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Isolation and characterization of a cDNA encoding the low molecular weight insulin-like growth factor binding protein (IBP-1)

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IGF-I and IGF-II are growth-stimulating peptides with strong mitogenic properties. These polypeptide growth factors circulate in serum bound to specific binding proteins. We report the cloning and complete sequence of a cDNA encoding a low mol. wt IGF-binding protein from a human placenta cDNA library. We propose the designation IGF-binding protein 1 (IBP-1) for the gene and corresponding protein. Expression of the cDNA encoding IBP-1 in COS cells resulted in the synthesis of a 30-kd protein which binds IGF-I and is immunologically indistinguishable from the IGF-binding protein isolated from amniotic fluid or human serum. Northern blotting analysis demonstrated that expression of the IBP-1 gene is highly tissue specific and limited to placental membranes and fetal liver suggesting a rigid control. The IBP-1 gene is a single copy gene, located on chromosome 7. The results obtained suggest that most, if not all, lower mol. wt IGF-binding proteins originate from this gene.
Key words: IGF-binding protein/human placenta/human fetal liver/HEPG2 cell line/cDNA

Introduction

Insulin-like growth factors (IGF) I and II are small polypeptide hormones structurally related to insulin (Rinderknecht and Humbel, 1978a,b). The IGFs evoke a number of biological activities including cellular induction of DNA synthesis and cell multiplication (Rechler *et al.*, 1974). IGF-I is thought to mediate the growth-promoting effects of growth hormone (Van Wyk *et al.*, 1974), while IGF-II is primarily expressed in first trimester human fetal tissue (Scott *et al.*, 1985).

The biological effects of the IGFs are exerted through association to specific receptors. The IGF-I receptor binds IGF-I and IGF-II with high affinity and insulin with lower affinity, the IGF-II receptor binds IGF-II with high affinity, IGF-I with lower affinity but has no significant affinity for insulin (Massague and Czech, 1982).

Unlike insulin both IGFs circulate in plasma tightly bound to specific binding proteins (BP). Two major forms of IGF-BPs with apparent mol. wts of 150 and 30-35 kd have been identified in human plasma (Smith, 1984). In addition, a wide variety of IGF-BPs have been isolated from various biological sources, e.g. tissue extracts (brain, pituitary and cerebrospinal fluid) and cell culture media (human hepatoma cell line HEPG2). The mol. wts range from 24 to 150 kd (Wilkins and D'Ercole, 1985; Binoux *et al.*, 1986). Some of these proteins are related to the 150-kd BP (Baxter *et al.*, 1986a), some are thought to be derived from the low mol. wt IGF-BP by proteolytic processing (Huhtala *et al.*, 1986). The NH_2-terminal amino acids of the 30-35-kd BPs derived from amniotic fluid, human serum and the HEPG2 cell line are completely homologous, suggesting that these proteins are in fact identical (Drop *et al.*, 1984a; Pòvoa *et al.*, 1985). Recently, another 35-kd IGF-BP has been isolated from soluble extracts of term human placental/fetal membranes (Koistinen *et al.*, 1986). The protein has been designated placental protein 12 (PP12) and the preferential site of synthesis of PP12 is in the secretory/decidualized endometrium (Rutanen *et al.*, 1986). Of the 15 NH_2-terminal amino acids of PP12 that have been determined 13 are identical to the sequence of the low mol. wt BPs found in amniotic fluid, serum and HEPG2 cells (Koistinen *et al.*, 1986). Because of the confusing terminology, the abbreviation IGF-BP28 has been suggested for the lower mol. wt binding proteins and for similar proteins sharing the same NH_2-terminal amino acid sequence. The designation is based on electrophoretic mobility of these proteins in non-reduced SDS-PAGE (Baxter *et al.*, 1987).

In various bioassays partly purified BP fractions were shown to inhibit the insulin-like and growth-promoting activities of IGF (Drop *et al.*, 1979; Zapf *et al.*, 1979). *In vivo* the BPs increase the biological half-life of IGFs (Hintz, 1984). Human amniotic fluid is an abundant source of 30-35-kd IGF-BP and very high levels are found in fetal serum suggesting a possible role during embryonal and fetal development (Drop *et al.*, 1979, 1984b). However, the precise biological function of these proteins is still unclear.

As a first step towards a better insight into the function of IGF-BP we have screened a cDNA library with an antibody to the low mol. wt IGF-BP and obtained cDNA clones encoding this protein from two different cDNA libraries. We propose the designation IBP-1 for the gene and

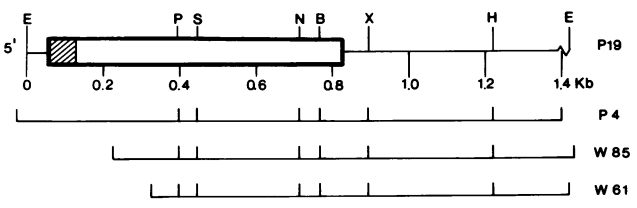

Fig. 1. Restriction map for human IBP-1. The four cDNA clones are aligned, p4 and p19 are representatives from the placenta cDNA library, w61 and w85 originate from the liver cDNA library. The composition restriction map is shown at the top of the figure. The open box represents translated regions. The putative leader sequence is shaded. Solid lines represent untranslated regions. The zigzag line represents the poly(A) tail. E = *Eco*RI, P = *Pst*I, B = *Bam*HI, H = *Hin*dII, S = *Sst*I, X = *Xba*I, N = *Nco*I.

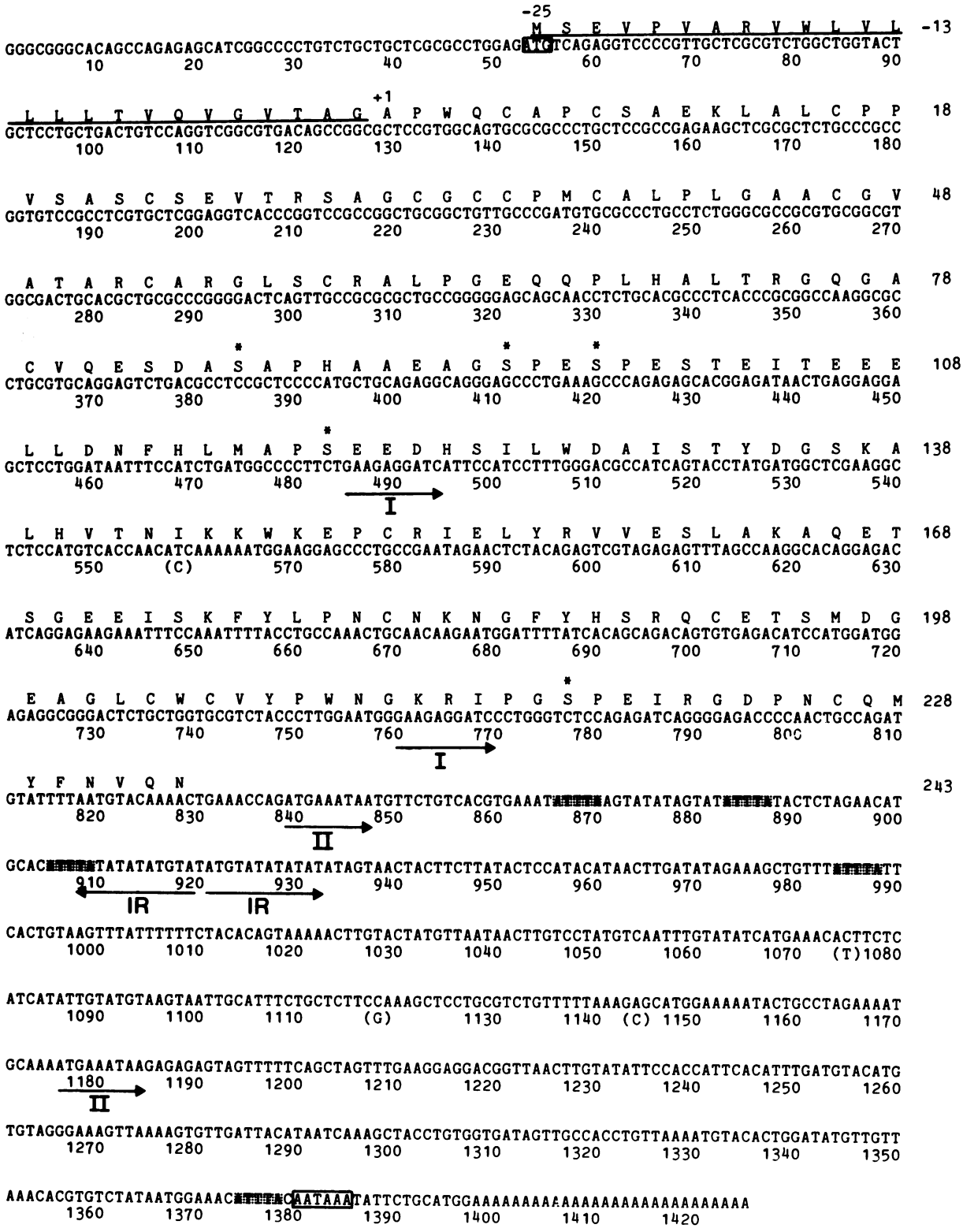

Fig. 2. Nucleotide and deduced amino acid sequence of human IBP-1. The putative initiating codon and the polyadenylating signal are boxed. A thin line represents the leader sequence. Also indicated are possible O-linked glycosylation sites (*), two repeated sequences (I and II) and an inverted repeat (IR). Differences between the placental cDNA sequence and the liver cDNA sequence are shown in parentheses.

corresponding protein. Northern blot analysis revealed that expression of the IBP-1 gene is highly regulated with specific expression in placental membranes and also in fetal and adult liver. In addition, we show that the IBP-1 gene is a single copy gene and is located on chromosome 7.

Results

Isolation of cDNA clones encoding IBP-1

We have previously reported the isolation of a polyclonal antibody raised against IGF–BP isolated from amniotic fluid

(Drop *et al.*, 1984a). The antibody was used to screen a human placental cDNA library in λ gt11. In this library 33 plaques which strongly cross-reacted with the polyclonal antibody were identified. Ten of them were rescreened and the inserts were isolated. The inserts, varying from 0.9 to 1.5 kb, were isolated and subcloned in the vector PTZ19. Restriction enzyme analysis of the subclones and Southern blot experiments showed that all clones but one originated from the same mRNA. In addition two other clones were isolated from a human-liver-derived cDNA library. Restriction enzyme analysis indicated that the clones isolated from the placenta library and the clones isolated from the liver library were colinear, supporting their candidacy as IGF−BP clones. Figure 1 shows the physical map and size of four representative cDNA clones.

Nucleotide sequence of IBP-1

The complete nucleotide sequence of the cDNA insert of two clones (p19 and w85) has been determined (Figure 2). The 1425-nt sequence of p19 contains one major open reading frame of 777 nucleotides. The potential initiation codon at position 53 is flanked by sequences matching Kozak's criteria for an initiation codon (Kozak, 1986). At the 3′ end the putative open reading frame is flanked by a translation termination codon (TGA) and a 596-nt-long 3′ untranslated sequence. At the 3′ end a canonical polyadenylation signal (AATAAA) is found 13 nt upstream of the poly(A) tail. The coding region contains a 10-nt-long repeat (Figure 2, I) and in the 3′ untranslated region a 9-nt-long repeat (Figure 2, II) and a 12-nt-long inverted repeat can be found (Figure 2, IR).

The nucleotide sequence is characterized by an unusually high content of CpG dinucleotides in the 5′ coding region. Between nucleotides 1 and 361 the content of C plus G is 74% and the level of the dinucleotide CpG is 94% of that expected on a random basis. Thus this region does not show marked suppression of the CpG dinucleotides as commonly seen in eukaryotic DNA (Gardiner-Garden and Frommer, 1987).

The 3′ untranslated region contains AT-rich sequences which are frequently found in transiently expressed genes of certain lymphokines, cytokines and proto-oncogenes. A common motif to these genes is the pentanucleotide sequence ATTTA, which has been proposed to destabilize the mRNAs of these genes (Shaw and Kamen, 1986).

Primary structure of the IBP-1 precursor

The open reading frame in cDNA clone p19 has a coding capacity for a protein of 259 residues, with a calculated M_r of 28 172 daltons. The proposed initiating methionine is the first amino acid of a series of highly hydrophobic amino acids, suggesting the presence of a signal peptide. A recently developed method for predicting signal sequence cleavage sites (Von Heijne, 1987) revealed a favourable signal peptide cleavage site after the glycine shown in Figure 2 as residue −1. The thus predicted NH_2 terminus of the mature protein is identical to the chemically determined NH_2-terminal sequence of the IGF-binding protein isolated from amniotic fluid (Pòvoa *et al.*, 1984a,b; Baxter *et al.*, 1986a) and the HEPG2 cell line (Pòvoa *et al.*, 1985). In view of these findings we propose that the ATG codon at position −25 in Figure 2 is indeed the ATG where translation initiation takes place.

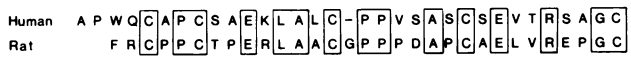

Fig. 3. Comparison of the deduced NH_2-terminal amino acid sequence of human IBP-1 with the NH_2-terminal rat IGF−BP. The conserved amino acid regions between the human and rat proteins are boxed.

The nucleotide sequences of the liver cDNA clones differ in four nucleotides from the placenta clones. Of these three are located in the 3′ untranslated region. The coding region bears a transition resulting in the incorporation of a Thr instead of an Ile (Figure 2). These differences may be caused by allelic variation of the IBP-1 gene.

The mature protein is unusually rich in tryptophans. We have identified six Trp residues in the amino acid sequence accounting for 2.6% of the total amino acid sequences. Other characteristic features are a cysteine-rich region with nine cysteine residues concentrated between residues 1 and 46. The primary structure of the protein encloses three regions (80−102, 105−127 and 141−178) with prominent α-helix properties, separated by short unordered segments. A short stretch of amino acids with the features of a phosphoryl binding site is located in the COOH-terminal part of the protein. Although the low mol. wt IGF−BP was found to be glycosylated (Bohn *et al.*, 1980; Koistinen *et al.*, 1987), the deduced amino acid sequence did not enclose N-linked glycosylation sites (N−T and N−S). However, at least five potential O-linked glycosylation sites (Takahashi *et al.*, 1984) were found in the NH_2-terminal two thirds of the molecule.

The IBP-1 protein did not show any significant homology to 4612 entries of the National Biomedical Research Foundation, protein data base release no. 11.0 and no. 26.0, suggesting that IBP-1 is a unique protein. Comparison of the amino acid sequence of IGF-I, IGF-II, insulin, IGF-I receptor and the IGF-II receptor with that of IBP-1 revealed no considerable homology.

Recently the NH_2-terminal amino acid sequence of the low mol. wt IGF−BP synthesized in rat BRL 3A cells has been presented (Mottola *et al.*, 1986). With the complete amino acid sequence available we demonstrate here that the NH_2-terminal part of human IBP-1 is strongly homologous with that of the rat IGF−BP (Figure 3), suggesting that the gene is conserved during evolution. In addition, no homology was found with the reported NH_2-terminal amino acid sequence of the rat high mol. wt IGF−BP (Baxter *et al.*, 1986b), indicating that these proteins are distinct.

Expression in COS-1 cells, immunoprecipitation and IGF-I binding

We constructed the expression plasmid pSV19 by inserting cDNA p19 (Figure 1) into expression vector pSV328 (Van Heuvel *et al.*, 1986). In pSV19 the IBP-1 coding sequence is under direction of the SV40 early promoter. Monkey COS-1 cells were transfected with this plasmid and pSV328 as a negative control. Samples of the medium of transfected COS-1 cells were used for non-reduced SDS−PAGE and subsequently transferred to nitrocellulose filters. The filters were incubated with either [^{125}I]IGF-I or antibody against low mol. wt IGF−BP. The results are shown in Figure 4. In lane B (pSV19) a band of 30 kd cross-reacting with the antibody is visible, which is absent in lane A (pSV328). This band migrates at the same position as the IGF−BP present in amniotic fluid (lane C). When incubated with [^{125}I]IGF-I

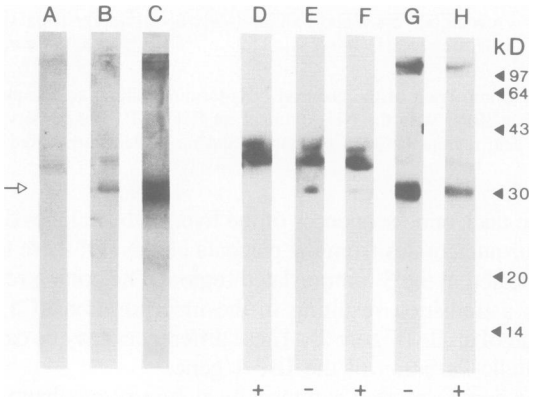

Fig. 4. Binding protein analysis in culture media from COS-1 cells transfected with pSV328 (**lanes A** and **D**), pSV19 (**lanes B, E** and **F**) and amniotic fluid as a positive control (**lanes C, G** and **H**). The IGF−BPs were separated by SDS−PAGE (12.5% acrylamide) and transferred onto nitrocellulose by Western blotting. The filters were incubated with >200 000 c.p.m. [^{125}I]IGF-I with (+) or without (−) 150 ng cold IGF-I, washed and autoradiographed (**lanes D−H**). After autoradiography the same filters were incubated with antibody against low mol. wt IGF−BP (**lanes A−C**). The immunoreactive bands were made visible by immunostaining as described for the screening of the cDNA libraries. The IBP-1 protein is indicated by an arrow.

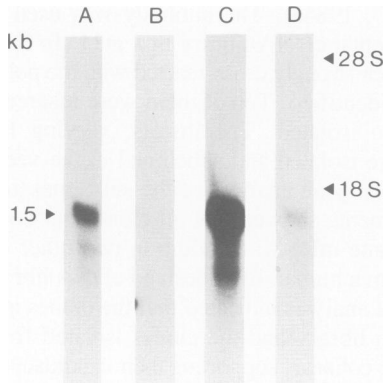

Fig. 5. Northern blot analysis of total mRNA isolated from HEPG2 cell line (**lane A**), human placenta (**lane B**), placental membranes (**lane C**) and human fetal liver (**lane D**). The samples containing 50 μg of RNA were electrophoresed on 0.8% agarose containing formaldehyde, transferred to nitrocellulose and hybridized with the 5′ *Eco*RI−*Pst*I fragment of IBP-1 cDNA as probe.

the same 30-kd band binds [^{125}I]IGF-I (lane E). This binding is specific because it can be displaced by cold IGF-I (lane F). Lanes C, G and H are control samples of IGF−BPs present in amniotic fluid showing a 30-kd band which reacts with the antibody (lane C) and with [^{125}I]IGF-I (lanes G and H without and with excess cold IGF respectively). Incubation with [^{125}I]IGF-II gave similar results (data not shown). Thus the IBP-1 cDNA encodes a IGF−BP which has the same M_r, immunoreactivity and IGF-binding properties as the IGF−BP present in amniotic fluid. In addition slower migrating bands of ~34 kd can be found in medium from pSV19 and control transfected cells (lanes A, B and D−F). These bands cross-react with the antibody (lanes A and B) and also bind [^{125}I]IGF-I which cannot be displaced by cold IGF (lanes D−F). They presumably represent non-specific cross-reacting proteins from COS-1 cells. The high mol. wt band in amniotic fluid which specifically binds IGF-I probably is the high mol. wt IGF−BP (lanes F and G).

Tissue specific expression of IBP-1 mRNA

Total RNA prepared from human fetal liver, the HEPG2 cell line, human placental tissue and placental membranes from term pregnancy were examined for IBP-1 expression by Northern blot analysis. Using a ^{32}P-labelled 5′ *Eco*RI−*Pst*I fragment derived from cDNA clone p19 as probe, a single transcript of ~1.5 kb was demonstrated in HEPG2 and in fetal-liver-derived RNA (Figure 5, lanes A and D). Since the cDNA clone p19 was isolated from a placental cDNA library it was surprising to find that RNA isolated from placental tissue did not show any expression (Figure 5, lane B). However, analysis of RNA isolated from placental membranes showed a strong hybridizing band of ~1.5 kb (Figure 5, lane C).

In addition, Northern blots of RNAs isolated from a wide range of human fetal tissues (14−16 weeks gestation) and adult liver and kidney were hybridized with IBP-1 and human actin probes. None of the tissues, with the exception of fetal and adult liver, showed hybridization to the cDNA probe, whereas all RNA samples tested were positive for actin expression (Table I). Apparently expression of the IBP-1 gene is highly tissue specific and of all tested tissues limited to liver and placental membranes. Expression of the IBP-1 gene in fetal liver was found to be 5−10 times higher than in adult liver (results not shown).

Table I. Northern blot analysis of total RNA from different tissues hybridized to human placental IBP-1 cDNA and to actin cDNA

RNA[a]	IBP-1	Actin
Liver	++	+
Kidney	−	+
Lung	−	+
Heart	−	+
Brain	−	+
Adrenal	−	+
Tongue	−	+
Muscle	−	+
Jejunum	−	+
Thymus	−	+
Spleen	−	+
Pancreas	−	+
Liver[b]	+	+
Kidney[b]	−	+

Northern blots were kindly provided by Dr M.Jansen.
[a]Total RNA was isolated from fetal tissues at 14−16 weeks gestation.
[b]The marked RNAs originated from adult tissues.

The IBP-1 clones hybridize to a single mRNA of ~1500 nt. This suggests that clone p4 which contains an insert of 1470 bp may represent a full-length cDNA clone.

Genomic DNA analysis

To determine the size and the number of IBP-1 genes present in the human genome, Southern blot analysis was performed using a 5′-(*Eco*RI−*Pst*I) and a 3′-(*Pst*I−*Eco*RI) fragment of clone p19. Figure 6 demonstrates that both probes hybridize with single restriction fragments. In most cases the probes recognized different restriction fragments. However, in *Eco*RI-digested DNA the 5′ probe hybridized with two fragments from which the larger also hybridized to the 3′ probe. In *Pst*I-digested DNA an additional 2.7-kb fragment

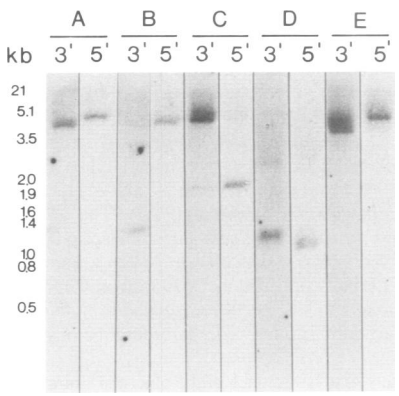

Fig. 6. Southern blots of genomic DNA hybridized to 3′ and 5′ fragments of IBP-1 cDNA. About 10 μg genomic DNA was digested with *Hin*dIII (**lane A**), *Bgl*II (**lane B**), *Sst*I (**lane C**), *Pst*I (**lane D**) and *Eco*RI (**lane E**). After digestion the samples were electrophoresed on 0.8% agarose and subsequently transferred to nitrocellulose and hybridized using either the 3′ *Pst*I–*Eco*RI fragment or the 5′ *Eco*RI–*Pst*I fragment of IBP-1 cDNA as probe.

Table II. Segregation of human IBP-1 gene in rodent–human hybrids

Human chromosome	Chromosome/IBP-1				% discordancy
	+/+	+/−	−/+	−/−	
1	9	6	6	8	41
2	4	2	11	12	45
3	8	2	7	12	31
4	9	4	6	10	34
5	10	2	5	12	24
6	12	4	3	10	24
7	14	1[a]	1[b]	13	7
8	10	7	5	7	41
9	9	6	6	8	41
10	7	6	8	8	48
11	7	5	8	9	45
12	10	6	5	8	38
13	9	6	6	8	41
14	11	9	4	5	45
15	8	5	7	9	41
16	9	5	6	9	38
17	14	9	1	5	34
18	8	1	7	13	28
19	11	7	4	7	38
20	11	7	4	7	38
21	12	7	3	7	34
22	12	7	3	7	34
X	8	9	7	5	52

[a]Exception may be due to small deletion of chromosome 7 not detected by cytogenetic analysis in this clone.
[b]Weak hybridization signal.

was found, which weakly hybridized to the 3′ probe and probably encodes only a small part of the cDNA. Genomic DNA digested with *Bgl*II reveals fragments of 1.2 and 4.5 kb, which hybridize to the 5′ and 3′ probes respectively. These data suggest that the IBP-1 gene is a single copy gene spread over at most 5.7 kb in genomic DNA.

Chromosomal localization

Southern blot analysis of genomic DNA isolated from 29 human–rodent somatic cell hybrids was used for the chromosomal localization of the IBP-1 gene. Filters containing *Pst*I- and *Sst*I-digested DNA from hybrids and their parental cell lines were hybridized with ^{32}P-labelled IBP-1 cDNA clone w85 (Figure 1). Specific 1.1- and 1.2-kb *Pst*I and 5- and 2.1-kb *Sst*I hybridizing fragments were clearly distinguishable in size from rodent cross-hybridizing IBP-1 fragments (results not shown). The presence or absence of human-specific *Pst*I and *Sst*I fragments was correlated with the presence or absence of human chromosomes in the hybrids. The results of this concordance analysis are summarized in Table II. The human IBP-1 fragments appeared to segregate with chromosome 7 with high (93%) concordance. Only two exceptions were observed. One discordant clone did contain chromosome 7, but did not hybridize to the IBP-1 probe. This may be due to a small deletion of chromosome 7 not detected by cytogenetic analysis in this clone, a phenomenon which is known to occur in hybrid cells. The other discordant clone hybridized only weakly with the probe. For all other human chromosomes high discordance scores were obtained ranging from 24 to 52%, confirming the assignment of the IBP-1 gene to human chromosome 7.

Discussion

IGF–BPs with apparent mol. wts of 24–150 kd have been isolated from different biological sources. The designation IGF–BP28 has been proposed for IGF–BPs with an apparent mol. wt of 28 kd under non-reducing conditions and for BPs with the same NH_2-terminal sequence (Baxter *et al.*, 1987). In this paper we describe the isolation of several clones from λ gt11 cDNA libraries using an antibody raised against the low M_r IGF–BP from amniotic fluid. All cDNA clones were found to derive from at most two alleles of the same single copy gene. These results strongly suggest that most, if not all, lower mol. wt IGF–BPs originate from this gene. In view of this finding and the confusing terminology for IGF–BPs, we propose the designation IBP-1 (IGF-binding protein 1) for the gene and corresponding protein.

The IBP-1 cDNA sequence predicts a mol. wt of 25 kd for the 234-amino-acid-long mature protein. The M_r found under non-reducing conditions is 30 kd. It has been reported that the protein is glycosylated. If true, this cannot be due to N-glycosylation because putative N-glycosylation sites are absent from the primary sequence of the protein. Although no definitive consensus sequence for O-glycosylation is known, recently some structural similarities in O-linked glycosylation sites have been observed (Takahashi *et al.*, 1984). Several such sites are found in the NH_2-terminal part of IBP-1. Thus, it is possible that O-linked glycosylation is partly responsible for the observed difference in M_r. Besides this discrepancy there is a difference between the M_r under non-reducing (30 kd) and reducing conditions (34 kd). This is probably due to the high cysteine content of the protein, enabling the formation of several S–S bridges, with as a consequence a faster migration in the gel.

Isolation of the BP from placental membranes, PP12, also yields lower mol. wt peptides which are thought to arise through proteolytic processing (Huhtala *et al.*, 1986). The presence of two putative proteolytic processing sites in the primary sequence of IBP-1 supports this finding. Some of the PP12-derived fragments were still able to bind IGF. They were shown to have the same NH_2-terminal sequence as PP12, suggesting that the IGF-binding site is located in the NH_2-terminal part of the protein. From our data it appears

that this part of the protein is very rich in cysteines. Cysteine-rich regions are frequently found in the binding portion of hormone receptors and are thought to define ligand specificity (Ullrich *et al.*, 1984, 1985; Yamamoto *et al.*, 1984). Similarly, the cysteine-rich region at the NH_2-terminal part of IBP-1 might be the portion of the molecule which binds IGF.

There is some evidence that IBP-1 may be involved in IGF–IGF receptor interaction. Binding studies with IGF-I on cultured fibroblasts demonstrated that these cells synthesize and secrete a 35-kd IGF–BP, which is associated with the cell surface. This BP is immunologically indistinguishable from IBP-1 (Clemmons *et al.*, 1987). Addition of increasing concentrations of IGF-I resulted in augmented binding of IGF-I to this cell-surface-associated BP and to the IGF-I receptor (Elgin *et al.*, 1987). In the primary sequence of IBP-1 as presented here no hydrophobic region which could serve as a membrane anchor is evident. Recently, the human GH-binding protein was found to be a truncated form of its receptor (Leung *et al.*, 1987). Similarly, evidence has been presented that a rat high mol. wt IGF–BP might be a truncated form of the rat IGF-II receptor (Morgan *et al.*, 1987). The lack of significant homology between IBP-1 and either type of IGF receptor proves that IBP-1 is not derived from one of the growth factor receptors. However, it cannot be excluded that IBP-1 might be associated with one or both types of IGF receptors and plays a role in IGF binding. IBP-1, the IGF-I and the IGF-II receptor all bind IGF-I as well as IGF-II, albeit with different affinities. An intriguing consequence of the difference in primary structure between IBP-1 and both receptors could be that the relatively small IGF peptides contain distinct binding sites for each of these three proteins.

The primary structure of the IBP-1 cDNA shows that the sequence ATTTA occurs several times in the 3′-non-coding region. This sequence is present in a similar position in several hormone mRNAs and has been shown to decrease mRNA half-life (Shaw and Kamen, 1986).

Northern blot analyses of RNA from different fetal and adult organs show that expression of the IBP-1 gene is highly tissue specific. Very large amounts of mRNA are found in placental membranes, with at most a very low expression in the placenta itself. Of the other organs tested only fetal and, to a lesser extent, adult liver were positive for IBP-1 mRNA. This type of expression correlates with the high amounts of IGF–BP that were found in amniotic fluid and in fetal serum. Thus, synthesis of IBP-1 is primarily taking place during fetal life, suggesting a specific role of this protein during fetal development. This is in accordance with the observation that synthesis of IGF–BP PP12, which presumably is identical to IBP-1, depends on progesterone stimulation (Rutanen *et al.*, 1985). As is generally known, progesterone levels increase during pregnancy, reaching a maximum shortly before birth.

The availability of complete cDNA encoding human IGF-binding protein IBP-1 will enable the investigation of the function of IBP-1 as a mediator in the complex action of insulin-like growth factors (IGF-I and IGF-II), the intriguing regulation of the gene and its putative role in fetal development.

Materials and methods

Isolation of cDNA clones and sequencing

A polyclonal antibody to the low mol. wt IGF–BP isolated from human amniotic fluid was raised and purified as described previously (Drop *et al.*, 1984a). The antibody preparation was depleted for *Escherichia coli* and λgt11 proteins and by incubation with nitrocellulose filters that had been lifted from confluent lysis plates of *E.coli* Y1090/λgt11, induced with 10 mM isopropyl β-*d*-thiogalactopyranoside (IPTG). The antibody was further absorbed against human serum albumin immobilized on nitrocellulose filters. The thus purified antibody was used to screen a human placenta cDNA library in λgt11 (kindly provided by Dr J.Groffen, Children's Hospital, Los Angeles) and a λgt11 library of RNA isolated from human liver (Chandra *et al.*, 1983) according to the procedure described by Young and Davis (1983).

Approximately 4×10^5 clones of the placental library and $\sim 0.5 \times 10^5$ clones of the liver library were screened with the purified antibody. The plates were overlaid with nitrocellulose filters (Millipore HATF) saturated with 10 mM IPTG, and they were incubated for 2 h at 37°C. The filters were removed, rinsed with Tris-buffered saline (TBS; 10 mM Tris–HCl, pH 7.5/150 mM NaCl) at room temperature and incubated with 3% BSA in TBS for 30 min at room temperature. The filters were incubated overnight with a 1:125 dilution of antibody in TBS, 3% BSA, 0.02% sodium azide for 16 h at 4°C. Subsequently the filters were washed and incubated for 60 min at room temperature with horseradish peroxidase conjugated goat anti-rabbit IgG (Tago) diluted 1:200 in 3% BSA in TBS. The filters were washed and stained with amidophenyl and naphthol AS-MX phosphate in 0.2 M Tris– HCl pH 9.2, 10 mM $MgCl_2$ at room temperature.

Isolation and analysis of DNA from positive phages was performed using standard methods (Maniatis *et al.*, 1982). Restriction fragments were subcloned in the vectors PTZ18 or PTZ19 (Pharmacia) and sequenced according to the chain termination method (Sanger *et al.*, 1977). In regions that lacked convenient restriction sites, appropriate clones were generated by *Bal*31 nuclease digestion.

DNA analysis

Genomic DNA was extracted from cultured HEPG2 cells (Blin and Stafford, 1976) and was digested with various restriction endonucleases (BRL, NEN, Boehringer) according to the suppliers' directions, electrophoresed in 0.7% agarose, and transferred to nitrocellulose filters according to the method of Southern (1975).

Chromosomal mapping of the IBP-1 gene was performed using Southern blots containing DNA from a panel of human–rodent somatic cell hybrids segregating human chromosomes. DNA was extracted from 25 human–hamster and four human–mouse hybrids, digested with *Pst*I or *Sst*I and subsequently separated on 0.7% agarose gels and blotted onto nitrocellulose filters. The resulting blots were hybridized to the IBP-1 cDNA clone w85. Chromosome analysis of hybrid cells was carried out using reverse (R) banding with acridine orange after heat denaturation. The same batches of cells were used for karyotyping and DNA extraction.

Northern analysis

RNA was extracted from fetal liver, HEPG2 cells, placental membranes and placenta tissue from term pregnancy by the thioguanidyl method as described (Ullrich *et al.*, 1977). The RNA was denatured with dimethylsulfoxide and glyoxal, electrophoresed in 1% agarose and transferred to nitrocellulose filters (Thomas, 1980). The filters were hybridized with fragments derived from IBP-1 cDNA clones. In addition, filters containing poly(A)$^+$ RNAs of various fetal tissues of 12–16 weeks gestation (kindly provided by Dr M.Jansen, Wilhelmina Children's Hospital, Utrecht) were hybridized with the same fragments.

Transfection of COS-1 cells and IGF–BP analysis

The cDNA clone p19 encoding the IBP-1 gene was inserted into the *Eco*RI site of pSV328, which expresses cloned inserts using the simian virus 40 (SV40) early promoter (Van Heuvel *et al.*, 1986). COS-1 cells (Gluzman, 1981) were transfected with the expression vector by incubation with 100 μg/ml DEAE–dextran for 2 h (McCutchan and Pagano, 1968) followed by treatment with 100 μM chloroquine in Dulbecco's MEM (DMEM) for 4 h. After this treatment the cells were fed with DMEM plus 5% fetal calf serum for 24 h. Subsequently, the cells were washed extensively with DMEM and incubated for 72 h with DMEM without serum.

The culture medium was concentrated ~20-fold using centricon microcon-

centrators (Amicon) and desalted on Sephadex G25 columns (Pharmacia). Samples of culture medium were used for SDS–PAGE (12.5% acrylamide) and transferred onto nitrocellulose filters (Millipore HATF) by Western blotting. Production of IBP-1 protein in culture media was determined by binding with [^{125}I]IGF-I (Hossenlop *et al.*, 1986) and by cross-reaction with the antibody against the low mol. wt IGF–BP. The nitrocellulose filters were incubated with >200 000 c.p.m. [^{125}I]IGF-I (kindly provided by Dr M.Jansen, Wilhelmina Children's Hospital, Utrecht) for 16 h at 4°C with or without 150 ng cold IGF, washed and autoradiographed. After autoradiography the same filters were incubated with antibody against low mol. wt IGF–BP. The immunoreactive bands were made visible by immunostaining as described for the screening of the cDNA libraries.

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References

Baxter,R.C., Martin,J.L., Tyler,M.I. and Howden,M.E.H. (1986a) *Biochem. Biophys. Res. Commun.*, **139**, 1256–1261.

Baxter,R.C. and Martin,J.L. (1986b) *J. Clin. Invest.*, **78**, 1504–1512.

Baxter,R.C., Martin,J.L. and Wood,M.H. (1987) *J. Clin. Endocrinol. Metab.*, **65**, 423–442.

Binoux,M., Hossenlop,P., Hardouin,S., Seurin,D., Lasarre,C. and Gourmelen,M. (1986) *Horm. Res.*, **24**, 141–151.

Blin,N. and Stafford,D.W. (1976) *Nucleic Acids Res.*, **3**, 2303–2308.

Bohn,H. and Kraus,W. (1980) *Arch. Gynecol.*, **229**, 279–291.

Chandra,T., Stackhouse,R., Kidd,V.J. and Woo,S.L.C. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 1845–1848.

Clemmons,D.R., Han,V.K.M., Elgin,R.G. and D'Ercole,A.J. (1987) *Mol. Endocrinol.*, **1**, 339–347.

Drop,S.L.S., Valiquette,G., Guyda,H.J., Corvol,M.T. and Posner,B.I. (1979) *Acta Endocrinol.*, **90**, 505–518.

Drop,S.L.S., Kortleve,D.J. and Guyda,H.J. (1984a) *J. Clin. Endocrinol. Metab.*, **59**, 899–907.

Drop,S.L.S., Kortleve,D.J., Guyda,H.J. and Posner,B.I. (1984b) *J. Clin. Endocrinol. Metab.*, **59**, 908–917.

Elgin,R.G., Busby,W.H., Jr and Clemmons,D.R. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 3254–3258.

Gardiner-Garden,M. and Frommer,M. (1987) *J. Mol. Biol.*, **196**, 261–282.

Gluzman,Y. (1981) *Cell*, **23**, 175–182.

Hintz,R.L. (1984) *J. Clin. Endocrinol. Metab.*, **13**, 31–42.

Hossenlop,P., Seurin,D., Segovia-Quinson,B., Hardouin,S. and Binoux,M. (1986) *Anal. Biochem.*, **154**, 138–143.

Huhtala,M.L., Koistinen,R., Palomäki,P., Partanen,P., Bohn,H. and Seppälä,M. (1986) *Biophys. Res. Commun.*, **141**, 263–270.

Koistinen,R., Kalkkinen,N., Huhtala,M.L., Seppälä,M., Bohn,H. and Rutanen,E.M. (1986) *Endocrinology*, **118**, 1375–1378.

Koistinen,R., Huhtala,M.L., Stenman,U.H. and Seppälä,M. (1987) *Clin. Chem. Acta*, **164**, 293–303.

Kozak,M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2850–2854.

Leung,D.W., Spencer,S.A., Cachianes,G., Hammonds,R.G., Collins, C., Henzel,W.J., Barnard,R., Waters,M.J. and Wood,W.I. (1987) *Nature*, **330**, 537–543.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Massague,J. and Chech,M.P. (1982) *J. Biol. Chem.*, **257**, 5038–5045.

McCutchan,J.H. and Pagano,J.S. (1968) *J. Natl. Cancer Inst.*, **41**, 351–357.

Morgan,D.O., Edman,J.C., Standring,D.N., Fried,V.A., Smith,M.C., Roth,R.A. and Rutter,W.J. (1987) *Nature*, **329**, 301–307.

Mottola,C., MacDonald,R.G., Brackett,J.L., Mole,J.E., Anderson,J.K. and Czech,M.P. (1986) *J. Biol. Chem.*, **261**, 11180–11188.

Pòvoa,G., Enberg,G., Jörnvall,H. and Hall,K. (1984a) *Eur. J. Biochem.*, **144**, 199–204.

Pòvoa,G., Roovete,A. and Hall,K. (1984b) *Acta Endocrinol.*, **107**, 563–570.

Pòvoa,G., Isaksson,M., Jörnvall,H. and Hall,K. (1985) *Biochem. Biophys. Res. Commun.*, **128**, 1071–1078.

Rechler,M.M., Podskalny,J.M., Goldfine,J.D. and Wells,C.A. (1974) *J. Clin. Endocrinol. Metab.*, **39**, 512–521.

Rinderknecht,E. and Humbel,R.E. (1978a) *J. Biol. Chem.*, **253**, 2769–2776.

Rinderknecht,E. and Humbel,R.E. (1978b) *FEBS Lett.*, **89**, 283–286.

Rutanen,E.M., Koistinen,R., Wahlström,T., Bohn,H., Ranta,T. and Seppälä,M. (1985) *Endocrinology*, **116**, 1304–1309.

Rutanen,E.M., Menabawey,M., Isaka,K., Bohn,H., Chard,T. and Grundzinskas,J.G. (1986) *J. Clin. Endocrinol. Metab.*, **63**, 675–679.

Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.

Scott,J., Cowell,J., Robertson,M.E., Priestley,L.M., Wadey,R., Hopkins,B., Pritchard,J., Bell,G.I., Ball,L.B., Graham,C.F. and Knott,T.J. (1985) *Nature*, **317**, 260–262.

Shaw,G. and Kamen,R. (1986) *Cell*, **46**, 659–667.

Smith,G.L. (1984) *Mol. Cell Endocrinol.*, **34**, 85–89.

Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503–517.

Takahashi,N., Takahashi,Y. and Putman,F.W. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 2021–2025.

Thomas,P.S. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5201–5205.

Ullrich,A.J., Shine,J., Chirgwin,J., Picket,R., Tischer,E., Rutter,W.J. and Goodman,H.M. (1977) *Science*, **196**, 1313–1319.

Ullrich,A., Coussens,L., Hayflick,J.S., Dull,T.J., Gray,A., Tam,A.W., Lee,J., Yarden,Y., Libermann,T.A., Schlessinger,J., Downward,J., Mayes,E.L.V., Whittle,N., Waterfield,M.D. and Seeburg,P.H. (1984) *Nature*, **309**, 418–425.

Ullrich,A., Gray,A., Tam,A.W., Yang-Feng,T., Tsubokawa,M., Collins, C., Henzel,W., Le Bon,T., Kathuria,S., Chen,E., Jacobs,S., Francke,U., Ramachandran,J. and Fujita-Yamaguchi,Y. (1986) *EMBO J.*, **5**, 2503–2512.

Van Heuvel,M., Bosveld,J.L., Mooren,A.T.A., Trapman,J. and Zwarthoff, E.C. (1986) *J. Gen. Virol.*, **67**, 2215–2222.

Von Heijne,G. (1987) *Nucleic Acids Res.*, **14**, 4683–4690.

Van Wyk,J.J., Underwood,L.E., Hintz,R.L., Clemmons,D.R., Viona,S.J. and Weaver,R.P. (1974) *Recent. Prog. Horm. Res.*, **30**, 259–318.

Wilkins,J.R. and D'Ercole,A.J. (1985) *J. Clin. Invest.*, **75**, 1350–1358.

Yamamoto,T., Davis,C.G., Brown,M.S., Schneider,W.J., Casey,M.I., Goldstein,J.L. and Russell,D.W. (1984) *Cell*, **39**, 27–38.

Young,R.A. and Davis,R.W. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 1194–1198.

Zapf,J., Schoenle,E., Jagars,G., Sand,I., Grunwald,J. and Froesch,E.R. (1979) *J. Clin. Invest.*, **63**, 1077–1084.

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Note added in proof

These sequence data will appear in the EMBL/GenBank/ DDBJ Nucleotide Sequence Databases under the accession number Y00856.