Identification of Five Different Insulin-like Growth Factor Binding Proteins (IGFBPs) from Adult Rat Serum and Molecular Cloning of a Novel IGFBP-5 in Rat and Human*

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Five different insulin-like growth factor binding proteins (IGFBPs) were isolated from adult rat serum using gel filtration, ligand affinity chromatography, and two steps of reversed-phase high performance liquid chromatography. Three of them were identified as IGFBP-2, -3, and -4 by their amino-terminal amino acid sequences. One of the remaining two proteins was the most homologue of the partially characterized IGFBP isolated originally from human cerebrospinal fluid, while the other appeared to be a novel member of the IGFBP family. IGFBP-1 was not found in the adult rat serum under our experimental procedures. cDNAs encoding the novel IGFBP were isolated and characterized from a rat ovary and a human placenta library. The mature protein predicted for both species contains 252 amino acids including 18 cysteines that were located in the homologous positions as IGFBP-1, -2, -3, and -4. We propose to name this protein IGFBP-5. Northern analysis of IGFBP-5 mRNA in rat tissues demonstrated that transcription of this gene is highly active in kidney, although the mRNA was detectable in all tissues examined. Alignment of the amino acid sequences of the five rat IGFBPs revealed a 47-60% similarity, indicating that their individual genes diverged from a single ancestral gene by successive gene duplications in a short time frame during evolution. The chromosomal localizations of IGFBP-1, -2, -3, -4, and -5 genes in human have been determined using polymerase chain reaction on somatic cell hybrid DNAs of human and hamster, and the results showed that they were located on chromosomes 7, 2, 17, and 5, respectively.

Insulin-like growth factors (IGF-I and IGF-II) have important functions in regulating cell growth, differentiation, and metabolism (Sara and Hall, 1990). The primary structures of the IGFs have sequence similarity with two other polypeptide hormones, insulin and relaxin (Rinderknecht and Humbel, 1978a, 1978b; James et al., 1977; Schwabe and McDonald, 1977). But unlike the latter two hormones, which are mainly produced in the pancreas and ovary, respectively, IGFs are synthesized in multiple tissues throughout the body (Zapf et al., 1984; Baxter, 1986), and their productions are stimulated by the pituitary hormones such as growth hormone, follicle-stimulating hormone, luteinizing hormone, thyroid-stimulating hormone, and adrenocorticotropic (Sara and Hall, 1990). Aside from their local production in various tissues, the IGFs are also present in blood bound to carrier proteins called IGF binding proteins (IGFBPs) (Zapf et al., 1975; Moses et al., 1976; Hinzl and Liu, 1977). The function of the IGFBPs is not clear. They may serve either to provide a storage pool and prolong the biological half-life of the IGFs in circulation, inhibit the action of the IGFs by complexing with the growth factors, or potentiate the action of the IGFs (Baxter and Martin, 1989a).

Gel-filtration analysis of adult rat and human serum under neutral conditions resolved the IGFBPs into two molecular size complexes of approximately 150 and 50 kDa (Zapf et al., 1975; Moses et al., 1976; Hinzl and Liu, 1977). SDS-PAGE fractionation of adult human serum IGFBPs, followed by Western ligand blotting with labeled IGF-I or IGF-II, revealed five bands with relative molecular masses of 41.5, 38.5, 34, 30, and 24 kDa (Hossenlopp et al., 1986). Using partial amino acid sequence information to construct oligonucleotide probes, four distinct classes of IGFBPs have been identified by molecular cloning.

The first class of BP whose complete primary structure was deduced from cDNA clones isolated from the libraries of a human HEP-G2 hepatoma cell line (Lee et al., 1988), human placenta (Brinkman et al., 1988a), as well as human (Brewer et al., 1988; Jukunen et al., 1988) and rat decidua (Murphy et al., 1990) was named IGFBP-1. Human IGFBP-1 gene has also been characterized (Brinkman et al., 1988b; Curbage et al., 1989) and its gene locus is mapped to the p12-p13 site on chromosome 1 (Brinkman et al., 1988a; Alitalo et al., 1989; Ekstrand et al., 1990). The mature protein has a molecular mass of 28-30 kDa on SDS-PAGE under nonreducing conditions, and Hossenlopp et al. (1990) has shown that it corresponds to the 30-kDa IGFBP in adult human serum by Western ligand blot.

The second class of IGFBP whose complete primary structure was determined from cDNA clones isolated from a rat BRL-3A cell line library (Brown et al., 1989), an adult rat liver library (Margot et al., 1989), and a human fetal liver.
library (Binkert et al., 1989) was named IGFBP-2. In addition, the gene encoding the human protein has been mapped to chromosome 2 (Agarwal et al., 1990). This BP exhibited a mass of 32–35 kDa on SDS-PAGE under nonreducing conditions, and it corresponds to the 34-kDa IGFBP in adult human serum (Hossenlopp et al., 1990). Both IGFBP-1 and -2 have an Arg-Gly-Asp sequence near the carboxyl terminus which may serve to attach the proteins to the cell surface integrin receptors (Rouslahti and Pierschbacher, 1987).

The third class of BP corresponds to the IGF-binding subunit within the 150-kDa IGF-binding complex in serum (Baxter and Martin, 1989b). Its complete primary structure was deduced from human (Wood et al., 1988), pig (Shimasaki et al., 1990a) and rat (Shimasaki et al., 1989; Albiston and Herington, 1990) cDNA libraries and named IGFBP-3. The 150-kDa IGF-binding complex in serum is composed of three components: an IGFBP-3 bound to either an IGF-I or IGF-II and an acid-labile 80-kDa protein which can only bind to IGFBP-3 in association with IGF under neutral conditions (Baxter and Martin, 1989b). Both the IGFBP-3 and the 80-kDa acid-labile subunit are glycosylated. Different glycosylated forms would explain why IGFBP-3 exhibits two bands at 15.5 and 38.5 kDa in adult human serum under SDS-PAGE.

The fourth class of BP was isolated from human bone cell-conditioned medium (Mohan et al., 1989) and adult rat serum (Shimokawa et al., 1989). Its complete primary structure was subsequently deduced from a rat liver and a human placenta library and named IGFBP-4 (Shimasaki et al., 1990b). Unlike the other three IGFBPs, this BP contains two extra cysteines in the midportion of the molecule in addition to the 18 homologous cysteines found in the other BPs. Moreover, it contains one potential Asn-linked glycosylation site.

While searching for additional IGFBPs in adult rat serum, we uncovered two more BPs whose complete primary structures have not been reported. One of the BPs has an amino-terminal amino acid sequence similar to the IGFBP found in human cerebrospinal fluid (Roghani et al., 1989) and human serum (Zapf et al., 1990) as well as culture medium conditioned by the SV40-transformed human fibroblast cell line AG2804 (Martin et al., 1990). The other BP has an amino-terminal amino acid sequence which has not been reported before. Using the amino-terminal amino acid sequenced information to construct a hybridization probe by polymerase chain reaction, we have now cloned the cDNAs encoding this novel BP from a rat ovary and a human placenta library. The complete primary structures deduced from their cDNA sequences are described. Based on the recommendation of the nomenclature committee (Dror, 1989), we propose to name this protein IGFBP-5.

**MATERIALS AND METHODS**

**Synthesis of Human IGF-II**—Human IGF-II was synthesized by a solid-phase peptide synthesis procedure (Ling et al., 1984) using a t-butoxy carbonyl-4-(N,N-dimethylaminomethyl)phenylacetamidomethyl resin on a model 990 peptide synthesizer (Beckman, Palo Alto, CA). Derivatized amino acids used in the synthesis were of L configuration and were purchased from Peninsula Laboratories, Inc. (Belmont, CA). The N'-amino functional group was protected exclusively with the t-butoxy carbonyl group. The side-chain protecting groups were as follows: Bzl for Asp, Thr, Ser, Glu; p(2,6-Cl)Bzl for Cys; 2-(2,6-Cl)Bzl for Tyr; 2-(2,6-Cl)Bzl for Lys; and tosyl- for Arg. After the last residue was coupled onto the growing peptide chain, the protected peptide resin was treated with the low-high hydrogen fluoride cleavage procedure (Ling et al., 1984) using a t-- Bzl for Asp, Thr, Ser, Glu; p(2,6-Cl)Bzl for Cys; 2-(2,6-Cl)Bzl for Tyr; 2-(2,6-Cl)Bzl for Lys; and tosyl- for Arg. After the last residue was coupled onto the growing peptide chain, the protected peptide resin was treated with the low-high hydrogen fluoride cleavage procedure (Ling et al., 1984) using a t--

**Binding Assay—**Column fractions containing the IGFBPs from gel-filtration fractionation of the serum proteins were located by a binding assay using IGF-I and the bound and free labeled peptide separated by activated charcoal according to a previously published procedure (Zapf et al., 1975).

**Preparation of Ligand Affinity Chromatography Column**—6 ml (90 nmol of active ester sites) of Affi-Gel 15 (Bio-Rad) was transferred to a 1.6 × 6-cm glass column sealed with a coarse-fritted disc at the bottom. The gel was washed with 10 ml of cold water four times to remove the isopropyl alcohol, and 6 ml of 0.1 M HEPES buffer, pH 7.4, containing 2 mg of synthetic IGF-II (288 nmol) was added and the column rocked gently in the cold room overnight. Synthetic IGF-II rather than recombinant human IGF-I was used for the affinity chromatography because an earlier affinity column prepared with IGF-I (Shimokawa et al., 1989) was found to be unstable after repetitive use and would lose its binding capacity to the IGFBPs after three applications. To block the unreacted active sites, 1 ml of 1 M ethanolamine, pH 8.0 (1 mmol), was added and the mixture continued rocking for 24 h. The reacted gel was washed with 10 column volumes each of 0.1 M HEPES buffer; 20 mm sodium phosphate, 0.5 M NaCl, pH 7.4; followed by 0.5 M acetic acid, pH 3.0; and finally by 20 mm sodium phosphate, 130 mm NaCl, pH 7.4 (PBS) before application of the sample.

**Purification of IGFBP-5 from Adult Rat Serum**—Adult rat serum was purchased from Chemicon (Temecula, CA). Purification of rat IGFBP-5 was carried out by a procedure involving gel filtration of the 30% acetic acid (v/v) soluble protein in adult rat serum followed by an affinity chromatography-purified IGFBPs in adult rat serum. The experimental conditions are given under "Materials and Methods." Roughly six peaks of IGFBPs denoted by open bars I to VI were resolved in the chromatogram.

![FIG. 1. Reversed-phase HPLC fractionation of the IGF-II affinity chromatography-purified IGFBPs in adult rat serum.](image-url)

The crude peptide was extracted with 5 M guanidine HCl in 0.1 M NH₄OAc, and the pH of the extract was maintained at 5 with HOAc. After filtering off the resin, the solution was diluted with 0.1 M NH₄OAc to 2 M guanidine HCl at a peptide concentration of 1 mg/ml. The peptide was cyclized by air oxidation by stirring at room temperature for 24 h while maintaining the pH at 8.4 with 10% concentrated NH₄OH. After oxidation, the pH was adjusted to 5 and ligand affinity-dialyzed against 10 M acetic acid at 4 °C for 48 h to remove the guanidine salt. The recovered dialysate was lyophilized and the crude product gel filtered on a Sephadex G-50 fine column in 1 M acetic acid to remove the polymeric by-product. The recovered monomeric product was purified batchwise by three steps of HPLC using the conditions as described before (Ling et al., 1986), and the active substance was monitored by a ['H]thymidine incorporation bioassay using Balb/c 3T3 cells (Maciag et al., 1979). The purified product has the correct amino acid composition, and its sequence was verified by amino acid sequence analysis using a model 470A gas-phase protein sequenator (Applied Biosystems, Inc., Foster City, CA). IGF-I Binding Assay—Column fractions containing the IGFBPs from gel-filtration fractionation of the serum proteins were located by a binding assay using [125I]IGF-I and the bound and free labeled peptide separated by activated charcoal according to a previously published procedure (Zapf et al., 1975).
After all the sample had been pumped through the column, the gel bed was washed with 200 ml of PBS buffer containing 0.5 M NaCl at the same flow rate. The adsorbed proteins were eluted with 0.5 M acetic acid, pH 3.0, at 3.5 ml per h, and 1-ml fractions were collected.

The IGFBPs in the eluate fractions were located by UV absorbance at 280 nm.

The recovered IGFBPs from the affinity column were pooled and, after dialysis with an equal volume of water, pumped directly onto a 0.7-× 25-cm Aquapore RP-300, 10-mm particle size, C6 column (Applied Biosystems, Inc., Santa Clara, CA) at a flow rate of 3 ml per min. After loading, the adsorbed proteins were separated in an acetonitrile-322 gradient HPLC system (Beckman, San Ramon, CA) using a linear gradient of 18–36% acetonitrile in the 0.1% trifluoroacetic acid (v/v) solvent system (Uiterdijk et al., 1989) in 180 min at a flow rate of 3 ml per min. The column effluent was monitored by UV absorbance at 210 nm. A representative chromatogram of this HPLC step is presented in Fig. 1, which shows roughly six peaks of IGFBPs being separated. Each peak was further fractionated by another HPLC step on a 1 × 25-cm Vydac, 5-μm particle size, C6 column (Separations Group, Hesperia, CA) using a linear gradient of 14–26% acetonitrile in the 0.1% triethylammonium phosphate (v/v) solvent system (Uiterdijk et al., 1989) in 120 min at a flow rate of 1 ml per min. Under these HPLC conditions, each of peaks I, III, and IV from Fig. 1 was further separated into two peaks as shown in Fig. 2, A, B, and C, whereas peaks I, V, and VI yielded essentially a single peak (data not shown). Each of the recovered HPLC peaks was subjected to microsequence analysis in an ABI model 470A gas-phase protein sequenator as described (Esch, 1984).

Preparation of a Probe for cDNA Library Screening by PCR—Using the amino-terminal amino acid sequence of the purified rat IGFBP-5, we prepared a specific DNA probe for the corresponding region by PCR (Saiki et al., 1985). Two synthetic oligonucleotide mixture primers with all possible codon combinations for the PCR were designed, 5'-CT(GTAC)(CTAC)(CTAC)(AGCT)CA(CT);TG(CT)CA(AGCT)TG(CT)TG(CT)GA(AGCC)-(ACGT)TG(3') and 3'-AG(CG)(AG)(AG)(AG)(AG)GC(CT)GG(ACGT)CC(ACGT)AC(ACGT)AC(ACGT)AC(AG)AC(ACGT)AC(3'), which encoded the sequence Phe-Val-His-Cys-Glu-Pro-Cys at the amino-terminal and Glu-Pro-Gly-Cys-Gly-Cys at the carboxyl-terminal of the available IGFBP-5 amino acid sequence. The Gly-Cys-Gly-Cys-Cys sequence at the aminoterminal region is invariant in all of the IGFBPs that have been cloned so far (see Fig. 6). The oligonucleotides were synthesized by a Cyclone Plus DNA synthesizer (Milligen/Biosearch, Novato, CA).

PCR was performed by a TwinBlock system (Ericomp, San Diego, CA) with a GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer Cetus, Norwalk, CT) using a PMSG-stimulated rat ovary cDNA library (Esch et al., 1989) as template. Annealing reactions were performed at 60 °C for 30 s, followed by a 30 s extension at 72 °C and 15-s denaturation at 94 °C. After 35 cycles of amplification, a PCR-derived fragment of 98 bp was purified, kinased by ATP, and then cloned into the EcoRV site of pBluescript SK+ (Stratagene, San Diego, CA). The DNA sequence of the amplified fragment was determined by the double strand dideoxy chain termination method (Hatfield et al., 1984).

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purchased from BIOS (PCRableDNA, New Haven, CT), and the
be less than 250 bp and having no introns between the pair of
targets for amplification by PCR were designed by the supplier. The targets for
Clontech Laboratories (Palo Alto, CA).

The PCR-amplified fragment was labeled by a random priming
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Cloning  and  Sequencing

Cloning and Sequencing of the Rat and Human IGFBP-5—The PCR-amplified fragment was labeled by a random priming
method using [32P]dCTP and used to isolate cDNA clones encoding rat IGFBP-5. The same PMSG-primed rat ovary cDNA library (Ech et al., 1987) used for the PCR was screened with this probe by
Rehmsendorf and inserted into M13mpl9 for sequence analysis of both strands by primer-
directed double strand deoxyxide chain termination sequencing along a
template DNA using synthetic oligonucleotides (17-mers) based on

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\text{FIG. 3. Schematic representation of the rat IGFBP-5 cDNA clones (A) and the nucleotide and deduced amino acid sequence of rat IGFBP-5 (B). The shaded box denotes the signal sequence and the open box denotes the mature protein region. The solid lines represent the entire cDNA insert of the clones. The nucleotides are numbered at the right and amino acids in one-letter code are numbered throughout.}
\]

or and Sakaki, 1986) using Sequenase (United States Biochemical
Co.), and its deduced amino acid sequence was found to be identical to the
amino-terminal amino acid sequence of the purified rat IGFBP-5.

cDNA Cloning and Sequencing of the Rat and Human IGFBP-5—The PCR-amplified fragment was labeled by a random priming
method using [32P]dCTP and used to isolate cDNA clones encoding rat IGFBP-5. The same PMSG-primed rat ovary cDNA library (Ech et al., 1987) used for the PCR was screened with this probe by

Rehmsendorf and inserted into M13mpl9 for sequence analysis of both strands by primer-
directed double strand deoxyxide chain termination sequencing along a
template DNA using synthetic oligonucleotides (17-mers) based on

\[
\text{FIG. 4. Schematic representation of the human IGFBP-5 cDNA clone (A) and the nucleotide and deduced amino acid sequence of human IGFBP-5 (B). The shaded box denotes the signal sequence and the open box denotes the mature protein region. The solid lines represent the region which had been sequenced, and dotted lines represent regions that had not been sequenced. The nucleotides are numbered at the right and amino acids in one-letter code are numbered throughout.}
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Molecular Cloning of Rat and Human IGFBP-5

**RESULTS AND DISCUSSION**

Fractionation of the IGF-II affinity chromatography-purified IGFBPs in adult rat serum by reversed-phase HPLC yielded roughly six peaks as shown in Fig. 1. Each peak was further purified by another HPLC step to give two peaks (a and b) for peaks II, III, and IV of Fig. 1, as presented in Fig. 2, while peaks I, V, and VI yielded essentially only one peak (data not shown). Table I shows the results of microsequence analysis of the amino-terminal amino acid sequences of the IGFBPs in the HPLC peaks recovered from Figs. 1 and 2. The amino acid sequence of peak I is identical to the amino-terminal sequence of rat IGFBP-3 (Shimassaki et al., 1989; Albiston and Herrington, 1990). Peak IIA contains two sequences corresponding to the amino terminus of the novel IGFBP-5 and the minor sequence corresponding to the amino terminus of IGFBP-3. Different molar ratios of these proteins in the sequencing sample allowed us to determine their individual amino acid sequences. Peak IIb has the same sequence as the amino-terminal sequence of IGFBP-3. The identity of the IGFBPs in peak IIIa is similar to that of peak IIa, whereas peak IIIb contains a major sequence corresponding to the amino-terminal sequence of IGFBP-3 and two minor sequences corresponding to the truncated forms of IGFBP-3 starting at positions -162 and -165 of rat IGFBP-3, respectively. The origin of the two amino-terminal truncated forms of IGFBP-3 is not clear. They might be related to truncated IGFBP-3 proteins present in human late pregnancy serum (Hossenlopp et al., 1990b). Rat IGFBP-1 was not found in the adult serum under experimental conditions.

**Fig. 5.** Amino acid sequence comparison between rat and human IGFBP-5. Amino acids are shown in one-letter code, and only amino acids that differ from rat IGFBP-5 are presented in the human structure. Signal peptide sequences are shown in boldface. A single gap in the signal peptide is inserted in the rat sequence to allow maximal homology alignment.

**Fig. 6.** Alignment of the amino acid sequences of the five rat IGFBPs. Amino acids are shown in one-letter code and gaps are inserted to allow maximal homology alignment. Identical residues at the corresponding positions in more than three IGFBPs are shown in bold. The locations of all the cysteines are marked by asterisks except for the additional two cysteines (underlined) present only in IGFBP-4. The amino acid sequences at the mid-portion one-third of each IGFBP could not be aligned and were therefore grouped by parentheses.

**Fig. 7.** Pairwise comparison of the amino acid sequence similarity between rat IGFBP-1, -2, -3, -4, and -5. Numbers represent the percentage of similarities in the amino acid sequence of two proteins based on the method of Needleman and Wunsch (1970) and the data were calculated by using the GAP program (University of Wisconsin Genetics Computer Group (Devereux et al., 1984)) with the GAP weight parameter being set to 3.0 and the GAP length weight parameter being set to 0.1.

Sprague-Dawley rats. Tissues were frozen in liquid nitrogen immediately after excision and stored at -80 °C. Total RNA was prepared by the guanidine isothiocyanate extraction method (Chirgwin et al., 1979) and poly(A)+ RNA was selected by oligo(dT) cellulose column chromatography.

**Northern Analyses—Poly(A)+ RNA** were electrophoresed on a 0.66 M formaldehyde-agarose gel and then transferred onto nylon membrane filters. Hybridization was performed at 68 °C for 15 h with a 32P-labeled cDNA probe containing a portion of the coding region (300 bp of SacII-HindIII fragment) of the cDNA clone RBP5-501 in a solution of 50% formamide, 6 x SSPE (60 mM NaCl, pH 7.0, 1.08 M NaCl, 6 mM EDTA), 0.5% SDS, 5 x Denhardt’s solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.2 mg/ml yeast tRNA, and 0.2 mg/ml denatured salmon sperm DNA.

The filter was washed with vigorous agitation in 300 mM NaCl, 30 mM sodium citrate, 0.1% SDS for 15 min at room temperature and then incubated in 15 mM NaCl, 1.5 mM sodium citrate, 0.1% SDS for 1 h at 65 °C. Autoradiography was performed on an x-ray film with an intensifying screen at -80 °C. Molecular weight estimation of the adenylated RNA standards. The same filter was used for both IGFBP-5 and rat β-actin Northern analyses.

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From this thorough analysis of the IGFBPs in adult rat serum, two BPs whose complete primary structures have not been reported before were identified. Since the length of the amino acid sequence obtained from IGFBP-5 (Peak IIIa) was suitable for generating the corresponding DNA fragment by
Table II

Chromosome complement of human/hamster somatic cell hybrid panel (upper panel) and chromosome mapping data of human IGFBP-1, -2, -3, -4, and -5 genes (lower panel)

Percentage numbers are the percent of the cell population containing the noted human chromosome. In the upper panel, − denotes the indicated human chromosome was not detected by Giemsa staining and + denotes more than 75% of the cells contain the indicated chromosome. D and Dq denote deletion at 5p15.1-5p15.2 and multiple deletions in 5q, respectively. + and − in the lower panel indicate positive and negative results by PCR on the location of the human IGFBP genes, respectively.

| DNA pools extracted from human and hamster hybrid cell lines | 004* | 324 | 423 | 734 | 750 | 803 | 860 | 867 | 940 | 212 | 567 | 683 | 756 | 811 | 983 | 862 | 900 | 337 | 854 | 904 | 967 | 968 | 1006 | 1049 | 1079 | 1099 | 104* |
|------------------------------------------------------------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Human chromosome                                          |      |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 1                                                          | +    | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   | +   |
| 2                                                          | +    | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | +   | +   | −   | −   | −   | +   | −   | −   | −   | +   | −   | +   |
| 3                                                          | +    | +   | +   | +   | −   | −   | +   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   | −   | −   | −   | −   | −   | −   | +   | −   | +   |
| 4                                                          | +    | −   | −   | +   | −   | −   | +   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   | −   | −   | −   | −   | −   | −   | +   | −   | +   |
| 5                                                          | +    | −   | −   | +   | D  | +   | +   | +   | Dq | +   | +   | D  | +   | +   | D  | +   | +   | +   | +   | D  | +   | +   | +   | +   | +   | +   |
| 6                                                          | +    | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   | −   | −   | −   | −   | +   | −   | −   | −   | −   | −   | −   | −   | +   | −   | +   |
| 7                                                          | +    | −   | −   | −   | +   | −   | +   | −   | −   | −   | −   | −   | −   | −   | +   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   | +   |
| 8                                                          | +    | −   | −   | −   | +   | +   | +   | +   | +   | D  | +   | +   | +   | D  | +   | +   | +   | +   | +   | D  | +   | +   | +   | +   | +   |
| 9                                                          | +    | −   | −   | −   | +   | −   | +   | −   | −   | −   | −   | −   | −   | −   | +   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   | +   |
| 10                                                         | +    | −   | −   | −   | +   | +   | +   | Dq | +   | +   | D  | +   | +   | D  | +   | +   | +   | +   | +   | D  | +   | +   | +   | +   | +   |
| 11                                                         | +    | −   | −   | +   | +   | Dq | +   | +   | D  | +   | +   | D  | +   | +   | D  | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| 12                                                         | +    | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   | −   | −   | −   | −   | −   | +   | −   | +   |
| 13                                                         | +    | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   | +   |
| 14                                                         | +    | −   | −   | −   | −   | +   | −   | −   | +   | +   | +   | Dq | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| 15                                                         | +    | −   | −   | −   | −   | +   | −   | −   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| 16                                                         | +    | −   | −   | −   | +   | −   | +   | −   | +   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   |
| 17                                                         | +    | −   | −   | −   | +   | −   | +   | −   | +   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   |
| 18                                                         | +    | +   | −   | −   | −   | +   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   |
| 19                                                         | +    | −   | −   | −   | +   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   |
| 20                                                         | +    | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   |
| 21                                                         | +    | +   | −   | −   | +   | −   | +   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   |
| 22                                                         | +    | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   |
| X                                                          | +    | −   | −   | −   | −   | +   | −   | −   | +   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   |
| Y                                                          | +    | −   | −   | −   | −   | −   | −   | −   | +   | −   | +   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   |
| IGFBP-1                                                    | +    | −   | −   | −   | −   | −   | −   | −   | +   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   |
| IGFBP-2                                                    | +    | −   | −   | −   | −   | −   | −   | −   | +   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   |
| IGFBP-3                                                    | +    | −   | −   | −   | −   | −   | −   | −   | +   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   |
| IGFBP-4                                                    | +    | −   | −   | −   | −   | −   | −   | −   | +   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   |
| IGFBP-5                                                    | +    | −   | −   | −   | −   | −   | −   | −   | +   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | +   | −   |

* DNA pool 004 is from human and pool 104 is from hamster cells only.
and blotted onto the nylon membrane filter. The same filter was used for the detection of these six clones. Each of these clones was sequenced, and its DNA sequence and deduced amino acid sequence were also present in the molecule, while the middle portion of the molecule is the most divergent.

The most favorable alignment of the amino acid sequences between the five rat IGFBPs revealed a 47–60% similarity as shown in Fig. 7. The relatively high sequence homology and the narrow range in the percentage similarities among the five rat BPs indicate that their individual genes were derived by successive gene duplication in a short time period during evolution.

To determine whether these five IGFBP genes are closely linked on the same chromosome as is the case with the globin genes (Spritz and Forget, 1983), the chromosomal localizations of IGFBP-1, -2, -3, -4, and -5 genes in human were mapped with PCR technology using somatic cell hybrids of human and hamster. Each pair of PCR primers used to map the individual human IGFBP genes was designed to avoid the predicted location of introns between the primers according to the exon-intron organization of the human IGFBP-1 (Brinkman et al., 1988b; Cubbage et al., 1989) and IGFBP-3 gene (Cubbage et al., 1990). The predicted sizes of the PCR products for the IGFBP-1, -2, -3, -4, and -5 genes were 151, 204, 184, 167, and 189 bp, respectively. The results from the PCR amplification are summarized in Table II, which shows that human IGFBP-1, -2, -3, -4, and -5 genes are located in chromosomes 7, 2, 7, 17, and 5, respectively. Only one (683) out of the 25 human-hamster hybrid DNA pools gave an inconsistent result with the assignment of the IGFBP-1 and IGFBP-3 genes, suggesting that this pool of DNA probably contains a part of chromosome 7 not observable by Giemsa staining of the somatic cell hybrids. To determine whether the IGFBP-1 gene is closely linked to the IGFBP-3 gene on chromosome 7 would require more detailed gene mapping.

The presence of IGFBP-5 mRNA in various tissues collected from 3-month-old male rats was ascertained by Northern analysis, and the results are shown in Fig. 8. A very intense band migrating at 6.0 kb was detected in the kidney extract. However, this band was also detectable in all tissues examined, including testis, intestine, adrenal, stomach, spleen, heart, lung, brain, and liver. This finding is unique for IGFBP-5 because the other IGFBP mRNAs are present in the most abundant concentration in the liver. Since the concentration of IGFBP-5 in adult rat serum is much less than that of IGFBP-3 and -4, this may be a reflection of its lower mRNA content in the liver. But because the mRNA is also present in many other tissues, especially in the kidney, IGFBP-5 may serve to regulate the local action of the IGFs in an autocrine or paracrine manner.
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REFERENCES

Schwabe, C., and McDonald, J. K. (1977) Science 197, 914–915