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Sequences of liver cDNAs encoding two different mouse insulin-like growth factor I precursors

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ABSTRACT

Complementary DNAs encoding mouse liver insulin-like growth factor I (IGF-I) have been isolated and sequenced. Alternative RNA splicing results in the synthesis of two types of mouse IGF-I precursor that differ in the size and sequence of the COOH-terminal peptide. The sequences of the signal peptides, IGF-I moieties and the first 16 amino acids of the COOH-terminal peptides or E-domains of the two precursors are identical. The sequence difference results from the presence in preproIGF-IB mRNA of a 52 base insertion which introduces a 17 amino acid segment into the COOH-terminal peptide of preproIGF-IB and also causes a shift in the reading frame of the mRNA. As a consequence of this insertion, the COOH-terminal 19 and 25 amino acids of mouse preproIGF-IA and -IB, respectively, are different. The sequences of mouse and human preproIGF-IA are highly conserved and possess 94% identity. In contrast, the sequences of mouse and human preproIGF-IB are quite different in the region of the COOH-terminal peptide. A comparison of the sequences of mouse and human preproIGF-IB mRNA indicates that they are generated by different molecular mechanisms.

INTRODUCTION

Insulin-like growth factor I (IGF-I) is a single-chain polypeptide of 70 amino acids (1). In vitro, it stimulates the proliferation of a wide variety of cultured cells that are primarily of mesodermal origin (3-5). In addition, it can cause some cells to differentiate including chondrocytes (6), myoblasts (7), osteoblasts (8) and erythroid precursors (9). In vivo, IGF-I regulates postnatal skeletal growth (5,10). The synthesis of IGF-I by many tissues (11) though suggests that it may have other functions as well. The sequences of cDNA clones encoding human IGF-I (12-14) indicate that it is generated by proteolytic processing of precursors of 130 or 172 amino acids that have been designated preproIGF-IA and -IB, respectively. The two precursors arise by alternative processing of a common primary transcript. As a consequence, the sequences of the cDNAs encoding the two human IGF-I precursors are identical in the 5'-untranslated region and the translated portion encoding the signal peptide, IGF-I and the first 16 amino acids of the COOH-terminal peptide.

They then diverge and the nucleotide sequences encoding the remainder of the COOH-terminal peptide (19 amino acids in preproIGF-IA and 61 in -IB) as well as the 3'-untranslated region are different and unrelated. These observations suggest that alternative RNA processing and the synthesis of different precursors might play a role in regulating the synthesis and/or properties of IGF-I. Unfortunately, the human is not particularly amenable for studies which could clarify these complexities of IGF-I biosynthesis. The mouse is a much more experimentally tractable organism and as a first step in developing this system, we have cloned and characterized cDNAs encoding the precursors to mouse IGF-I.

MATERIALS AND METHODS

Double-stranded cDNA was prepared from liver poly(A)$^+$ RNA (isolated from 8-week-old male BALB/c mice) as described by Gubler and Hoffman (15). After methylation of internal EcoRI sites and the addition of EcoRI linkers, the cDNA was ligated into the EcoRI site of λgt10 (16). The phage were packaged and recombinants selected by plating on E. coli strain BNN102 as described by Huynk et al. (16). Recombinants containing inserts encoding mouse IGF-I were identified by hybridization (17) with the nick-translated insert from the human preproIGF-IA cDNA, phigf1 (13). Phage were purified by successive rounds of plating and hybridization. DNA was prepared from seven of the phage and their EcoRI inserts were cloned into M13mp19 and sequenced on both strands (18).

Ten micrograms of liver poly(A)$^+$ RNA from 8-week-old male BALB/c mice were denatured with glyoxal (19) and, after electrophoresis through a 1.2% agarose gel, transferred to a nitrocellulose filter (20). ^{32}P-labeled and glyoxal-denatured fragments of a HindIII digest of lambda DNA and a HaeIII digest of ØX174 DNA were included as size standards. Nitrocellulose filter strips were hybridized with nick-translated migf1-1 cDNA or oligonucleotides labeled with [γ^{32}-P]ATP and T4 polynucleotide kinase. The oligonucleotide probes were: 1. a 20-mer whose sequence, 3' C TGA GTC TTC CTT CAT GTA A 5', was complementary to preproIGF-IA mRNA from the third nucleotide of codon 83 thru the first nucleotide of codon 90 and hybridized only to this mRNA under the conditions used; 2. a 25-mer whose sequence, 3' TTG TTC TTT TGC TTC GAC GTT TCC T 5', was unique to preproIGF-IB mRNA and complementary to this mRNA from codon 93 thru the first nucleotide of codon 101; and 3. a 25-mer whose sequence, 3' TTTTCCTCGAGGCCCCCTACTTACG 5', was complementary to that of the variant 5'-untranslated region. The hybridization conditions used with the

cDNA probe have been described previously (17). The oligonucleotide probes were hybridized with the filters at 42°C overnight in a solution of 4XSSC (SSC is 0.15M NaCl and 0.15M sodium citrate), 40 mM sodium phosphate (pH 6.8), 2X Denhardt's solution (1X=0.02% bovine serum albumin, 0.02% Ficoll 400 and 0.02% polyvinylpyrrolidone), 0.1% sodium dodecyl sulfate and 300 μg/ml of sonicated and heat-denatured salmon testes DNA and containing $1\text{-}2X10^6$ cpm/ml of the labeled oligonucleotide. The filters that were hybridized with the cDNA probe were washed in 0.1XSSC and 0.1% sodium dodecyl sulfate at 55°C and those hybridized with the oligonucleotide probes were washed in 2XSSC and 0.1% sodium dodecyl sulfate at 50°C before autoradiography.

RESULTS

Isolation and Sequences of cDNA Clones Encoding Mouse IGF-I Precursors

Complementary DNA clones encoding mouse IGF-I were isolated from a library prepared with liver poly(A)$^+$ RNA isolated from 8-week-old male BALB/c mice by hybridization with the insert from the human preproIGF-IA cDNA clone, phigf1 (13). Sixty-six of 200-250,000 recombinants hybridized with this probe whereas none hybridized with the insert from the human preproIGF-II cDNA, phigf2 (13). Nor did the human preproIGF-II cDNA probe hybridize to any RNA species on a Northern blot of mouse liver poly(A)$^+$ RNA. Thus, in contrast to adult human or Rhesus monkey liver (13, 21, 22, L.B.R. unpublished), little if any preproIGF-II mRNA accumulates in adult mouse liver.

Seven of the phage that hybridized with the human preproIGF-IA cDNA probe were plaque-purified and their EcoR1 inserts sequenced. Unexpectedly, a number of different nucleotide sequences were observed. The sequences of migf1-2, -3 and -9 were identical in the regions in which they overlapped and contained an open reading frame which predicted the sequence of mouse preproIGF-IA, a 127 amino acid protein possessing 94% identity with human preproIGF-IA (Fig. 1A and 2). Mouse IGF-I would be generated by removal of the signal peptide and proteolytic processing at a single basic amino acid, Arg 71. The initiation codon was assigned to the fourth Met codon in the sequence. The first and second Met codons (underlined in Fig. 1A) are followed by a termination codon in the 5'-untranslated region. Initiation of translation at the third Met codon, which is in the same reading frame as IGF-I, would yield a protein of 137 amino acids, including a 32 residue signal peptide. While we cannot exclude initiation at this Met codon, the fourth may be preferred as it conforms more closely to the consensus initiation sequence, CCA/GCCATG(G), proposed by Kozak (23).

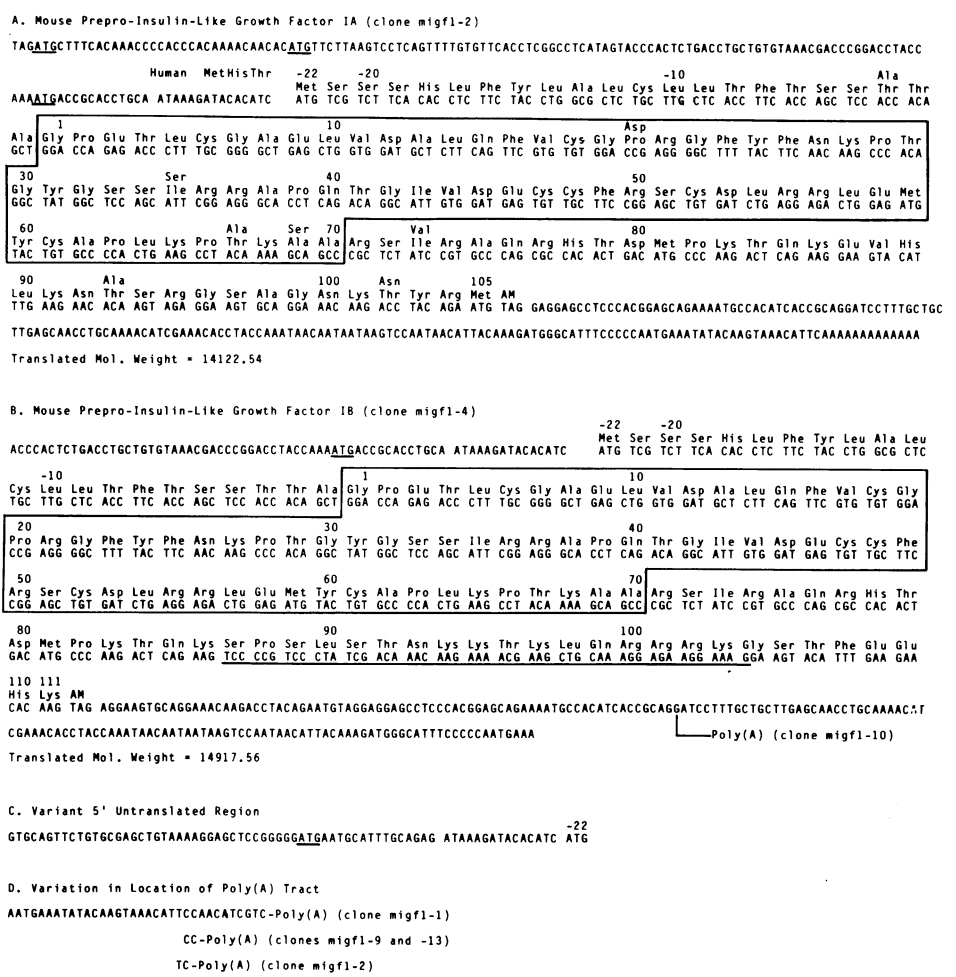

Figure 1. Sequence of mouse cDNAs encoding preproIGF-IA and -IB. The predicted amino acid sequence is numbered from the amino terminus of IGF-I. The region of each precursor corresponding to IGF-I is boxed. The B-domain of IGF-I comprises residues 1-29, the C-domain residues 30-41, the A-domain residues 42-62 and the D-domain residues 63-70. A. Sequence of a cDNA encoding preproIGF-IA. The sequence of clone migf1-2 is indicated. The amino acid residues of human preproIGF-IA which differ from those of mouse preproIGF-IA are presented above the mouse sequence. The ATGs in the 5'-untranslated region are underlined. Isolates migf1-1, -3 and -9 also code for this precursor: migf1-3 has 71 bp and 210 bp of 5'- and 3'-untranslated region, respectively, but lacks a poly(A) tract; migf1-9 extends from the third base of the codon for Tyr 24 to the poly(A) tract; migf1-1 has a variant 5'-untranslated region (Fig. 1C) and a poly(A) tract. B. Sequence of a cDNA encoding preproIGF-IB. The sequence of clone migf1-4 is indicated. The 52 bp insert is underlined. Isolate migf1-10 has 67 bp of 5'-untranslated region and a short 3'-untranslated region of 79 bp that ends with a poly(A) tract. Clone migf1-13

has a variant 5'-untranslated region (Fig. 1C) and ends with a poly(A) tract. C. Sequence of the variant 5'-untranslated region. The sequence of this region of migf1-13 is indicated. This region of migf1-1 is 60 bp. The sequence of the variant 5'-untranslated region diverges from the normal sequence 15 bp upstream of Met -22. D. Location of the poly(A) tract in cDNAs encoding IGF-I. The location of the poly(A) tract in migf1-10 is indicated in Fig. 1B.

The sequence of another cDNA, migf1-1, was identical to those described above except that it diverged in the 5'-untranslated region at a point 15 base pairs (bp) upstream from Met -22 (Fig. 1A and 1C) which is also the position of an intron in the human gene (22). As discussed below, the cDNAs and hence mRNAs of this type occur with low frequency and could result from the failure to completely remove the intron in the 5'-untranslated region. Messenger RNAs that contain this variant 5'-untranslated region also have an in-frame ATG (underlined in Fig. 1C) whose environment corresponds poorly to the consensus sequence (23).

The inserts of migf1-4 and -10 encoded a second mouse IGF-I precursor of 133 amino acids that has been designated preproIGF-IB (Fig. 1B). The sequences of these cDNAs were identical to those encoding preproIGF-IA except for the presence of a 52 bp insertion following the codon for Lys 86. This is also the site at which human preproIGF-IA and -IB diverge (Fig. 2) and corresponds to the position of an intron in the human gene (14,22). This 52 bp insertion introduces a unique 17 amino acid segment into the COOH-terminal peptide of preproIGF-IB and in addition alters the reading frame of the mRNA. As a consequence of the insertion and shift in reading frame, the COOH-terminal 19 and 25 amino acids of mouse preproIGF-IA and -IB, respectively, are different. As the sequences of the mRNAs encoding preproIGF-IA and -IB are identical except for the 52 base insertion, they most likely result from alternative splicing of a common precursor. The 52 bp insert ends with an AG dinucleotide as do all introns (24) suggesting that the presence of the insert might result from failure to completely remove the intron at this position. However, the region upstream from this AG lacks the polypyrimidine-rich character of most splice acceptor sites including the one in the intron at this position in the human gene (22,24). Consequently, we believe that the mRNAs encoding preproIGF-IB contain a small exon ending by chance in an AG and that this exon is absent in those mRNAs encoding preproIGF-IA. We also observed a cDNA, migf1-13, that has both the 52 bp insert and the variant 5'-untranslated region described above (Fig. 1B and 1C).

Five of the seven cDNAs that were sequenced had a poly(dA) tract;

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          -25          -22     -20                                  -10
Human A  Met His Thr Met Ser Ser Ser His Leu Phe Tyr Leu Ala Leu Cys Leu
Human B  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Mouse A              --- --- --- --- --- --- --- --- --- --- --- --- ---
Mouse B              --- --- --- --- --- --- --- --- --- --- --- --- ---

                                                  1
Human A  Leu Thr Phe Thr Ser Ser Ala Thr Ala Gly Pro Glu Thr Leu Cys Gly
Human B  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Mouse A  --- --- --- --- --- --- Thr --- --- --- --- --- --- --- --- ---
Mouse B  --- --- --- --- --- --- Thr --- --- --- --- --- --- --- --- ---

                10                                            20
Human A  Ala Glu Leu Val Asp Ala Leu Gln Phe Val Cys Gly Asp Arg Gly Phe
Human B  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Mouse A  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Mouse B  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                                30
Human A  Tyr Phe Asn Lys Pro Thr Gly Tyr Gly Ser Ser Ser Arg Arg Ala Pro
Human B  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Mouse A  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Mouse B  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

         40                                            50
Human A  Gln Thr Gly Ile Val Asp Glu Cys Cys Phe Arg Ser Cys Asp Leu Arg
Human B  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Mouse A  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Mouse B  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                           60                                      70
Human A  Arg Leu Glu Met Tyr Cys Ala Pro Leu Lys Pro Ala Lys Ser Ala Arg
Human B  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Mouse A  --- --- --- --- --- --- --- --- --- --- --- Thr --- Ala --- ---
Mouse B  --- --- --- --- --- --- --- --- --- --- --- Thr --- Ala --- ---

                                        80
Human A  Ser Val Arg Ala Gln Arg His Thr Asp Met Pro Lys Thr Gln Lys Glu
Human B  --- --- --- --- --- --- --- --- --- --- --- --- --- --- Tyr
Mouse A  --- Ile --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Mouse B  --- Ile --- --- --- --- --- --- --- --- --- --- --- --- --- Ser

                90                                            100
Human A  Val His Leu Lys Asn Ala Ser Arg Gly Ser Ala Gly Asn Lys Asn Tyr
Human B  Gln Pro Pro Ser Thr Asn Lys Asn Thr Lys Ser Gln Arg Arg Lys Gly
Mouse A  --- --- --- --- --- Thr --- --- --- --- --- --- --- --- Thr ---
Mouse B  Pro Ser Leu Ser Thr Asn Lys Lys Thr Lys Leu Gln Arg Arg Arg Lys

             105                      111
Human A  Arg Met AM
Human B  Trp Pro Lys Thr His Pro Gly Gly Glu Gln Lys Glu Gly Thr Glu Ala
Mouse A  --- --- AM
Mouse B  Gly Ser Thr Phe Glu Glu His Lys AM

Human B  Ser Leu Gln Ile Arg Gly Lys Lys Lys Glu Gln Arg Arg Glu Ile Gly

                                                        147
Human B  Ser Arg Asn Ala Glu Cys Arg Gly Lys Lys Gly Lys OP
```

Figure 2. Comparison of the sequences of human and mouse IGF-I precursors. The sequences of human preproIGF-IA and -IB are from Jansen et al. (12) and Rotwein (14), respectively. Dashes indicate identity with the sequence of human preproIGF-IA.

however, it was located at four different positions (Fig. 1). This heterogeneity presumably is due to the presence of a variant polyadenylation signal, AATATA, or unknown sequence in the case of migf1-10 (Fig. 1B).

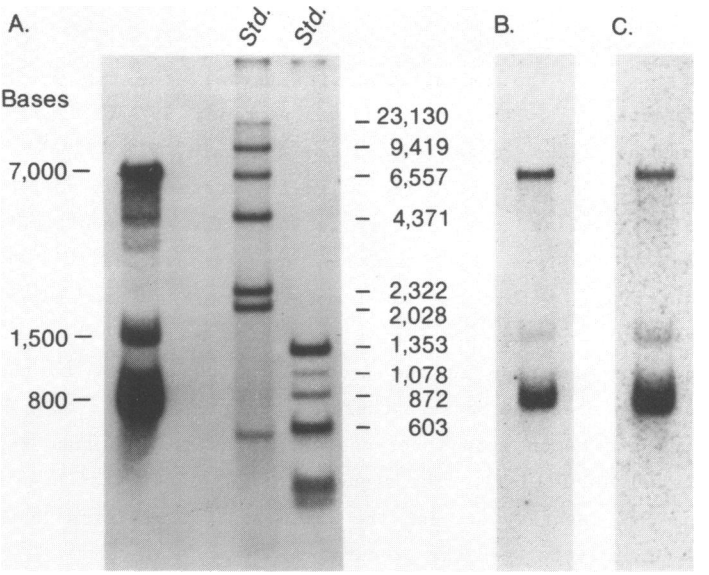

Figure 3. Northern analysis of mouse liver preproIGF-IA and -IB mRNA. A. Hybridization with the ^{32}P-labeled cDNA insert of clone migf1-1. B. Hybridization with a ^{32}P-labeled 20 base oligonucleotide specific for preproIGF-IA mRNA. C. Hybridization with a ^{32}P-labeled 25 base oligonucleotide specific for preproIGF-IB mRNA. The sizes of the transcripts and the glyoxal-denatured DNA standards are indicated.

Analysis of Mouse IGF-I mRNA

Hybridization of the cDNA, migf1-1, to a Northern blot of adult mouse liver poly(A)$^+$ RNA revealed transcripts of about 800, 1,500 and 7,000 bases (Fig. 3A). The smallest and most abundant group of transcripts ranges in size from 700-1,000 bases. Presumably the cDNAs that we have isolated and sequenced belong to this class. Also, as the size of migf1-2 (Fig. 1A) is 696 bp exclusive of the poly(A) tract and linkers, it could correspond to a nearly complete copy of the mRNA. Oligonucleotides specific for either preproIGF-IA or -IB mRNA also hybridized to all three size classes (Fig. 3). Hybridization of the oligonucleotide specific for preproIGF-IB mRNA to a replica of the cDNA library from which the clones were isolated indicated that 21 of 66 (32%) of the IGF-I cDNA clones encoded preproIGF-IB, suggesting that preproIGF-IA mRNA and presumably protein are about twice as abundant as preproIGF-IB. Only 2 of 66 (3%) of the clones hybridized to a 25 base oligonucleotide specific for the variant 5'-untranslated region and no signal was observed on hybridization of this probe to a Northern blot of mouse liver poly(A)$^+$ RNA.

DISCUSSION

The sequences of mouse and human preproIGF-IA are highly conserved (Fig. 1A, Fig. 2). The overall identity between the two proteins is 94% (119/127 residues): within the signal peptide, 95% (21/22); IGF-I, 94% (66/70); and the COOH-terminal peptide or E-domain, 91% (32/35). As one of the substitutions in the latter region is the conservative replacement of an isoleucine for a valine, the homology is 94%. The sequence homology between the E-domains of the type IA IGF-I precursor suggests that this region has an important function. Within the IGF-I moiety, there are two substitutions in the eight residue D-domain (see Fig. 1 legend for boundaries), one each in the B- and C-domains and none in the A-domain. In contrast to the extensive sequence identity observed between these molecules, there is only 66% identity between mouse and human growth hormone (25), the protein that regulates the serum levels of IGF-I (3-5). The sequence of IGF-II is also highly conserved and there is 94% identity between the human and rat proteins; however, there is only 85% identity between the precursors (26).

The nucleotide sequence homology in the regions encoding mouse and human preproIGF-IA is 89.5%. The sequences of the 5'- and 3'-untranslated regions are less highly conserved, possessing 43% and 80% homology, respectively.

The low nucleotide sequence homology in the 5'-untranslated region of the mouse and human IGF-I precursor mRNAs and the high degree of homology in the region encoding the putative signal peptide supports the contention that synthesis of the human precursors is initiated at Met -25 or -22 and not at a Met codon further upstream and that synthesis of the mouse precursors is initiated at Met -22.

Alternative RNA splicing in mouse and human liver generates a second IGF-I precursor, preproIGF-IB. A comparison of the sequences of mouse and human preproIGF-IB mRNA indicates that they arise by different molecular mechanisms and in fact do not encode homologous proteins. Human preproIGF-IA and -IB mRNA result from the use of an alternative 3'-most exon (22,27) whereas the two mouse mRNAs result from the presence/absence of a 52 base insertion. The amino acid sequences encoded by the 52 base insert of

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Mouse B TCC CCG TCC CTA TCG ACA AAC AAG AAA ACG AAG CTG CAA AGG AGA AGG AAA G
         *  * *  ** * * **  **  *** *** **  *** ***     **  **  **  *
Human B TAT CAG CCC CCA TCT ACC AAC AAG AAC ACG AAG TCT CAG AGA AGG AAA GGT T
```

Figure 4. Comparison of the nucleotide sequence of the 52 bp insert in mouse preproIGF-IB cDNA with the corresponding region of human preproIGF-IB cDNA. Asterisks indicate identical residues.

mouse preproIGF-IB mRNA and by the corresponding region of human preproIGF-IB mRNA have 59% homology (Fig. 2) (9/17 amino acids are identical and one represents a conservative replacement). In addition, 32 of the 52 nucleotides (62%) are identical (Fig. 4). This striking homology suggests that these sequences are from the corresponding regions of the mouse and human IGF-I genes and are bounded by splice acceptor and donor sites in the mouse genome and only by an acceptor site in the human. We are presently isolating the mouse IGF-I gene to elucidate the molecular basis for the generation of the different IGF-I encoding mRNAs.

IGF-I has an important role in postnatal skeletal growth and its presence in adults suggests that it has other functions as well. The data presented here, which includes that of Rotwein and his colleagues (14,27), indicate that the sequence of IGF-I is contained within three different types of precursor that are homologous in the IGF-I domain but differ in the length and sequence of the flanking COOH-terminal peptide. As precursors to IGF-I have not been isolated and sequenced, it is unknown if all three types of precursor are processed to IGF-I. The synthesis of an mRNA encoding an homologous precursor, preproIGF-IA, by mouse and human liver suggests that this form of the precursor may serve a similar function in both animals. Moreover, as we have recently indentified cDNAs encoding precursors of this type in the guinea pig, we believe that this form represents the typical IGF-I precursor. The presence of multiple IGF-I transcripts and protein precursors indicates that the biogenesis of IFG-I can be regulated at several levels: transcription, RNA processing and protein processing. The availability of oligonucleotide probes and antibodies specific for mouse preproIGF-IA and -IB mRNA and protein will allow us to examine the role of each level of regulation in the expression of this gene as well as to address the functions of the two precursors during development.

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