The liver-specific promoter of the human insulin-like growth factor II gene is activated by CCAAT/enhancer binding protein (C/EBP)

M.A. van Dijk, R.J.T. Rodenburg, P. Holthuizen and J.S. Susсенbach*
Laboratory for Physiological Chemistry, State University of Utrecht, Vondellaan 24a, 3521 GG Utrecht, The Netherlands

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ABSTRACT
The human gene coding for insulin-like growth factor II (IGF-II) contains four promoters (P1—P4), that are subjected to tissue-specific and development-dependent regulation. Expression of promoter P1 is detected only in adult liver tissue, whereas promoter P3 is the major IGF-II promoter in fetal liver and is further expressed in other fetal tissues and in adult non-hepatic tissues. C/EBP is a tissue- and development-specific transcription factor that is expressed predominantly in adult liver, adipose tissue and lung. The effect of C/EBP on the expression of constructs containing IGF-II promoter P1 or P3 linked to the luciferase gene was investigated in cotransfection assays using Hep3B cells. We found that promoter P1 can be activated by C/EBP, whereas this transcription factor has no effect on the expression of promoter P3. By gel retardation and DNasel footprinting it was demonstrated that C/EBP can bind to a region of P1 located between 82 and 109 basepairs upstream of the cap site. Furthermore, we showed that deletion of this C/EBP binding region strongly reduces the ability of C/EBP to stimulate transcription from P1. These results indicate that C/EBP is a major component in the specific activation of the human IGF-II promoter P1 in adult liver.

INTRODUCTION
The human gene coding for insulin-like growth factor II (IGF-II) spans a region of 30 kb located on the tip of the short arm of chromosome 11 and consists of 9 exons 1—9, of which exons 7, 8 and the first part of exon 9 code for the IGF-II precursor protein. The 5' part of the gene consists of non-coding leader exons 1—6, of which exons 1, 4, 5 and 6 are preceded by a promoter (P1—P4). Transcription from these promoters leads to the formation of IGF-II mRNAs with different untranslated leaders (1, 2, 3). Two functional polyA addition signals in exon 9 contribute further to the heterogeneity of the IGF-II mRNAs (Fig. 1A). The IGF-II promoters are active in a variety of embryonic and adult tissues and are subjected to developmental and tissue-specific regulation of expression. The best example of differential regulation of the IGF-II gene is found in the human liver. During fetal development promoters P2, P3 and P4 are active yielding high levels of IGF-II mRNA, primarily derived from promoter P3. After birth the activity of these promoters declines and promoter P1 becomes active in adult liver, yielding a 5.3 kb mRNA (Fig. 1A) (3).

In a previous study we have performed an initial characterization of the four human IGF-II promoters (4). We found that luciferase reporter constructs bearing promoter P1 fragments show low levels of activity when transiently introduced into various eukaryotic cell lines of hepatic and non-hepatic origin. In contrast, constructs bearing promoter P3 exhibit a more widely distributed pattern of expression and are highly expressed in the hepatoma cell line Hep3B. The absence of readily detectable levels of promoter P1 derived 5.3 kb mRNA in cell lines and the exclusive expression pattern of promoter P1 in adult liver tissue suggests that P1 activity is restricted to terminally differentiated hepatocytes, and thus is dependent on the action of one or more transcription factors that are active in adult liver only. Although analysis of the nucleotide sequence of promoter P1 does not reveal the presence of binding sites of known liver-specific transcription factors (see (5) for a review), a potential candidate for the activation of promoter P1 in adult liver is the CCAAT/Enhancer binding protein (C/EBP) (6, 7). This transcription factor is expressed predominantly in terminally differentiated hepatocytes, adipocytes and lung (8), and binds to sites in the DNA that share the loose sequence homology T^P_G/N^T/C/AA^P/T (9). It has been shown that C/EBP is required for the activation of the promoter of the liver-specific serum albumin gene (10) and binds to regulatory sites in this and several other genes that are expressed only in liver (11) and adipose tissue (12).

We report here that the transcription factor C/EBP binds to the human IGF-II promoter P1. Furthermore, we demonstrate in cotransfection experiments with Hep3B cells that whereas promoter P3 can not be activated, C/EBP can trans-activate constructs carrying promoter P1 linked to the luciferase gene. The activation is diminished upon removal of the C/EBP binding.

* To whom correspondence should be addressed
site from P1-luciferase constructs, indicating that the effect of C/EBP is mediated through this site.

MATERIALS AND METHODS

Materials

Enzymes were purchased from Boehringer Mannheim and used according to the manufacturer's protocol. 32P-labelled nucleotides were purchased from Amersham, Buckinghamshire (UK).

Plasmid DNAs

The vectors bearing fragments of promoter P1 of the human IGF-II gene coupled to the firefly luciferase gene were constructed as described (4). Briefly, a SmaI/NcoI fragment (−889 to +52 relative to the cap site of P1) was inserted in the HindIII/NcoI sites of pSLA3 (4). The NcoI site in pSLA3 contains the initiation codon of the luciferase gene. Subsequently, the −184, −147 and −54 P1 constructs were made using appropriate restriction sites within promoter P1 (Fig. 1B). The promoter P3 construct was made by insertion of a BglII/Sall fragment from P3 (−1220/+134) into the BamHI/Sall sites of pSLA3. For the cotransfection assays we used pMSV-C/EBPwt (10), which was a kind gift from Drs. S.L. McKnight and Z. Cao (Baltimore) and pBD9 (10a) (a kind gift of Dr. G.W.M. Swart (Nijmegen)).

Oligonucleotides

The oligonucleotides used in the competition studies are derived from the C/EBP-binding site located from +1 to +20 in the promoter of the human factor IX (13) and from the C/EBP binding site in IGF-II promoter P1 (Fig. 3B). The sequence of the factor IX oligonucleotide is: 5'-GACCTTACACTTTTACAATCTGCTAG-3' and 3'-CTCGGAATGGTGAAGTTGATGAGCAGTC-5'. As a non-specific oligonucleotide we used a double-stranded oligonucleotide containing a xE2 site (E2). The sequence of this oligonucleotide is: 5'-CGCGCGTGGACGGGTGCAGCAGC-3' and 3'-TCGAGCCGCCACCTGCCCCAGCGAG-5'.

Transfections

Hep3B cells (15) were transfected at 60% confluence in 25 cm² flasks using the calcium phosphate coprecipitation method (16). Each transfection mixture contained 3 μg of the indicated luciferase reporter plasmid with or without 3 μg of pMSV-C/EBPwt (10). To keep the amount of DNA constant, pUC12 DNA was added to the transfection mixtures without pMSV-C/EBPwt. To correct for differences in transfection efficiency, 1 μg of pRSV-LacZ was included in each transfection. Cells were harvested 48 h after transfection. Preparation of enzyme extracts and luciferase- and β-galactosidase assays were performed as described (17, 18). Luciferase levels were determined using a Lumac/3M Biocounter M2010A.

Preparation of extracts

For the preparation of whole cell extracts, two subconfluent 75 cm² flasks of Hep3B cells were transfected with 20 μg of either pMSV-C/EBPwt or pUC12 DNA. 48 hr after transfection the cells were harvested by scraping and washed twice with phosphate-buffered saline. After pelleting the cells in a microfuge, the cell pellet was frozen on dry ice and thawed by resuspension in 5 volumes of lysis buffer (10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid) pH 7.9, 400 mM sodium chloride, 100 mM EGTA (ethylene glycol tetraacetic acid), 0.5 mM dithiothreitol, 5% glycerol and 0.5 mM phenylmethylsulphonic acid). Cell debris was removed by a 5 min. ultracentrifugation step at 100,000×g. The supernatant was placed at 70°C for 5 min. after which insoluble proteins were removed by a second 5 min. ultracentrifugation step at 100,000×g. Partially purified wild type rat C/EBP was produced in E. coli as described (19).

Gel retardation and DNasel footprinting

Conditions for DNasel footprinting and gel retardation were as described (20). For DNasel footprinting a XhoI/NcoI fragment (−184/+52) of IGF-II promoter P1 was labelled at the NcoI site at the top or bottom strand using either Klenow DNA polymerase and α-32P-dCTP or T4 polynucleotide kinase and γ-32P-ATP. The oligonucleotide probes for the gel retardation assays were labelled with T4 polynucleotide kinase and γ-32P-ATP.

RESULTS

In a previous study we found that the −889 P1 construct was expressed at a low level in Hep3B cells (4). 5' deletion analysis revealed that promoter P1 was activated approximately two-fold upon truncation from −889 to −184. This indicated the presence of a repressor upstream of −184. Further truncations of promoter P1 from −184 to −54 showed a gradual decrease in promoter activity.

The first indication that C/EBP might regulate the activity of the promoter P1 of the human IGF-II gene came from transient expression assays. When Hep3B cells were cotransfected with a construct bearing a fragment of promoter P1 (−889/+52) coupled to the luciferase gene, and the expression plasmid pMSV-C/EBPwt (which encodes full length C/EBP) we observed an approximately 3 to 4-fold higher luciferase value than found when Hep3B cells were transfected with the same promoter P1 construct and pUC12 DNA (Fig. 2). This stimulation is in the same order of magnitude as reported for the C/EBP binding D domain of the albumin promoter (20a). In addition, the expression of the plasmid pBD9 carrying nine copies of the D domain fused to the HSVtk promoter (10a) and linked to the CAT gene was stimulated over 50-fold by C/EBP (results not shown). To demonstrate that the major fetal IGF-II promoter P3 was not activated by C/EBP, a construct containing promoter P3 coupled to the luciferase gene was also tested. As shown in Fig. 2 this promoter could not be activated by C/EBP. This indicates that the stimulatory effect of C/EBP is significant and specific for P1. Since analysis of the nucleotide sequence of promoter P1 did not reveal the presence of an obvious C/EBP binding site we used truncated promoter P1 constructs to localize the site(s) in P1 required for the activation by C/EBP (Fig. 1B). Hep3B cells were cotransfected with the truncated P1 constructs and either pMSV-C/EBPwt or pUC12. In all transfections containing IGF-II P1 constructs in combination with pMSV-C/EBPwt we found an increased luciferase activity compared to those containing IGF-II P1 constructs and pUC12. The luciferase values in the transfections containing the −889 constructs are elevated 3.3-fold in the presence of pMSV-C/EBPwt (Fig. 2). When this construct is truncated to −184 and −147, the luciferase levels are elevated 5.7 and 6.1-fold, respectively. The reduced activation by C/EBP of the −889 construct compared to the −184 construct might be due to the presence of a repressor element upstream of −184 (4). The presence of this element in promoter P1 results in a twofold reduction of promoter activity. This suggests that
the twofold decrease in activation by C/EBP of the P1 construct -889 compared to the P1 constructs -184 and -147 may be due to the presence of this repressor element. As mentioned above, the -184 construct is the most active promoter P1 construct in transient assays. A further truncation of promoter P1 to -54 reduces the activating effect of pMSV-C/EBPwt to 2-fold. The observation that the 6-fold elevation of luciferase levels obtained with the -184 and -147 constructs was reduced to 2-fold with the -54 construct indicates that the element(s) responsible for this differential response are located between 147 and 54 bp upstream of the cap site of IGF-II P1.

Subsequently, we prepared whole cell extracts from Hep3B cells transfected with or without pMSV-C/EBPwt. As a control, we also prepared an extract from E. coli containing a C/EBP expression construct (19). Since C/EBP, in contrast to most of the other proteins within the cell, is stable at high temperatures (6), these extracts can be partially purified by a heat treatment (see Materials and Methods). We used these heat-treated Hep3B extracts and partially purified rat C/EBP synthesized in E. coli to identify the site binding C/EBP by DNase footprinting. When partially purified C/EBP from E. coli was used we observed a footprint located from basepairs -88 to -109 on the top strand of P1 and from basepairs -82 to -105 on the bottom strand (Fig. 3A). A similar footprint was formed when extract from pMSV-C/EBPwt transfected Hep3B cells was used, but not with extract from pUC12 transfected Hep3B cells. The observation that the -82 to -105 region of promoter P1 is protected against DNase cleavage by E. coli and pMSV-C/EBPwt transfected Hep3B extracts indicates that the same factor is present in both extracts. Since this factor is heat-stable and not detected in an extract from pUC12-transfected Hep3B cells, we conclude that the -82 to -109 footprint results from the binding of C/EBP.

In lanes representing the footprint reactions with rat C/EBP produced in E. coli, we observed a second, weaker footprint located from -49 to -79 (Fig. 3A). This footprint is not detected with pMSV-C/EBPwt transfected Hep3B extract, which could indicate that this footprint is not caused by C/EBP but by an endogenous heat-stable E. coli protein. With Hep3B extract we also observed a footprint located from -31 to -54, which is not formed with E. coli extract (Fig. 3A). This footprint is caused by the binding of a heat-stable factor that most likely corresponds to the factor binding to the element PE1-1, which was previously identified by DNase footprinting (4).

Comparison of the nucleotide sequence of the C/EBP binding site in promoter P1 with a recently defined C/EBP consensus sequence (9) reveals that in the lower strand of P1, from positions -91 to -99, a sequence is present that fits the consensus except for one mismatch (Fig. 3B). To investigate the binding properties of the promoter P1 C/EBP binding site we used a double-stranded oligonucleotide containing basepairs -87 to -106 for gel retardation analysis. This oligonucleotide was labelled and subsequently incubated with 0.1 μl of either of the extracts used for DNase footprinting, in the presence or absence of different competitor oligonucleotides. To confirm our results we also performed gel retardation assays with a labelled oligonucleotide containing the high affinity C/EBP-binding site of the factor IX promoter. Fig. 4 illustrates that a low mobility heat-stable protein-DNA complex is formed when either of the labelled oligonucleotides was incubated with the partially purified rat C/EBP synthesized in E. coli or with the pMSV-C/EBPwt transfected Hep3B extract. This complex is not observed when these oligonucleotides were incubated with the extract from pUC12 transfected Hep3B cells. The low mobility protein-DNA complex formed with the P1 oligonucleotide is only detected in extracts that also protect the -82 to -109 region of P1 against DNase cleavage. This is a further indication that complex formation and protection against DNase cleavage are caused by the same factor. The observation that DNA-protein complex...
formation is effectively abolished by the addition of excess unlabeled oligonucleotides containing the C/EBP binding site from IGF-II promoter P1 or from the factor IX promoter, but not by addition of an excess of the non-specific xE2 oligonucleotide (Fig. 4), indicates first: that indeed C/EBP is involved in the formation of the observed protein-DNA complex, and second: that the C/EBP-II oligonucleotide has a weaker affinity for C/EBP than the factor IX oligonucleotide. Furthermore, the absence of a shifted protein-DNA complex in lanes 11 and 22 (Fig. 4) indicates that Hep3B cells do not produce endogenous C/EBP. C/EBP is however produced in Hep3B cells when these cells are (co)transfected with pMSV-C/EBPw. On the basis of these results we conclude that the six-fold activation of the −184

DISCUSSION

By DNaseI footprinting we have shown that C/EBP is able to bind specifically to a site in IGF-II promoter P1. This C/EBP site is located between positions −82 to −109 and is homologous to the C/EBP consensus sequence (9) in six out of seven residues. The binding of C/EBP to this region of promoter P1 is confirmed by gel retardation analysis. When a labeled oligonucleotide containing the C/EBP-II P1 region from −87 to −106 is used in a gel retardation assay we observed the formation of a low mobility heat-stable complex with partially purified C/EBP synthesized in E. coli (Fig. 4, lane 2). A similar heat-stable complex is formed with an oligonucleotide containing the factor IX C/EBP binding site (Fig. 4, lane 21), indicating that the same factor which is required for the formation of the shifted protein-DNA complex with the P1 oligonucleotide is also able to bind the factor IX oligonucleotide. When these oligonucleotides are incubated with extract from pMSV-C/EBPw transfected Hep3B cells, we observed the formation of a complex with a slightly lower mobility than the complex formed with C/EBP produced in E. coli (lanes 12 and 23). The slightly lower mobility of the complex formed with Hep3B extract compared to the complex formed with E. coli extract might be due to posttranscriptional modification of C/EBP produced in Hep3B cells. Some indication that C/EBP might be modified in eukaryotic cells comes from comparison of the molecular weight of C/EBP predicted on the basis of the nucleotide sequence of the cDNA (38 kD) with the molecular weight of C/EBP purified from rat liver (42 kD) (7).
The formation of a shifted protein-DNA complex was not observed with an extract from pUC12 transfected Hep3B cells (Fig 4, lanes 1, 11 and 22), indicating that Hep3B cells do not contain detectable levels of endogenous C/EBP. That the factor present in the shifted protein-DNA complex formed with C/EBP-E. coli extract is similar to the factor present in extract from pMSV-C/EBPwt transfected Hep3B cells was further confirmed by competition assays. Excess unlabelled IGF-II P1 or factor IX oligonucleotide competes in a similar manner for the formation of the shifted protein-DNA complex in pMSV-C/EBPwt transfected Hep3B cell extract and C/EBP-E. coli extract (Fig. 4). These competition studies further indicate that the affinity of the IGF-II P1 oligonucleotide for C/EBP is about 5-fold lower than the affinity of the factor IX oligonucleotide. Nevertheless, this C/EBP binding site clearly has a function in the regulation of IGF-II promoter P1 activity, as is demonstrated by transient expression assays. A luciferase construct bearing an IGF-II P1 fragment containing the region from +52 to −147 or −184 is activated 6-fold by C/EBP in a transient expression assay in Hep3B cells. When the P1 fragment is truncated to −54 the stimulation observed upon cotransfection with pMSV-C/EBPwt is only 2-fold. Since other C/EBP binding sites were not detected in the region from −147 to −54, we conclude that the ability of promoter P1 to be activated by C/EBP in this assay is dependent on the presence of the C/EBP binding site located between positions −82 and −109. The 6-fold stimulation observed with the −147 and −184 constructs is reduced to 3.3-fold with the −889 constructs. Previous observations have indicated the presence of a repressor element between −889 and −184 (44). The twofold decrease in activation by C/EBP of the −889 construct compared to the −184 and −147 constructs could be due to the presence of this repressor element. Promoter P3 is not expressed in adult liver and does not contain consensus C/EBP sites. The observation that C/EBP does not activate this promoter indicates that the observed activation of promoter P1 is specific.

As described by Nye and Graves (21), the precise sequence requirements for the binding of C/EBP are not known, although most published C/EBP sites are homologous to the consensus sequence TNC/CAAT/G published by Ryden and Beamon (9). In spite of the predicted frequent occurrence of this consensus in the genome, C/EBP activates only certain promoters. It has also been observed that the ability of C/EBP to activate transcription in transient transfection assays is dependent on the cell type used in the assay. The activation observed in HepG2 cells was higher than in L cells (10). This indicates that for C/EBP to efficiently activate transcription there are more conditions to be met than only the presence of a binding site. These conditions may include the presence of other (transcription) factors and/or the position of the C/EBP binding site with respect to the binding sites of other transcription factors. The latter option could also explain how a sub-optimal C/EBP binding site such as the site identified in this study can still be functional: other transcription factors binding in the vicinity of the C/EBP binding site might stabilize the binding of C/EBP. A similar stabilizing effect has been observed between Oct1 and Sp1 (22). The 5.3 kb mRNA derived from IGF-II promoter P1 is only detected in terminally differentiated human hepatocytes. In the liver, C/EBP is present predominantly in these cells (8). The activation of promoter P1 by C/EBP, whereas P3 is not activated, makes it likely that this transcription factor plays a specific role in the regulation of promoter P1 in adult liver.

It has been observed that the presence of promoter P1 is species-dependent. The IGF-II genes in rat and mouse show extensive structural homology (23, 24, 25, 26, 27). The human leader exons 4, 5 and 6 and the coding exons are very well conserved. However, exons homologous to the human exons 1, 2 and 3 are either absent (in rat) or partially present as pseudo exons (in mouse) (28). As a consequence analogs of promoter P1 are absent in rat and mouse. Recently, however, an IGF-II mRNA was detected in adult sheep liver, indicating the presence of a functional promoter P1 in sheep (29). In man and sheep the serum level of IGF-II remains high after birth, while the IGF-II level in rodents fall to nearly undetectable levels after birth (reviewed in (30)). The presence of promoter P1, therefore, seems to correlate with the persistence of high serum IGF-II levels after birth. The species-dependent presence of this promoter is intriguing. By studying the regulation of this promoter a better insight might be obtained in the functional significance of this phenomenon.

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