-IA cDNA. The remaining five clones had a different map and are called IGF-IB cDNA. The two largest in the latter group were selected for DNA sequence analysis.

Nucleotide Sequence Analysis. Fig. 1 illustrates a map of the IGF-IB cDNA and depicts the approach to DNA sequencing. Both isolates gave identical results over shared regions. The DNA sequence and the amino acid translation appear in Fig. 2. The aggregate IGF-IB cDNA consists of 1136 nucleotides, including 42 deoxyadenosine residues of the poly(A) tract. This agrees well with the size of the major mRNA determined by filter hybridization (1100–1200 nucleotides; see Fig. 4). The sequence can be divided into three sections. A 5′ untranslated region comprises the initial 182 nucleotides. An initiation codon and an open reading frame of 585 nucleotides (195 codons) follow the 5′ untranslated sequences. A 3′ untranslated region of 369 nucleotides follows the TGA (opal) termination codon. The 3′ untranslated region is rich in adenine and thymidine residues and contains several near-consensus polyadenylylation signals. The signal that is used, AATAAA starting at position 1078, is of consensus type (40). By comparison with the genomic sequence (unpublished data), the poly(A) tail is added commencing at position 1099.

The 585-nucleotide open reading frame begins with the second in-phase ATG codon. The first ATG at nucleotides 84–86 is followed immediately by an in-frame opal terminator. The open reading frame shown in Fig. 2 encodes a putative IGF-I precursor of 195 amino acids with a molecular weight of 21,841, assuming that the ATG codon at bases 183–185 initiates protein synthesis. At present, no direct evidence exists concerning the position of translation initiation. The mature IGF-I protein sequence is encoded by nucleotides 327–536 and is cross-hatched in Fig. 1 and underlined in Fig. 2. The 70 IGF-I codons are followed by a predicted carboxyl-terminal extension of 77 amino acids and a stop codon.

Comparison of Two IGF-I cDNA Sequences Reveals Two IGF-I Protein Precursors. In Fig. 3 the IGF-IB cDNA sequence is compared with the IGF-IA cDNA of Jansen *et al.* (15). The DNA sequences are identical over 413 nucleotides, except for one difference, a conservative third position change in a glycine codon (nucleotide 452 in Fig. 2). The DNAs then diverge. When analyzed in terms of protein sequence, the point of divergence follows a lysine residue 16 amino acids after the IGF-I region. In the gene, this corresponds to an exon-intron junction (unpublished observations). Both cDNAs predict proteins containing the same initial 134 amino acids, and in both cDNAs open reading frames continue beyond the point of divergence. The IGF-IA sequence of Jansen *et al.* (15) contains an additional 19 amino acids, for a total length of 153; the IGF-IB sequence contains an additional 61 for a total of 195 residues. The two carboxyl-terminal peptide extensions show no amino acid homology with each other or with any other protein in the National Biomedical Research Foundation Protein Sequence Data Bank.

RNA Hybridization Studies. Both IGF-IA and -IB cDNAs hybridize to RNA transcripts in human liver. In Fig. 4, an autoradiogram after hybridization of either the unique 3′ end of IGF-IA cDNA (lane A) or the unique 3′ end of IGF-IB

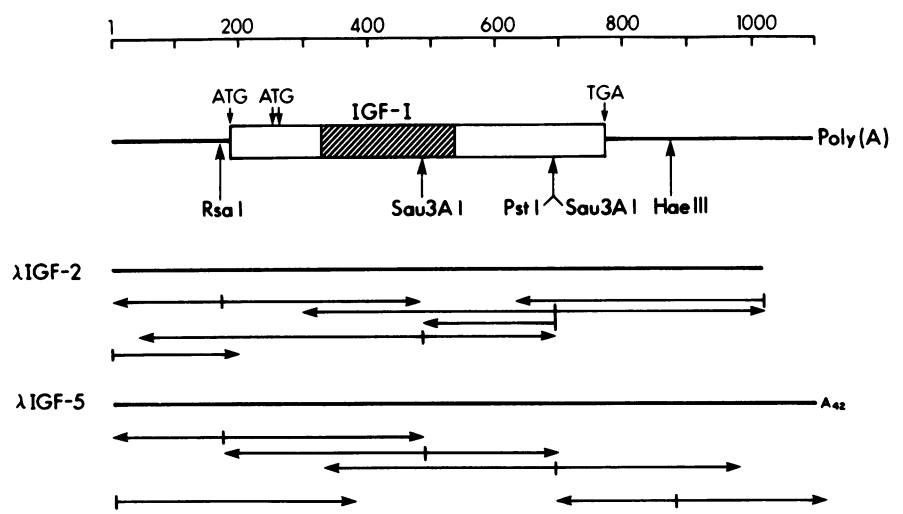

FIG. 1. Restriction map of human IGF IB-cDNA: cDNAs were isolated from a human liver library in λgt11 by screening with the 42-base oligonucleotide probe. Potential initiation codons are indicated. The 585-base open reading frame is depicted by the box; 5′ and 3′ untranslated regions are indicated by thin lines. The 70-codon IGF-I region is cross-hatched. Selected restriction enzymes are indicated. The strategy for DNA sequence determination by the dideoxy-chain-termination method is indicated below each of the clones sequenced.

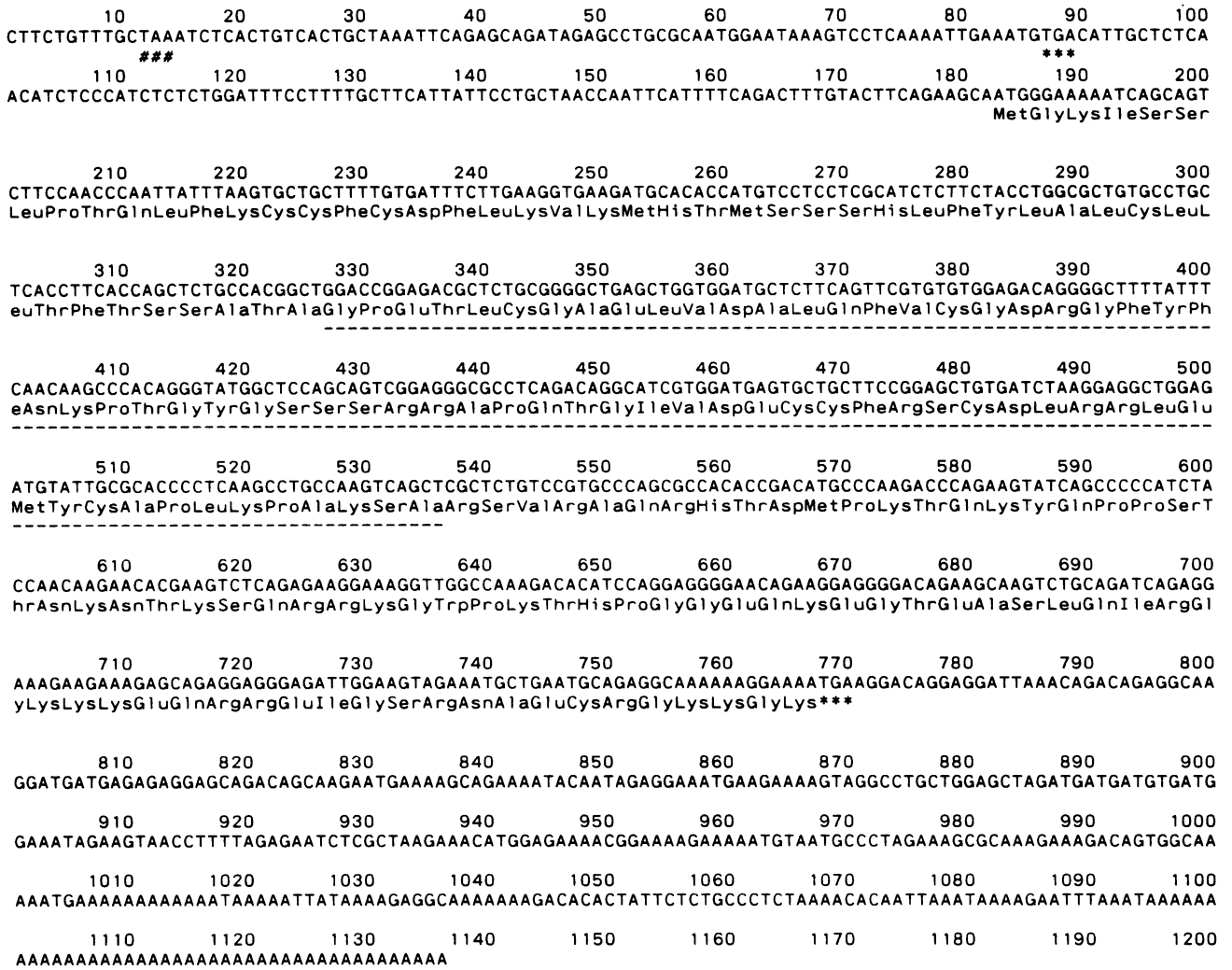

FIG. 2. DNA sequence of IGF-IB (1136 nucleotides): A translation of the 585-base open reading frame starting at nucleotide 183 is shown. The molecular weight of this putative IGF-I precursor is 21,841. In-phase termination codons are indicated by ### and ***. The region encoding IGF-I extends from nucleotides 327 to 536 and is underlined.

cDNA (lane B) to human liver polyadenylylated RNA shows a major band of ≈1100 nucleotides. Other larger bands can be seen of 1.7, 3.7, and 6.3 kilobases, potentially representing partially processed precursor mRNAs or, alternatively, other IGF-I-related mRNAs. Parallel experiments using the entire IGF-IA or IGF-IB cDNA yielded similar results (not shown).

DISCUSSION

In this report, I describe the characterization of two different cDNAs encoding human IGF-I, isolated from a liver cDNA library with an oligonucleotide probe. The DNA sequence reported defines an mRNA of 1136 nucleotides and represents a second type of IGF-I mRNA, IGF-IB, identical in its amino-terminal and IGF-I coding regions to a previously described cDNA (15) but diverging at the 3′ end. Both cDNAs encode large IGF-I precursor peptides with identical amino but divergent carboxyl extensions. Both must undergo substantial protein processing to release mature IGF-I into the circulation. The nature and regulation of these processing events are as yet unknown but the 70-amino acid IGF-I molecule must be cleaved from precursors of either 153 or 195 amino acids.

Although the isolation of IGF-IB cDNA from the human liver library does not prove the presence of the IGF-IB peptide precursor (as the isolation of IGF-IA cDNA only infers the biosynthesis of IGF-IA protein), several lines of evidence support the existence of IGF-IB mRNA. First, minimizing the possibility of an artifact in cDNA construction, five IGF-IB cDNA clones of different length were isolated; the two sequenced were identical except for the extent of their 5′ and 3′ ends. Similarly, two IGF-IA cDNAs were obtained from the library, matching the previously published sequence (ref. 15; unpublished results). Second, the unique 3′ region of IGF-IB cDNA hybridizes to human liver mRNA species of similar size to those hybridizing to IGF-IA cDNA, demonstrating that both messages are expressed in liver RNA. Third, and most compelling, in the human *IGF-I* gene the exons encoding the 3′ ends of IGF-IA and IGF-IB mRNA lie on a single contiguous strand of DNA (unpublished results). Each IGF-I mRNA is thus encoded by the same gene. The existence of at least two IGF-I mRNA species in the face of current evidence for only one human *IGF-I* gene (17, 18) supports a model encompassing alternative RNA processing leading to the formation of IGF-I mRNA. Such alternative processing is not unprecedented and has been described for several genes (19, 20, 22, 23). In one example, the calcitonin/calcitonin gene-related peptide (CGRP) gene, as yet unknown tissue-specific signals govern the exclusive expression of calcitonin in the medullary cells of the thyroid and CGRP in several areas of the central nervous system (41). The proteins making up the contractile apparatus in muscle exhibit a more complex pattern of expression. Multiple forms of muscle protein mRNAs (22, 23)

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 1' CTTCTGTTTGCTAAATCTCACTGTCACTGCTAAATTCAGAGCAGATAGAGCCTGCGCAAT
61' GGAATAAAGTCCTCAAAATTGAAATGTGACATTGCTCTCAACATCTCCCATCTCTCTGGA

121' TTTCCTTTTGCTTCATTATTCCTGCTAACCAATTCATTTTCAGACTTTGTACTTCAGAAG
                                                    :::::::::
  1"                                                CTTCAGAAG

181' CAATGGGAAAAATCAGCAGTCTTCCAACCCAATTATTTAAGTGCTGCTTTTGTGATTTCT
     :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
 10" CAATGGGAAAAATCAGCAGTCTTCCAACCCAATTATTTAAGTGCTGCTTTTGTGATTTCT

241' TGAAGGTGAAGATGCACACCATGTCCTCCTCGCATCTCTTCTACCTGGCGCTGTGCCTGC
     :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
 70" TGAAGGTGAAGATGCACACCATGTCCTCCTCGCATCTCTTCTACCTGGCGCTGTGCCTGC

301' TCACCTTCACCAGCTCTGCCACGGCTGGACCGGAGACGCTCTGCGGGGCTGAGCTGGTGG
     :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
130" TCACCTTCACCAGCTCTGCCACGGCTGGACCGGAGACGCTCTGCGGGGCTGAGCTGGTGG

361' ATGCTCTTCAGTTCGTGTGTGGAGACAGGGGCTTTTATTTCAACAAGCCCACAGGGTATG
     :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
190" ATGCTCTTCAGTTCGTGTGTGGAGACAGGGGCTTTTATTTCAACAAGCCCACAGGGTATG

421' GCTCCAGCAGTCGGAGGGCGCCTCAGACAGGCATCGTGGATGAGTGCTGCTTCCGGAGCT
     :::::::::::::::::::::::::::::::: ::::::::::::::::::::::::::
250" GCTCCAGCAGTCGGAGGGCGCCTCAGACAGGTATCGTGGATGAGTGCTGCTTCCGGAGCT

481' GTGATCTAAGGAGGCTGGAGATGTATTGCGCACCCCTCAAGCCTGCCAAGTCAGCTCGCT
     :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
310" GTGATCTAAGGAGGCTGGAGATGTATTGCGCACCCCTCAAGCCTGCCAAGTCAGCTCGCT

541' CTGTCCGTGCCCAGCGCCACACCGACATGCCCAAGACCCAGAAGTATCAGCCCCCATCTA
     :::::::::::::::::::::::::::::::::::::::::::: : :: : : : :
370" CTGTCCGTGCCCAGCGCCACACCGACATGCCCAAGACCCAGAAGGA--AGTACATTTGAA

601' CCA-ACAAGAACACGAAGT-----CTCAGAGAAGGAAAGGTTGGCCAAAGACACATCCAG
       : :::: : : : :::          :::: : ::: ::    ::::: : :
428" GAACGCAAGTAGAGGGAGTGCAGGAAACAAGAACTACAGGATGTAGGAAGACCCTCCTGA

655' GAGGGGAACAGAAGGAGGGGACAGAAGCA-AGTCTGCAGATCAGAGGAAAGAAGAAAGAG
     : : ::: ::    : :  :: : :: :  : :::    ::  :  :   :  : : :
488" GGAGTGAAGAGTGACATGCCACCGCAGGATCCTTTGCTCTGCACGAGTTACCTGTTA-AA

714' CAGAGGAGGGAGATTGGAAGTAGAAATGCTGAATGCAGAGGCAAAAAAGGAAAAT----G
     :  :::  :  :  :: :  ::  :::  ::     :: :  ::    :
547" CTTTGGA-ACACCTACCAAAAAATAAGTTTGATAACATTTAAAAGATGGGCGTTTCCCCC

770' AAGGACA-GGAGGATTAAACAGACAGAGGCAAGGATGATGAGAGAGGAGCAGACAGCAAG
     :: :: :   :  : ::::::  :  :        : : ::: ::   :::    ::::
606" AATGAAATACACAAGTAAACATTCCAACATTGTCTTTAGGAGTGATTTGCACCTTGCAAA

829' AATGAAAAGCAGAAAATACAATAGAGGAAATGAAGAAAAGTAGGCCTGCTGGAGCTAGAT
     ::::
666" AATGGTCCTGGAGTTGGTAGATTGCTGTTGATCTTTTATCAATAATGTTCTATAGAAAAG


889' GATGATGTGATGGAAATAGAAGTAACCTTTTAGAGAATCTCGCTAAGAAACATGGAGAAA
949' ACGGAAAAGAAAAATGTAATGCCCTAGAAAGCGCAAAGAAAGACAGTGGCAAAAATGAAA
1009' AAAAAAAAATAAAAATTATAAAAGAGGCAAAAAAAGACACACTATTCTCTGCCCTCTAAA
1069' ACACAATTAAATAAAAGAATTTAAATAAAAA
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FIG. 3. Comparison of two human IGF-I cDNAs. On the top line (single quotation mark) of the comparison is the sequence presented in Fig. 2 (IGF-IB) and on the bottom line (double quotation mark) is the IGF-IA cDNA of Jansen *et al.* (15). The sequences are identical over 413 nucleotides except for a conservative third position substitution within a glycine codon (at amino acid 42 of IGF-I). The DNA sequences diverge 3′ to the IGF-I region (nucleotide 585 of top sequence). IGF-IB cDNA encodes an additional 61 amino acids, while the IGF-IA encodes 19.

can be found in the same tissue at a given time. Similarly, the two IGF-I mRNAs are concurrently expressed in liver, since both cDNA types hybridize to human liver RNA and since both were isolated from the λgt11 liver library. Discovery of the steps involved in IGF-I mRNA expression in different tissues awaits further study. The availability of distinct probes derived from the 3′ end of each type of cDNA should facilitate such an analysis and make feasible experiments designed to look at tissue-specific IGF-I mRNA processing and regulation by specific hormonal mediators such as growth hormone (13, 14, 42, 43).

The existence of mRNAs encoding two different IGF-I protein precursors suggests a second level of regulation, differential processing of each peptide to mature IGF-I. It also suggests that the finding of large IGF-I-immunoreactive species in several human cell lines (13, 14, 44) may be another

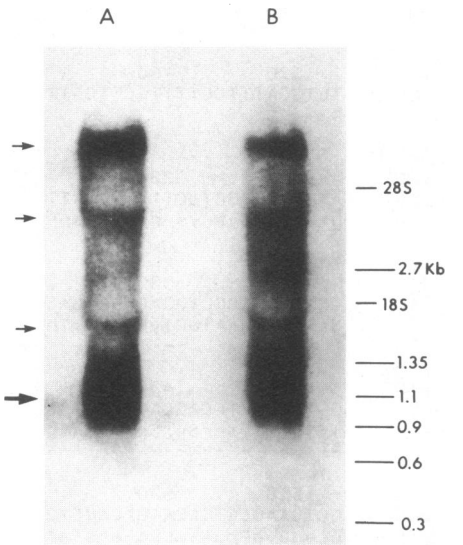

FIG. 4. Autoradiogram of human liver polyadenylylated RNA demonstrating mature IGF-I mRNA and larger forms. Ten micrograms of RNA was denatured with glyoxal, electrophoresed, and transferred to nitrocellulose as described in *Materials and Methods*. The filter was hybridized to ^{32}P-labeled IGF-I cDNA probes comprising the unique 3′ end of IGF-IA cDNA [*Bam*HI site to the poly(A) tract (15); lane A] or the unique 3′ end of IGF-IB cDNA [*Pst* I site to the poly(A) tract; lane B]. The major message is ≈1.1 kilobases (kb) long (large arrow). Larger bands of 1.7, 3.7, and 6.3 kb can be seen (small arrows).

consequence of tissue-specific regulation of IGF-I biogenesis. In addition, this observation raises the possibility that the amino and carboxyl peptides may have biological functions and that IGF-I, like pro-opiomelanocortin, may be a polyprotein (45) in which the biosynthesis of each component peptide is regulated in a tissue-specific way. Finally, the availability of the two IGF-I cDNA probes will facilitate characterization of the entire human *IGF-I* gene.

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