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## cDNA cloning and mRNA expression of the six mouse insulin-like growth factor binding proteins

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### Abstract

The insulin-like growth factor binding proteins (IGFBPs) comprise a family of six distinct proteins which modulate insulin-like growth factor action. We have isolated cDNAs encoding the six mouse IGFBPs (mIGFBPs). In addition, we studied the mRNA expression of the six mIGFBPs during development and in various adult tissues. Our results show that each of the six mIGFBPs is highly homologous to their human and rat counterparts, whereas only the N and C terminal ends are conserved between the six mIGFBPs. Northern blotting revealed that mIGFBP-2, -3, -4 and -5 genes are already expressed at gestational day 11.5, suggesting a role for these mIGFBPs in embryonal development. In liver, a peak of mIGFBP-1 mRNA expression was found around birth, suggesting a function for mIGFBP-1 in the newborn mouse. Finally, tissue-specific expression of the six mouse IGFBP genes was observed in adult tissues suggesting different roles or modes of actions in adult life.

**Keywords:** Insulin-like growth factor; Insulin-like growth factor binding proteins; Cloning; Expression; Development; Murine

### 1. Introduction

Insulin-like growth factors (IGFs) are peptides displaying important functions in regulating cell proliferation, differentiation and metabolism (Sara and Hall, 1990). The IGFs are synthesized and secreted by many tissues and interact with the cell by binding to specific membrane receptors (Rosenfeld and Hintz, 1986; Daughaday and Rotwein, 1989). In the body fluids, the IGFs are found in complex with a specific group of proteins, the IGF binding proteins (IGFBPs). These IGFBPs have an affinity for IGFs equal to that of IGF-receptors and are involved in the modulation of IGF action in different tissues (Hardouin et al., 1987; Baxter and Martin, 1989). The IGFBPs belong to a family of at least six different proteins, designated IGFBP-1 to -6. At present, the cDNAs encoding the six

rat and human IGFBPs have been isolated and characterized revealing that the six IGFBPs are clearly distinct, but share regions with strong homology (Drop et al., 1992). Furthermore, IGFBP gene expression is tissue specific and developmentally regulated (Rechler and Brown, 1992).

Proposed roles of the IGFBPs are to distribute the IGFs over the various body fluid compartments and to protect the body from the hypoglycemic effects of IGFs. Another function of the IGFBPs appears to be the modulation of IGF binding to their receptors, thereby playing an important role in mediating IGF action (Clemmons, 1992). However, the precise role of the six different IGFBPs is still poorly understood. Using *in situ* hybridization techniques, we have shown recently that the six mouse IGFBPs are expressed in a tissue and cell specific manner during development, suggesting distinct roles for the six mouse IGFBPs in embryonal and fetal life (Schuller et al., 1993). Here we describe the isolation and characterization of the cDNAs encoding the six mouse IGFBPs. We have studied the mRNA expression of the six mouse IGFBPs during the second half of gestation and

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postnatally by examining total RNA extracted from whole conceptuses (day 11–18) and from pre- and postnatal liver. In addition, we determined the mRNA expression in various adult mice tissues.

## 2. Materials and methods

### 2.1. Isolation of cDNA clones

For isolation of mouse IGFBP-1 cDNA clones,  $1 \times 10^6$  phage plaques of a liver cDNA library in lambda gt11 (Promega, Madison, WI) were lifted onto nitrocellulose filters and hybridized with a  $^{32}\text{P}$ -labelled Pst1-Xba1 (490 bp) fragment of a human IGFBP-1 cDNA clone, p19 (Brinkman et al., 1988) at 59°C according to standard procedures (Maniatis et al., 1982). For isolation of the cDNAs encoding mouse IGFBP-2, -3, -4, -5 and -6, mouse liver and kidney cDNA libraries in lambda gt11 and a mouse liver cDNA library in lambda zap (kindly provided by Dr. F. van Leuven, University of Leuven, Belgium) were screened. Complementary DNA fragments specific for each of the IGFBPs were amplified by PCR as described before (Schuller et al., 1993),  $^{32}\text{P}$ -labelled and used as probe at 65°C. After washing, filters were exposed to X-ray film with an intensifying screen at -80°C for 3 days. Positive phage plaques were purified by re-screening and DNA isolated according to standard procedures (Maniatis et al., 1982). All cDNA inserts were excised with EcoR1/Not1, inserted into pBluescript KS<sup>+</sup> (Stratagene) and sequenced according to the dideoxy chain termination method (Sanger et al., 1977) using the M13 forward and reverse primers or specifically designed oligonucleotide primers, synthesized on a DNA synthesizer (Applied Biosystems).

### 2.2. Northern analysis

Tissues (liver, kidney, lung, heart, spleen, brain, muscle, testis and ovary) were obtained from adult BALB/c mice, frozen in liquid nitrogen and stored at -80°C until use. Likewise, mouse conceptuses (11–18 days gestation) were frozen in liquid nitrogen and stored. In addition, liver was isolated from 16- and 18-day-old mouse fetuses and from mice 1, 3, 7, 14 and 28 days postnatally. Total RNA was extracted by the guanidinium thiocyanate method as described by Chomczynski and Sacchi (1989). RNA was denatured with dimethylsulfoxide and glyoxal, electrophoresed in 1% agarose and transferred to nylon membranes (GeneScreen, Du Pont de Nemours, Germany). Hybridization was performed with  $^{32}\text{P}$ -labelled mIGFBP cDNA fragments at 42°C in a RNA hybridization buffer containing 50% formamide, 10% dextran sulphate, 100 µg/ml ssDNA, 10× Denhardt's, 0.1% Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 50 mM Tris (pH 7.5) and 0.1% SDS.

### 2.3. Quantification of blots

Autoradiograms were quantified by densitometric scanning using a Bio-Rad video densitometer model 620.

## 3. Results

### 3.1. Isolation of mIGFBP cDNA clones

Screening of mouse liver and kidney cDNA libraries using IGFBP specific probes resulted in the isolation of several independent clones. Sequencing of the inserts and comparison of the nucleotide sequence with that of the six rat IGFBPs revealed that we had isolated several cDNA clones for each of the six mouse IGFBPs. Furthermore, for all six mouse IGFBPs, at least one complete cDNA was isolated. The nucleotide sequences and predicted amino acid sequences of the six mouse IGFBP cDNAs are shown in Fig. 1. Comparison of the amino acid sequence of the six mouse IGFBPs showed conservation of 18 cysteine residues in all mIGFBPs, with the exception of mIGFBP-4 which has two additional cysteines and mIGFBP-6 which only contained 16 cysteine residues. Furthermore, a high degree of homology between the six mIGFBPs was observed in both the N-terminal and C-terminal regions, whereas the middle part of the IGFBPs is less well conserved. Both mIGFBP-1 and -2 contain an Arg-Gly-Asp sequence at their C-terminal end (Fig. 2). Comparison of the predicted amino acid sequences of mIGFBP-1, -2, -3, -4, -5 and -6 with the corresponding human IGFBP showed a homology of 73%, 89%, 81%, 93%, 97% and 73%, respectively. The predicted amino acid sequences showed an even higher degree of homology with the rat IGFBPs (94%, 96%, 93%, 99%, 99.6% and 94%, respectively). In mIGFBP-2, an additional glycine at position 118 was found and mIGFBP-3 lacked an alanine at position 7, when comparing them with their respective rat counterparts (Margot et al., 1989; Shimasaki et al., 1989). Furthermore, mIGFBP-6 has an insertion of 9 amino acids (residues 14–22), which is not found in rat IGFBP-6 (Shimasaki et al., 1991) (Fig. 2).

### 3.2. Developmental mIGFBP mRNA expression

The mRNA expression of the six mIGFBP genes during development was examined in total RNA extracted from whole conceptuses (11–18 days gestation) and in total RNA extracted from pre- and postnatal liver. As is shown in Fig. 3, mRNA expression of mIGFBP-2, -3, -4 and -5 was already detectable in embryos at day 11.5. Expression of mIGFBP-1 mRNA was hardly detectable and first seen in 13.5 day embryos (not shown). Transcripts for mIGFBP-6 were not detected even after an exposure time of 2 weeks (not shown).





mIGFBP-5		mIGFBP-6	
1	CCCTTTCACACACTCGCTCTCTGCGCCGCCCGAGGTAAAGCCAGACTCCGAGAAATGGTATC	1	CACGAGAAAGCCGGGTGGCCAGAGTGGGTGGCCCTCCAGTCCACCAGTTAAGGGACTGCCTTTTCC
72	S V V L L L L A A Y A V P A Q G L G S F V H C E	73	CTCCCTATCCACCCTGGCTTTTAAAGGGCCGGCGAGACTCTGCTTCTTAAAGAGAGGCCCGCAGCAGCA
144	P C D E K A L S M C P S P L G C E L V K E P G	145	GCTAGACTGACTCTCGAAAGAGTGTACAGGGCAAAACTCTGACCAATGACCTGGGATGGCTGCCACA
216	C G C M T C A L A E G Q S C G V Y T E R C A Q	217	Q P L L M L L M L L F A A G S G S A L A G C P G
288	TGTGGCTGTCATGACTTGGCCCTGGCGGAGGAKAGTCTGTGGCTTACACAGGAGCGTGGCCG	289	CAGCCGCTGCTCATGCTGCTAATGCTGTGTTGCTGCGGCTCAAGGTCAGGGTCCGCTTAGCGGGTCCCGGC
360	G L R C L P R Q D E E K P L H A L L H G R G V C	361	C G P G M Q T G C R G G C V E E D A G S P A D
432	GGTTTGGCTGCCCTCCCGGAGGATGAGAGAGCCGCTGCAGCCCTGCTGCACGCGCGCGGGGTTGC	433	TGCGGGCTGGATGCAGACTGGTGTGCGGGGGCTGGGTGGAGGAGCAAGACGCGGGATCGCTGCAGC
504	L N E K S Y G E Q T K I E R D S R E H E P T T	505	G C T E A G G C L R R E G Q P C G V Y S P K C A
576	CTCAAGAAAGACTACGGCGACCAACCAAGATAGAGAGACTCTCGGAAACACGAGGAACCCACC	577	GGCTGTACAGAGCGGAGGCTGTCTCAGAGAGAGGACAAACGTCGCGGGTCTACAGGCTAAGTGGCC
648	S E M A E E T Y S P K V F R P K H T R I S E L K	649	P G L Q C Q P R E N E E T P L R A L L I G Q G R
720	TCCGAGTGGCTGAGAGAGCCGAGAAAGCTGACCCAGTCCAGTTTGTGGGGGTGCAGAGACACT	721	CCAGGACTCGAGTGCACACCCCGAGAACTACAAAGGAGGAAACCCCAAGGAGGTGGCTCCCGCT
792	A E A V K K D R R K K L T Q S K F V G G A E M T	793	C Q R A R G P S E E T T K E S K P Q G G A S R S
864	GCTAGGCTGTGAAAGAGCCGAGAAAGCTGACCCAGTCCAGTTTGTGGGGGTGCAGAGACACT	865	TGTCAGCCCAAGAGGCGCTGGAGGACTACAAAGGAGGAAACCCCAAGGAGGTGGCTCCCGCT
	A H P R V I P A E N R Q E S E Q G P C R R H M	937	R D T N H R D R Q K N P R T S A A P I R P M P V
	GCCCACCCAGTCAITCCCTGCACCTGAGATGACACGGATCCGAAACAGGCCCTGCCGACACACATG	1009	CGTGACACAAACCCAGAGACCAGGCAAGAAATCCACGGACCTCTGCTGCCCTATAGGCCCAATCTGTT
	E A S L Q E F K A S P R M V P R A V Y L P N C D	1081	Q D S E M G P C R R H L D S V L Q Q L Q I E V F
	GAAAGCTCCCTCCAGGAGTTCAAAGCCAGCCACGCAATGGTCCCGCTGCTGATACVYLPNCD		CAAGTATGATGATGGGCCCTGCCGACACACTTGGATTCAGTACTGCCAGCGCTCCAGACTGAGGCTTC
	R K G F Y K R K Q C K P S R G R K R G I C H C V		R G G A R G L Y V P H C D L R G F Y R K Q C R
	CGCAAGGATTCACAAAGAAAGCAGTGTAGGCCCTCCCGTGGCGCAACGTTGCACTGCTGCTGCTG		CGAGGGGAGCAGTGGGCTCTATGTGCCAAACTGTGACCTCAGAGCTTCTACCGCAAGCAGCAGTGTCT
	D K Y G M K L P G M E Y V D G D F Q C H A F D S		S S Q G N R R G P C W C V D P M G Q P L P V S P
	GACAAGTACGGAATGAAAGTGGCGGCAAGAGTACGTGGAGTGGGACTTTCAGTGGCCAGCCCTCCAGC		TCTTGGCAGGGGAAATCGCCGTGGCCCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
	S N V E END		D G G S T Q C S A R S S G END
	AGTAACGTTGAGTGACCGCTCCCT		GATGGTCAAGAAAGCAGTCAAGTCTGCCAGAGGAGTGGGTGGAGGCCCGGAGGAGCTCCAGGAGGCA
			GCAGGAACAGGGGCTGTAAATTTGAGCTGTATGTGAAGCAATGAATACTGTGTCGCAAGACCAACCTTC
			ACGCCACCCCAATGCTCCCTGTCCCTTGGCCCTTACACAGTAGTTAGAAAGATTGCTGTTGGCTGT
			GTACTAATAAACAAGTATTGGGGGGTGTCTCTGGCTTAAAAAAMAAAAAAAAAAAA

Fig. 1. Nucleotide sequence and predicted amino acid sequence of mIGFBP--5 and -6 (c). Putative initiation codons and polyadenylation signals are underlined.

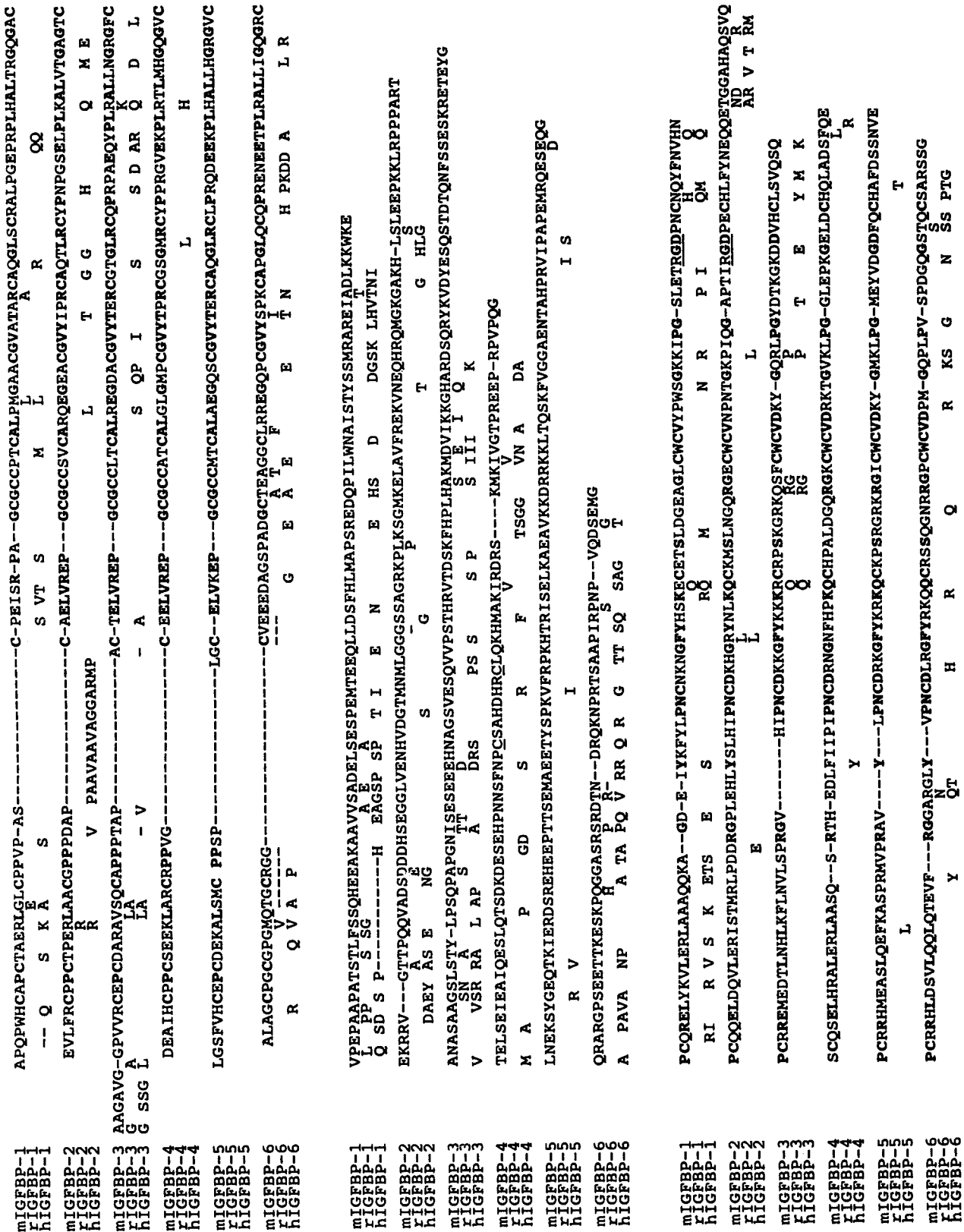


Fig. 2. Comparison of the predicted amino acid sequences of the six mouse, rat and human IGFbPs. The human and rat amino acid sequences are reported with kind permission from Shimasaki et al. (1991). Residues shared by at least four of the six IGFbPs are printed in bold letters. Only the residues that differ from the mouse sequence are shown for the human and rat IGFbPs. A dash denotes the absence of a specific residue.

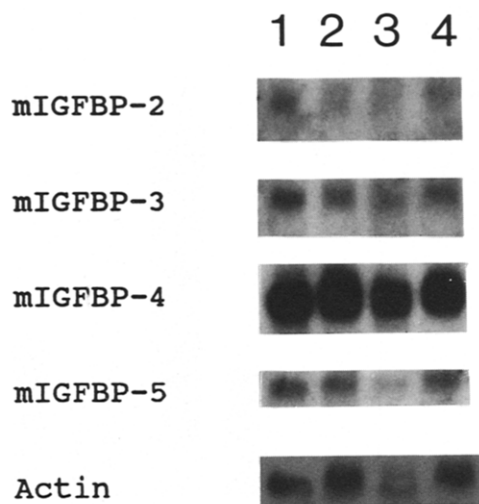


Fig. 3. Messenger RNA expression of mIGFBP-2, -3, -4 and -5 in total RNA isolated from mouse embryos. RNA (20  $\mu$ g) was applied to each lane. The RNA samples were obtained from embryos at 10.5 (lane 1), 12.5 (lane 2), 13.5 (lane 3) and 14.5 (lane 4) days of gestation.

In total RNA extracted from fetal and postnatal livers (Fig 4a), expression of mIGFBP-1 was detectable at day 16.5 of gestation and showed a dramatic increase at the first day after birth. Expression of mIGFBP-1 was still high at day 3 postnatally, but decreased to lower levels thereafter. Expression of mIGFBP-2 was also detectable in fetal livers and increased dramatically after birth. However, the expression of mIGFBP-2 decreased only slightly after postnatal day 3 and remained abundant thereafter. Expression of mIGFBP-3 transcripts was observed from day 18.5 of gestation. The levels of mIGFBP-3 mRNA expression slowly increased to a maximum at 1 week postnatally. Expression of mIGFBP-4 was observed from fetal day 16.5 and did not change dramatically over the period examined. An interpretation of these results based on densitometric measurements is given in Fig. 4b. Expression of mIGFBP-5 and -6 mRNA was not observed, even after an exposure time of 2 weeks. Finally,  $\beta$ -actin mRNA expression was found to decrease after birth. This

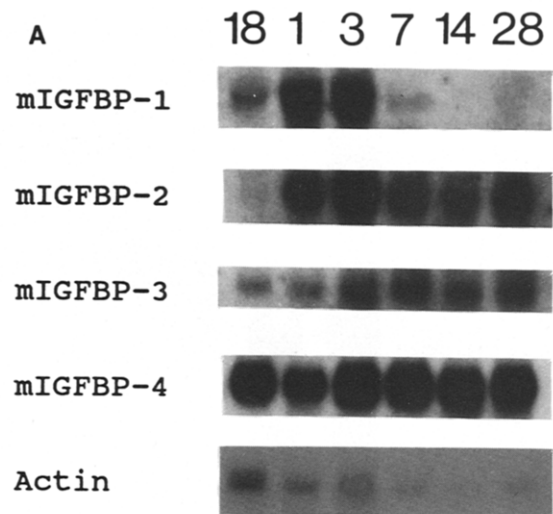


Fig 4. (a) Messenger RNA expression of mIGFBP-1, -2, -3 and -4 in fetal and postnatal mouse liver. In each lane 20  $\mu$ g of total RNA was applied isolated from 18 day fetal liver (18) and from liver obtained 1, 3, 7, 14 and 28 days after birth (1, 3, 7, 14 and 28, respectively).

observation has also been made during brain development in mouse (Lazarini et al., 1991), demonstrating that  $\beta$ -actin expression cannot always be used to show equality of the amount of RNA applied.

### 3.3. Tissue specific mIGFBP mRNA expression in adult mice

Total RNA from different organs of adult BALB/c mice was examined for IGFBP gene expression by Northern blot analysis. A summary of these results is given in Table 1. Expression of IGFBP-1 was detected solely in liver. High expression of mIGFBP-2 was found in liver, but also in kidney, lung, brain, spleen, testis and ovary. Transcripts for mIGFBP-3 were detected mainly in kidney, liver, lung, heart, spleen and muscle. Expression of mIGFBP-4 was highest in liver, kidney and spleen, but also detectable in lung, heart, brain and muscle. Expression of mIGFBP-5 was abundant in kidney, muscle and

Table 1

Messenger RNA expression of the six mIGFBPs in adult mouse tissues

	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6
Liver	+	++	+	++	-	+/-
Kidney	-	++	++	++	++	-
Lung	-	+	+	+	+	+
Heart	-	+/-	+	+	+	+
Spleen	-	+	+	++	+/-	+/-
Brain	-	+	+/-	+	+	+/-
Muscle	-	-	+	+	++	+
Testis	-	+	-	-	+	+
Ovary	-	+	+/-	-	++	+

+, detected; -, not detected; +/-, only detected after an exposure time of >10 days.

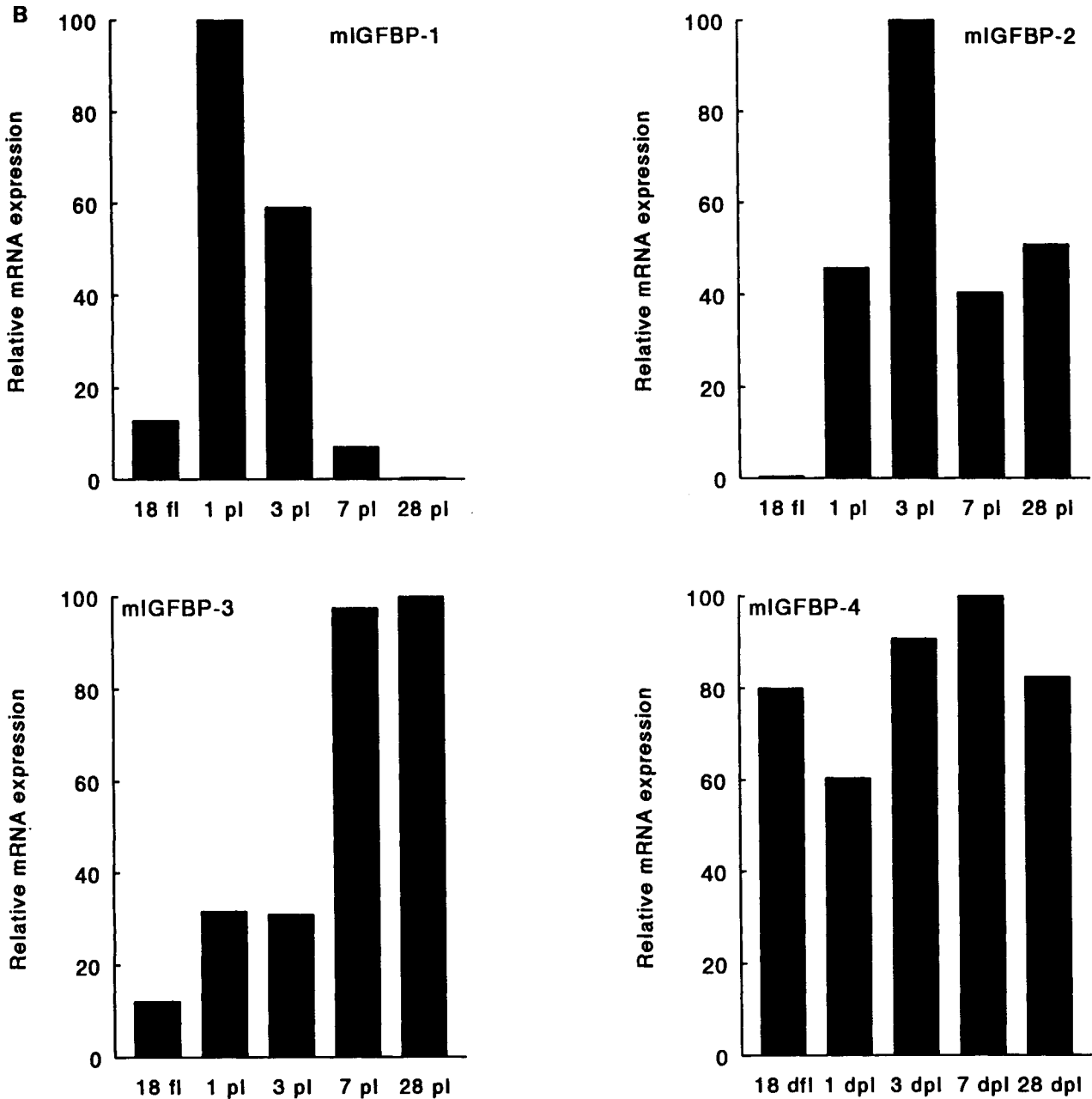


Fig. 4. (b) Relative hybridization of mIGFBP-1, -2, -3 and -4 based on densitometric measurements. Highest mRNA level of each mIGFBP in liver was set to 100%. Results are presented as a percentage of the maximum expression level. 18 fl represents 18 days fetal liver; 1, 3, 7, 14 and 28 pl represent 1, 3, 7, 14 and 28 days postnatal liver.

ovary, being lower in lung, heart, brain and testis, whereas mIGFBP-6 was expressed mainly in lung, heart, muscle, ovary and testis (Table 1).

#### 4. Discussion

Our data show that the six mIGFBPs are highly homologous in both the C-terminal and N-terminal region.

Furthermore, 18 cysteine residues were found spatially conserved in all mIGFBPs, with the exception of mIGFBP-4 which has two additional cysteines and mIGFBP-6 which lacks two cysteine residues. Thus, we conclude that the distribution of the cysteine residues in the mIGFBPs is completely analogous to that of the human IGFbPs (Drop et al., 1992). In addition, every murine IGFbP is highly homologous to its human and rat



counterpart. This also holds true for the central part of the IGFBPs where no obvious conservation is found between different IGFBPs from one species. In relation to the functions of the mIGFBPs, these observations may indicate that the N- and C- terminal regions contain domains important for all six mIGFBPs, for instance binding of IGF, whereas the middle part may represent a region that is important for the unique features of each of the mIGFBPs, such as cell specific attachment, sensitivity to proteases and protein interactions. Recently, the isolation of a mIGFBP-2 genomic clone has been reported (Landwehr et al., 1993). The predicted amino acid sequence of this clone was identical to the mIGFBP-2 cDNA clone reported here, with the one exception being a His residue at position 99, where we found an Asp residue. Whether this difference represents a cloning artefact or a natural polymorphism remains to be investigated.

In whole embryos, transcripts for mIGFBP-2, -3, -4 and -5 were already detectable at 11.5 days gestation, whereas low levels of mIGFBP-1 mRNA were first found in 13.5-day-old embryos and expression of mIGFBP-6 was below detection limits. Using *in situ* hybridization, we have shown that mRNAs for mIGFBP-2, -4 and -5 are expressed in various tissues of mid-gestational mouse embryos and mRNA expression of mIGFBP-1 was detected in liver. Expression of the mIGFBP-3 and -6 genes was found in fetal stages (Schuller et al., 1993). Furthermore, Cerro et al. (1993) recently showed by *in situ* hybridization techniques that IGFBP-1, -3, -4 and -6 were expressed at least as early as rat embryonic day 14. Our finding that mRNA expression of mIGFBP-6 was below detection limits in 20 µg total RNA extracted from whole embryos suggests that the mRNA expression of IGFBP-6 observed in rat embryonal liver and a previously unrecognized cell population surrounding developing cartilage (Cerro et al., 1993) is either at a low level or that these cells contribute to only a small percentage of the embryo and therefore the expression of mIGFBP-6 mRNA is not detectable in total RNA extracted from a whole embryo. Taken together, these observations indicate that all IGFBPs are expressed in mid-gestational embryos.

Using gene disruption techniques, it has recently been shown that both IGF-I and IGF-II are essential for normal embryonic and fetal mouse development. Homozygous disruption of the IGF-I or IGF-II genes resulted in severe growth retardation (birth weight 60% of normal littermates). Double mutants carrying both disrupted IGF-I and IGF-II genes displayed an even more severe growth deficiency, reaching only 30% of the normal birth weight (DeChiara et al., 1990; Liu et al., 1993). Comparison of embryonal growth kinetics revealed that the growth retardation induced by IGF-I gene disruption was first seen at embryonal day 13.5, whereas that induced by IGF-II disruption was already seen at embryonal day 11.5 (Baker et al., 1993). Thus, these observations indicate that all six mIGFBP genes are expressed at the stage of development

when IGF action is essential. Therefore, the IGFBPs may play a significant role in mediating the IGF effects in embryonal growth and development.

In fetal and postnatal liver, a striking mRNA expression pattern was observed for mIGFBP-1, being highest at days 1 and 3 after birth. Expression of mIGFBP-2 mRNA also increases after birth and decreases after postnatal day 3, but the expression stays high thereafter. These findings confirm the observations of Babajko et al. (1993) who showed that the transcriptional activity of the rat IGFBP-1 and -2 genes in liver are 25 times and 5 times greater at birth than at 16 days gestation. At birth, hepatic mRNA levels of rIGFBP-1 and -2 were increased approximately 50-fold and 5–10-fold, decreasing to approximately 20% by day 6 for rIGFBP-1, whereas the levels of rIGFBP-2 mRNA remained constant. These observations suggest a distinct role for mIGFBP-1 produced by the liver during the first days after birth. A possible role for transiently high levels of IGFBP-1 in early postnatal life was suggested to be the modulation of the growth promoting activities of IGFs, prior to growth hormone sensitivity (Cerro et al., 1993). Although this possibility exists, transcripts for the growth hormone receptor have been detected at earlier stages in rat liver (Berry et al., 1993). Furthermore, mRNA of the growth hormone-responsive serpin gene Spi 2.1 is expressed in fetal rat liver. In addition, the same deoxyribonuclease I hypersensitive sites were present in the 5' flanking region of the Spi 2.1 gene in DNA isolated from fetal and postnatal rat livers, suggesting that the gene is transcriptional competent in fetal life (Berry et al., 1993). Therefore, the possibility exists that the growth promoting effects of IGFs are also already under growth hormone control in late fetal and early postnatal life. Another possibility is that the high IGFBP-1 mRNA levels reflect the nutritional changes of the newborn mice, since for instance fasting has also been shown to induce both IGFBP-1 and -2 mRNA expression in rat liver (Murphy et al., 1990; Ooi et al., 1990; Orłowski et al., 1990).

The mRNA expression of the six mIGFBP mRNAs in various adult mouse tissues, as studied by Northern blotting, revealed tissue specific expression patterns for each of the six mIGFBPs, which were partly overlapping, but distinct. In general, the mRNA expression patterns of the six mIGFBPs as described here, are in agreement with previous observations for the human and rat IGFBPs (Rechler and Brown, 1992), although additional sites of low IGFBP mRNA expression have been reported.

In summary, we have isolated and characterized the cDNAs encoding the six mIGFBPs. Northern blotting revealed that mIGFBP-2, -3, -4 and -5 genes are already expressed at gestational day 11.5 and that at least five of the six mIGFBP genes are expressed at 13.5 days gestation, suggesting roles for the IGFBPs in organogenesis and embryonal development. Furthermore, the peak in especially mIGFBP-1 mRNA expression found in liver

shortly after birth suggests an important role for this protein in the newborn mouse. Finally, the tissue specific expression patterns of the six mIGFBP genes in adult mice, suggest distinct roles or modes of actions for the different mIGFBPs in adult life.

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