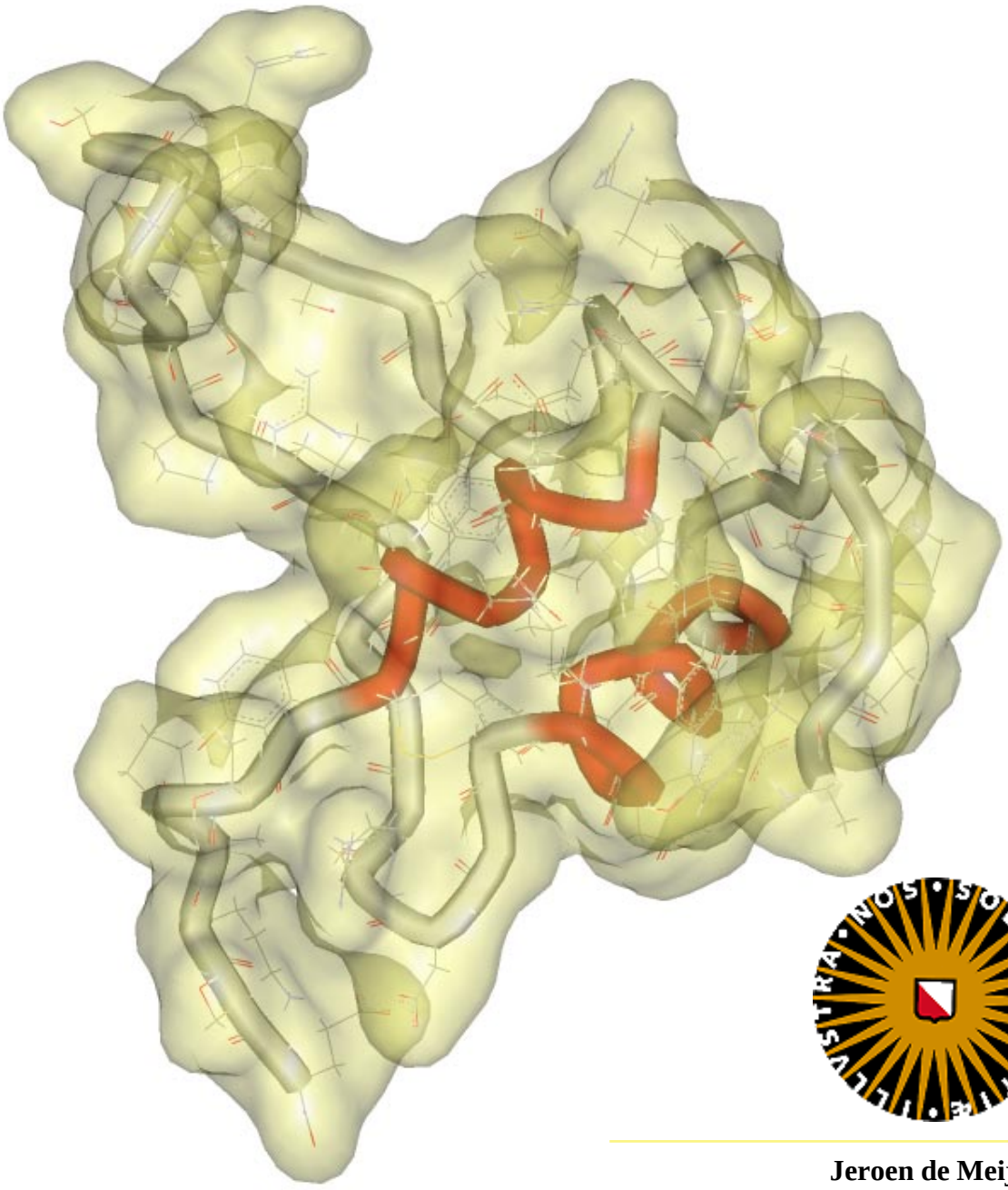
The role of the upstream region in the cell- and tissue-specific regulation of IGF-II promoter P3



Jeroen de Meijer
Sep-1996/Dec-1997

"Anyone who teaches knows there are two questions to steer clear of on exams: The one about evolution, to which there is no certifiable answer; and the one about gene control, to which every conceivable answer is correct. Or so it seems at times."

MARYLIN KOZAK (1988)

Front page illustration:
Insulin-like growth factor II

The role of the upstream region in the cell- and tissue-specific regulation of IGF-II promoter P3

The role of the upstream region in the cell- and tissue-specific regulation of IGF-II promoter P3

J. de Meijer 1997

A report of a 9 month research period at the Department of Physiological Chemistry, University of Utrecht, under supervision of drs. L.E.G. Rietveld.

Abstract

The insulin-like growth factor-II plays an important role in growth. It is developmentally- and tissue-specifically regulated and has a complex promoter structure. The IGF-II gene contains 4 promoters. Promoter P3 is active in the foetal tissues and liver and in various tumour cells. P3 is not active in the adult liver though. Rietveld et al. (1997) and Van Dijk et al. (1992a) have shown that the proximal region of promoter P3 supports basal transcription activity. They have identified several important regions and proteins essential for this basal promoter activity. This implies that the more upstream region is involved in cell specificity. To investigate the role of proteins binding to this upstream region in the developmental- and tissue-specific regulation of P3, experiments were performed in order to:

- Determine the regions that are important for cell-specific regulation of P3 by transfection of 3 cell-lines (Hep3B, HeLa and 293) with the deletion constructs
- Determine protein-binding to P3 with the use of EMSA analysis and footprinting

The results support the findings of Van Dijk et al. (1992a) and Rietveld et al. (1997) that two regions can be distinguished; A proximal region (-509/-136) important for basal promoter P3 activity, and a distal region (-1225/-509). This distal region shows differences in protein binding between 293 and Hep3B, and contains hypersensitive sites. It's not clear whether these proteins have a role in the cell specific regulation of P3 activity. .

It is possible that the cell specific regulation of P3 takes place at a different level (i.e. not at the transcriptional level); for instance DNA folding or methylation. To further clarify the cell specific regulation of P3, additional research needs to be done.

Contents

Abstract 6
Contents 7
Introduction 9
1.1 Transcriptional regulation 9
1.2 Tissue and cell specific regulation 12
1.3 IGF-II 13
1.4 The structure of the IGF-II gene 13
1.5 The IGF-II mRNAs 15
1.6 The translation of IGF-II mRNAs 16
1.7 IGF-II receptors and binding proteins 17
1.8 RNA cleavage 17
1.9 Parental imprinting 18
1.10 Promoter P3 19
Material & Methods 21
2.1 Cell culture 21
2.2 Materials 21
2.3 Plasmids 21
2.4 DNA digestion and purification 22
2.5 Synthesis of blunt ends and ligation 22
2.6 Dephosphorylation 22
2.7 Phenol extraction and alcohol precipitation 22
2.8 Calcium competent E. coli DH5a and transformation 23
2.9 Miniprep 23
2.10 Maxiprep 23
2.11 Sequencing 24
2.12 Transfections 25
2.13 Labelling of probes 25
2.14 Nuclear and whole cell extracts 26
2.15 Electrophoretic mobility shift assay (EMSA) 26
2.16 Footprinting and GA ladder 27
Results 28
3.1 HeLa/Hep3B transfections 28
3.11 Optimization of the DEAE transfection method 28
3.12 Transfection of HeLa and Hep3B 29
3.2 EMSA analysis and footprinting with HeLa and Hep3B WCE 29
3.3 Truncated promoter constructs 33
3.4 Transfection of 293 and Hep3B 35
3.5 EMSA analysis with 293 and Hep3B nuclear extracts 38
Discussion 40
4.1 293 versus Hep3B 40
4.2 HeLa versus Hep3B 42
4.3 Concluding remarks 44
References 46

Supplement 55
1 Buffers and media 55
2 Transfection data 56
2.1 293 56
2.2 Hep3B 59
Acknowledgements 62

1 Introduction

Growth is a regulated process. Malfunctions in growth regulation can lead to several disorders such as cancer, and can even result in the death of the organism. An important role in growth regulation is played by growth factors, such as EGF, PDGF and IGF. Regulation of the expression of these growth hormones is of vital importance for proper development and growth. Regulation can occur at several levels; at the transcriptional, translational or posttranslational level (binding to other proteins, protein degradation). Transcription, especially the initiation of mRNA synthesis, is well established to be a major point of regulation. In this study, regulation at the transcriptional level of the human insulin-like growth factor II (IGF-II) promoter P3 is the subject of investigation.

1.1 Transcriptional regulation

The regulation of mRNA synthesis from a gene is governed by proteins interacting with that gene. A gene can possess several regulatory domains, a proximally situated promoter and several, more upstream located, enhancer regions. The promoter is located directly upstream of the transcription initiation site, and the complex that binds to this region can be influenced by other proteins binding elsewhere.

Usually a gene possesses one promoter. However, there are some exceptions which all contain two promoters, like the PDGF receptor gene (Vu et al. 1989), the mouse carbonic anhydrase gene (Fraser et al. 1989), the human progesterone gene (Kastner et al. 1990), the mouse α-amylase 1^a gene (Schibler et al. 1982, Shaw et al. 1985), the glucokinase gene (Magnuson et al. 1989), and the muscle phosphofructokinase gene (Yamasaki et al. 1991). The apolipoprotein gene contains three promoters (Shellness et al. 1984), and the IGF-II gene contains four promoters (Van Dijk et al. 1991). In the case of IGF-II, translation of the mRNAs derived from the four promoters results in the same prepropeptide, whereas in the case of for instance the PDGF receptor gene, the mRNAs give rise to two different receptors (one being truncated, missing the first 5 exons and thereby part of the extracellular domain) depending on the promoter used. In bovine the papilloma virus type 1, two isoforms from one gene were found, one of these isoforms being an activator, the other one being a repressor of BPV-1-mediated

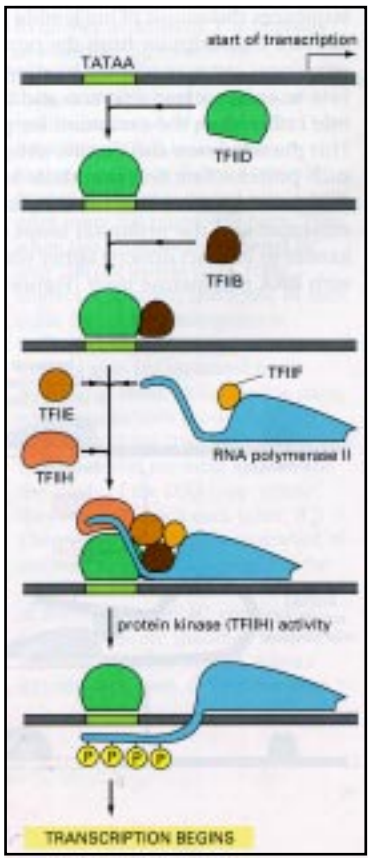


Figure 1;
The assembly of the general transcription factors required for the initiation of transcription by RNA polymerase II.
In the first stage, TFIID binds specifically to a TATA sequence. Next, TFIIB enters the complex, followed by RNA polymerase II escorted by TFIIF. TFIIE and TFIIH assemble into the complex next. In the presence of ATP, TFIIH phosphorylates RNA polymerase II, which then releases the polymerase so that it can initiate transcription. (adapted from Alberts et al.)

transformation of mouse C127 cells (Lambert et al. 1987). In the yeast invertase gene, the two promoters also give rise to functionally different enzymes (Carlson et al. 1982).

The advantage of multiple promoters lies probably in the regulation of the gene in various tissue and/or developmental stages, although this can also be achieved with one promoter using tissue- and/or developmentally-specific trans-acting factors. One example of such tissue specific regulation is the glucokinase gene. This gene has two differentially regulated promoters that produce the same protein, glucokinase. Glucokinase has a different function in the liver then in the β cell; In the β cells it has a role in the regulation of insulin secretion which is controlled by the concentration of glucose, whereas in the liver it helps to facilitate the uptake and conversion of glucose by the liver which is regulated by insulin. This differential regulation is made possible because the gene is transcribed from a different promoter in the liver than in the β cell (Magnuson et al. 1989).

Transcription from a promoter is initiated by the binding of the basal transcription machinery. This macinery consists of multiple proteins that form one complex (***Figure 1***). With footprinting experiments it has been made plausible that the complex is assembled by binding of the proteins one by one in a specific order to the promoter site (Zawel et al. 1993). The initial start of the assembly is the binding of TFIID (transcription factor II D) to the promoter site at a TATA-box. This is an A/T rich region, mostly containing the consensus sequence TATAA. This initial binding is followed by the subsequent binding of TFIIA, TFIIB, TFIIF, polymerase II and TFIIE (for a review see Nikolov et al. 1997). Next to the TATA containing promoters, there are TATA-less promoters most of wich possess a cis-acting element called the initiator (Inr) element. This consensus element is recognized by either TFIID or TFIIi, which then nucleates the formation of a pre-initiation complex, which contains RNA polymerase II itself and other known general transcription factors. There is also evidence that RNA polymerase II itself can recognize and bind the Inr (Carcamo et al. 1991). In the absence of a TATA box, TFIID is still required for a stable initiation complex (Smale et al. 1990). A proposed model is that another protein, binding to the Inr, tethers TFIID to the promoter through protein-protein interactions, thus enabling the subsequent assembly of the initiation complex. Roy et al. (1993) and Manzano-Winkler et al. (1996) suggest that TFIIi plays a role in recruiting TFIID to the promoter. In spite of the strong evidence they provide,

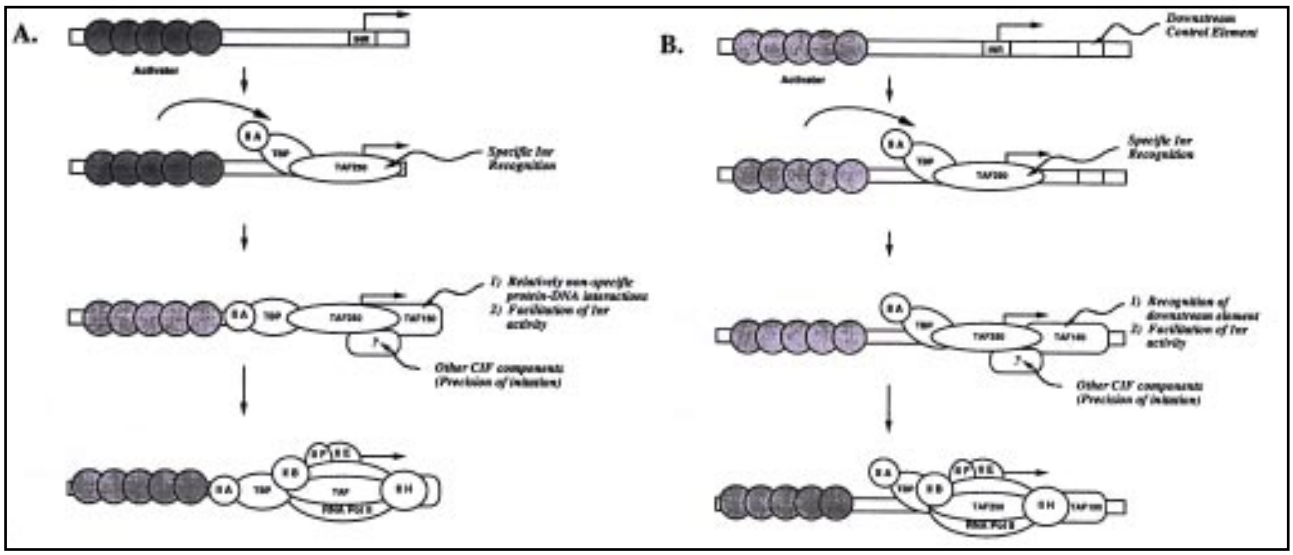


Figure 2;
Working models for Inr-mediated transcription (see text).
A: The Promoter lacks a downstream element and a TBP-DNA interaction is therefore required.
B: The promoter contains a downstream element. A TBP-DNA interaction is not required. (adapted from Smale et al. 1997)

the relevance of TFIIi for the activity of Inr elements remains unclear (Smale et al. 1997).

A model for Inr-mediated transcription is depicted in ***Figure 2***. Smale et al. (1997) have suggested that Inr activity requires factors such as TAF150 and other CIF (Cofactor of Inr Function) components. In panel A, the promoter does not contain a functionally important element downstream of the Inr and therefore stable TFIID binding depends on a sequence-independent (as there is no TATA-box) interaction between the TBP (TATA binding protein) subunit of TFIID and the -30 region of the promoter. In panel B, the promoter does contain a functionally important downstream element. Smale et al. (1997) suggest that in such a situation, TAF150 recognizes this downstream element and positions the transcription complex on the promoter in that way. For further information on the Inr-mediated transcription see the article of Weis et al. (1997) and the review of Smale et al. (1997).

The assembled basal transcriptional complex regulates the basal activity of the promoter. This basal promoter activity can, however, be influenced by factors binding to their respective recognition sites in the more upstream enhancer region. These enhancer binding sites can be situated relatively far from the transcription initiation site. In some cases, the distance to the initiation site or the orientation of the enhancer region has does not even influence the enhancer activity. The question that now arises is how these factors are still able to influence the transcription complex. The most plausible theory at the moment is that the factors binding to the enhancer region are exerting their function by looping out the intervening DNA and thereby making it possible to influence the basal transcription complex by protein protein interactions (Lyubchenko et

al. 1997, Mastrangelo et al. 1991, Perros et al. 1996, Rippe et al. 1995, Su et al. 1990, 1991, Valentin-Hansen et al. 1996).

1.2 Tissue and cell specific regulation

The phenotypic differences that distinguish the various kinds of cells are largely due to differences in the expression of genes. As stated earlier, the expression of genes can be controlled at various points in the path from gene to protein; during transcription, RNA processing, RNA transport, translation or mRNA degradation. For most genes, transcriptional control is paramount. When you take into consideration that transcriptional control does not leave intermediates this emphasis on transcriptional control seems logical. because regulation by RNA cleavage ,for instance, or protein degradation, can leave mRNA fragments or protein degradation products which might interfere with other cell processes.

There are several mechanisms for cell specific regulation. At the transcriptional level cell specific regulation is normally achieved by cell specific transcription factors which activate or enhance the expression of a cell specific gene. An example of such cell specific regulation is the PEPCK gene which is regulated by several tissue-specific enhancers (Tontonoz et al. 1995). But also the structure of the DNA can be important. The folding of the DNA, for instance, can be in such a way that the initiation complex can not bind the DNA (Croston et al. 1993), like in the mammalian MMTV LTR promoter (Richard-Foy et al. 1987, Archer et al. 1991). Genes present in the heterochromatin (very condensed DNA), for example, can not be transcribed. The most striking example of inactivation of genes by DNA condensation is the random inactivation and condensation of one of the two female X-chromosomes. One way to activate inactive chromatin is by (protein induced) supercoiling of the DNA, that will result in partial unwinding and thereby giving transcription factors an opportunity to bind (Freeman et al. 1992). Methylation of the C in DNA CG doublets can also inhibit transcription. For instance, the transcriptional activity of the human *p53* tumour suppressor gene promoter is inactivated upon methylation (Schroeder et al. 1997). Because methylation is inherited by virtue of a methyl-directed methylating enzyme, it can play a role in cell specific regulation, as methylation is not lost in the progeny of the cell (for a review on methylation see Siegfried

et al. 1997). It also seems likely that methylation is involved in genetic imprinting (as will be discussed later in this paper)(Sasaki et al. 1993).

Although controls on the initiation of gene transcription are the predominant form of regulation for most genes, other controls can act later in the pathway from RNA to protein, modulating the amount of gene product that is made on a cell specific basis. Examples of suchs later controls are the premature termination of transcription (Jones et al. 1989), alternative splicing, resulting in different forms of a protein from the same gene (Black et al. 1992), RNA editing, RNA degradation (Sachs 1993) and the regulation of translation. (See Alberts et al. 1994).

An example of a tissue specific and developmental-stage dependent gene is the insulin-like growth factor-II gene (IGF-II), which will be the focus of this study.

1.3 IGF-II

There are two insulin-like growth factors (IGFs); IGF-I and IGF-II. They stimulate the growth of tissue of mesodermal origin (Froesch et al. 1985). IGFs were discovered in a search for factors mediating the effect of growth hormone (Salmon et al. 1957). They are thought to have a role in the liver regeneration and foetal liver growth and in tumour genesis and differentiation (Zvibel et al. 1995). The function of IGF in embryonic growth has been demonstrated by using transgenic mice. In these mice, the IGF-II gene was knocked out by gene targeting. The mice developed normally, but were notably smaller after birth than their wild type litter mates (60% of the normal size). This suggests that IGF-II plays not only an important developmental role in determining the extent of embryonic growth, but also that IGF-II does not appear to have a role in cell differentiation (DeChiara et al. 1990 and 1991). Furthermore, although in human the levels of IGF-II in serum remain high after birth, they are lower than in the IGF-II levels present in the foetal human organism (Daughaday et al. 1989). Therefore, a role for IGF-II in postnatal growth may be feasible.

1.4 The structure of the IGF-II gene

As the name implies, IGFs show a high homology with pro-insulin, both structurally and physiologically (Rechler et al. 1974). IGF-II is a polypeptide of 67 amino acids which can be divided in several domains

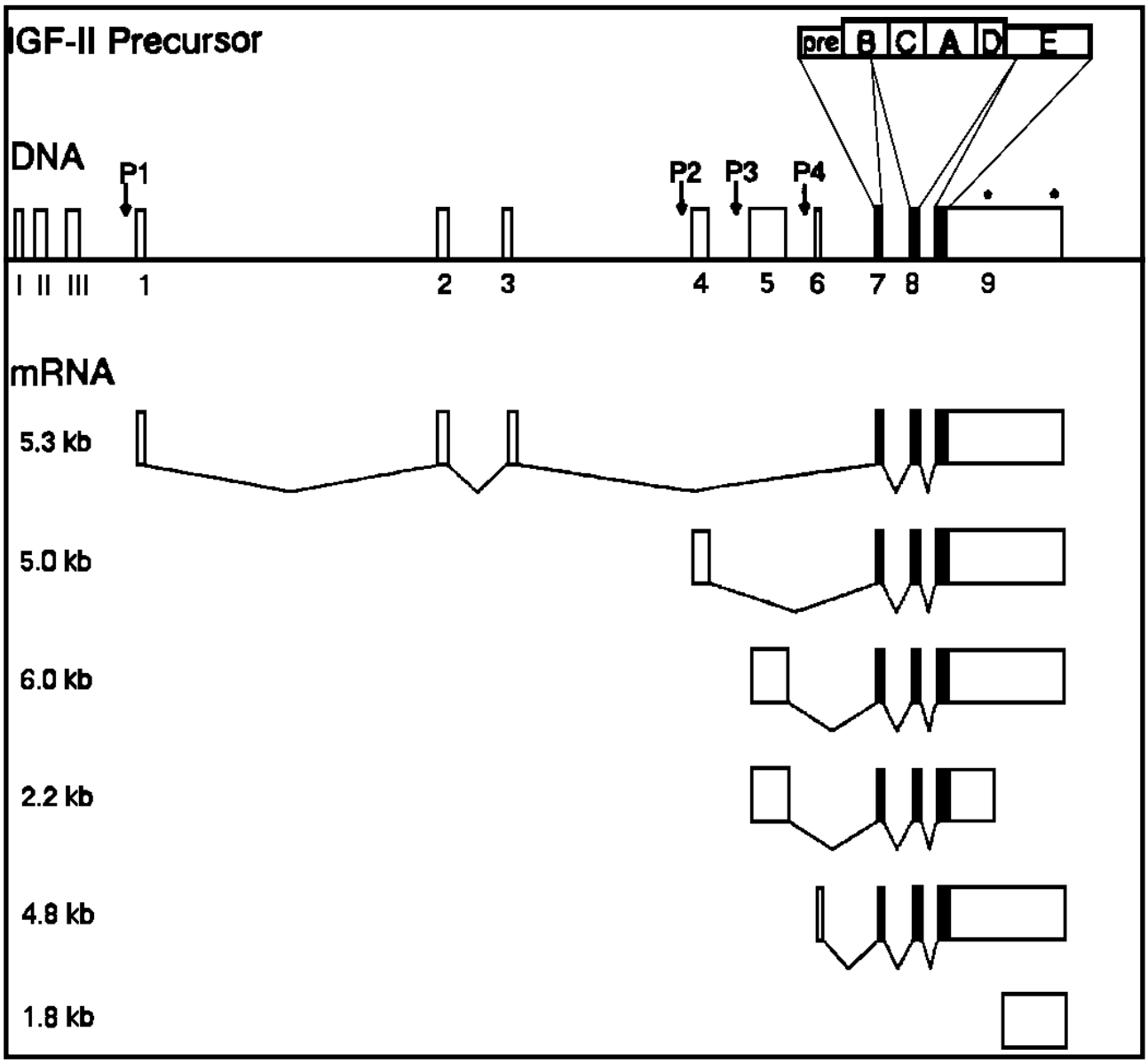


Figure 3;
The structure of the human IGF-II gene, the corresponding mRNAs and the IGF-II precursor protein. The numbered boxes indicate the three exons of the insulin gene (I-III) and the nine exons (1-9) of the IGF-II gene. The location of the four promoters for IGF-II (P1-4) and the polyA addition signals (*) are also indicated. In the upper part, the domain structure of the IGF-II precursor protein is depicted. In the lower part, the IGF-II mRNA species are depicted. The open boxes represent the exon sequences that are contained within the various mRNAs. The solid boxes indicate the regions coding for the IGF-II precursor protein. (adapted from Van Dijk 1992b)

(***Figure 3***). Starting from the amino-terminus, the IGF proteins consist of the peptide domains B, C, A and D. Domains A and B are structurally homologous to the insulin A and B chains. Domain C is analogous to the connecting peptide in pro-insulin which is removed upon processing of pro-insulin to insulin, but remains present in the mature IGF-I and IGF-II. Domain D is not present in insulin. The IGF-II precursor consists of a 24 amino acid signal peptide, a 67 amino acid mature IGF-II gene and a 89 amino acid carboxy terminal called the E domain (Bell et al. 1984, Jansen et al. 1985).

The IGF-II gene is located on the tip of the short arm of chromosome 11 and spans about 30 kb (Pagter-Holthuizen et al. 1985). 1.4 kb upstream of this gene, the insulin gene is located (O'Malley et al. 1988). Both genes are in the same transcriptional orientation. The human

IGF-II gene can give rise to a variety of mRNAs, which all have the same coding region, but differ in their 5' leader sequence (***Figure 3***). Furthermore, the presence of two polyadenylation signals also contributes to this variety (Holthuizen et al. 1990, Pagter-Holthuizen et al. 1987 and 1988). The IGF-II gene contains 9 exons, of which the exons 7, 8 and part of 9 contain the coding sequence for the precursor of IGF-II, while exons 1 to 6 are non-coding. Four promoters are located upstream of exons 1, 4, 5 and 6,named P1, P2, P3 and P4 respectively. Downstream of the coding region in exon 9, a 4 kb 3'-untranslated region is located which contains the two functional polyadenylation signals (Holthuizen et al. 1991, Sussenbach et al. 1992).

1.5 The IGF-II mRNAs

The IGF-II promoters (P1 to P4) show a distinct tissue- and developmentally-specific activity. In foetal tissues, there is a high expression of IGF-II mRNAs from promoters P2, P3 and P4, of which P3 is the most active promoter. After birth, these promoters remain active albeit, at a lower level. The exception to this is the liver. In the foetal liver promoter P3 is active, and at a lower level of activity promoters P2 and P4 (Jansen et al. 1987). In the adult liver, however, promoter P1 is active and promoters P2, P3 and P4 are down-regulated (Pagter-Holthuizen et al. 1985). Promoter P2 activity has only been assessed in some human tumour cell lines (Ikejiri et al. 1991). In hepatocarcinoma (tumour) cell lines, like Hep3B, promoter P1 is shut off in favour of promoter P3.

As tumourgenesis and growth are indissolubly connected, it was not surprising that the involvement of IGF-II in tumourgenesis was both suggested and found. IGF-II has been shown to stimulate the growth of several tumours, among which Wilms' tumour (Scott et al. 1985, Reeve et al. 1985), leiomyosarcomas and leiomyomas (Gloudemans et al. 1990), liposarcoma (Tricoli et al. 1986), rhabdomyosarcoma (Minniti et al. 1994), and colon carcinoma (Tricoli et al. 1986). The exact role of IGF-II, however, is not yet understood. In most tumours, though, high levels of IGF-II are found, mainly derived from the P3 promoter. One can speculate that this suggests a switch from the adult status (promoter P1 expression) to the foetal status (promoter P3 expression).

1.6 The translation of IGF-II mRNAs

Differences in the amount of IGF-II are not necessarily refelcted in an equal disparity in promoter activity. Dissimilarities can occur as a result of distinct turn over rates or variable translatability of the mRNAs. Such dissimilarity seems even more likely when one takes into consideration that the IGF-II gene gives rise to a wide variety of mRNAs with different leader exons and different poly-adenylation signals. The mRNAs from promoters P1, P2 and especially P3 have very long leaders, 586 nt, 408 nt, and 1171 nt respectively, whereas promoter P4 has a short leader of only 109 nt. Promoter P4 is therefore much more efficiently translated than the mRNAs derived from the other promoters, in particular promoter P3. Although the P3 mRNA is poorly translated (Nielsen et al. 1990, de Moor 1994a and 1994b), Nielsen et al. (1995) have shown that the translatability can be selectively enhanced depending on the growth state of the cells. In dense cell populations, P3 mRNA is very poorly translated, but when the concentration of cells is low, i.e. when the cells are growing exponentially, its translation occurs more efficiently. This means that besides the regulation at the transcriptional level, there is also regulation at the translational level, which may be coupled to the growth-speed of cells.

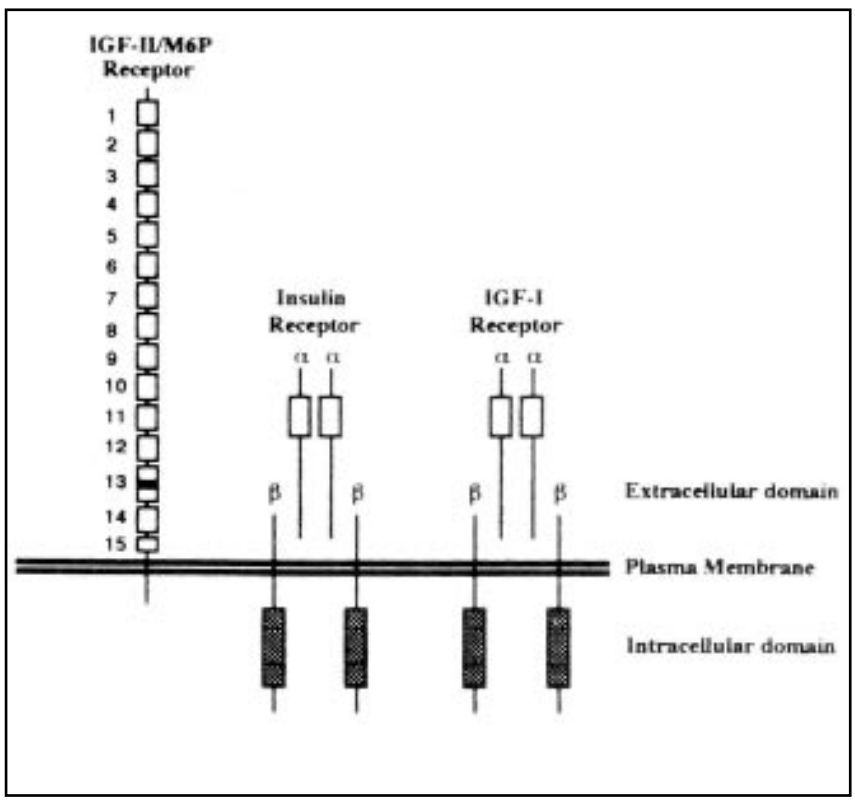


Figure 4;
The receptor structure of the type II IGF receptor, the insulin receptor and the type I IGF receptor.
The repeat sequences in the extracellular domain of the M6P/ IGF-II receptor are indicated by the open boxes. The solid bar in repeat 12, corresponds to a 43 aa insertion. The open boxes in the extracellular domains of the insulin and the IGF-I receptor correspond to cysteine-rich regions. The shaded boxes in the cytoplasmatic portion of these receptors represent tyrosine kinase domains. The à and á subunits of the insulin and the IGF-I receptor are connected by disulphide bonds. (adapted from Nissley et al. 1991)

1.7 IGF-II receptors and binding proteins

There are two types of IGF receptors. Both IGFs can bind to each type IGF receptor, although the affinity will differ (***Figure 4***). The type I receptor can bind both IGF-I and IGF-II, but the affinity for IGF-I is much higher than for IGF-II. The type I receptor can also bind insulin, but only with low affinity. The type II receptor preferably binds IGF-II and does not bind insulin. This receptor is identical to the human cation-dependent mannose-6-phosphate receptor (M6P) (Oshima et al. 1988). IGF-II exerts its effects mainly through the type I receptor (LeRoith et al. 1995). The importance of the type 1 receptor was shown by mice with a null mutation in the type I receptor gene. They showed a severe growth deficiency (45% of normal size) and died at birth due to failure of the respiratory system (Liu et al. 1993).

Unlike most hormones, IGF-II is not stored in glands and released to the blood in high amounts upon stimulation, which would result in a high and rapid rise (and decline thereafter) of the hormone. Most IGFs reside in the blood plasma. Like insulin, IGF has an effect on the blood sugar level. Because the concentration of IGF in the blood plasma is about three times higher than that of insulin, this could result in hypoglycaemia. For that reason, IGF does not circulate freely in the plasma, but is bound by other proteins; the IGF binding proteins (IGFBPs). The binding of IGF to these proteins leads to an increase of the half-life of IGF, but also blocks the insulin-like effect of IGF. So far, six IGFBP have been identified, designated IGFBP-1 to -6. Although the six IGFBPs are clearly distinct, they share regions with strong homology (Drop et al. 1992). Furthermore, IGFBP gene expression is tissue specific and developmentally regulated (Rechler et al. 1992, Schuller et al. 1993, 1994)

1.8 RNA cleavage

Besides the IGF-II RNAs derived from P1 to P4, an additional 1.8 kb RNA is present on a northern blot. This RNA is derived from the 3' untranslated part of exon 9 (De Pagter-Holthuizen et al. 1988). It has been shown, that the 1.8 kb RNA is formed by endonucleolytic cleavage of IGF-II mRNAs and not by transcription from a separate promoter in exon 9 (Meinsma et al. 1991). Scheper et al. (1996) have

shown that this cleavage is responsible for the growth factor induced degradation of IGF-II mRNAs. Besides the stable 1.8 kb RNA, the cleavage also results in an unstable RNA molecule (possibly because it lags the poly(A)-tail). This would suggest that the the cleavage provides a regulatory mechanism to rapidly decrease the levels of normally very stable IGF-II transcripts. The stability of the 1.8 kb RNA, however, could also indicate that the 1.8 kb RNA has its own physiological role in the cell. This is supported by the observation that in mammalian cells, 3' UTRs can have an effect on differentiation and tumourgenesis (Rastinejad et al. 1993a, Rastinejad et al. 1993b)

1.9 Parental imprinting

The IGF-II gene is subject to parental imprinting, which means that there is a differential expression of the paternal (from the father) and maternal (from the mother) alleles of a gene. Only the paternal IGF-II gene is active. The maternal IGF-II gene is silent in most tissues (DeChiara et al. 1991, Vu et al. 1994). Located 100 kb downstream of the IGF-II gene, is the H19 gene, which has an opposite imprint; the maternal allele is active and the paternal allele is silent. When the H19 gene is deleted, the IGF-II gene loses its imprint (Leighton et al. 1995). The theory has been postulated that both genes are regulated by the same two enhancers, 10 kb downstream of H19 (Bartolomei et al. 1993). Normally these enhancers would regulate H19, but when the H19 gene is disrupted, for instance because of a methylation on the paternal chromosome, the enhancers begin to regulate IGF-II. This would explain the opposite imprint. So, H19 is preferentially expressed over IGF-II (because of a strong promoter and/or its location closer to the enhancers), unless expression of H19 is not possible for some reason.

Furthermore, whereas the IGF-II promoters P2, P3 and P4 are subject to maternal imprinting, promoter P1 is not, which is in normal tissue independent of the type of tissue examined. In tumour cells, however, imprinting is sometimes lost. The parental imprint of IGF-II is in agreement with the idea that imprinting is used to regulate foetal development, as promoter P1 is only active in the adult liver tissue. Methylation is thought to play a major role in imprinting. In the mouse, differences in the parental allelic methylation of the mouse promoter P1 have been observed (Sasaki et al. 1992). As the mouse promoter P1 is homologous to the human promoter P2, this is would support the suggestion of Vu et

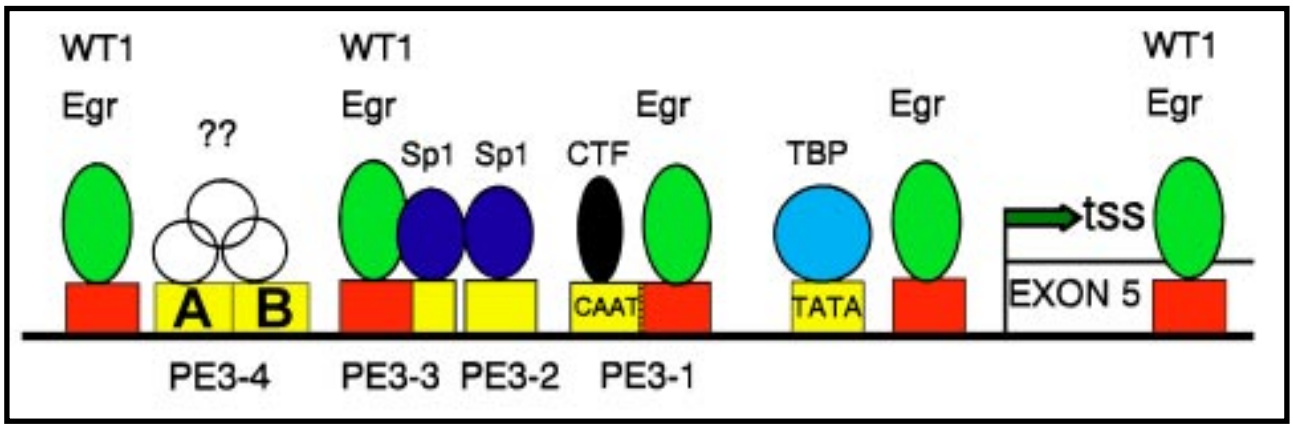


Figure 5;
Cis-elements and transacting factors that participate in activation or repression of the human IGF-II promoter P3.
Only the proximal 250 nt are shown. The transcription start site (tss)(+1) is indicated by an arrow. The positions of the various cis-elements are indicated by boxes. The transacting factors are represented by ellipses. TBP represents the TATA-box binding protein. Footprinting analysis using protein extracts from HeLa cells revealed protection of proximal elements PE3-1 (-64/-90), PE3-2 and PE3-3 (-103/-146), and element PE3-4 (-172/-192), which is protected by a multiprotein complex (Rietveld et al. 1997). (adapted from Steenbergh et al. 1997)

al. (1994) that the signal for imprinting is located between promoter P1 and promoter P2, which would have as a result that promoter P1 is not imprinted.

1.10 Promoter P3

The P3 promoter spans 1300 bp upstream of exon 5 and is very G/C rich (80%). P3 contains several sites in the proximal region (***Figure 5***); such as a TATA-box at -25, a CCAAT-box at -78 and several Sp1 sites.

Van Dijk et al. (1991) have shown, using deletion constructs, that the proximal region (downstream of -180) only yields a low level of promoter activity in HeLa and Hep3B, and that the region from -180 to -1300 is necessary to obtain high levels of promoter activity. Extension of the promoter fragment from -180 to -290 resulted in a strong increase in promoter activity in both HeLa and Hep3B. The region from -289 to -1 seems to be responsible for the basal transcription activity, whereas the upstream region from -1300 to -289 seems responsible for the tissue specific transcription. This thesis is supported by a recent study of Rietveld et al. (1997) which shows that the region between -289 and -183 is essential for basal P3 activity.

The proximal region is subject of intense investigation. Van Dijk et al. (1991) found four additional footprints beside the TATA-box in the proximal region of P3 (-184/+52), designated PE3-1 to PE3-4; PE3-1 being the most proximal. Functional analysis has shown that Nuclear Factor I (NF1) can bind to the PE3-1 region, and Sp1 to the PE3-2 region. The factors binding to PE3-3 and PE3-4 still remain unidentified. It has been shown,however, that truncation of P3 between -289 and -183 results in a strong decrease in P3 activity, which suggests that factors binding to PE3-4 are of major importance for basal P3 activity (Van Dijk et al.

1992a, Rietveld et al. 1997). Rietveld et al. (1997) have shown that the PE3-4 region binds three specific proteins which are involved in basal P3 activity; designated protein 3, 4 and 5. They have also shown that the PE3-4 region can be divided into two non-overlapping boxes: box A (-193/-188), which binds proteins 4 and 5, and box B (-183/-172), which binds protein 3. Box A is important for P3 activity. The importance of box B is not yet known.

Schneid et al. (1993) have examined cell specific protein binding of Hep3B and SW613 nuclear extracts with P3 constructs, using EMSA analysis. Hep3B preferentially expresses IGF-II from promoter P3, whereas SW613 preferentially uses promoter P4. However, no qualitative differences in binding were observed between Hep3B and SW613, although minor quantitative differences in cell type specific binding could be seen in the -1231/-1063 region of promoter P3. Besides the work of Schneid (1993), not much is known about the distal region of P3 and its role in cell specificity. This study will mainly focus on the upstream 'enhancer' region. The goal of this study is to try to identify the specific factors in the distal region of P3 that may be responsible for the cell type specificity of the IGF-II promoter P3 in the cell lines Hep3B and 293.

2 Material & Methods

For the composition of media and buffers, see supplement 1.

2.1 Cell culture

The human hepatoma cell line Hep3B was cultured in α-modified minimal essential medium (αMEM), whereas the cell lines 293 and HeLa were cultured in Dulbecco's modified Eagle's medium (DMEM). All cells were cultured in 162 cm^2 flasks in the appropriate medium, supplemented with 100 µg/ml streptomycin, 100 IU/ml penicillin, 10% foetal calf serum, and 300µg/ml glutamine. To keep the cells at about 60-70% confluency cells were sub-cultured regularly. The medium was refreshed every other day.

2.2 Materials

Enzymes were purchased from Boehringer Mannheim (Mannheim, Germany). Labelled nucleotides were purchased from Amersham (Buckinghamshire UK). Primers were obtained from Pharmacia Biotech. All culturing plastics were from Costar.

2.3 Plasmids

Four constructs containing promoter P3 fragments were available;

- BglII/SalI -1225/+135 in pSla3 (Hup3)
- BssHII/SalI -509/+135 in pSla3 (Hbs)
- AvaI/SalI -136/+135 in pSla3 (Has)
- BglII/EcoRI -1225/-289 in pEMBL6 (*p*BE18)

Hup3 was constructed by insertion of the BglII/SalI fragment of promoter P3 into a BamHI/SalI digested pSla3 vector.

To construct Hbs, Hup3 was digested with BssHII, followed by filling in the BssHII site and subsequent digestion with SalI. This fragment was then placed into the pSla3 vector, which was digested with BamHI, filled in at the BamHI site, and subsequently digested with SalI. The Has construct was made in the same way as Hbs, Instead of digestion of Hup3

with BssHII (blunt), however, digestion for the Has construct was done with AvaI (blunt).

The *p*BE18 construct was made by placing the BglII(blunt)/EcoRI digested P3 fragment into a BamHI(blunt)/EcoRI digested *p*EMBL6 vector.

*p*Tk-Luc was constructed by placing the -105/+51 region of the Herpes Simplex Virus Thymidine kinase promoter into *p*Sla3.

2.4 DNA digestions and purification

Digestions were done with 2 U restriction enzyme per µg DNA at 37 °C for one hour in the appropriate buffer (Boehringer Mannheim).

The digestion products were separated on an 0.7%-1% agarose gel (depending on the fragment or vector size), cut out, and purified using the QIAEX II Agarose Gel Extraction method of QIAGEN (Hilden, Germany).

2.5 Synthesis of blunt ends and ligation

Sticky ends were filled in by adding 2.5 mM dNTP and 2 U Klenow polymerase per 10 µg digested, DNA and leaving it at 37 °C for 30 minutes.

Ligation was done with an equilar amount of DNA fragment and vector in 10 µl containing 10 mM ATP, 1x ligation buffer, and 2 µl ligase (1 U/µl) overnight (o/n) at room temperature (R.T.) for blunt end ligation or at 16 °C for sticky end ligation.

2.6 Dephosphorylation

For dephosphorylation, 10 µg DNA was put at 37 °C for 15 minutes in 1x dephosphorylation buffer and 1 U CIAP (calf intestine alkalic phosphatase)(Boehringer Mannheim), followed by a 15 minute incubation at 55 °C. This procedure was repeated and 1 U CIAP was added.

2.7 Phenol extraction and alcohol precipitation

To the DNA solution, an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added, mixed thoroughly, and centrifuged

for 5 minutes. The lower phase was then discarded. This procedure was repeated with an equal volume of chloroform:iso-amylalcohol (24:1).

For precipitation, 2.5 volumes of ice-cold 96% ethanol and 0.1 volume of 3M NaAc pH 5.2 was added, mixed, and centrifuged for 15 minutes at 4 °C. The pellet was washed with 70% ethanol, dried under vacuum and dissolved in TE buffer.

2.8 Calcium competent E. coli DH5a and transformation

E. coli DH5α calcium competent cells were made according to the procedure described by Inoue et al. (1990). These cells were transformed by slowly defrosting them on ice, adding the ligate and leaving it on ice for an additional 30 minutes. Subsequently the cells were shocked at 42 °C for 1 minute, and put back on ice for 5 minutes. Next, the transformed cells were cultured in 800µl SOB medium for 1 hour at 37 °C and plated out on LB +amp (25 µg/ml) plates (*p*Bluescript transformed cells on XIA plates) and grown overnight at 37 °C.

2.9 Miniprep

Colonies were ented in 3 ml LB medium +amp (25µg/ml) and grown overnight at 37 °C. Cells were collected by centrifugation and resuspended in approximately 100µl LB medium. To lyse the cells, 300 µl TENS was added while continuously mixing. Next, 150µl 3M NaAc pH 5.2 was added, mixed, and centrifuged for 5 minutes. After this the supernatant was collected. The plasmids were obtained by alcohol precipitation as described above (see paragraph 2.7).

2.10 Maxiprep

Maxiprep was performed according to the procedure set out by Sambrook et al. (1989). Shortly, colonies were ented in 0.5 or 1 l LB medium and grown o/n. The cells were collected by centrifugation (20', 3000 rpm, 4 °C) and resuspended in 10 ml solution I (0.05M glucose, 0.025 M Tris pH 8.0, 0.01 M EDTA). Next, the cells were lysed by adding 20 ml solution II (0.2M NaOH, 1% SDS) (10', room temperature). Chromosomal DNA, high molecular weight RNA and protein/membrane complexes were precipitated by adding 15 ml solution III

(3 M KAc, 11,5% glacial acetic acid)(10' on ice). This precipitate was pelleted by centrifugation (15', 4000 rpm, 4 °C) after which the supernatant (containing the plasmid DNA) was filtrated through a tissue. The DNA was pelleted by adding 30 ml iso-propanol and subsequent centrifugation (15', 5000 rpm, R.T.). Next, the pellet was dried and washed. The plasmid DNA was purified twice by ultra centrifugation (16 hr, 70000 rpm, 20 °C) in a CsCl-ethidiumbromide gradient, according to the procedure described by Sambrook et al. (1989). After centrifugation, the DNA was recovered by alcohol precipitation and washed. The concentration and purity of plasmid DNA was measured by means of photo spectrometry at 260/280 nm.

2.11 Sequencing

The sequence of the obtained new constructs was examined by sequencing. Sequencing was done with the Boehringer Mannheim sequence kit, according to the manufactures instructions. Briefly; 1 µl (25 ng) primer was annealed to 3 µg DNA (in a 10 µl volume) by adding 2 µl annealing buffer and 1 µl 1M NaOH (bringing the total volume to 14 µl) and subsequent incubation for 3 minutes at 100 °C. Next, the DNA chains were extended by adding 3 µl labelling mix, 1 µl α32P dATP (5 µCi/µl), and 2 µl T7 polymerase (1 U/µl) and subsequent incubation for 5 minutes at R.T.. Next, 4.5 µl of this mixture was added to 2.5 µl A-, C-, G-, or T-stopmix, and put at 37 °C for 5 minutes. Next, 4 µl stop/loading buffer was added, and the samples were heated to 80 °C for 2-3 minutes. 3 µl samples were subjected to electrophoresis on 6% (w/v) polyacrylamide urea gel. After drying the gel, the bands were visualized by autoradiography. For sequencing, the following primers were used:

-BssH-Rev (-985/-1004 of P3):
5'-CGCCTAAGCCTAGCCAAGGG-3'
-BstE-Rev (-729/-749 of P3):
5'-TCCTACCTGCATGGCCGAGCT-3'
-Luc-Start (+11/+36 relative to SalI of pSla3):
5'-CCTTTTCTTTATGTTTTTGGCGTCTTC-3'

The 3' border of both the Hhs and Hbs insert was examined with Luc-Start, which primes 3' of the SalI site in pSla3. The 5' border of the Hhs construct was examined with BssH-Rev, which primes 3' of the -1087

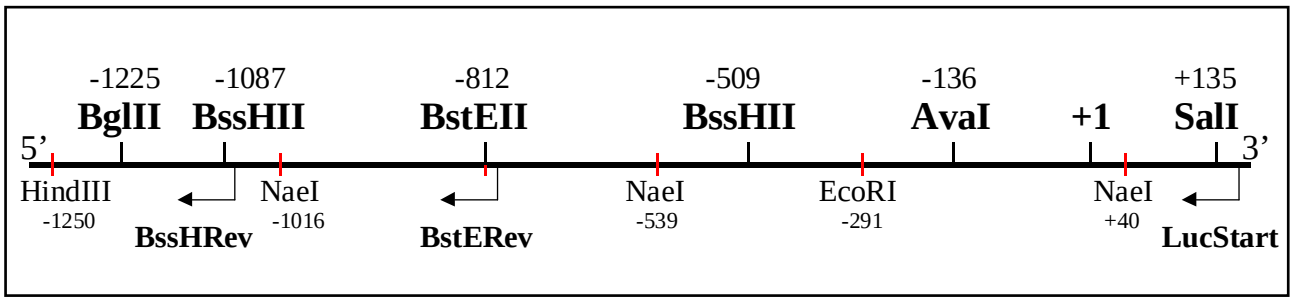


Figure 6;
The promoter P3 with the restriction sites used for the construction of the constructs (black) and the restriction sites used for the construction of the probes used for EMSA analysis (red). The position of the primers for sequencing are also depicted

BssHII site. The 5' border of the Hes construct was examined with BstE-Rev, which primes 3' of the -812 BstEII site. (***Figure 6***)

2.12 Transfections

Cells were transfected in 6 cm dishes at 60-70% confluency ($12 \cdot 10^5$ cells/dish). The 293 and Hep3B cells were transfected using the BES buffered calcium phosphate coprecipitation method (Sambrook et al. 1989), the HeLa cells were transfected using the DEAE method (Sambrook et al. 1989). All cells were transfected with 2 µg/dish luciferase reporter plasmid and with 500 ng/dish RSV-LacZ (to correct for differences in transfection efficiency), which was put on the cells for a period of 4 hours (in the case of HeLa cells, 2 hours). After 4 hours, the Hep3B cells were shocked for 1 minute with 10% DMSO. The 293 cells were not shocked. The HeLa cells were shocked after 2 hours fro 5 minutes with 22.5% DMSO (see chapter 3.11). After 48 hours, with a refreshment of the medium after 24 hours, the cells were harvested in lysis buffer. Subsequently, luciferase and β-galactosidase assays were performed as described by Sambrook et al. (1989) and Van Dijk et al. (1991). Luciferase levels were measured on a Lumac/3M Biocounter M2010A (Landgraaf, The Netherlands).

2.13 Labelling of probes

The probes used for EMSA analysis were end-labelled. This end-labelling was done by adding 2 µl Klenow DNA polymerase (1 U/µl) and 50 µCi α32P dATP or dGTP to the DNA digest. This was incubated for 30 minutes at 37 °C. Probes were purified on a 5% (w/v) acrylamide gel, cut out, and eluted from the gel o/n at 37 °C in 700 µl elution buffer. The supernatant was precipitated by adding 1 volume of iso-propanol and subsequent centrifugation for 15 minutes. Next, the

precipitate was washed with 70% ethanol and, after drying, dissolved in 100 µl TE.

2.14 Nuclear and whole cell extracts

Nuclear extracts (NE) of Hep3B and 293 cells were made according to the method described by Li et al. (1991). Shortly, cells were harvested by centrifugation and washed twice with phosphate buffered saline (PBS) and once with 500 µl ice-cold buffer A. Next, the cells were swelled in 500 µl buffer A for 10 minutes and lysed by gently passing the cell suspension though a 28-gauge needle. The nuclei were collected by centrifugation, resuspended in buffer C, and incubated at 4 °C for 30 minutes while mixing gently. Next, an equal amount of buffer D was added to the cell suspension and the cell debris was pelleted by centrifugation. The supernatant was used as nuclear extract. The protein concentration was determined using the Bradford protein quantitation assay (BioRad, Brussels).

Whole cell extracts (WCE) of Hep3B and HeLa cells were made as following; Cells were washed with PBS, harvested and collected by centrifugation. The cell pellet was frozen at -80 °C, resuspended in 100 µl 420 mM KCl, 20 mM Hepes·KOH pH 7.6, 1.5 mM $MgCl_2$, 10% glycerol, and 1 mM DTT, incubated on ice for 20 minutes and centrifuged for 10 minutes. The supernatant was used as whole cell extract. The protein concentration was determined using the Bradford protein quantitation assay (BioRad, Brussels).

2.15 Electrophoretic mobility shift assay (EMSA)

EMSA binding reactions were performed as described by Pruijn et al. (1987). Shortly, binding reactions were performed by mixing 10.000 cpm of DNA probe with binding buffer after which 2 µg protein extract was added. The total reaction volume was 15 µl, which was incubated on ice for 60 minutes. Electrophoretic analysis of bound and unbound probe was done on a 6% (w/v) polyacrylamide gel using 0.5x TBE. After drying, the gel the bands were visualized by autoradiography using intensifier screens.

2.16 Footprinting and GA ladder

A solution containing a probe-saturating amount of extract (which was determined with EMSA), 1x binding buffer, 1 µg dIdC and 10.000 count/µl probe was made and brought to a 75 mM NaCl concentration. The total volume was adjusted to 15 µl. To this solution, 35 µl of DNAse dilution was added (containing 1x binding buffer, 75 mM NaCl and DNAse I), bringing the total volume to 50 µl. This mixture was put at 25 °C for 90 seconds, after which 3 µl stopbuffer was added. Next the mixture was mixed and put on ice. After this the mixture was phenol extracted two times and alcohol precipitated. The pellet was dried by vacuum centrifugation for 5 minutes and dissolved in 4 µl sequencing buffer. The sample was put at 96 °C for 3 minutes and put on a 8% (w/v) polyacrylamide urea gel. After drying the gel, the bands were visualized by autoradiography.

A GA ladder was made by mixing 100.000 cpm of probe with 7 µg CT-DNA in a total volume of 20 µl. Next, 2 µl PIP-formiate pH 2.0 was added after which the mixture was incubated at 37 °C for 10 minutes. Subsequently, 100 µl PIP was added and the solution was incubated at 95 °C for 5 minutes, after which 100 µl TENS, 5 µl CT-DNA and 700 µl 96% ethanol was added. This was precipitated together with the footprint samples. Further handling was the same as the footprint samples.

3 Results

3.1 HeLa/Hep3B transfections

To investigate whether there are cell specific factors that bind to the upstream region of P3, the parts of the upstream region that are important for P3 activity had to be identified first. This was done by using several truncated P3 constructs. Promoter fragments were placed into the *p*Sla3 vector in front of the luciferase gene, to allow easy determination of the promoter activity in transient transfection experiments. Three promoter constructs were used; the full P3 promoter (Hup3, BglII/SalI -1225/+135), BssHII/SalI (Hbs, -509/+135), and AvaI/SalI (Has, -136/+135). These constructs were transfected into HeLa and Hep3B cells, of which HeLa cells do not express IGF-II endogenously, and Hep3B cells do express IGF-II mRNA, predominantly derived from promoter P3.

3.11 Optimization of the DEAE transfection method

Since HeLa cells are difficult to transfect, the appropriate transfection method, the DEAE transfection described by Sambrook et al.(1989), had to be optimized. In order to do this, HeLa cells were transfected under various conditions; with or without a 22.5% DMSO shock, with different batches of DEAE dextran and with full or serum-free medium. The results are depicted in ***Figure 7***. When full serum was used without a DMSO shock, the luciferase activities were low. When a DMSO shock was applied however, the luciferase activity increased more then six fold with both DEAE batches. In addition, the use of serum free medium led to a further significant increase in luciferase activity (batch D1, two-fold, batch D2, four-fold increase). The differences between the two batches of DEAE are not large, although two transfections with D2 are very high. In conclusion; the D2 batch combined with a 22.5% DMSO shock, and with the use of serum free medium gave the best result. There was, however, not enough D2 available. As a result we needed to use the D1 batch, which also gave acceptable results with serum free medium and a 22.5% DMSO shock, for the transfection of HeLa cells. The DNA was left on the cells for 2 hours in these experiments, as described in paragraph 2.12.

Methode	Luciferase	Average
D1-M+S	1414	1337
	1788	
	1027	
	1117	
D2-M+S	853	3057
	1006	
	5134	
	5235	
D1+M+S	472	660
	848	
D2+M+S	567	797
	1026	
D1+M-S	62	84
	106	
D2+M-S	102	183
	264	

Figure 7;
The optimization of the DEAE transfection method.
The first column depicts the method used, the second the result of the individual experiments, whereas the third depicts the average result. D1=DEAE batch provided by Holthuizen; D2=DEAE batch provided by the Academic Hospital Utrecht; -M/+M=transfection in serum free medium / in full serum respectively; +S/-S= with/without a 5 minute 22.5% DMSO shock.

	HeLa (SD)	Hep3B (SD)
-1225/+135	35 (±7)	132 (±0 *)
-509/+135	54 (±9)	106 (±25)
-136/+135	3 (±0)	9 (±0)
TkLuc	100 (±9)	100 (±2)

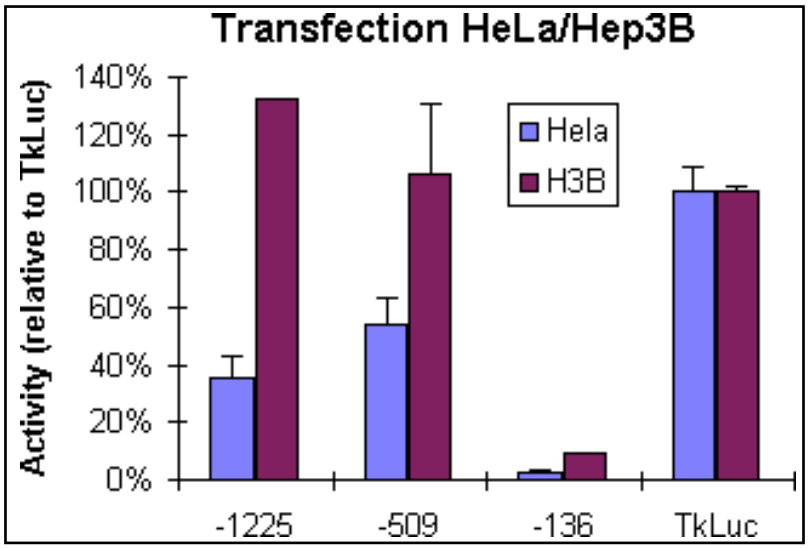


***Figure 8**;*
The results of transient transfections of HeLa and Hep3B cells with 3 promoter constructs and TkLuc. The values are the luciferase activity in %. The values are indicated relative to TkLuc. (only one value, so SD=0)*

3.12 Transfection of HeLa and Hep3B

Transfection of HeLa and Hep3B cells with the three promoter constructs led to the results depicted in ***Figure 8***. The activity of the -1225 P3 construct was, relative to TkLuc, almost four times as high in Hep3B compared to HeLa. Upon truncation of the promoter from -1225 to -509, the activity of the construct increased with 54% in HeLa, whereas in Hep3B the activity decreased with 20%. When the promoter was truncated to -136 the promoter activity was completely abolished. It should be noted, however, that these results are based on just one experiment. With a new batch of HeLa cells, the DEAE transfections failed to work. As it would take a lot of time to optimize the transfection method for a new batch of cells, it was decided to switch to a cell line that was easier to transfect that, similar to HeLa, does not express P3 endogenously; the 293 cell line (see paragraph 3.4).

This single experiment does, however, show that the region between -1225 and -509 seems to be an interesting area to search for cell-type specific factors by EMSA analysis and footprinting.

3.2 EMSA analysis and footprinting with HeLa and Hep3B whole cell extract

To analyse protein binding to the upstream region, the PstI/BssHII probe was constructed, labelled at the BssHII site (see ***Figure 15***). The PstI site is located in the *p*BE18-polylinker upstream of BglII (-1225). EMSA analysis with this probe showed four protein DNA complexes of which one was only present with HeLa WCE (see arrow,

Figure 10;
Footprint analysis with HeLa and Hep3B (H3B) whole cell extracts using the PstI/BssHII probe. The first and last lane are a GA-ladders. The first set of six lanes is with HeLA WCE (60 μg), the second set with Hep3B WCE (50 μg) and the third set of three lanes is with free probe (no extract added). The amount of DNAse-I used is indicated above each lane in U. The bars on the left indicate the position relative to the P3 transcription start site, based on the GA-ladder.

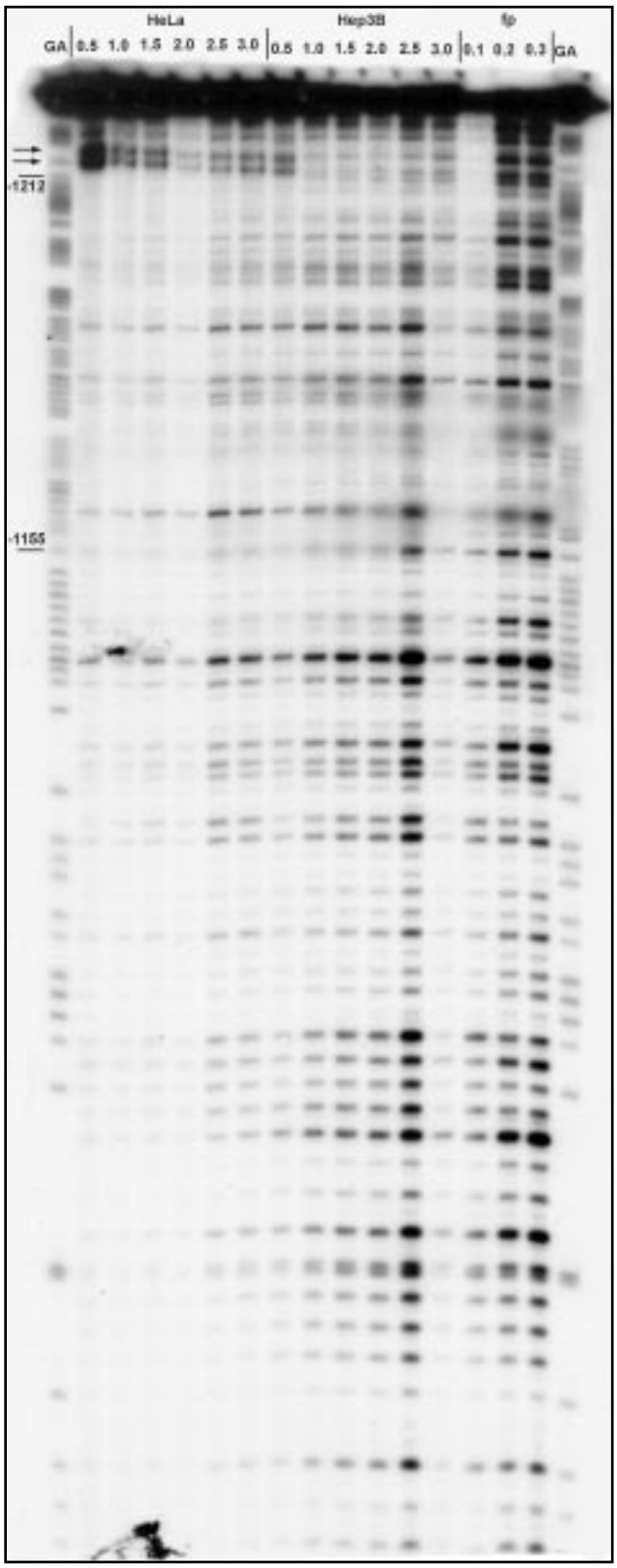


Figure 9;
EMSA analysis with HeLa and Hep3B (H3B) whole cell extracts using the PstI/BssHII probe. The first six lanes are with HeLa WCE, the next six lanes with Hep3B WCE in increasing amounts. The last lane is with free probe (no extract added). The amount of WCE used is indicated above each lane.

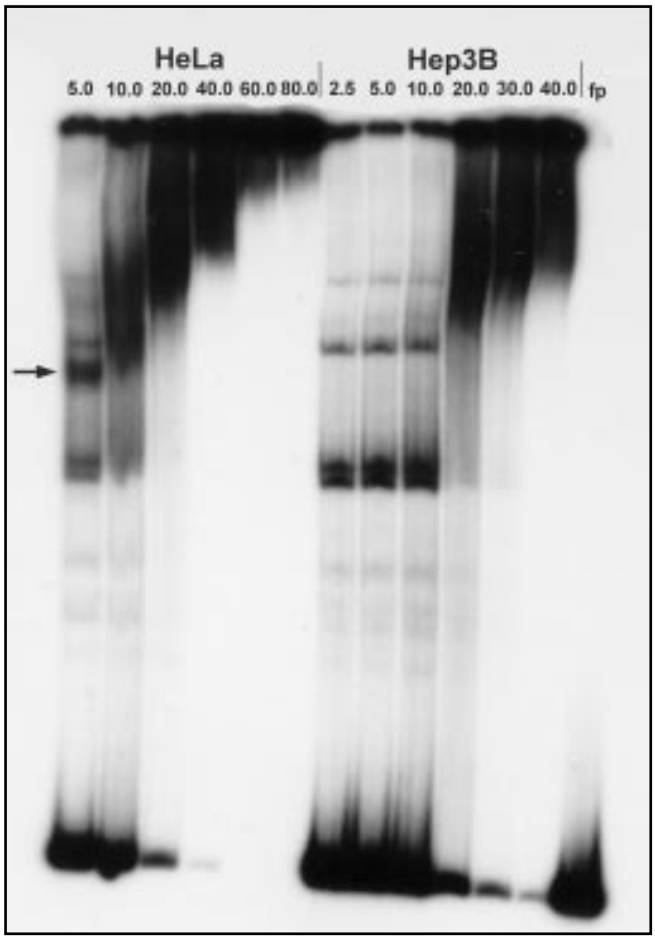


Figure 9). This complex was not visible with Hep3B WCE. To determine the probe saturation point needed for footprinting, the shift was done with an increasing amount of WCE. Subsequently, with a probe saturating amount of WCE (60μg HeLa WCE, 50μg Hep3B WCE), DNAse-I footprinting was performed. No clear footprints could be seen.

Figure 12;
Footprint analysis with HeLa and Hep3B (H3B) whole cell extracts using the HindIII/NaeI probe. The first and last lane are a GA-ladders. The first set of six lanes is with HeLA WCE 60 µg), the second set with Hep3B WCE (50 µg) and the third set with free probe (no extract added). The amount of DNAse-I used is indicated above each lane in U. The bars on the right indicate the position relative to the P3 transcription start site, based on the GA-ladder.

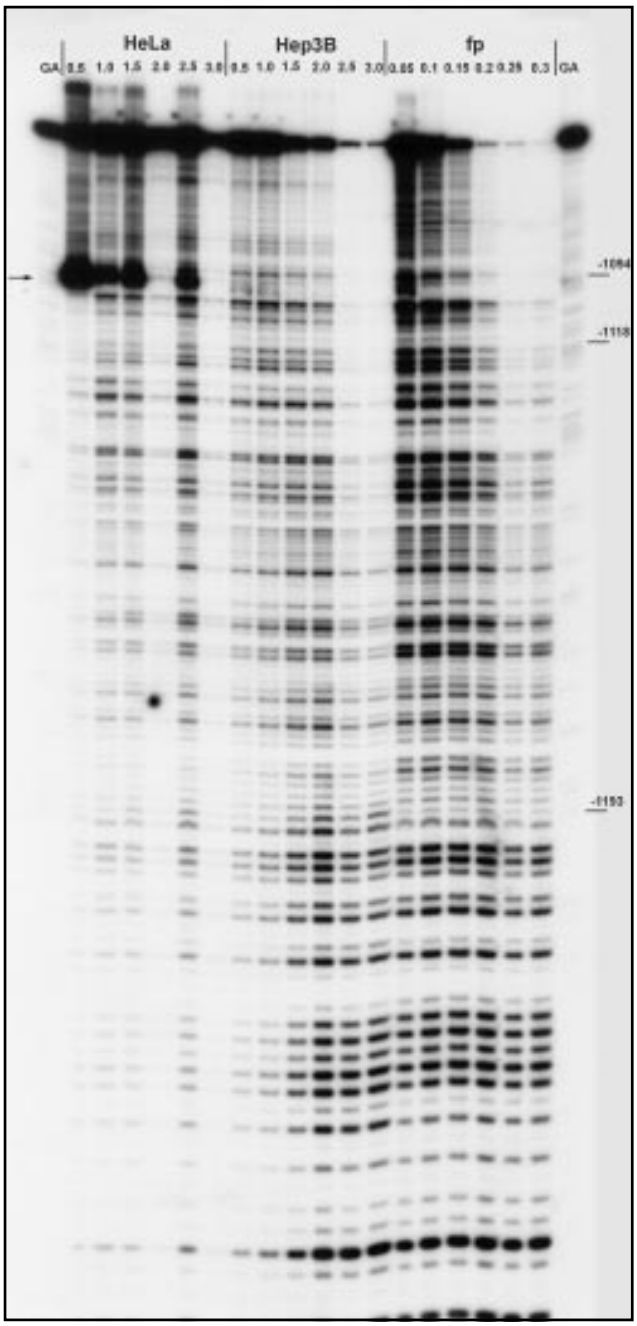
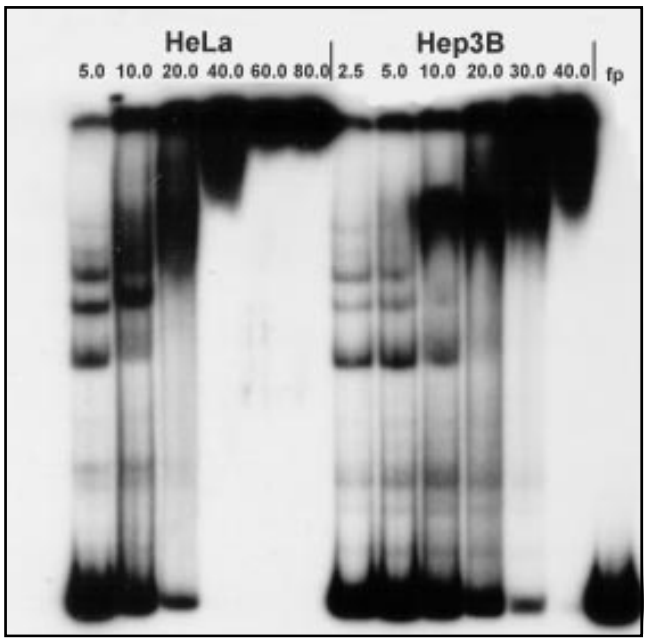


Figure 11;
EMSA analysis with HeLa and Hep3B (H3B) whole cell extracts using the HindIII/NaeI probe. The first six lanes are with HeLa WCE, the next six lanes with Hep3B WCE in increasing amounts. The last lane is with free probe (no extract added). The amount of WCE used is indicated above each lane



There was, however, more then one hypersensitive site with HeLa WCE that seemed to be absent with Hep3B WCE (see arrows, ***Figure 10***). Although the exact location was difficult to determine, they seemed to be located at about -1215. Because this hypersensitive site was located very close to the end of the probe, a second probe, HindIII/NaeI (***Figure 15***) was created, labelled at the 5' end (at HindIII which is located in the *p*BE18-polylinker).

Again four protein DNA complexes could be seen, this time without any differences between HeLa and Hep3B (***Figure 11***). Footprinting with this probe showed a hypersensitive site around -1094 in HeLa, but not in Hep3B (see arrow, ***Figure 12***). The hypersensitive sites wich

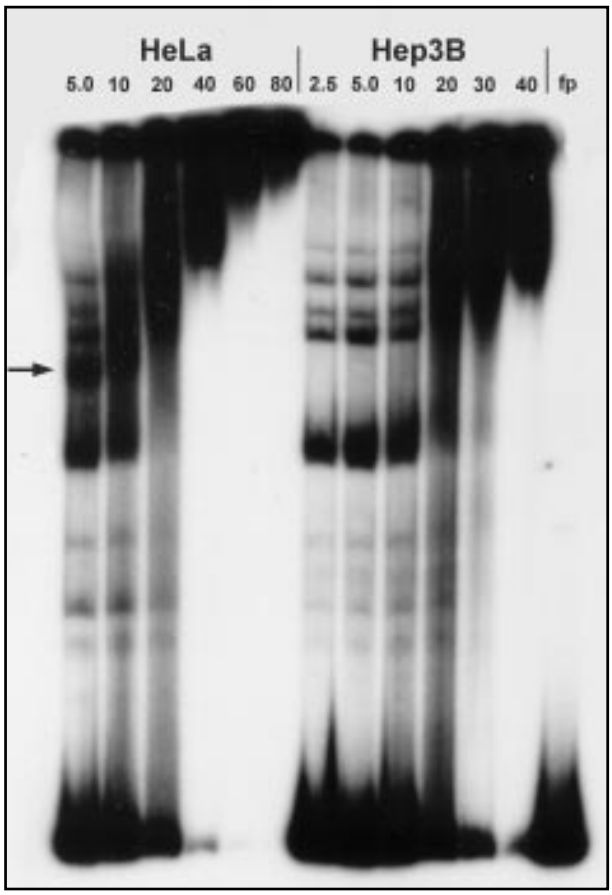


Figure 13;
EMSA analysis with HeLa and Hep3B whole cell extracts using the HindIII/BssHII probe. The first six lanes are with HeLa WCE, the next six lanes with Hep3B WCE in increasing amounts. The last lane is with free probe (no extract added). The amount of WCE used is indicated above each lane.

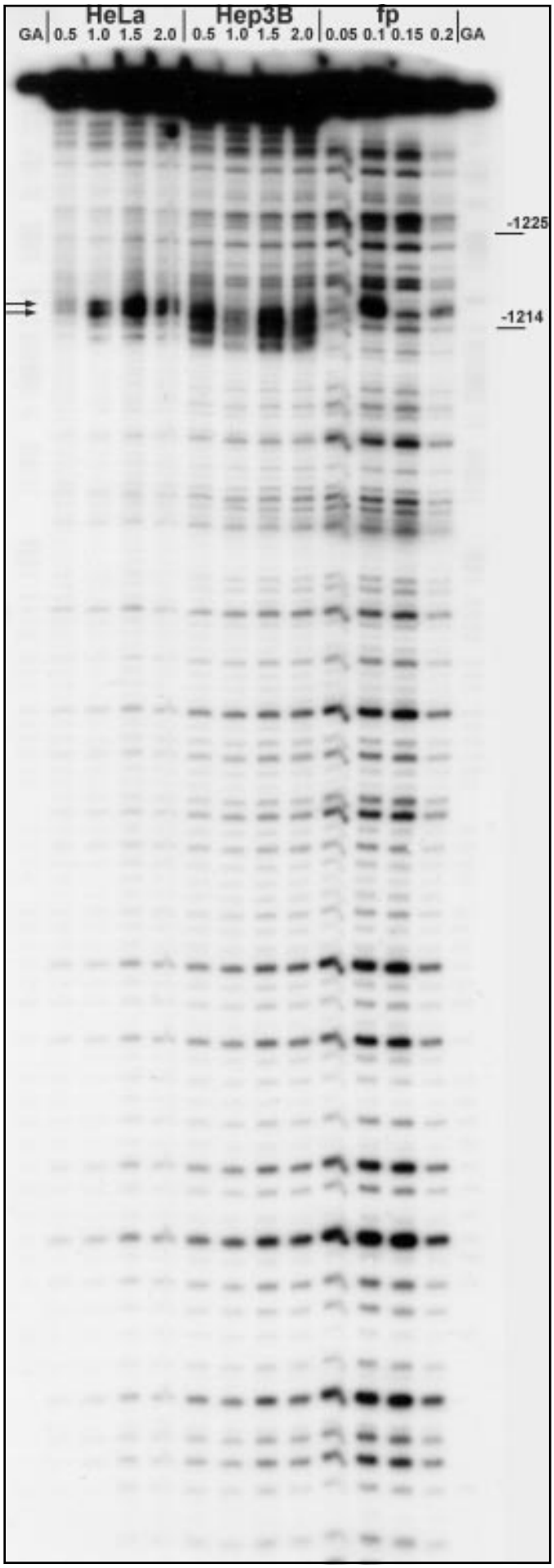


Figure 14;
Footprint analysis with HeLa and Hep3B (H3B) whole cell extracts using the HindIII/BssHII probe. The first and last lane are a GA-ladders. The first set of four lanes is with HeLA WCE (60 μg), the second set with Hep3B WCE (50 μg) and the third set with free probe (no extract added). The amount of DNAse-I used is indicated above each lane in U. The bars on the right indicate the position relative to the P3 transcription start site, based on the GA-ladder.

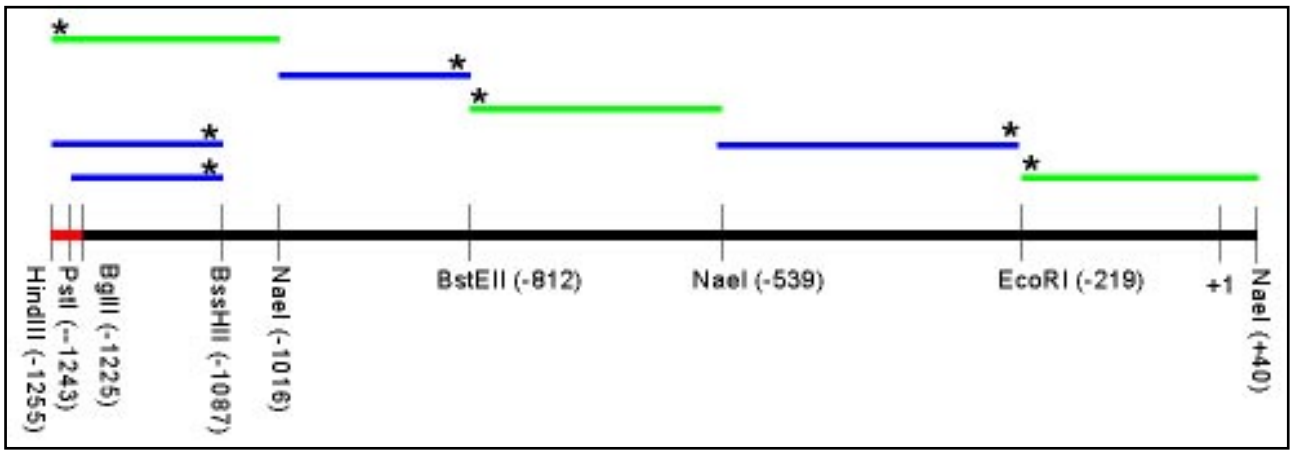


Figure 15;
*Map of the probes used for EMSA analysis and footprinting. The * marks the site of labelling. The P3 promoter is depicted in black, and the pBE18 region in red.*

were detected with the PstI/BssHII probe, were to close to the beginning of this probe to be seen on the footprint. Because of this, a third probe, that extended further upstream then the first probe, HindIII/BssHII, was constructed, labelled at the BssHII site (HindIII is located in the *p*BE18-polylinker upstream of -1225)(***Figure 15***).

The shift showed eight protein DNA complexes, of which one is present with HeLa WCE but not with Hep3B WCE (see arrow, ***Figure 13***). The footprint with this probe showed the hypersensitive site also seen with the PstI/BssHII probe (see arrows), at the same position (***Figure 14***).

3.3 Truncated promoter constructs

As the upstream region seems to be important, we examined this region more closely. In order to do this, constructs between the Hup3 (-1225/+135) and the Hbs (-509/+135) construct had to be made, that would divide this relatively large region into smaller parts. The following two intermediate constructs were made: BssHII/SalI (Hhs, -1087/+135) and BstEII (Hes, -812/+135).

At first, it was attempted to directly clone the fragments into *p*Sla3 (***Figure 16***). This was attempted for the -1087/+135 fragment by partially digesting the Hup3 plasmid with BssHII (because Hup3 contains two BssHII sites), isolating the correct fragment on gel, filling in the BssHII site and subsequent digestion with SalI. The resulting fragment (BssHII(blunt)/SalI) was ligated into *p*Sla3, which was digested with BamHI, filled in at the BamHI site and subsequently cut with SalI (*p*Sla3 BamHI(blunt)/SalI). For the -812/+135 fragment, the Hup3 plasmid was digested with BstEII, filled in at the BstEII site and subsequently digested with SalI. Subsequently, this BstEII(blunt)/SalI fragment was

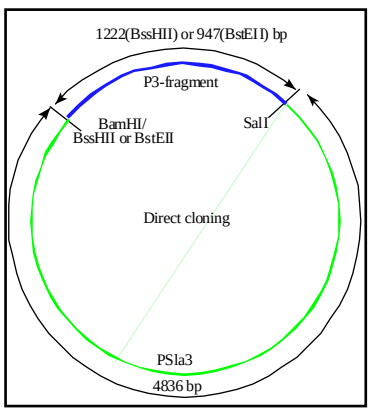


Figure 16;
A map of the construct that would be the result of direct cloning into pSla3. The blue line is the P3 fragment, the green line is pSla3.

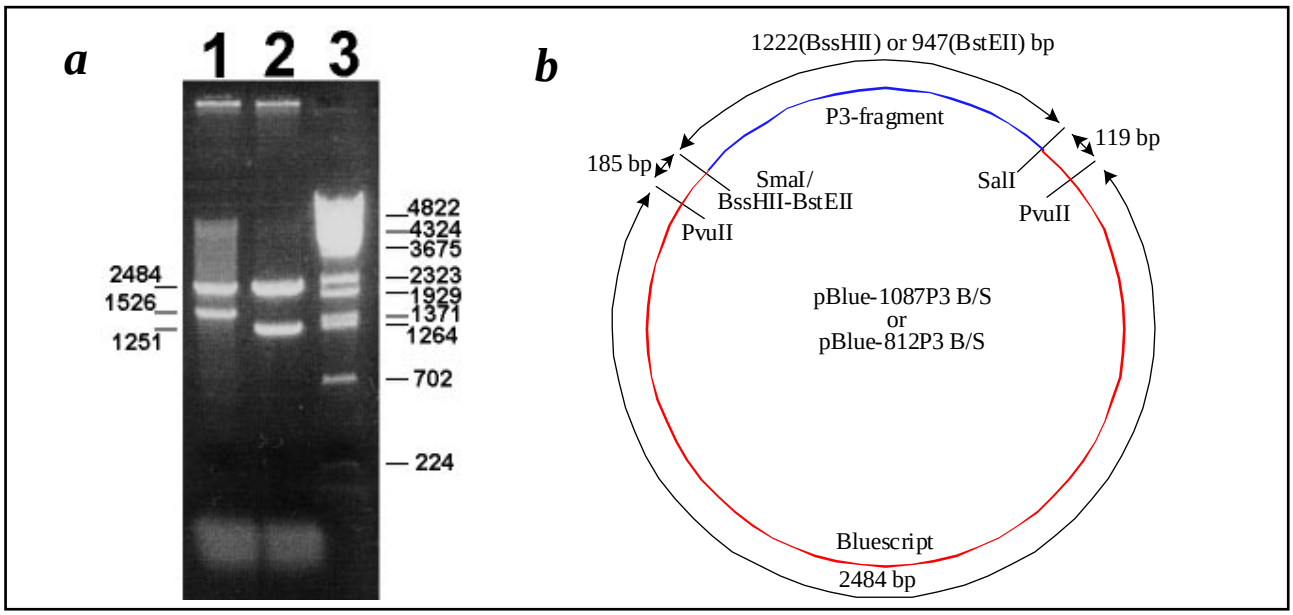


Figure 17;
a) PvuII digestion of the pBluescript vectors containing the BssHII/SalI (lane 1) or BstEII/SalI (lane 2) P3 fragment as described before. Lane 3 contained a lambda-BstE-II marker.
b) Map of the construct. Blue line is P3 fragment, red line is pBluescript

ligated into *p*Sla3 BamHI(blunt)/SalI. This, however, did not result in the desired constructs, possible because of problems with filling in the BamHI site of the *p*Sla3 vector. Filling in of the BstEII or BssHII site of the fragment did not cause the problems as filling in these sites gave no problems in the subcloning experiments discussed below. To abolish the need to fill in the BamHI site of the vector, subcloning via *p*Bluescript was attempted.

Again, the -1087/+135 fragment was obtained by partially digesting the Hup3 plasmid containing the -1225/+135 construct with BssHII, and isolating of the correct fragment on gel. The BssHII site was filled in using dNTP and Klenow polymerase. Next, this fragment was digested with SalI, and the resulting BssHII/SalI fragment isolated on gel. The promoter constructs were subcloned into a dephosphorylated SmaI/SalI digested *p*Bluescript (ks) vector. The resulting construct (*p*Blue-1087P3 B/S) was transformed into calcium competent DH5α *E. coli* cells as described in paragraph 2.8. The transformed bacteria were plated on XIA plates after which positive (white) colonies were selected and miniprepped. The miniprep DNA was cut with PvuII to verify the correctness of the constructs (***Figure 17***). The promoter fragment was cut out of the *p*Bluescript vector with BamHI/SalI and ligated into a dephosphorylated BamHI/SalI digested *p*Sla3 plasmid.

The -812/+135 fragment was obtained by digesting the Hup3 plasmid containing the -1225/+135 construct with BstEII. The BstEII site was filled in and the fragment subsequently digested with SalI. The -812/+135 fragment was handled in the same way as the -1087/+135 construct, resulting in a P3-fragment-*p*Bluescript vector (*p*Blue-812P3 B/S) and the desired P3-fragment-*p*Sla3 vector.

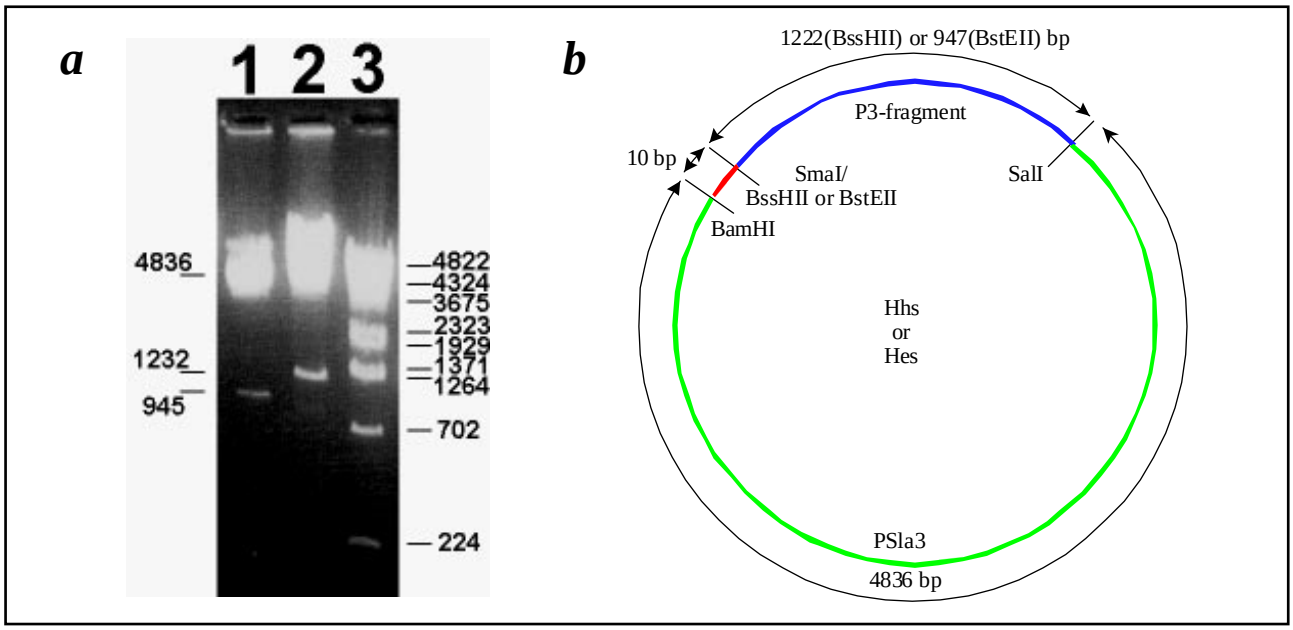


Figure 18;
a) BamHI/SalI digestion of the BstEII/SalI (lane 1) and BssHII/ SalI (lane 2). Lane 3 contains a lambda-BstE-II marker.
b) Map of the construct. The blue line is the P3-fragment, the red line is pBluescript, the green line is pSla3.

The two *p*Sla-P3 constructs were transformed into DH5α *E. coli*. Positive clones were miniprepped, after which the miniprep DNA was cut with BamHI/SalI to verify the correctness of the construct (***Figure 18***).

Finally, the constructs were sequenced. The 5' end of the -1087/+135 construct was sequenced with the BssHRev primer, and the 3' end with the LucStart primer. The 5' end of the -812/+135 construct was sequenced with the BstERev primer and the 3' end with the LucStart primer. The constructs proved to be correct. Both constructs had a 8 bp BamHI/SmaI *p*Bluescript addition at the 5' border. (The borders between fragments and vectors are indicated with a |)

- 5'-border:

	*p*Sla3	*p*Bluescript	P3 fragment
Hhs	5'-ATCATGTCTG	GATCCCCC	CGCGCGGAGGGGCGAA-3'
Hes	5'-ATCATGTCTG	GATCCCCC	GTAACCATTATCCAGA-3'

- 3'-border:

	P3 fragment	*p*Sla3
Hhs	5'-AAGGCGGCCCCGC	GTCGACCA-3'
Hes	5'-AAGGCGGCCCCGC	GTCGACCA-3'

3.4 Transfection of 293 and Hep3B

In order to analyse the promoter activity of the truncated promoter constructs, the constructs were transfected into the 293 and Hep3B cell lines. The activity of the promoter could easily be determined by measuring the luciferase levels. LacZ was used to correct for differences in

	293 (SD)	Hep3B (SD)
-1225/+ 135	1645 (± 44)	383 (± 17)
-1087/+ 135	1912 (± 123)	744 (± 24)
-812/+ 135	804 (± 15)	459 (± 52)
-509/+ 135	535 (± 24)	404 (± 31)
-136/+ 135	1 (± 0)	1 (± 0)
TkLuc	100 (± 3)	100 (± 9)

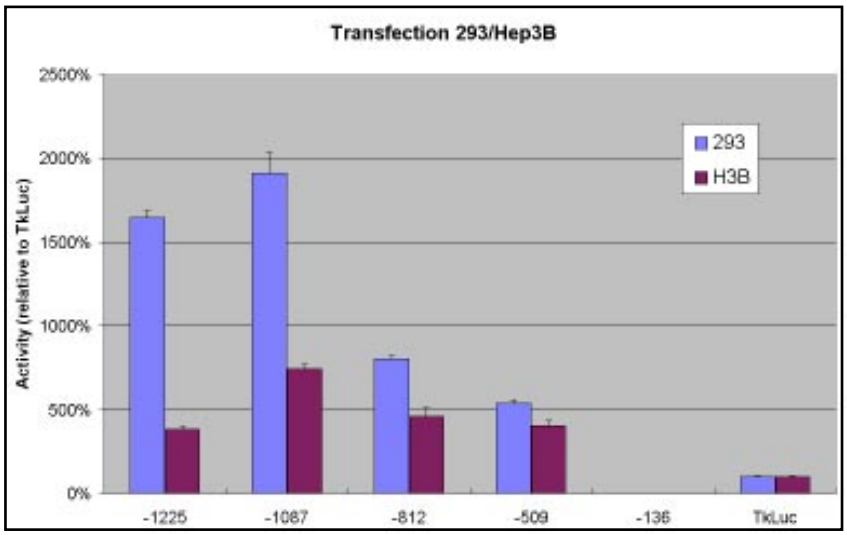


Figure 19;
The results of the transient transfections of 293 and Hep3B cell lines with 5 promoter constructs and TkLuc. The results are the avarage of 7 individual experiments. The values are relative to TkLuc (in %).

transfection efficiency. The activity of the TkLuc plasmid, which contains the basal Tk-promoter region, was used as a reference to compare the two cell lines.

The results of the transfections into 293 and Hep3B are shown in ***Figure 19***. The pattern of increase and decrease of activity was the same in both cell lines. The activity of the -1225 P3 construct, however, showed a 4.3 fold higher activity in 293 then in Hep3B cells. When the promoter was truncated to the -1087 position, the activity of the promoter increased in both cell lines. In Hep3B, however, the promoter activity increased with 94% whereas the activity in 293 showed a much smaller increase in promoter activity (16%). When the promoter was truncated even further to -812, the promoter activity in both 293 and Hep3B decreased approximately two-fold. A further truncation of the promoter to -509, resulted in a small decrease in promoter activity (33% and 12% for HeLa and Hep3B, respectively).

When the promoter construct was truncated to -136, the promoter activity decreased almost to zero in both cell lines. It should be noted, however, that the activity of the -509 P3 construct was roughly equal to, or slightly higher than the -1225 P3 construct in Hep3B, whereas the activity in 293 was about one third of the activity of the full construct.

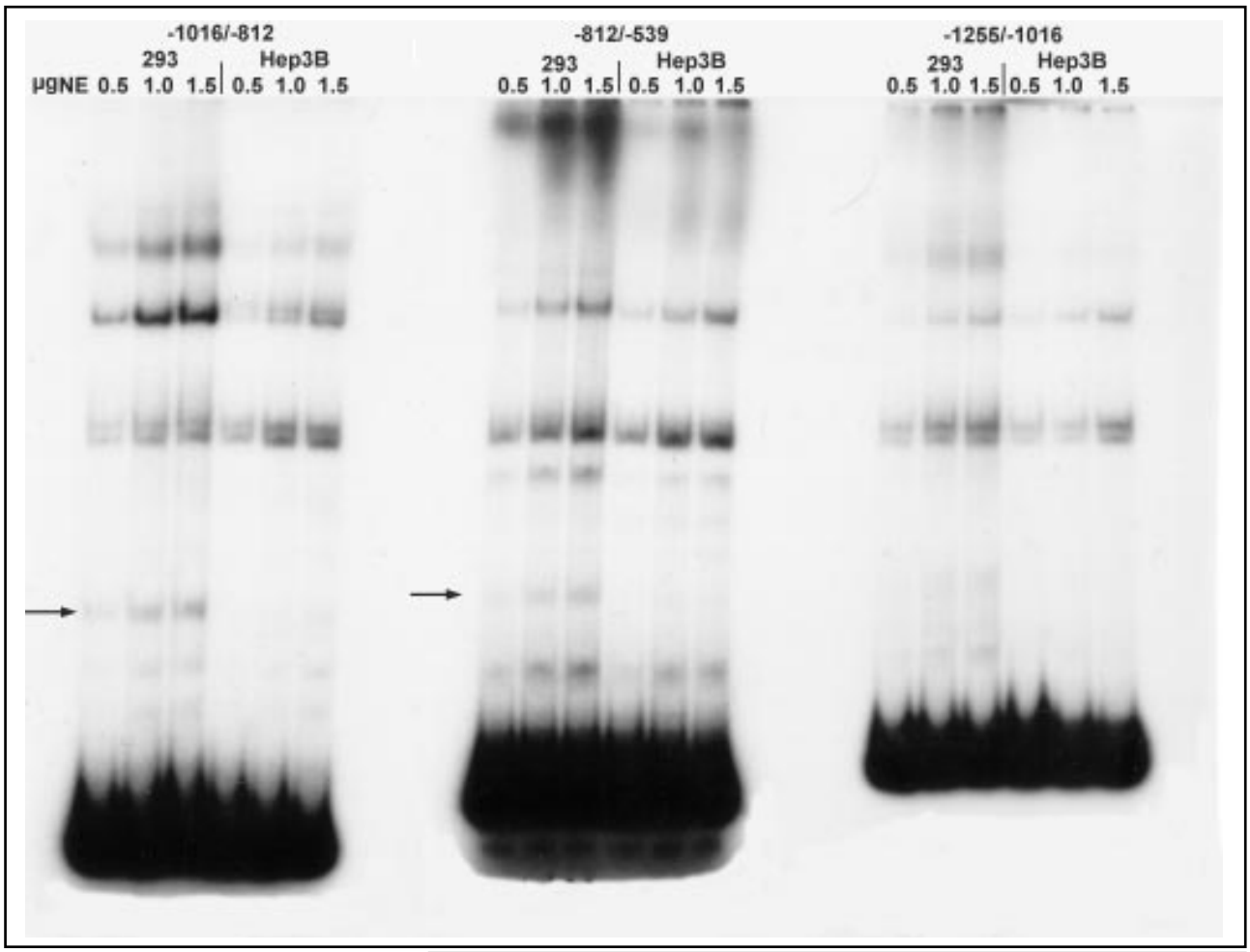


Figure 20;
EMSA analysis with 293 and Hep3B (H3B) nuclear extracts, using three probes spanning the upstream region of the P3 promoter. The first lane is with 0.5 µg NE, the second with 1.0 µg NE and the third with 1.5 µg NE. The first set of three lanes are with 293 NE, the next set of three lanes with Hep3B NE. The used probe is indicated above each set of six lanes.

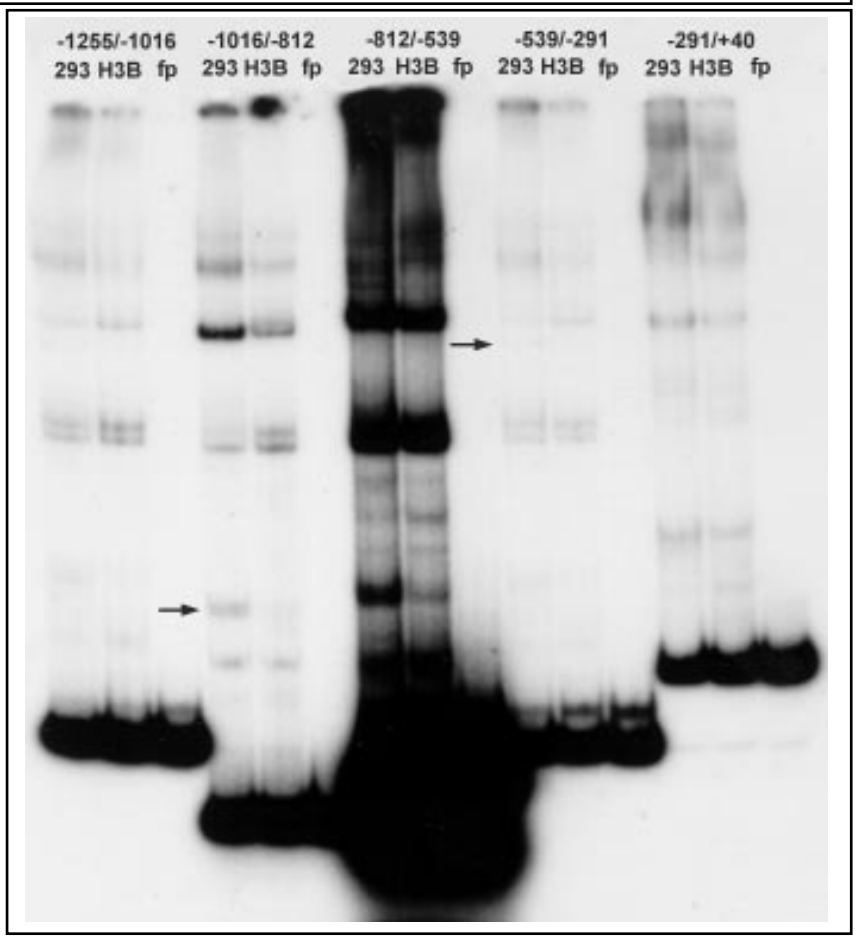


Figure 21;
EMSA analysis with 293 and Hep3B (H3B) nuclear extracts using 5 different probes spanning the whole P3 promoter. The first lane is with 293 NE, the second with Hep3B NE and the third with free probe (no extract added). The used probe is indicated above each set of three lanes. 2 µg NE was used.

3.5 EMSA analysis with 293 and Hep3B nuclear extracts

To analyse protein binding to the promoter in 293 and Hep3B cells, an EMSA analysis with nuclear extracts of both cell lines was performed (***Figures 20, 21 and 22***). In order to do this, probes of about 250 bp were isolated, which together span the entire P3 promoter region.

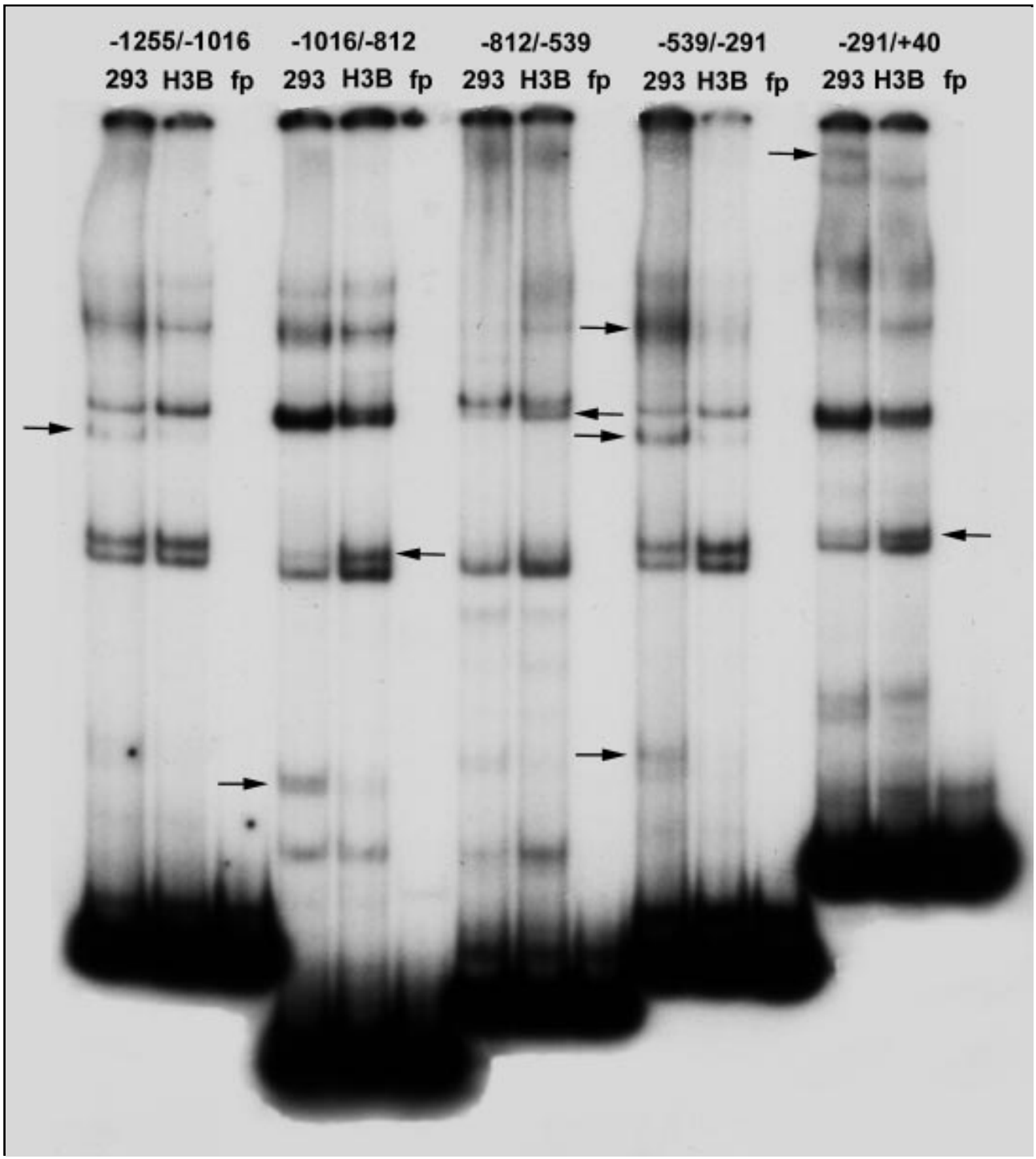


Figure 22;
EMSA analysis with 293 and Hep3B (H3B) nuclear extracts, using five different probes spanning the whole P3 promoter. The first lane is with 293 NE, the second with Hep3B NE and the third with free probe (no extract added). The used probe is indicated above each set of three lanes. 2 µg NE was used.

The following 5 probes were isolated after digestion of *p*Sla3 promoter constructs:

1 HindIII/NaeI -1225/-1016 (+ 30 bp of *p*BE18 polylinker)
2 NaeI/BstEII -1016/-812
3 BstEII/NaeI -812/-539
4 NaeI/EcoRI -539/-291
5 EcoRI/NaeI -291/+40

Probe 1 was end labelled at the HindIII site, probes 2 and 3 were end-labelled at the BstEII site, whereas probes 4 and 5 were end-labelled at the EcoRI site (***Figure 15***).

Figure 20 shows the first EMSA, performed using the first three probes. With the -1225/-1016 probe, four protein DNA complexes were seen, with no differences between 293 and Hep3B. With the -1016/-812 probe, seven complexes could be distinguished, although some were very faint. One complex seemed to show cell type specificity (see arrow). With the -812/-539 probe, five complexes were found of which one was absent with Hep3B NE (see arrow).

The EMSA in ***Figure 21*** is identical to the EMSA in ***Figure 22***, except for the differences in activity of the probes used. This was corrected in the EMSA shown in ***Figure 22***. No differences in binding can be seen between these two EMSAs.

Six protein DNA complexes were detected with the -1255/-1016 probe. One complex was more abundant in 293 cells than in Hep3B cells (see arrow). Using the -1016/-812 probe, seven protein DNA complexes could be distinguished. One was more abundant in 293 compared to Hep3B whereas another was more abundant in Hep3B compared to 293 (see arrows). With the -812/-539 probe, six DNA protein complexes were seen showing a small quantitative difference (see arrow). The -539/-291 probe displayed seven complexes, of which three were more abundant in 293 compared to Hep3B (see arrows). When using the -291/+40 probe, five protein DNA complexes could be seen with one complex that was more abundant in 293 compared to Hep3B.

In summary, one can conclude that six DNA protein complexes are more abundant with 293 NE and three DNA protein complexes are more abundant with Hep3B NE. It is important to note, however, that these differences are quantitative and not qualitative.

4 Discussion

IGF-II has a high level of complexity, having four promoters, two polyA signals, RNA cleavage and being tissue- and developmental-specific expressed. The mechanism of specific regulation of promoter P3 is one of the many things that is still unknown. It is known, however, that the promoter can be divided into two regions; a downstream region (-289/+1), which supports basal promoter activity, and an upstream region (-1300/-289), which is responsible for cell type specificity and provides enhanced promoter activity (Van Dijk et al. 1991). The specific factors binding to the proximal region of promoter P3 and their cognate binding sites have also been identified (Van Dijk et al. 1991, Rietveld et al. 1997). At the moment, however, little is known about the upstream region of promoter P3. In this study, we tried to characterize the regions which are involved in tissue specific activation in this upstream region of promoter P3. Three cell lines were used as a model system: two that do not express P3 endogenously (HeLa and 293), and one that does (Hep3B). To clarify the importance of the different parts of the upstream region in cell type-specific regulation, various constructs containing P3 fragments were used in transient transfection assays with HeLa, 293 and Hep3B cells. To investigate protein binding to different regions of P3, EMSA analysis and footprinting were performed with probes spanning the whole promoter P3 region.

4.1 293 versus Hep3B

Transient transfections with the full P3 construct (-1225/+135) resulted in high levels of P3 activity in both 293 and Hep3B cells. In order to compare the activities of P3 in the two cell lines, the activity of the TkLuc construct was set to 100%. Relative to TkLuc, the activity of the -1225/+135 construct was more than four times higher in 293 cells than in Hep3B cells. Truncation of the promoter from -1225 to -1087 resulted in a slight increase of activity (16%) in 293 cells. In Hep3B cells, it resulted in an approximately two-fold increase (94%). Because deletion of this region resulted in a higher P3 activity, this suggests that an important protein (or proteins) binds to this region which has a repressing effect on P3 activ-

ity, especially in Hep3B. In EMSA analysis, the -1225/-1087 region was encompassed by the -1225/-1016 probe. One quantitative difference in protein binding could be seen between the two cell lines (see arrow, ***Figure 22***). This protein DNA complex seemed to have a low abundancy in both cell lines. Upon truncation of the promoter from -1087 to -812, both Hep3B and 293 constructs showed an approximately two fold decrease in activity (38% and 58% respectively). This suggests that elements are located within this region that can activate transcription of IGF-II in both 293 cells and Hep3B cells. The -1016/-812 probe, which spans the truncated region, showed quantitative differences in protein binding in EMSA analysis. One protein DNA complex was more abundant in 293 cells than in Hep3B cells, whereas another protein DNA complex was more abundant in Hep3B cells than in 293 cells (see arrows, ***Figure 22***).

The overall effect of these two truncations (i.e. truncation of the promoter region from -1225 to -812) was not the same in 293 and Hep3B cells. In 293 cells, it resulted in a two-fold decrease in P3 activity, whereas in Hep3B cells the effect of this truncation was a slight increase in P3 activity (20%). The two probes, -1225/-1016 and -1016/-812, span exactly this truncated region. Although truncation to -812 had only a small effect on P3 activity in Hep3B, as the stimulating and repressing effects compensated for each other, the factors binding to this region in Hep3B can be important for cell-type specific P3 activity. In 293 cells, however, truncation of P3 to -812 led to a two-fold decrease in P3 activity. This means that overal the effect of the proteins binding to this region on P3 promoter activity, is activating. It is possible that one or both of the protein DNA complexes that are more abundant in 293 cells in this region (indicated by the arrows in ***Figure 22***) are responsible for the activating effect. One would expect, however, that factors important for cell-type specific activity would have an inhibiting effect in 293, as 293 does not express P3 endogenously.

Further truncation of P3 from -812 to -509 had a small effect on P3 activity in 293 cells (34% decrease) and only a slight effect on P3 activity in Hep3B cells (12% decrease). This suggests that the proteins binding in this region are not significantly important for P3 activity in these two cell lines. The probe that spans this region (-812/-539) did not show any significant differences in protein bind-

ing between 293 and Hep3B cells, except for a, low abundant, additional complex with Hep3B NE, which suggests that no cell-type specific factors bind to this region. Truncation of the promoter from -509 to -136 resulted in a severe decrease in promoter activity in both cell lines. This suggests that important factors for P3 activity bind in this region.

Rietveld et al. (1997) have shown for HeLa (which, like 293, has no endogenous P3 activity) and Hep3B cells, that the region from -289 to the transcription start site is essential for basal promoter activity. As the PE3-4 region, described in paragraph 1.10, is situated between -193/-172, the -136 P3 construct does not contain this region. Since both boxes A and B of this element have been shown to be important for basal promoter activity (Rietveld et al. unpublished), the deletion of these elements could account for the severe decrease in P3 activity upon truncation of the P3 promoter to -136. The probe spanning the major part of the truncation (-539/-291) showed three quantitative differences in protein binding in EMSA analysis (arrows, ***Figure 22***). When using the -291/+40 probe, two small differences in binding could be seen; one being more abundant with 293 NE and one being more abundant with Hep3B NE (see arrows, ***Figure 22***). The differences are very small, which is in agreement with the fact that this region supports basal promoter activity in Hep3B and HeLa cells (Rietveld et al. 1997).

In summary, one can distinguish two regions. The first distinguishable region is -1225/-509, which contains cell-type specific elements. In EMSA analysis with 293 NE, multiple DNA-protein complexes could be seen in this region that were more abundant with 293 NE and some that were more abundant with Hep3B NE. In transient transfection assays, in 293 cells only a stimulating effect could be seen, whereas in Hep3B cells both a stimulating (from -812 to -1087) and an inhibiting (from -1087 to -1225) effect could be seen. This suggests that between -1087 and -812 a non cell-type specific activator (or activators) is binding, and that between -1225 and -1087 a Hep3B specific inhibitor (or inhibitors) binds. It could also be possible that a non cell-type specific inhibitor binds to the -1225/-1087 region together with a 293 specific factor which would abolish the effect of the inhibitor. The second distinguishable region is -509/-136, and is important for promoter activity in both cell lines.

4.2 HeLa versus Hep3B

Because only one transient transfection with HeLa and Hep3B cells was succesful, the results could not be reproduced, which makes the following results only indicative. Transfection with the full P3 construct resulted, relative to TkLuc (set to 100%), in a high P3 activity in Hep3B cells and relatively low P3 activity in HeLa cells. This is the opposite of what was seen in the transfections with 293 and Hep3B cells. In those transfections, promoter activity in 293 cells was more than four times higher than in Hep3B cells. It should be noted that the activity of all constructs is very low in HeLa cells. This is possibly because HeLa cells proved to be very difficult to transfect in our experiments. Upon truncation of the construct to -509, the activity in HeLa cells increased with 54%, whereas the activity in Hep3B cells decreased slightly by 20%. However, in the 293 versus Hep3B experiments, the activity in 293 cells decreased three fold upon truncation from -1225 to -509. The activity with Hep3B did, like in the HeLa/Hep3B experiment, change only slightly. Upon further truncation of the promoter to -136, promoter activity was almost completely abolished, which supports the findings of the 293/Hep3B experiment.

All three probes used in the shifts and footprints with HeLa and Hep3B WCE, were located in the -1225/-1016 region (more or less the region of the first truncation; -1225 to -1087). The PstI/BssHII probe showed an additional protein DNA complex with HeLa WCE in EMSA analysis and a hypersensitive site in footprint analysis around position -1215. Because this hypersensitive site was very close to the non-labelled end of the probe, a nwe probe was constructed. This probe (HindIII/NaeI) was labelled at the opposite side, which placed the hypersensitive site more to the labelled end of the probe. With this probe (HindIII/NaeI), however, no difference in protein binding could be seen in EMSA analysis, even though the probe spaned the region of the first probe. The EMSA with the same probe, but with 293 NE, did show a complex that was more abundant compared to Hep3B. This could be explained by the difference in extract used, although theoretically a WCE should contain all nuclear proteins as well. Except for this difference, both EMSAs with NE and WCE using the -1225/-1016 probe, showed the same pattern of complexes. In footprinting analysis, the hypersensitive site around -1215 could not be seen. However, another hypersensi-

tive site around position -1094 was found with the HindIII/NaeI probe. The HindIII/BssHII probe was created in a second attempt to place the hypersensitive site at -1215 further from the end of the probe. In EMSA analysis, this probe showed an additional protein-DNA complex with HeLa WCE. In footprinting the hypersensitive site around -1215, also found with the PstI/BssHII probe, could be seen. As both probes are almost identical, this was to be expected. Since the three probes span only a small part of the -1225/-509 region, it is not possible to correlate the shift or the footprint with the transfections for HeLa versus Hep3B.

In summary; the Hep3B WCE and Hep3B NE give, as would be expected, comparable results in EMSA analysis. HeLa WCE and 293 NE, however, do not give comparable results, although neither of them express P3 endogenously. The transient transfections with Hep3B in the HeLa/Hep3B and the 293/Hep3B experiments give comparable results. The P3 activity does not change significantly upon truncation from -1225 to -509, and decreases to zero upon further truncation in both experiments. Compared to TkLuc, the activity of the constructs showed a significant difference between the HeLa/Hep3B and the 293/Hep3B experiments, which is rather remarkable. The 293 and HeLa transfection are not very comparable. The P3 activity increased significantly in HeLa cells (54% increase) upon truncation to -509, whereas in 293 cells the P3 activity decreased considerable (76% decrease). Van Dijk et al. (1991) have also performed transfections with truncated P3 promoter constructs in HeLa and Hep3B. They found that upon truncation from -1300 to -565 the promoter P3 activity in HeLa decreased with 57%. This is comparable to our findings for 293 cells, but not with our findings for HeLa cells. One possible explanation is that HeLa cells proved to be very hard to transfect on our assay, resulting in not reproducible results. For Hep3B, Van Dijk et al. (1991) found a 87% decrease upon truncation of the promoter from -1300 to -565. In both our HeLa/Hep3B experiment and in our 293/Hep3B experiments, the promoter activity remained more or less the same. Additional transfections need to be done to explain this difference.

4.3 Concluding remarks

To gain more insight in the factors binding promoter P3, and in their possible role in cell specific regulation, footprints with the five probes used in EMSA analysis with NE need to be done. One should pay attention to the fact that the used probes do not overlap each other and that it is possible that factors binding to these regions can not bind to either probe. Therefore additional probes should be constructed that overlap the probes used in this study. The EMSA should also be repeated with a different batch of constructs and NE.

Although we focused on the binding of cell-type specific factors to promoter P3, cell-type specificity is not necessarily a result of cell specific enhancers or repressors binding to P3. They could also be a result of regulation at a different level. For instance, it could be possible, that cell type specificity is the result of cell type specific DNA methylation, tertiary DNA structure, or factors binding to regions located further upstream or downstream of promoter P3.

Preliminary results, however, do not show a difference in the status of methylation of promoter P3 between Hep3B en 293 cells. Nonetheless, other possibilities of cell-type specific regulation deserve further investigation. Especially since our results do not present clear evidence for cell-type specific regulation by proteins binding to promoter P3. It will be clear that a lot of research still needs to be done to understand the rationale behind the cell type specific regulation of P3, let alone of the whole IGF-II gene.

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Supplement

1 Buffers and media

Buffer A:	10 mM Hepes pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT
Buffer C:	20 mM Hepes pH 7.9, 25% glycerol v/v, 420 mM KCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, o.5 mM PMSF
Buffer D:	20 mM Hepes pH 7.9, 20% glycerol, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT
Bindingbuffer:	20 mM Hepes KOH pH 7.6, 1 mM MgCl2, 75 mM KCl, 1 µg poly (dI-dC), 1 mM DTT, 0.018% NP-40
Elutionbuffer:	0.5 M NH4Ac, 10 mM MgAc, 1 mM EDTA pH 8.0, 0.1% SDS
LB medium:	10 g/l bacto-tryptone, 5 g/l bacto-yeast extract, 10 g/l NaCl, pH 7.0
Ligation buffer (10X):	0.5 M Tris pH 7.6, 100 mM MgCl2, 100 mM DTT
Lysisbuffer:	1M DTT, 1% Triton X100, 15% glycerol, 25 mM Tris pH 7.8
TBE (10X):	108 g/l Tris, 55 g/l boric acid, 20 mM EDTA
TE:	10 mM TRIS, 1 mM EDTA
TENS:	0.1 M NaOH, 1% SDS in TE pH 8.0(0.01% Tris, 0.001% EDTA)
SOB medium:	20 g/l bacto-tryptone, 5 g/l bacto-yeast extract, 0.5 g/l NaCl, 2.5 mM KCl, 10 mM MgCl2
XIA plates:	20 mg/ml Xgal (5-bromo-4-chloro-3-indolyl-ß-D-galactosidase), 200 mg/ml IPTG (isopropylthio-ß-D-galactosidase), 25 mg/l Amp.

293	luc	lacZ	luc/lacZ	Average
-1221	641020	4.8	133545.8	
	674160	4.64	145293.1	139419.5
-1083	1143300	5.6	204160.7	
	1722600	6.54	263394.5	233777.6
-809	618910	7.06	87664.31	
	587750	6.48	90702.16	89183.23
-507	466240	4.64	100482.8	
	534840	5.22	102459.8	101471.3
-135	690	5.46	126.3736	
	720	4.44	162.1622	144.2679
TkLuc	263430	7.34	35889.65	
	276370	6.74	41004.45	38447.05
RSVLuc	4195700	2.6	1613730	
	3952800	2.74	1442627	1528179
-1221	1454640	2.86	508615.4	
	1720380	3.18	541000	524807.7
-1083	7473300	7.46	1001782	
	6323300	7.12	888103.9	944943.4
-809	1271620	4.4	289004.5	
	1306800	4.8	272250	280627.3
-507	1393580	3.98	350145.7	
	1197300	3.2	374156.3	362151
-135	2060	3.84	536.4583	
	2040	3.84	531.25	533.8542
TkLuc	464200	4.62	100476.2	
	523540	5.08	103059.1	101767.6
RSVLuc	10526600	2.2	4784818	
	12984800	2.3	5645565	5215191
-1221	941700	7.42	126913.7	
	1000280	5.26	190167.3	158540.5
-1083	1515300	11.72	129291.8	
	2335600	14.8	157810.8	143551.3
-809	1049640	9.5	110488.4	
	814420	8.02	101548.6	106018.5
-507	531460	10.76	49392.19	
	503680	9.32	54042.92	51717.56
-135	500	8.56	58.41121	
	560	10.3	54.36893	56.39007
TkLuc	46680	6.24	7480.769	
	42200	6.24	6762.821	7121.795
RSVLuc	10053600	6.44	1561118	
	12637600	7.44	1698602	1629860

-1221	1043660	5.76	181191	
	1102960	6.06	182006.6	181598.8
-1083	1779520	9.24	192588.7	
	1960560	9.54	205509.4	199049.1
-809	900020	7.12	126407.3	
	781200	7.56	103333.3	114870.3
-507	511480	7.98	64095.24	
	359840	5.9	60989.83	62542.53
-135	720	7.28	98.9011	
	780	8.12	96.05911	97.48011
TkLuc	56180	5.32	10560.15	
	70300	6.3	11158.73	10859.44
RSVLuc	7565100	4	1891275	
	12966000	4.3	3015348	2453311
-1221	1710100	2.9	589689.7	
	1563280	2.68	583313.4	586501.5
-1083	1673220	2.62	638633.6	
	1367760	2.24	610607.1	624620.4
-809	614280	1.66	370048.2	
	624420	1.66	376156.6	373102.4
-507	447060	2.46	181731.7	
	485060	2.36	205533.9	193632.8
-135	500	2.46	203.252	
	500	2.12	235.8491	219.5505
TkLuc	41180	2.14	19242.99	
	45940	1.9	24178.95	21710.97
RSVLuc	5677000	0.64	8870312	
	5387500	0.64	8417968	8644140
-1221	4844500	3.6	1345694	
	5936100	4.58	1296091	1320893
-1083	7321600	3.88	1887010	
	7251400	4.6	1576391	1731700
-809	1766200	2.52	700873	
	1693920	2.56	661687.5	681280.3
-507	1158780	3.26	355454	
	1071080	2.58	415147.3	385300.6
-135	900	4.58	196.5066	
	880	4	220	208.2533
TkLuc	194880	3.64	53538.46	
	194640	3.3	58981.82	56260.14
RSVLuc	12594000	1.12	11244642	
	15559800	1.5	10373200	10808921

-1221	3991600	2.38	1677142	
	4308100	2.74	1572299	1624721
-1083	4195100	2.86	1466818	
	4048500	3.06	1323039	1394928
-809	1253420	2.18	574963.3	
	1283220	2.26	567796.5	571379.9
-507	812760	2.44	333098.4	
	715260	2.34	305666.7	319382.5
-135	900	3.6	250	
	920	4.78	192.4686	221.2343
TkLuc	120500	3.28	36737.8	
	96980	2.28	42535.09	39636.45
RSVLuc	8473100	0.72	11768194	
	9993200	0.56	17845000	14806597

Verzamelstaat

	Average	SD	%	SD +/-
-1225	648068.9	17261.23	1645%	44%
-1087	753224.5	48549.32	1912%	123%
-812	316637.4	5896.025	804%	15%
-509	210885.5	9456.796	535%	24%
-136	211.5758	9.506684	1%	0%
TkLuc	39400.49	1046.332	100%	3%
RSVLuc	6440886	973201.5	16347%	2470%

Hep3B	luc	lacZ	luc/lacZ	Average
-1221	2728	0.503	5423.459	
	2536	0.428	5925.234	5674.346
-1083	5712	0.61	9363.934	
	7362	0.617	11931.93	10647.93
-809	1228	0.243	5053.498	
	1859	0.353	5266.289	5159.893
-507	2714	0.273	9941.392	
	3465	0.365	9493.151	9717.271
-135	29	0.287	101.0453	
	25	0.336	74.40476	87.72503
TkLuc	4538	0.477	9513.627	
	5223	0.624	8370.192	8941.91
RSVLuc	20676	0.153	135137.3	
	8945	0.089	100505.6	117821.4
-1221	7293	0.195	37400	
	10281	0.205	50151.22	43775.61
-1083	65806	0.6	109676.7	
	99067	1.015	97602.96	103639.8
-809	5316	0.199	26713.57	
	5940	0.223	26636.77	26675.17
-507	9337	0.223	41869.96	
	9328	0.219	42593.61	42231.78
-135	22	0.154	142.8571	
	26	0.188	138.2979	140.5775
TkLuc	21443	0.387	55408.27	
	21627	0.417	51863.31	53635.79
RSVLuc	203910	0.134	1521716	
	183640	0.126	1457460	1489588
-1221	3828	0.062	61741.94	
	3636	0.06	60600	61170.97
-1083	37668	0.255	147717.6	
	47196	0.314	150305.7	149011.7
-809	12660	0.115	110087	
	13478	0.153	88091.5	99089.23
-507	20974	0.179	117173.2	
	13639	0.178	76623.6	96898.39
-135	23	0.119	193.2773	
	24	0.135	177.7778	185.5275
TkLuc	3020	0.171	17660.82	
	2709	0.167	16221.56	16941.19
RSVLuc	91759	0.115	797904.3	
	161568	0.176	918000	857952.2

-1221	4653	0.071	65535.21	
	6264	0.077	81350.65	73442.93
-1083	30216	0.195	154953.8	
	40280	0.246	163739.8	159346.8
-809	19329	0.158	122335.4	
	24789	0.185	133994.6	128165
-507	14112	0.137	103007.3	
	18631	0.222	83923.42	93465.36
-135	44	0.108	407.4074	
	48	0.116	413.7931	410.6003
TkLuc	1044	0.051	20470.59	
	1792	0.051	35137.25	27803.92
RSVLuc	19102	0.044	434136.4	
	14996	0.028	535571.4	484853.9
-1221	10632	0.283	37568.9	
	14770	0.267	55318.35	46443.63
-1083	28641	0.283	101204.9	
	24264	0.301	80611.3	90908.12
-809	4574	0.109	41963.3	
	7040	0.114	61754.39	51858.84
-507	4681	0.126	37150.79	
	5433	0.128	42445.31	39798.05
-135	21	0.103	203.8835	
	22	0.1	220	211.9417
TkLuc	1809	0.088	20556.82	
	2117	0.114	18570.18	19563.5
RSVLuc	314292	0.097	3240123	
	272304	0.047	5793702	4516912
-1221	46090	0.175	263371.4	
	45099	0.172	262203.5	262787.5
-1083	87482	0.17	514600	
	78493	0.147	533966	524283
-809	21415	0.094	227819.1	
	26073	0.082	317963.4	272891.3
-507	20972	0.079	265468.4	
	20286	0.085	238658.8	252063.6
-135	39	0.094	414.8936	
	41	0.088	465.9091	440.4014
TkLuc	1044	0.102	10235.29	
	1792	0.086	20837.21	15536.25
RSVLuc	19102	0.058	329344.8	

	14996	0.066	227212.1	278278.5
-1221	47786	0.212	225405.7	
	53679	0.213	252014.1	238709.9
-1083	87408	0.215	406548.8	
	96716	0.266	363594	385071.4
-809	50195	0.181	277320.4	
	41752	0.135	309274.1	293297.3
-507	34684	0.133	260782	
	35420	0.164	215975.6	238378.8
-135	67	0.09	744.4444	
	69	0.125	552	648.2222
TkLuc	6531	0.151	43251.66	
	6553	0.121	54157.02	48704.34
RSVLuc	181728	0.047	3866553	
	260128	0.058	4484965	4175759

Verzamelstaat

	Average	SD	%	SD +/-
-1225	104572.12	4702.843	383%	17%
-1087	203272.69	6506.605	744%	24%
-812	125305.24	14319.99	459%	52%
-509	110364.75	8565.304	404%	31%
-136	303.57081	31.02038	1%	0%
TkLuc	27303.842	2578.234	100%	9%
RSVLuc	1703023.8	426692	6237%	1563%

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First, I would like to start by thank everybody I forget to mention below. Next, I want to thank John Sussenbach, who made it possible for me to do this study. It is too bad we did not have the time to talk more about our interests (I really do like astronomy!), but we were both too busy I guess.

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I won't thank the building (it's the worst building I've ever worked in), although the view from the lab was great in the summer :). I do want to thank Pierre Abbink Spaink, for letting me use the printer that so beautifully produced the cover, and my sister Lucy, for proof-reading this report.

As the acknowledgement is THE place for things you can not account for scientifically, a little philosophical reverie; Thinking about the complexity of the regulation of the IGF-II gene, and about gene regulation in general, you will be filled with an overwhelming admiration for mother nature; it works! And this admiration and the great satisfaction once another piece of the regulation machinery has been unravelled will provide the energy to continue the struggle for knowledge so typical of mankind.

My results were not easy to interpret, and the wildest theories passed my mind. And theoretically, they could all be correct. That is why I added Mrs. Kozak quote on one of the first pages of this report.

Thank you all!