The liver-specific promoter of the human insulin-like growth factor-II gene contains two negative regulatory elements

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Abstract The adult liver-specific IGF-II promoter P1 is activated by CCAAT/Enhancer Binding Proteins α and β. Here we present evidence that promoter P1, in addition to positively regulating elements, contains two elements of 67 nucleotides that form an inverted repeat (IR) and suppress P1 activity. The two IR elements are specifically bound by a protein (inverted repeat binding factor, IRBF). The amounts of IRBF in various cell lines correlate with the levels of suppression of P1 activity, suggesting that this factor is responsible for the suppression of P1 mediated by the IR elements.

Key words: Human; Insulin-like growth factor; IGF-II; Transcription; Promoter; Suppressor; Liver

1. Introduction

Human insulin-like growth factor II (IGF-II) is a 67 amino acids long protein that influences both proliferation and differentiation in a cell type and development-dependent manner. Since the functions of IGF-II are so diverse, it is not surprising that the regulation of IGF-II production is very complex. At the transcriptional level, regulation takes place by differential activation of four distinct promoters (P1-P4) which give rise to a family of IGF-II encoding mRNAs [1]. In fetal liver IGF-II transcription is directed by the promoters P2, P3, and P4, of which P3 is the most active one. After birth, these promoters are down-regulated and promoter P1 is activated [2]. We have reported previously that an important class of regulators of the adult liver-specific promoter P1 is the CCAAT/enhancer binding protein (C/EBP) family of transcription factors [3,4]. These factors are present at high levels in adult liver, whereas fetal liver produces little or no C/EBP factors [5,6]. Furthermore, we found that the distal region of P1 exerts a negative effect on P1 promoter activity [3,7], indicating that, in addition to positive regulation by C/EBP factors, negative regulation of P1 activity also takes place. In the present study we have investigated the putative negative regulatory activity present in the distal region of P1. Interestingly, the distal region of P1 contains two 67 bp elements located at positions −877 to −812 (element IR1) and at positions −243 to −177 (element IR2), respectively, which are oriented as an inverted repeat. The effect of the IR elements on P1 activity has been examined and a putative role for the IR elements in tissue-specific expression is discussed.

2. Materials and methods

2.1. Plasmid construction

The luciferase reporter gene construct containing the TK promoter (TK-Luc) and the P1-luciferase constructs SN-Luc, BsN-Luc, and PsN-Luc containing P1 fragments ending at position +54 and beginning at positions −892, −342, and −207, respectively, have been described [4,7]. Mutant P1-luciferase construct dBP-Luc in which the fragment from positions −337 to −208 is deleted, was constructed by inserting a *Sma*I–*Bst*EII P1 fragment (positions −892 to −338), which was made blunt using Klenow polymerase, in front of the −207 to +54 P1 fragment in PsN-Luc, of which the *Pst*I site was made blunt using T4 polymerase. Several P1 fragments were cloned in front of the TK promoter in TK-Luc. Construct SP-TK-Luc contains the *Sma*I–*Pvu*II fragment of P1 (positions −892 to −163) cloned into the *Pvu*II site in front of the TK promoter. Construct PS-TK-Luc contains the same *Sma*I–*Pvu*II fragment in an inverted orientation. Similarly, constructs SB-TK-Luc and BS-TK-Luc contain the *Sma*I–*Bst*EII P1 fragment (positions −892 to −338) in two orientations, and constructs BP-TK-Luc and PB-TK-Luc contain the *Bst*EII–*Pvu*II P1 fragment (positions −342 to −163) in two orientations. Constructs BP2-TK-Luc and PB2-TK-Luc contain two tandemly arranged *Bst*EII–*Pvu*II P1 fragments in two orientations. The P1 subclone pNci3 contains the *Nci*I P1 fragment from positions −893 to −685, which was filled in using Klenow polymerase and cloned into the *Sma*I site of pUC18. The expression vector for C/EBPα, CMV-C/EBPα, has been described [8].

2.2. Transient transfection assays

Hep3B [9] and PLC/PRF/5 [10] cells were cultured in α-modified Minimum Essential Medium, and IN157 cells [11] were cultured in Dulbecco's Modified Eagles Medium. Media were supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 300 μg/ml glutamine. Cells were grown in 25-cm^2 flasks to 50–70% confluence and transfected using a modified calcium-phosphate precipitation protocol [12]. Each flask was transfected with 2 μg of luciferase construct, 0.5 μg of expression vector, and 0.25 μg of pRSV-LacZ, which served as an internal control for transfection efficiency. The amount of DNA was kept constant by the addition of carrier DNA up to 10 μg of DNA per flask. Preparation of cell extracts, β-galactosidase assays, and luciferase assays were performed as described [12,13]. Luciferase values obtained with P1-luciferase constructs were approx. 100-fold higher than background values obtained with a promoterless luciferase vector, and 5- to 10-fold lower than values obtained with TK-Luc. Each transfection presented in this report was performed with duplicate samples and has been repeated at least three times using different batches of plasmid DNA.

2.3. Electrophoretic mobility shift assays

The P1 fragment containing the upstream IR1 elements was isolated from pNci3 by digestion with *Bam*HI and *Eco*RI and radioactively labelled using Klenow polymerase and [α-^{32}P]dATP. A fragment containing IR2 was obtained by isolating the *Bst*EII–*Pvu*II fragment from BsN-Luc and labelled using [α-^{32}P]dGTP. The following oligonucleotides were synthesized on a Pharmacia/LKB Gene Assembler Plus and purified using a Sephadex G-50 column:

IR2 upper: 5′-GATCCTGGCTGGGCCTGGGGCTGGCATGGGCTGTGGCTGCAGACCACTGCCAGCTTGGGCCTCGAGGCCAGGT-3′

IR2 lower: 5′-TCGAACCTGGCCTCGAGGCCCAAGCTGGCAGTGGTCTGCAGCCACAGCCCATGCCAGCCCCAGGCCCAGCCAG-3′

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Double-stranded (ds) IR2 oligonucleotide was prepared by annealing the IR2 upper and lower oligonucleotides, and was purified on a non-denaturing 19:1 polyacrylamide gel. The sequence of the ds IR2 oligonucleotide maps at positions −243 to −177 relative to the transcription start site in P1. The sequence of the P1 CBS upper and lower oligonucleotides have been described [4]. Purified ds oligonucleotide was radioactively labelled using [γ-^{32}P]ATP and T4-kinase. Protein extracts from cell lines were prepared as described [14,15]. EMSA reaction mixtures contain 20 mM HEPES-KOH (pH 7.6), 1 mM $MgCl_2$, 60 mM KCl, 1 μg poly(dI.dC), 1 mM DTT, 0.018% NP40, and were used as described [7]. Bound and unbound probes were separated on a non-denaturing 37.5:1 polyacrylamide gel and visualised by autoradiography.

3. Results

3.1. The distal region of p1 exerts a negative effect on p1 activity

The negative regulatory activity exerted by the distal region of P1 was examined by introduction of long and short P1-reporter constructs (Fig. 1A) into the human cell lines Hep3B, PLC/PRF/5, and IN157, respectively, which exhibit differential IGF-II expression. In the hepatoma cell line PLC/PRF/5 low P1 activity can be detected, whereas P2, P3 and P4 are silent [4]. In contrast, the hepatoma cell line Hep3B exhibits high endogenous P3 activity and little or no P1 activity [4].

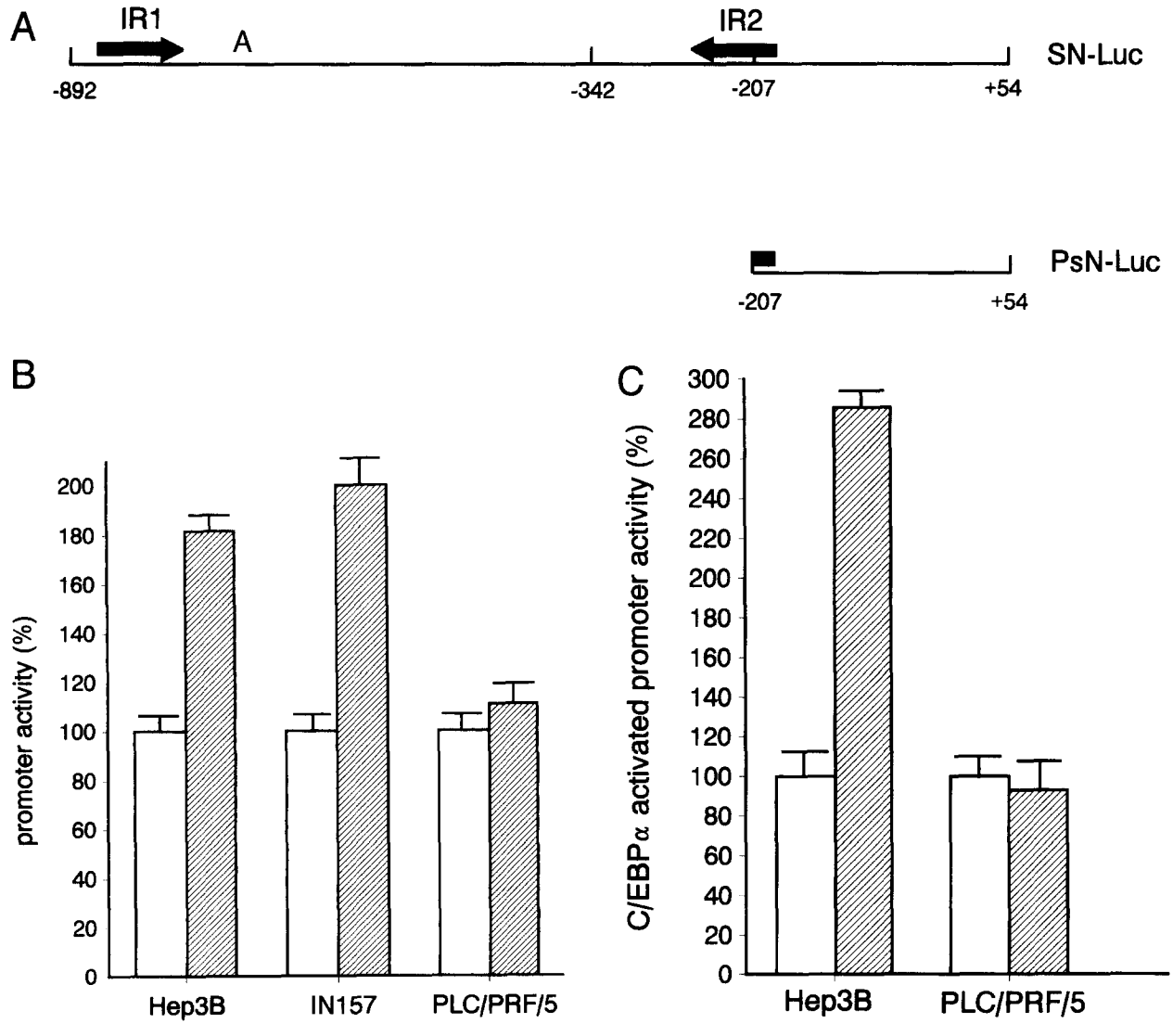

Fig. 1. Schematic representation of IGF-II P1 luciferase constructs and their activities. (A) IGF-II-P1-luciferase constructs SN-Luc and PsN-Luc used in transient transfection experiments. Numbers indicate nucleotide positions relative to the transcription start site (+1). The positions of the two IR elements in P1 are −877 to −812 (element IR1) and −243 to −177 (element IR2). (B) Expression of IGF-II-P1-luciferase gene constructs SN-Luc and PsN-Luc transiently transfected into the cell lines Hep3B, IN157, and PLC/PRF/5, respectively. For details of the transfection assays see Section 2. Activities of the luciferase constructs carrying either a long P1 fragment (SN-Luc, open bars) or a truncated P1 fragment (PsN-Luc, filled bars) are indicated as % of promoter activity measured for SN-Luc, which was arbitrarily set to 100%. Standard errors are indicated. All data represent the mean and standard deviation of at least three samples. (C) Expression of IGF-II P1-luciferase gene constructs SN-Luc and PsN-Luc transiently co-transfected into Hep3B and PLC/PRF/5 cells together with an expression vector encoding the C/EBPα transcription factor. All reporter gene activities were normalised and expressed as percentage of the promoter activity of SN-Luc which value was arbitrarily set to 100%.

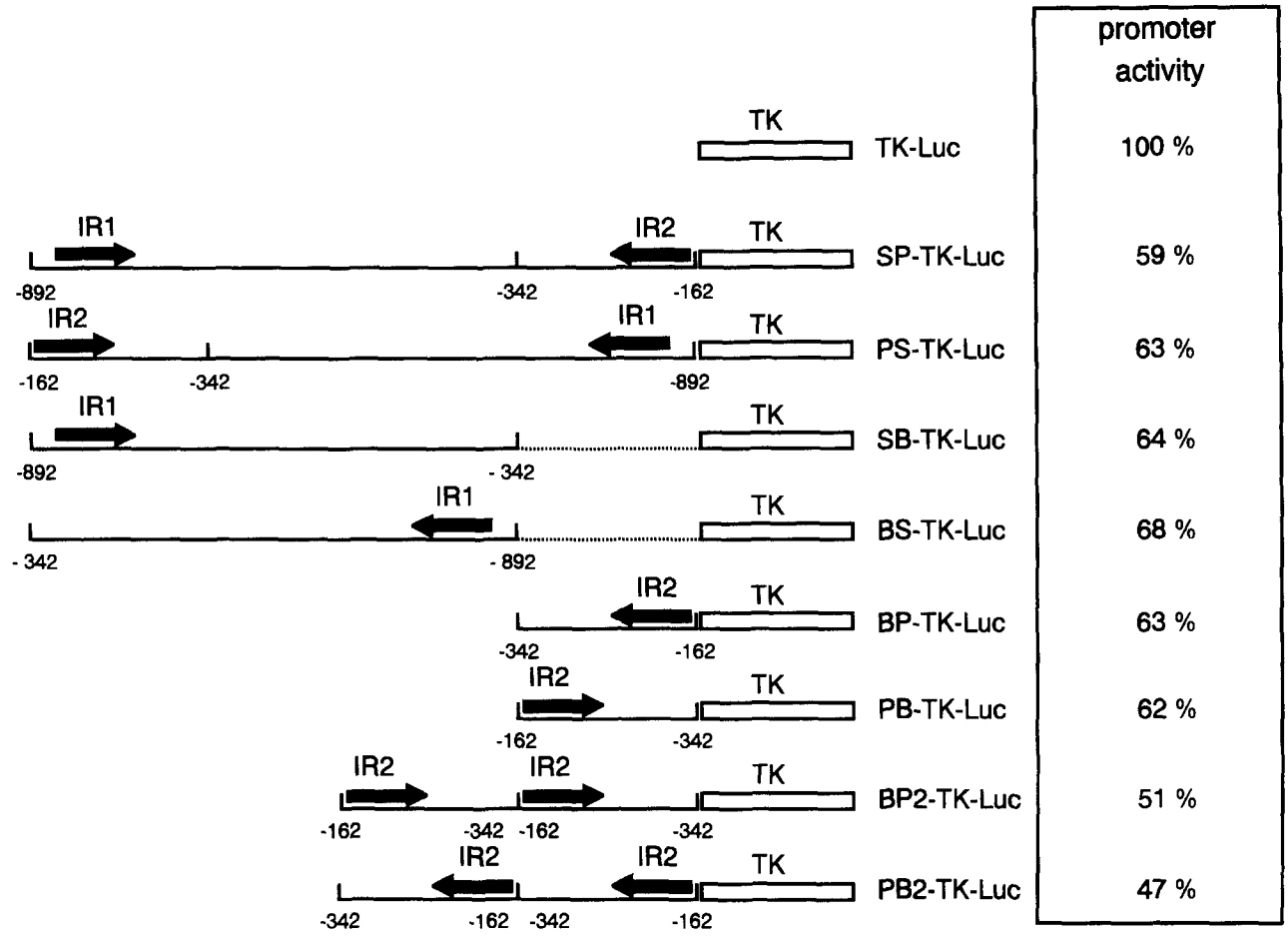

Fig. 2. Expression of IGF-II-P1-TK-luciferase gene constructs transiently transfected into Hep3B cells. For experimental details see Section 2. Constructs carrying P1 fragments with the IR elements in various orientations were cloned in front of the TK promoter. All reporter gene activities were normalised and expressed as percentage of the activity of the wild-type TK promoter (TK-Luc) which was arbitrarily set to 100%.

IN157 is a rhabdomyosarcoma cell line that shows an IGF-II expression pattern very similar to that of Hep3B [11]. Transfection of Hep3B cells with the P1 PsN-Luc truncated promoter construct that contains only 207 bp of P1 coupled to the luciferase reporter gene showed a 1.8-fold higher promoter activity than a construct containing 892 bp of P1 (SN-Luc) (Fig. 1B). This is in agreement with previous observations [3,7]. Transfection of IN157 cells with these P1 constructs also showed a 2-fold increase in the activity of the PsN-Luc construct compared to the SN-Luc construct. In contrast, in PLC/PRF/5 cells the activities of the short and long P1 fragments differ only marginally (Fig. 1B). These results indicate that deletion of the P1 region between positions −892 and −207 removes a region that exerts a negative effect on P1 activity in Hep3B and IN157 cells. Furthermore, this inhibitory effect on transcription of the SN-Luc P1 construct is cell type-dependent, since PLC/PRF/5 cells do not exhibit such an effect. Since the −892 to −207 region contains IR1 and most of IR2, two additional deletion mutants of SN-Luc were created to determine whether the IR elements were involved in the negative regulatory effect that is exerted by the distal P1 region in Hep3B and IN157 cells. In construct BsN-Luc the region from −892 to −342 containing IR1 was deleted, and in construct dBP-Luc the region from −342 to −207 is absent (Fig. 1A). In the latter case most of the IR2 element has been removed. Hep3B cells were transfected with these two single IR-deletion constructs, and the promoter activities of these P1 constructs were compared to the promoter activities of the long SN-Luc construct that contains both IR elements. Whereas deletion of both IR elements (PsN-Luc) leads to a 2-fold activation (Fig. 1B), deletion mutants carrying IR1 (dBP-Luc) or IR2 (BsN-Luc) show only a 1.2-fold higher promoter activity than SN-Luc. Obviously, the presence of a single IR element already leads to suppression of P1 activity.

Transfection experiments have shown that high P1 activity (10- to 15-fold induction) can be obtained in hepatoma cells when the cells are co-transfected with expression vectors encoding the C/EBP transcription factors C/EBPα or C/EBPβ [4]. We tested whether IR-mediated suppression of P1 could still occur when P1 activity is highly stimulated by C/EBPα. Hep3B and PLC/PRF/5 cells were transiently co-transfected with either SN-Luc or PsN-Luc, together with an expression vector encoding the liver-enriched transcription factor C/EBPα (Fig. 1C). In PLC/PRF/5 cells no P1 suppression is observed in the presence of C/EBPα, which is similar to the results obtained in PLC/PRF/5 cells in the absence of C/EBPα (Fig. 1B). In Hep3B cells co-transfected with a C/EBPα expression plasmid, suppression of transcription of the SN-Luc construct was again observed (Fig. 1C). This shows that C/EBPα does not interfere with the IR-effect, and that IR-mediated suppression of P1 occurs even when P1 activity is highly stimulated by C/EBP transcription factors.

3.2. The negative effect on transcription of the distal P1 region can be transferred to the thymidine kinase promoter

In general, enhancer and suppressor elements act relatively independent of their orientation and position. Furthermore, the effects that these elements exert on transcription are often transferable to heterologous promoters. To further examine the effect of the IR elements on transcription, we tested whether the negative effect exerted by the P1 distal region could be transferred to a heterologous promoter. Therefore, the distal P1 region (positions −892 to −162) containing both IR elements was coupled in two orientations to the thymidine kinase (TK) promoter fused to the luciferase reporter gene (SP-TK-Luc and PS-TK-Luc, Fig. 2). Transfection of Hep3B cells with these hybrid promoter constructs containing the IR elements showed a 40% reduction in promoter activity as compared to the wild-type TK promoter activity (Fig. 2). This level of suppression of TK promoter activity is comparable to the level of suppression of IGF-II promoter P1 containing the IR elements in their natural context. Transfection of various hybrid TK constructs containing P1 fragments in which either IR1 or IR2 was present still showed a 30–40% suppression of TK promoter activity, irrespective of the orientation of the fragments. Thus, also in front of the heterologous TK promoter the presence of IR elements gives rise to suppression of promoter activity, which is similar to the results obtained with the IGF-II P1 promoter constructs.

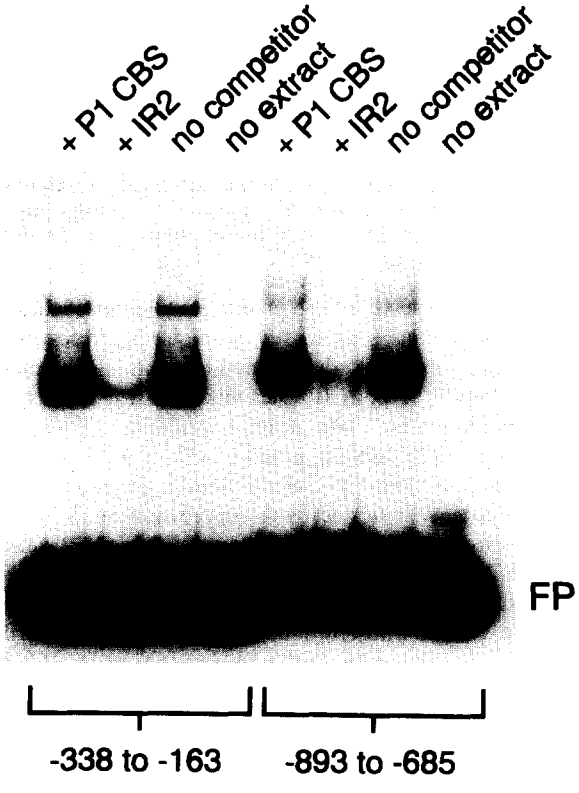

Fig. 3. Formation of DNA-protein complexes with probes containing the IR elements and Hep3B nuclear extracts. EMSA of end-labelled IR element containing probes with 2.5 μg of Hep3B nuclear extract. The 176 bp long IR2 probe contains P1 sequences from positions −338 to −163, and the 209 bp IR1-probe contains P1 sequences from positions −893 to −685. Competition analyses for binding were performed by addition of a 50-fold molar excess of unlabelled ds oligonucleotides containing the IR2 element (positions −243 to −177) or a non-specific oligonucleotide (P1 CBS) representing P1 at positions −110 to −87. Non-bound probe is indicated (FP).

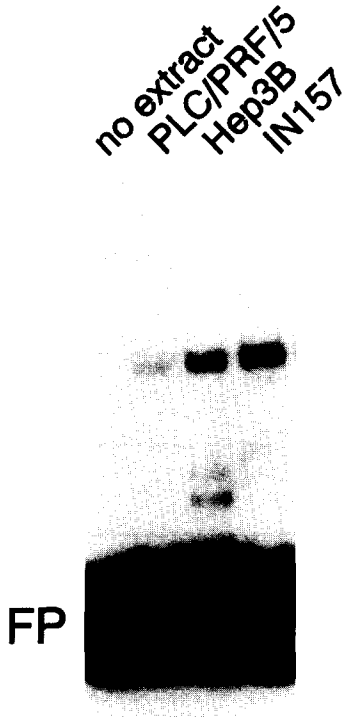

Fig. 4. Formation of protein-DNA complex with the IR2 element and nuclear extracts of various cell lines. EMSA of end-labelled ds oligonucleotide fragment (positions −243 to −177), representing the IR2 element. 5 μg of nuclear extract from the PLC/PRF/5, Hep3B, and IN157 cell lines, respectively, were incubated with excess labelled probe. Non-bound probe is indicated (FP).

3.3. Elements IR1 and IR2 can be bound by a specific protein

The mechanism by which enhancer and silencer elements usually influence transcription is by recruiting factors that specifically bind to these elements, and exert their effect on basal transcription. To test whether proteins can bind to the IR elements, an Electrophoretic Mobility Shift Assay (EMSA) was performed using radioactively labelled P1 fragments carrying either the IR1 element or the IR2 element. Incubation of the two IR element containing probes with Hep3B nuclear extract resulted for each of the DNA probes in the formation of one major protein-DNA complex and two minor more slowly migrating complexes (Fig. 3). These complexes were efficiently competed away after addition of an unlabelled 67 bp ds oligonucleotide carrying only the IR2 basepair sequence. This demonstrates that the IR element present in each probe is responsible for the protein-DNA complexes that are formed with Hep3B nuclear extract and IR containing fragments. Addition of an unlabelled non-specific ds oligonucleotide (P1 CBS) does not influence binding of Hep3B protein to the IR elements, which shows that binding to the IR elements is specific (Fig. 3). The major protein that binds to the IR elements will be referred to as inverted repeat binding factor (IRBF).

In order to optimise conditions for IRBF binding, the effects of varying salt and EDTA conditions were tested. It was found that IRBF binding is lost at KCl concentrations of 300

mM and higher, therefore 60 mM of KCl was used in further IRBF binding studies. Addition of 1 mM of EDTA to the binding reaction mixture already inhibits IRBF binding, which shows that IRBF binding is depending on divalent cations (results not shown).

3.4. *The levels of IRBF correlate with the levels of suppression of transcription of P1*

The level of suppression of transcription of P1 was found to vary among different cell lines; in Hep3B and IN157 cells promoter activity is inhibited to approx. 50%, whereas in PLC/PRF/5 cells hardly any inhibition can be observed. Since the IR elements that are involved in P1 suppression were found to be specifically bound by IRBF protein, the question arises whether there is a correlation between the level of suppression and the amount of IRBF in these cell lines. Therefore, EMSA experiments were performed using the ds IR2 oligonucleotide as a probe and nuclear extracts prepared from the cell lines PLC/PRF/5, Hep3B, and IN157, respectively. In these EMSA experiments a large excess of probe was used to ensure that all IRBF present in the nuclear extracts could bind to the probe. It was found that the amount of IRBF in PLC/PRF/5 nuclear extract is much lower than the IRBF levels in Hep3B and IN157 nuclear extracts (Fig. 4). This differential expression of IRBF correlates very well with the relative levels of suppression of P1 activity in these cell lines (Fig. 1B) and suggests that binding of IRBF to the IR elements is responsible for the inhibition of promoter activity. The level of P1 suppression is therefore primarily determined by the amount of IRBF that can bind to the IR elements.

4. Discussion

The proximal region of the liver-specific IGF-II promoter 1 contains two 67 bp regions, which are orientated as an inverted repeat (IR) spaced by 568 bp. In this report we present evidence that these two IR elements act as cell type-specific negative regulators of P1 activity. Analysis of the promoter activity of P1 constructs carrying deletions of one or both IR elements by transient transfection of Hep3B cells demonstrates that the presence of the IR elements leads to approximately 45% suppression of P1 promoter activity. The suppressing effect of the IR elements could be transferred to the Thymidine Kinase promoter, resulting in a 35%–50% reduction in promoter activity compared to wild-type TK promoter activity, irrespective of the relative orientation and spacing of the IR elements.

Incubation of either IR element with Hep3B nuclear extract results in the formation of a major protein-DNA complex, which is caused by binding of the IRBF. The level of IRBF varies among different cell lines; Hep3B and IN157 cells contain large amounts of IRBF, whereas PLC/PRF/5 cells express low levels of IRBF, which shows that the level of IRBF expression is cell type-dependent. The amounts of IRBF that are present in extracts prepared from Hep3B, IN157, and PLC/PRF/5, correlate with the levels of suppression of P1 activity mediated by the IR elements in these cell lines. In the cell lines Hep3B and IN157, the IR elements suppress P1 activity up to 50%. However, in the cell line PLC/PRF/5 only 10% suppression is observed. These observations suggest that the level of IRBF determines the level of IR-mediated suppression of P1 promoter activity.

In several hepatoma cell lines that we have tested for IGF-II expression, activity of P1 is either completely lost or reduced to a low level [4]. In many hepatoma cell lines such as Hep3B, the fetal IGF-II promoters have been reactivated. Low P1 activity in hepatoma cell lines is most likely caused by down-regulation of C/EBP transcription factors, a phenomenon that has been observed in proliferating liver cells such as hepatoma cells, in vitro cultured primary hepatocytes and regenerating liver cells [16,17]. In co-transfection experiments of hepatoma cells, the levels of P1 activity can be enhanced by the addition of exogenous C/EBPα, which is due to a strong C/EBP binding site in the proximal region of P1. Although C/EBPα enhances the promoter activity up to 10-fold, the addition of C/EBPα does not interfere with cell type-dependent IR-mediated suppression of P1 activity.

Hep3B and IN157 exhibit very high endogenous activity of the fetal IGF-II promoter P3 and little or no P1 activity, whereas in PLC/PRF/5 promoter P1 is exclusively active, albeit at a low level [4,11]. Although only a limited number of cell lines was tested in this study, it suggests that cell type-dependent IR-mediated suppression of P1 is operational in cells exhibiting an IGF-II expression pattern similar to fetal liver. The results presented in the present report support the notion that the activity of the liver-specific IGF-II promoter P1 is regulated by an interplay of positive and negative regulatory proteins.

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