Interactions between liver nuclear proteins and the human insulin-like growth factor binding protein 1 promoter in the course of development

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Insulin-like growth factor binding proteins (IGFBPs) modulate the bioavailability of the IGFs. Among the six IGFBPs known to date, IGFBP-1 is the most tissue-specific, its expression being limited to the liver and the endometrium. In the liver, IGFBP-1 gene expression is maximal during the perinatal period, with its peak corresponding to a transient rise in gene transcription activity. In this study, interactions between rat liver nuclear proteins and the human IGFBP-1 promoter have been analysed in the course of development, using in vitro DNase I protection and mobility shift assays. Only the interactions between DNA and proteins localized between nt −305 and −268 varied through the period studied (16 days in utero to 70 days postnatally). Three proteins, named Pa, PC1 and PC2, interacted with sequences between nt −295 and −285, nt −305 and −295 and nt −285 and −268, respectively. There was a marked perinatal increase in phenotype expression of Pa, which was parallel to that in IGFBP-1 gene transcription activity. In addition, DNA–Pa interactions and DNA–PC2 interactions were mutually exclusive. These results suggest that the interaction of Pa with its target sequence(s) prevents PC2 binding and thereby contribute towards increased IGFBP-1 gene transcription.

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Insulin-like growth factors (IGF-I and IGF-II) are involved in cell metabolism, proliferation and maturation and hence in fetal and postnatal growth and development in mammals (1). In biological fluids, the IGFs are non-covalently bound to high-affinity binding proteins (IGFBPs) that, like the IGFs, are produced ubiquitously but mainly by the liver (2, 3). Six distinct species of these IGFBPs are known to date (4) and they have been shown to control both the bioavailability of the IGFs and their binding to their target cell receptors. Although the six IGFBPs have liver production in common, there are significant differences in the developmental control and tissue specificity of their expression.

In terms of tissue specificity, IGFBP-1 expression is the most restricted. In the adult, apart from the liver, only the endometrium during the luteal phase and decidual tissue during pregnancy express IGFBP-1 (5–7). In situ hybridization studies have shown that in the fetus IGFBP-1 transcripts are limited to the liver (5, 8).

Hepatic transcription of the IGFBP-1 gene is controlled by liver-enriched trans-acting factors, such as hepatic nuclear factor 1 (HNF1) (9, 10) and albumin promoter D-site binding protein (DBP) (11), stimulated by cAMP (12) and glucocorticoids (13–15) and inhibited by insulin (14–17). Expression of IGFBP-1 also varies during development both in man (5, 18) and in the Rhesus monkey (19). In the rat, where maximal expression occurs during the perinatal period (8, 20, 21), increases in IGFBP-1 mRNA are correlated with enhanced transcription rates (21).

In this study we have sought to identify sequences within the human IGFBP-1 (h-IGFBP-1) promoter where DNA–protein interactions may vary during the perinatal period. To our knowledge, this is the first report to throw some light on the mechanisms involved in developmentally linked transcriptional regulation of the IGFBP-1 gene.

Materials and methods

Plasmids and oligonucleotides

pBP-p1-341rev is a eukaryotic expression vector in which CAT gene expression is driven by sequences spanning nt −341 to +1 of the h-IGFBP-1 promoter inserted in antisense orientation (10).

In pBP-p1-341/285–276rev, the h-IGFBP-1 promoter was mutated by the polymerase chain reaction (PCR)
Table 1. Sequences of the synthetic oligonucleotides used to analyse interactions between liver nuclear proteins and the human IGFBP-1 promoter.

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>BP-1235–210</td>
<td>5'-GTTGAAACTGGAATTACAAGGGCC-3'</td>
</tr>
<tr>
<td>BP-1268–248</td>
<td>5'-AACAAAGGTACACCCCTCC-3'</td>
</tr>
<tr>
<td>BP-1285–268</td>
<td>5'-GCTGTCTTTTTGACAAAA-3'</td>
</tr>
<tr>
<td>BP-1285–268mut</td>
<td>5'-GCTGTCTTTTTGACAAAA-3'</td>
</tr>
<tr>
<td>BP-1305–287</td>
<td>5'-AACAAACGTCATCCCCCTCCC-3'</td>
</tr>
<tr>
<td>BP-341/285-276rev</td>
<td>5'-CTGGGGAACCTGGAGG-3'</td>
</tr>
<tr>
<td>BP-1341/285-276rev</td>
<td>5'-CTGGGGAACCTGGAGG-3'</td>
</tr>
</tbody>
</table>

*Bold characters represent the mutation introduced into the sequence.

between nt −285 and −276, as described previously (10), using the mutated primers 5'- TTTTCTCTCAGGTTGGCCAGTGCTGCCC-3' (3' primer) and 5'-CTGGGGAACCTGGAGG-3' (5' primer). Bold characters indicate the mutated bases and those underlined indicate the XhoI restriction site introduced into the mutated promoter.

The oligonucleotide sequences used for gel mobility shift assays and as competitors in DNase I footprinting are shown in Table 1.

DNase I protection assay

Plasmids were linearized by HindIII digestion (the restriction site of which is six bases 3' of the h-IGFBP-1 promoter). 32P-labelled on one or other strand, either by filling-in with Klenow or by 5'-end labelling using T4 polynucleotide kinase, then digested with a second restriction enzyme, EcoNI- and EcoO1901-digested pBP-142rev (or pBP-1341/285–276rev) yield fragments of 320 and 136 bp, respectively, which are labelled at the 5' end of the promoter (close to nt −341). The fragments were purified by 3.5% polyacrylamide gel electrophoresis and dissolved in water to yield 10 000 000 cpm/μl.

DNase I protection assays were performed as described by Lichsteiner et al. (22). The reaction mixture contained 1 μg of 32P-labelled promoter fragment, 1 μg of poly-dl-dC and either 20 μg or 40 μg of crude nuclear extract (CNE) prepared according to Gorski et al. (23) or 100 μg of DNase-free BSA (Pharmacia) in 20 μl of 25 mmol/l HEPES (pH 7.6), 34 mmol/l KCl and 5 mmol/l MgCl2 buffer. After 15 min of preincubation at 0–4°C, 10−3 U/tube (CNFs) or 0.75 × 10−3 U/tube (BSA) DNase I (Pharmacia) was added and the incubation continued for 3 min at 0–4°C. The reaction was stopped by addition of 80 μl of 20 mmol/l TRIS·HCl (pH 8), 20 mmol/l EDTA, 250 mmol/l NaCl and 0.5% SDS, and treated with proteinase K (final concentration, 10 mg/ml) for 60 min at 45°C. After phenol/chloroform extraction and ethanol precipitation, the samples were dissolved in 6 μl of gel loading buffer (90% formamide, 0.1% xylene cyanol and 0.1% bromophenol blue).

The samples were then applied to 6–10% polyacrylamide gel/50% urea prepared in 1 × TBE (89 mmol/l TRIS borate, 89 mmol/l boric acid, 2 mmol/l EDTA, pH 8.3) and electrophoresis was run at 1750 V/40 mA for about 2 h. Finally, the gels were autoradiographed at −80°C.

**Gel mobility shift assay**

For the gel mobility shift assays (24), 50 ng/ml 32P-end-labelled double-stranded oligonucleotides was mixed with 50 μg/ml double-stranded poly-dl-dC and 0.5 mg/ml liver CNEs in a 20-μl reaction mixture containing 25 mmol/l HEPES (pH 7.6), 34 mmol/l KCl and 5 mmol/l MgCl2. After 30 min of incubation at 0°C, the reaction mixture was loaded onto 5% non-denaturing polyacrylamide gel prepared in 1 × TAE (40 mmol/l TRIS acetate, 1 mmol/l EDTA) and electrophoresis run at 100 V for 3 h at room temperature. The gels were then fixed with 50% methanol/10% acetic acid, dried and autoradiographed at −80°C.

**Results**

**Identification of an interaction between the h-IGFBP-1 promoter and liver nuclear proteins that varies with stage of development**

DNase I protection assays revealed sequences on the h-IGFBP-1 promoter (nt −341 to +1) that were protected by rat liver CNEs, including that between nt −54 and −76 (the cis element for HNF1) (9). Similar protection patterns were observed when the DNase I footprints were obtained with CNEs prepared from rat livers at different stages of development.

However, a DNase I cleavage site was identified (nt −282 (coding strand), −285 (non-coding strand)), the intensity of which changed in the course of development (Fig. 1). Compared with the BSA control, this cleavage was weak at 16 days in utero (d.i.u.), weaker at 20 d.i.u. and weakest at birth, then gradually strengthened to overtake the control (i.e. hypersensitive: HS −282/−285) at adulthood (Figs. 1B and C). On the basis of this observation, DNA–protein interactions at the sequences flanking this cleavage site were studied in greater detail.

**Three nuclear proteins interact with the sequences between nt −305 and −268**

In order to investigate the DNA–protein interactions at sequences between nt −305 and −268, five overlapping oligonucleotides were designed (BP-1305–287, BP-1295–268 and BP-1285–268, BP-1268–248, BP-1235–210; Table 1) and used for mobility shift assays.
Two shifted bands, Pa and PC1, were obtained with 
$^{32}$P-labelled BP-1$_{305-287}$ preincubated with CNEs from
newborn rat livers (Fig. 2, lane 1 and Fig. 3A, lane 1).
As both bands disappeared when a 30-fold excess of the
same unlabelled oligonucleotide was added to the
reaction medium, these DNA–protein interactions
were specific (Fig. 3A, lane 2).

Similarly, two shifted bands (Pb and PC2) corrsponding
to specific DNA–protein interactions were obtained when the same CNEs were incubated with
labelled BP-1$_{295-268}$ (Fig. 2, lane 6 and Fig. 3C, lane 1).
The most rapidly migrating band, PC2, was different from Pa and PC1 and probably represents a different
DNA–protein interaction, which is consistent with the
observation that it was not extinguished by a 30-fold
excess of unlabelled BP-1$_{305-287}$ (Fig. 3C, lane 2).
Conversely, the migration of the slowest band (Pb) was
identical to that of Pa and the band disappeared in the
presence of a 30-fold excess of cold BP-1$_{305-287}$ (Fig. 3C, lane 2), as did the Pa band when $^{32}$P-labelled BP-
1$_{305-287}$ was incubated with excess radio-inert BP-
1$_{295-268}$ (Fig. 3C, lane 5). This protein will therefore be
referred to as Pa for the remainder of the article. The
sequences between nt $-295$ and $-287$ (common to the
two oligonucleotides) are therefore necessary for
interaction between Pa and the IGFBP-1 promoter.
Interestingly, these same proteins, Pa and PC2,
interacted with the sequences between nt $-295$ and

Fig. 2. Mobility shift analysis of DNA–protein interactions between nt $-305$ and nt $-210$ of the hIGFBP-1 promoter. The $^{32}$P-labelled oligonucleotides BP-1$_{305-287}$ (lane 1), BP-1$_{235-210}$ (lanes 2, 3 and 5), BP-1$_{268-248}$ (lane 4) and BP-1$_{295-268}$ (lane 6) were incubated with 10 $\mu$g of proteins from newborn rat liver crude nuclear extracts.
Fig. 3. Mobility shift analysis of three DNA–protein interactions between nt –305 and nt –268 of the hIGFBP-1 promoter. The $^{32}$P-labelled oligonucleotides BP-1,305–287 (A), BP-1,235–210 (B) and BP-1,295–268 (C) were incubated with 10 µg of proteins from newborn rat liver crude nuclear extracts. Some experiments were done in the presence of a 30-fold excess of BP-1,305–287 (lane 2), BP-1,285–268 (lane 3) and BP-1,235–210 (lane 4), BP-1,305–287 (lane 1), BP-1,235–210 (lanes 2, 3 and 5), BP-1,268–248 (lane 4) or BP-1,295–268 (lane 5).

-268 and between nt –235 and –210 (cf Fig. 2, lane 5 with lane 6 and Fig. 3B with Fig. 3C).

The PC1 band persisted when mobility shift assays were performed with $^{32}$P-labelled BP-1,305–287 in the presence of a 30-fold excess of unlabelled BP-1,295–268 (Fig. 3A, lane 5) and the PC2 band persisted when gel shifts were carried out with $^{32}$P-labelled BP-1,295–268 and a 30-fold excess of cold BP-1,305–287 (Fig. 3C, lane

Fig. 4. Localization of sequences interacting with the nuclear protein responsible for HS –282/–285. DNase I footprinting was performed with 20 µg of rat liver crude nuclear extracts (CNEs) or 100 µg of BSA (A, B). Wild-type promoter sequences spanning nt –341 to –205 were labelled at their distal end and incubated with CNEs prepared from newborn (A) or adult (B) rat livers. The incubation was carried out without (lane 1) or with a 30-fold excess of unlabelled BP-1,305–287 (lane 2), BP-1,285–268 (lane 3) or BP-1,285–268mut (lane 4). (C) DNase I footprints were obtained with the same promoter sequences mutated between nt –285 and –276. The incubations were carried out with the CNEs indicated in the absence (lane 1) or presence of a 30-fold excess of cold BP-1,305–287 (lane 2), BP-1,285–268 (lane 3) or BP-1,285–268mut (lane 4).
2) or with BP-1,235–210 (Fig. 3B, lane 2). This supports the notion that PC1 and PC2 reflect interactions of different protein species with the oligonucleotides and suggests that the sequences not shared by BP-1,305–287 and BP-1,295–268, i.e., those between nt -296 and -305 and between nt -268 and -285, are necessary for the interactions of PC1 and PC2, respectively, with the IGFBP-1 promoter.

Lastly, PC3 interacted with sequences between nt -268 and -248 (Fig. 2, lane 4) and probably corresponds to a member of the cAMP-responsive element binding protein (CREB) family (Ref. 12 and our unpublished results).

**PC2 is responsible for HS -282/–285**

In DNase I protection assays performed with adult liver CNEs, the intensity of the band corresponding to the HS -282/–285 cleavage site was diminished to less than that of the control (BSA) in the presence of a 30-fold excess of unlabelled BP-1,285–268 (cf lanes 1 and 3 and BSA, Fig. 4B), but remained unchanged in the presence of a 30-fold excess of an oligonucleotide spanning the same sequence mutated between nt -285 and -276, BP-1,285–267mut (Fig. 4B, lane 4). The hypersensitivity of the cleavage at -282/–285 therefore appears to depend on the interaction of liver nuclear protein(s) with cis elements between nt -285 and -276, i.e., those interacting with PC2.

In agreement with this, DNase I footprint experiments carried out with a promoter fragment mutated between nt -285 and -276 yielded identical footprints for CNEs prepared from newborn and adult livers, but in the adult the cleavage site at -282/–285 was no more hypersensitive (Fig. 4C, lanes 1 and 3). Consequently, it was predictable that the footprints obtained with the mutated promoter remained unchanged in the presence of a 30-fold excess of unlabelled BP-1,305–287 or BP-1,285–267 (Fig. 4C, lanes 2 and 4).

There was a clearcut increase in the intensity of the DNase I cleavage at -282/–285 (cf lanes 1 and 2, Fig. 4A) in the presence of a 30-fold excess of radio-inert BP-1,305–287. By contrast, a 30-fold excess of unlabelled BP-1,287–268 had no effect on the -282/–285 cleavage in the presence of newborn liver CNEs (lane 3). These results suggest that the nuclear protein responsible for the hypersensitive cleavage at -282/–285 (PC2) is also present in hepatocyte nuclei at a time (from several hours to several days after birth) when, at least under our in vitro footprint conditions, it fails to interact with the IGFBP-1 promoter and that this absence of interaction depends on Pa and/or PC1 binding to sequences between nt -305 and -287.

**Phenotype expression of Pa is most intense during the perinatal period**

The possible interplay of Pa and/or PC1 in the interaction of PC2 with its cis element led us to check the levels of Pa and PC1 during development, particularly during the perinatal period (Fig. 5). Mobility shift assays revealed increased phenotype expression of Pa at this time (Fig. 5A). Densitometric quantification of the Pa and PC1 bands showed that during the perinatal period (from 20 d.i.u. to 5 days postnatally) Pa increased five times more than PC1 (the Pa/PC1 ratio rose from 0.57

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Fig. 5. Changes in phenotype expression of Pa and PC1 in the course of development. Comparison with IGFBP-1 gene transcription activity. (A) Mobility shift assays were performed with 32P-labelled BP-1,305–287 and 10 µg of crude nuclear extract proteins prepared from rat livers excised at 14 days in utero (lane 1) and several hours (lane 2) and 70 days (lane 3) postnatally. (B) The intensities of the bands corresponding to specific DNA–protein interactions (Pa and PC1) were determined by laser densitometry. Results are expressed in terms of Pa/PC1 ratios and compared with IGFBP-1 gene transcription activity (21) at each stage of development.
to 2.7). In addition, there was a close relationship between the Pa/PC1 ratios determined through development and the transcriptional activity of the IGFBP-1 gene, as measured by run-on (21) (Fig. 5B).

Discussion

In the liver, IGFBP-1 gene expression is abundant during the perinatal period (17–21) and we have shown previously that the increased amounts of IGFBP-1 mRNA in fetal liver towards the end of gestation are accompanied by increased gene transcription levels (21). Several mechanisms may account for amplified gene expression, like alterations of chromatin structure (25, 26), modifications of DNA methylation (27) or changes in the interactions between DNA and trans-acting factors at the promoter, leading to relief of repression or modulation of hormonal induction (28).

As part of our study of IGFBP-1 gene expression in the course of development, we examined DNA–protein interactions at the human promoter (nt −341 to +1) with a view to identifying changes that may occur during the perinatal period. We concentrated on interactions occurring at sequences between nt −305 and −285 because they exhibited a pattern that was dependent on stage of development. The most striking effect was a change in the sensitivity to DNase I cleavage at nt −282 (+strand)/−285 (−strand). Compared with controls, this site became hypersensitive in the presence of CNEs from adult rat livers.

Using oligonucleotides with overlapping sequences that encompass those between nt −305 and −268, three proteins were identified (Pa, PC1 and PC2) that interact with sequences between nt −295 and −287, nt −305 and −295 and nt −285 and −268, respectively. The relationship between the transcriptional activity of the endogenous IGFBP-1 gene and the relative abundance of Pa, as quantified from mobility shift assays, strongly suggest that this protein may be an activator of IGFBP-1 gene transcription. In the cross-competition experiments, Pa proved capable of interaction with at least two sequences within the human promoter: nt −295 to −287 and nt −235 to −210. By aligning BP-1_{305−287} (nt −295 to −287) and BP-1_{235−210} (nt −235 to −210), a putative target sequence for Pa could be deduced: (A/G)CTGGAC(C/T)T. This is 100% homologous with the sequences reported for LF-A1 (29), which has been described as a liver-enriched trans-acting factor, interacting with promoter regions of the α₁-antitrypsin and apolipoprotein A1, A4 and B1 genes whose transcriptional activity it increases (29–31). To our knowledge, the ontogeny of LF-A1 in the liver remains to be described.

In our cross-competition experiments (in vitro DNase I footprinting and mobility shift assays), the protein that interacted with sequences between nt −285 and −268 (PC2) and was responsible for the hypersensitive cleavage site at −282/−285 was also present in CNEs prepared from newborn rat livers (which do not generate the hypersensitive site). In addition, the hypersensitive cleavage could be obtained with these extracts in the presence of an oligonucleotide that prevents Pa and PC1 from interacting with the human promoter. These findings suggest that the interactions between Pa and/or PC1 and the IGFBP-1 promoter and between PC2 and the promoter are mutually exclusive and provide support for the model set out in Fig. 6 to describe the interplay of the three trans-acting factors that interact with the sequences spanning nt −305 to −268. Pa would activate IGFBP-1 gene transcription either as an intrinsic activator or by preventing the binding of PC2 (a transcription inhibitor).

The sequences with which PC2 interacts are 70% homologous with the consensus sequence reported for the insulin response element (32) and their mutation prevented the −282/−285 hypersensitive cleavage. Insulin inhibits transcription of the endogenous IGFBP-1 gene in the liver and in cultured hepatocytes and decreases the activity of IGFBP-1 promoter in transient transfection (Ref. 14 and our unpublished results). Also, the ratio of insulin to glucagon decreases during the perinatal period in the rat (33). It can therefore be inferred that PC2 may play a role as transcription repressor.

After this description of the interactions between rat liver nuclear proteins and the h-IGFBP-1 promoter, which vary with stage of development, the next step will be to define the three proteins Pa, PC1 and PC2 and to determine their functional roles in regulating IGFBP-1 gene transcription.

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