Structural analysis of the human insulin-like growth factor-II P3 promoter

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The expression of insulin-like growth factor-II (IGF-II) has been observed previously in many human cancers. The human IGF-II P3 promoter has been shown by others to give rise to abundant 6.0 kb and 2.2 kb fetal transcripts which are expressed in a variety of both paediatric and adult tumours. In order to determine the mechanism by which the P3 promoter is controlled, the promoter was analysed in cell lines using chlor-

amphenicol acetyltransferase (CAT) assay and DNAase I footprinting techniques. The data indicated that P3 is a complex promoter involving at least nine transcription factor binding sites. Furthermore, high levels of 5-methylcytosine detected in the P3 promoter of HeLa genomic DNA suggest that IGF-II gene expression may also be influenced by DNA methylation.

INTRODUCTION

Insulin-like growth factor-II (IGF-II) is a polypeptide hormone which stimulates cell growth via autocrine/paracrine mechanisms [1–7]. This growth factor is expressed in a variety of human cancers [8–17], although its precise role in the origin of these tumours is not known.

Several studies have indicated that the control of IGF-II biosynthesis may occur at several molecular levels [18–20]. One mechanism involves *trans*-acting factors interacting with the IGF-II promoters [18]. It has been observed that IGF-II has at least four independent promoters (P1–P4) which give rise to independent transcripts [21–24]. The P1 promoter is active in adult liver [13], while P2, P3 and P4 are fetal promoters [22,24]. The P3 and P4 promoters are active in fetal tissues but are simultaneously repressed at parturition [7,23]. The mechanism of rat P4 promoter regulation has been studied in HeLa cells and the promoter was found to consist of four Sp1 binding sites [18]. The IGF-II P3 promoter, which gives rise to abundant 6.0 kb and 2.2 kb transcripts, has also been examined for *trans*-acting factors. One Sp1 binding site was reported in the rat [25] and several other protected regions were detected in the human P3 promoter [26]. Differential utilization of the P3 and P4 promoters may occur, as it has been observed in smooth muscle tumours that the P3 promoter becomes more active relative to the P4 promoter during tumour progression [9]. The P3 promoter activation was suggested to occur as a consequence of *trans*-acting factors.

Transcription factors suggested to play a role in P3 regulation include the transcription factors that bind the early growth response element (ERG-1) GCGGGGCG, of which a sequence has been identified in the IGF-II P3 promoter [27]. The finding that the Wilms' tumour gene product (WT-Zf) binds the EGR-1 consensus sequence [28] leads to the possibility that WT-Zf may be a repressor of P3 promoter activity [29]. In addition, WT-Zf has recently been shown in co-transfection assays to repress the activity of the P3 promoter [30].

Other mechanisms of IGF-II regulation have been suggested by previous reports in which the IGF-II gene was found to be

imprinted in the mouse [20]. The significance of imprinting in the regulation of IGF-II gene expression remains unknown. Methylation at cytosine residues has been postulated to account for the differential expression of maternal and paternal IGF-II alleles [20].

In this paper, an analysis of the human IGF-II P3 promoter in HeLa cells reveals a complex promoter which consists of at least nine protected regions detected using the DNAase I footprinting assay. The methylation status of the HeLa P3 promoter was also examined and found to correlate with *in vivo* expression of the IGF-II gene.

MATERIALS AND METHODS

Construction of plasmids

Chloramphenicol acetyltransferase (CAT) plasmids were made by cloning sequentially deleted DNA fragments (Figure 1) into the pBLCAT3 vector [31]. DNA fragments analysed by DNAase I footprinting were a *Bgl*II–*Bst*EII fragment (–1232 to –812) and an *Eco*RI–*Hinc*II fragment (–291 to +130) which were cloned into the Bluescript vector. RNAase protection was done using an *Eco*RI–*Pst*I fragment (+981 to +1251) which contained 178 bp of the untranslated exon 5 (Figure 1).

CAT assays

HeLa (ATCC; CCL2) and HepG2 (ATCC; HB8065) cell lines were grown in MEM- α medium (Gibco) supplemented with 10% bovine serum. DNA transfections were done according to Chen and Okayama [32], with some modifications. Briefly, cells were seeded at a density of 3×10^5 cells/cm² in 6 cm diameter dishes. The cells were refed after 24 h and then transfected by the calcium phosphate procedure [32]. Cells were then incubated for 9 h (HepG2 cells) or 12 h (HeLa cells) at 37 °C in 5% CO₂ before being washed twice in PBS and twice in serum-free media. The cells were incubated for a further 24 h before being assayed for CAT activity as previously described [33]. Conversion of the ¹⁴C-labelled chloramphenicol substrate (Amersham) to its

Abbreviations used: IGF-II, insulin-like growth factor-II; ERG, early growth response element; CAT, chloramphenicol acetyltransferase; SV40, simian virus 40.

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Figure 1 Molecular cloning of P3 promoter fragments

(a) Structure of the IGF-II gene. Numbered exons are indicated by hatched boxes. Arrows indicate positions of promoters within the IGF-II gene complex. P3 occurs upstream of exon 5 and gives rise to 6.0 kb and 2.2 kb transcripts. (b) Restriction map of the P3 and P4 promoter regions. Numbers indicate positions of restriction sites in base pairs relative to the P3 transcription initiation site, indicated as zero. Restriction enzymes are as follows: H, *HindIII*; B, *BamHI*; Bg, *BglII*; Bst, *BstEII*; A, *AvaI*; Sc, *SacII*; E, *EcoRI*; Hc/S, *HincII-SalI*; P, *PstI*. The positions of the P3EI and P3EII regulatory elements are marked. (c) Derivation of promoter fragments for characterization. The fragments used for experiments are summarized. Each fragment was derived from a parental *HindIII-SalI* (3.5 kb) sequence. For CAT assays, fragments were cloned into the vector pBLCAT3. Fragments used for footprinting or RNAase protection were cloned into the Bluescript vector.

acetylated derivatives was measured by separation of products on t.l.c. plates. CAT assay experiments were done in duplicate and standardized by co-transfection of the plasmid pCH104, which contains the *lacZ* gene fused to the simian virus 40 (SV40) early region promoter.

For the purpose of comparing P3 promoter constructs in HeLa and HepG2 cells, plasmid uptake was measured directly for each plasmid, i.e. PSV2, pBLCAT3 and pBLCAT3.1-3.4, by transfection. Assay of plasmid uptake was done by transforming competent *Escherichia coli* (DH5 α) at an efficiency of $10^6/\mu\text{g}$ with Hirt supernatants extracted from the cultured cells as described previously [34].

DNAase I footprinting

Crude nuclear extracts were obtained by the method of Dignam et al. [35], except that HepG2 nuclei lysis buffer contained, in addition, 5 $\mu\text{g}/\text{ml}$ pepstatin, 5 $\mu\text{g}/\text{ml}$ leupeptin and 5 $\mu\text{g}/\text{ml}$ antipain (Sigma). End-labelling of DNA fragments was done with the Klenow fragment of DNA polymerase (Amersham) using 3000 Ci/mmol [α - ^{32}P]dATP (Amersham).

DNAase I footprinting was done as described by Briggs et al. [36] and modified by lowering the probe concentration to 1 fmol and increasing the amount of nuclear extract to 30 μg . Binding reactions were done at 0 $^{\circ}\text{C}$ for 10 min in the following

buffer: 20 mM Hepes (pH 7.5), 100 mM KCl, 20% glycerol, 1 mM dithiothreitol, 0.1% Nonidet P-40, 1% polyvinyl alcohol (Sigma) and 1 fmol of probe DNA. The double-stranded alternating DNA polymer poly(dI-dC) (Pharmacia) was used as competitor DNA. For HeLa binding reactions, 0.5 μg of poly(dI-dC) was used, whereas 1.0 μg was used for HepG2. The amount of DNAase I added to the footprinting reaction was 0.25 ng in the absence of extract and 5 ng when extract was added. After binding, an equal volume of 10 mM $\text{MgCl}_2/5$ mM CaCl_2 solution was added and the reaction brought to room temperature. DNAase I (Worthington dPFF) was added and the reaction was stopped after 1 min by the addition of 2 vol. of stop solution (20 mM EDTA, 1% SDS, 0.2 M NaCl). Proteinase K was added to a final concentration of 100 $\mu\text{g}/\text{ml}$ and the sample was incubated for 5 min at 37 $^{\circ}\text{C}$. The nucleic acids were extracted once with phenol/chloroform (1:1, v/v) and ethanol-precipitated. The pellet was resuspended in formamide loading dye and the products were analysed on a denaturing 6% acrylamide gel. Autoradiography was at -80 $^{\circ}\text{C}$ for up to 10 days.

RNAase protection

Cytoplasmic RNA was isolated from HeLa and HepG2 cells as described previously [37]. Labelling of the antisense probe was done as described by Melton et al. [38]. Linearized plasmid (0.5 μg) was incubated at 40 $^{\circ}\text{C}$ for 1 h; 10 $\times$ buffer, nucleotides, T7 polymerase, RNasin (Promega) and 3000 Ci/mmol [α - ^{32}P]UTP (Amersham) were added according to manufacturer's specifications. The gel-purified probe was hybridized to cytoplasmic RNA as described previously [39]. Hybridization was at 55 $^{\circ}\text{C}$ in 80% formamide, 200 mM Pipes (pH 6.4) and 5 mM EDTA. Annealed complexes were treated with RNAase A and T1 (Sigma), and the products were electrophoresed on denaturing 6% polyacrylamide gels and autoradiographed.

Methylation analysis

HeLa and HepG2 genomic DNA was isolated as previously described [37]. Restriction enzymes *MspI* and *HpaI* (Boehringer) were used at 10 units/ μg . After digestion, DNA was ethanol-precipitated and redissolved in a formamide loading dye before being electrophoresed through a denaturing 6% polyacrylamide gel. Electrophoretic transfer to Gene Screen was done as described by Saluz and Jost [40]. The DNA probe was nick-translated by 3000 Ci/mmol [α - ^{32}P]dCTP to a specific activity of 10^8 c.p.m./ μg . Hybridizations were at 65 $^{\circ}\text{C}$ in 0.25 M NaH_2PO_4 (pH 7.2), 1 mM EDTA and 7% SDS. Washing of filters was done as previously described [40], with the final washing step being at 65 $^{\circ}\text{C}$ in $0.1 \times \text{SSC}/0.5\%$ SDS ($1 \times \text{SSC} = 0.15$ M NaCl and 0.015 M sodium citrate).

RESULTS

Regulatory regions of the human IGF-II P3 promoter

To determine the DNA sequences involved in the regulation of the human IGF-II P3 promoter, sequential 5' deletions of the promoter were made and the DNA fragments were linked to the CAT coding sequence. The constructs were then transfected into HeLa and HepG2 cells in duplicate and after transient expression CAT activity was assayed. In HeLa cells two elements that simulated P3 promoter activity were identified (Figure 2). One

Figure 2 Deletional analysis of the IGF-II P3 promoter in HeLa and HepG2 cells

The level of CAT activity obtained by each construct is summarized. Two independent experiments were done using cells freshly thawed from liquid nitrogen storage. The CAT assay values were obtained from four assay measurements with HepG2 cells and five assay measurements with HeLa cells. $\square$, HepG2 cells; ▨ , HeLa cells. The percentage CAT conversion was measured as: acetylated CAT (c.p.m.)/total CAT (c.p.m.). PSV2 and pBLCAT3 are positive and negative control plasmids respectively.

element was located between -1232 and -812 (P3EII), while the other was between -291 and $+130$ (P3EI). On deletion of the DNA sequence containing P2 promoter sequences, little effect on P3 promoter activity was observed. In HepG2 cells a similar result was obtained (Figure 2), i.e. regulatory elements P3EI and P3EII were found. However, in HepG2 cells a further stimulatory effect of P2 promoter sequences on P3 promoter activity was also observed. The positions of the regulatory regions relative to IGF-II P3 promoter restriction sites are indicated in Figure 1.

The data in Figure 2 revealed, in addition to the regulatory regions of the P3 promoter, that each of the P3 constructs, when transfected into HepG2 cells, generally showed a 5–10-fold greater level of CAT activity relative to that in HeLa cells. In order to determine whether the difference in CAT activity between HeLa and HepG2 cells might have a more trivial explanation, plasmid uptake and stability as well as CAT enzyme stability were examined. To compare the plasmid uptake and stability in HeLa and HepG2 cells, each plasmid was transfected in duplicate and the Hirt supernatants were extracted and assayed for intact plasmids after transfection. No difference was found in the rate of plasmid degradation between the two cell lines. However, HepG2 cells were found to take up twice as many plasmid molecules as HeLa cells (results not shown). Furthermore, no significant difference was found in CAT enzyme stability between the two cell lines (results not shown). These data suggest that the P3 promoter is more active in HepG2 cells than in HeLa cells and therefore that HepG2 cells might contain additional factor(s) which stimulate the P3 promoter.

Detection of human IGF-II P3 promoter *trans*-acting factors

To determine the nature of transcription factors necessary for P3 promoter activity in HeLa cells, the promoter elements P3EI and

P3EII were examined for *trans*-acting factors by DNAase I footprinting. The conditions for footprinting described previously [36], i.e. 10 fmol of probe and 10 μ g of HeLa nuclear extract, were found to be satisfactory for the detection of the SV40 GC-box region (not shown). The same conditions were then used to identify protected regions in the IGF-II promoter, and only one GC-box was found to be protected (results not shown). A scan of the P3EI sequenced (-291 to $+130$; EMBL database accession no. X03562) revealed, however, that there are three Sp1-like consensus sequences, three EGR-1 consensus sequences and one potential CCAAT-box. By reducing the probe concentration to 1 fmol and increasing the amount of HeLa nuclear extract to 30 μ g, DNAase I footprinting of the P3EI DNA sequence (Figures 3a and 3b) revealed protected regions at the three Sp1-like sequences (sites 3–5), at a CCAAT-box (site 2), and at three other sequences (sites 1, 6 and 7). The data are summarized in Figure 3(c). The DNA sequences at sites 1, 6 and 7 do not correspond to consensus sequences of any known transcription factor [41]. Sites 1, 6 and 7 each have a high content of C-G nucleotide pairs and are similar in their DNA sequences. It is possible that each site is bound by a single class of transcription factor. Collectively these data indicate that the P3 promoter is a complex promoter and involves at least three different types of transcription factor.

DNAase I footprinting of the P3EII element revealed protection at two further sites (Figures 3e and 3f), and the approximate sequence boundaries are shown in Figure 3(d). The sequences protected resembled Sp1 binding sites. Sites 8 and 9 fulfilled the criteria of being potential Sp1 binding sites, as has been previously described [19].

Absence of detectable EGR-1-like binding activity in HeLa nuclear extract

The P3EI element was clearly active in HeLa cells as determined by CAT assay; however, whether or not EGR-1 binding activity was necessary for this promoter activity remained to be established. Figure 3(c) shows that there are three EGR-1 consensus sequences in the P3EI element, designated as EGR-1(a), (b) and (c). It was necessary to determine, by DNAase I footprinting, whether protection at the EGR-1 consensus sites could be observed with HeLa nuclear extracts. At the EGR-1(a) sequence, protection was observed (Fig. 3a) of a region that spanned a total of 34 nucleotides. This result was inconsistent with protection of a 9 bp EGR-1 consensus sequence GCGGGGGCG. The lack of EGR-1 binding, however, was confirmed by the absence of detectable protection at two other EGR-1 consensus binding sites, i.e. EGR-1(c) (Fig. 3a) and EGR-1(b) (Fig. 3b), which overlaps with an Sp1 binding site. On removing Sp1 binding activity, by competition, no protection was observed at EGR-1(b) (Figure 4, lane 4), further indicating that HeLa cells contain no detectable EGR-1 binding activity. As a control, CCAAT-box binding proteins were competed with an unlabelled CTF/NF1 [33] consensus sequence. The unlabelled double-stranded oligonucleotide CTF/NF1 failed to affect Sp1 binding activity; however, loss of protection at the P3 CCAAT-box was observed (Figure 4, lanes 6 and 7). The data confirmed the conclusion that Sp1 binding activity was specifically removed by the addition of the unlabelled Sp1 consensus sequence. Furthermore, the loss of protection at the Sp1 consensus sequence (site 4) was consistent with the absence of binding at the overlapping EGR-1 consensus sequence.

It was possible, however, that EGR-1 binding activity was simply not detectable by the footprinting assay when using crude

Figure 3 Characterization of the IGF-II promoter by DNAase I footprinting

DNAase I footprinting of the P3EI element of the P3 promoter was carried out as described in the Materials and methods section. Samples were run on two separate sequencing gels: (a) was stopped after 1.75 h of electrophoresis and autoradiographed for 3 days at -80°C ; (b) was stopped after 3.5 h and autoradiographed for 8 days at -80°C . Lane 1 shows a DNAase I digestion profile of fragment DNA in the absence of nuclear extract; lane 2 contains 30 µg of HeLa extract. A + G indicates DNA cleaved at adenine and guanine residues. Numbers 1–7 denote protected regions, whereas – indicates no detectable binding. The position of each EGR-1 consensus sequence is indicated. The protected regions found in the P3EI (c) and P3EII (d) elements are summarized. Sequences were obtained from the EMBL database (accession no. X03562). The approximate boundaries of binding sequences are indicated. The arrow indicates the transcription start site. Stippled boxes indicate HeLa protected regions. The EGR-1 consensus sequences are indicated by hatched boxes. The P3EII region was contained in a 420 bp *Bgl*–*Bst* fragment (nucleotides –1232 to –812 relative to the P3 transcription start site), and DNAase I footprinting of the sense strand is shown in (e) and (f). Electrophoresis was for 1.5 h (e) or 3.5 h (f). Lane 1 contained no nuclear extract (control), and lane 2 contained 30 µg of HeLa nuclear extract.

Figure 4 Analysis of EGR-1(b) by competition to remove binding at an overlapping GC-box

HeLa EGR-1-like binding activity was examined at the EGR-1(b) site by footprinting the P3EI antisense strand (nucleotides –291 to +130). Competition of DNA-binding activity was done by the addition of double-stranded oligonucleotides (the high-affinity Sp1 binding site GGGGCGGGGC or the CTF/NFI consensus sequence ATTTGGCTTGAAGCCAATATG) to the binding reaction before footprinting (lanes 2–7). Lanes 1–7, 30 µg of HeLa extract was added to each binding reaction; lanes 2–4, 0.5 ng, 2.5 ng and 12.5 ng of the Sp1 consensus sequence; lanes 5–7, 0.5 ng, 2.5 ng and 12.5 ng of the CTF/NFI consensus sequence; lane 8, DNAase I cleavage pattern of labelled DNA without nuclear extract.

nuclear extracts. To rule out this possibility, HepG2 crude nuclear extracts were used in a footprinting assay (Figure 5). Binding to the EGR-1(c) sequence was clearly observed and hence it was concluded that the footprinting conditions used here were of sufficient sensitivity to detect EGR-1 binding activity with crude nuclear extracts. It was concluded that EGR-1-like binding activity is not a necessary component for an active P3 promoter in HeLa cells.

Figure 5 Protection of an EGR-1 consensus sequence using HepG2 nuclear extracts

DNAase I footprinting of the P3 promoter P3EI element was done as described in the Materials and methods section. A + G indicates DNA cleaved at adenine and guanine residues. Lane 1, no nuclear extract; lane 2, 30 µg of HepG2 extract.

Absence of detectable IGF-II transcripts derived from the P3 promoter in HeLa cells

To determine the level of IGF-II P3 promoter activity in HeLa DNA, the P3-derived 6.0 kb and 2.2 kb transcripts were analysed in HeLa and HepG2 cells. Cytoplasmic RNA from the two cell lines was isolated and the P3-derived transcripts were quantified by RNAase protection assay. The probe used was an exon 5 fragment, which includes 178 bp of the 3' end of exon 5. Figure 6 shows that 5 µg of HepG2 RNA was sufficient to obtain a strong signal (arrowed), whereas with 100 µg of HeLa RNA no signal was detected. A similar result was obtained using an S1 nuclease protection technique (not shown). It was concluded

Figure 6 Quantification of exon 5 levels in HeLa and HepG2 cells by RNAase protection

Hybridization of [α - 32 P]UTP-labelled antisense transcripts derived from exon 5 to cell line RNA was done as described in the Materials and methods section. Lane 1, probe only; lane 2, 5 μ g of HepG2 total RNA; lane 3, 100 μ g of HeLa total RNA.

Figure 7 Methylation status of the IGF-II P3 promoter

Each lane contains 10 μ g of DNA restricted with *MspI* (lanes 1 and 3) or *HpaII* (lanes 2 and 4), each at 10 units/ μ g. DNA was separated on a 4% denaturing acrylamide gel and transferred to a Gene Screen membrane as described in the Materials and methods section. M is pBR322 digested with *HinfI*. Lanes 1 and 2, HeLa DNA; lanes 3 and 4, HepG2 DNA. The membrane was probed with a *BglII-SalI* 1363 bp fragment of the P3 promoter. The arrow indicates a 267 bp *MspI* fragment.

that, although HeLa cells contain sufficient transcription factors for promoter activity (as demonstrated above), the P3 promoter in HeLa genomic DNA was inactivated via a mechanism not involving transcription factors.

The human IGF-II P3 promoter is extensively methylated in HeLa genomic DNA

To determine whether methylation at cytosine residues may account for the inactive P3 promoter in HeLa genomic DNA, the P3 promoter was analysed using the methylation-sensitive enzymes *MspI*/*HpaII*. The P3 promoter when cut with *MspI* gives rise to a large number of relatively small fragments of which the largest is 267 bp (Figure 7, arrowed). On cutting HeLa DNA with *HpaII* and probing with a P3 promoter fragment

(*BglII-SalI*; -1232 to +130), the 267 bp band (Figure 7, lane 2) was not present, but high-molecular-mass bands occurred at the top of the gel. This result suggested that the HeLa promoter was extensively methylated. HepG2 DNA, however, appeared to be unmethylated (Figure 7, lanes 3 and 4). These data were consistent with IGF-II promoter activity being critically dependent on the methylation status.

DISCUSSION

IGF-II has been demonstrated to have an essential role in development [43,44] and may also be a contributing factor in the origin of certain tumours. An essential aspect of IGF-II biology is to determine the mechanisms by which IGF-II biosynthesis is controlled. The IGF-II fetal promoters, P2-P4, have been previously found to be developmentally repressed [7,22,23], although the mechanism of repression is not known.

Previous studies of the rat IGF-II gene have shown that the P4 promoter is regulated by the general transcription factor Sp1 [18]. The rat P4 promoter contains four Sp1 binding sites and shows marked sequence similarity to the human P4 promoter. In this paper an analysis of the transcription factors of the human P3 promoter revealed a promoter considerably more complex than the rat P4 promoter.

Deletion analysis of the human P3 promoter revealed that the promoter consists of at least two promoter elements, P3EI (-291 to +130) and P3EII (-1232 to -812). The two promoter elements were found in both HeLa cells and HepG2 cells. This result was consistent with a previous report which identified a strong stimulatory effect of the region -180 to -1300 on P3 promoter activity in both HeLa and Hep3B cells [26]. A further promoter element was found here in HepG2 cells upstream of P3EII which may correspond to P2 promoter sequences. This result suggested that the liver-derived HepG2 cells may contain P2 transcription factors which are not present in HeLa cells. This result was in contrast to that of others, who found that P2 had a negative effect in Hep3B cells [26]. The expression of a 5.0 kb transcript in only a few cell types including fetal liver may account for the presence or absence of P2-associated transcription factors [24].

Further evidence of IGF-II P3 promoter complexity was indicated by DNAase I footprinting analysis. Binding sites were detected in addition to those previously reported in the P3 promoter region [26]. A total of nine protected regions were found here, of which five were Sp1-like, one was a CCAAT-box, and a further three appeared to correspond to a novel transcription factor [2]. Most of the binding sites that were detected were only detectable by a modified footprinting assay designed to enhance the sensitivity of detecting DNA binding activity with crude nuclear extracts, i.e. by increasing the protein concentration and decreasing the probe concentration. The data obtained using this approach suggested that the binding activity at nearly all binding sites was of relatively weak affinity. The finding that the P3 promoter gave rise to abundant transcripts [7,22,23] most likely indicates that strong P3 promoter activity is the result of a cumulative effect of many weak DNA binding activities.

The role of EGR-1 consensus sequences in the IGF-II P3 promoter remains unclear. It can be concluded, however, that because HeLa cells contain little or no EGR-1-like binding activity, binding of a transcription factor to the EGR-1 consensus sequences cannot be a necessary prerequisite for P3 promoter activity. In addition, as P3 promoter activity was readily detected in HeLa cells in CAT assays, we cannot exclude the possibility that the binding of some factor(s) to the EGR-1 consensus may result in the repression of this promoter. The precise role of the

EGR-1 consensus sequence in the P3 promoter will be difficult to establish in different cell types, particularly as there are already three factors known to bind the sequence in humans, i.e. EGR-1 [45], the Wilms' tumour WT1 zinc-finger protein (WT-Zf) [28] and NGF-1C [46]. It is not known whether these factors may compete for the same binding site, of which some will bind with higher affinity than others. The EGR-1-like binding activity detected in HepG2 cells may be one of the factors contributing to P3 promoter activity being higher in HepG2 cells than in HeLa cells. The precise nature of the binding activity of the EGR-1-like binding activity in HepG2 cells is not known. The finding that P3 promoter activity is only 5-fold greater in HepG2 cells relative to HeLa cells suggests that binding to the EGR-1 consensus sequence most likely serves to modulate P3 promoter activity.

The precise function of the WT1 gene product WT-Zf in the control of the P3 promoter remains to be elucidated, although current evidence suggests that it may be a repressor of this promoter. WT-Zf has recently been shown in *in vitro* co-transfection assays to repress the activity of the P3 promoter [30]. Given the complexity of the IGF-II P3 promoter, however, the effect of WT-Zf on this promoter *in vivo* may be quite different. WT-Zf has also been shown to act as a repressor for the fos and murine promoters when EGR-1 sites are engineered into the promoter [29].

The observation that the IGF-II P3 promoter is extensively methylated in HeLa cells is most likely an example of methylation-associated gene inactivation [47]. Interestingly, the binding sites within the P3 promoter detected by DNAase I footprinting each contain at least one CpG, suggesting that there may be many potential targets for methylation-associated gene inactivation in the P3 promoter. It has been reported previously that cell lines are susceptible to high levels of *de novo* methylation [47]. In cell lines the genes that were highly methylated were also found to be inactivated. It is possible that the necessary signals that determine whether a gene becomes extensively methylated or not may be intimately associated with the signals that give rise to allelic imprinting.

The data presented here indicate that the IGF-II P3 promoter is under complex regulation. The presence of several transcription factor binding sites in the P3 promoter offers a potential mechanism by which *trans*-acting factors might cause the activation of the promoter. The regulation of the P3 promoter in IGF-II biosynthesis may represent only a subset of controls that regulate the rate of IGF-II biosynthesis. Other controls that have been found to influence IGF-II biosynthesis are at the level of translation [19]. It was reported that the P3-derived 6.0 kb transcript does not bind to polysomes isolated from a rhabdomyosarcoma cell line. This is in contrast with the P4-derived 4.8 kb transcript, which was bound to polysomes. Another report, however, has shown that polysomes derived from placenta bound at least 50% of all IGF-II transcripts [7]. As the P3 promoter gives rise to by far the most abundant 6.0 kb and 2.2 kb transcripts in placenta, it must be concluded that placental polysomes do bind the P3-derived transcripts. The simplest explanation for these observations is that polysomes from different cell types differentially bind the various IGF-II transcripts. The P3-derived 6.0 kb and 2.2 kb transcripts might therefore have an essential role in the regulation of IGF-II biosynthesis. The identification of several *trans*-acting factor binding sites in the P3 promoter may be useful in determining their role in the control of IGF-II gene expression.

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