

STRUCTURAL AND FUNCTIONAL ASPECTS OF SITE-SPECIFIC IGF-II mRNA CLEAVAGE

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Structurele en Functionele Aspecten van Plaats-Specifieke IGF-II mRNA Klieving

(met een samenvatting in het Nederlands)

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Wir wollen nicht nur wissen *wie* die Natur ist (und wie Ihre Vorgänge ablaufen), sondern wir wollen auch nach Möglichkeit das vielleicht utopisch und anmassend erscheinende Ziel erreichen, zu wissen, warum die Natur *so und nicht anders ist*.

Albert Einstein

Aan mijn ouders

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CHAPTER 1

Introduction: the IGF system

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1. General introduction.

Growth and development of multicellular organisms are complex processes that are strictly regulated by hormones and growth factors. Growth hormone (GH), which is synthesized in the pituitary gland, is responsible for the stimulation of postnatal growth in vertebrates. However, the growth stimulatory effect of GH is indirect, as evidenced by the initial observation by Salmon and Daughaday that GH *in vitro* did not correct a defect in the synthesis of matrix proteins of hypophysectomized rats, whereas the serum of GH-treated hypophysectomized rats did (Salmon and Daughaday, 1957). This has led to the so-called ‘somatomedin hypothesis’ (Daughaday *et al.*, 1972) which states that GH acts on skeletal tissues by inducing the formation of growth factors (=somatomedins) circulating in the blood and acting on peripheral tissue. Purification and subsequent amino acid sequence determination revealed the existence of two distinct molecules; the initial name somatomedins was replaced by insulin-like growth factors I and II (IGF-I and IGF-II) because of their structural and functional homology with insulin (Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978b). It is noteworthy that the synthesis of IGF-II is not under stringent control of GH, in contrast to IGF-I (Humbel, 1990).

Mature IGF-I and IGF-II are polypeptides of 70 and 67 amino acids, respectively, with 65% homology to each other and 43 and 41 % homology to pro-insulin (Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978b; Daughaday and Rotwein, 1989). The fully processed and most active form of IGF-II is 7.5 kDa (Kiess *et al.*, 1994), but larger forms of up to 20 kDa have also been isolated from serum (Zapf *et al.*, 1992; Kotani *et al.*, 1993; Hunter *et al.*, 1994). These large forms of IGF-II generally constitute less than 10% of the total IGF-II in human serum, but patients with certain tumors may have up to 75% of their serum IGF-II in the form of these larger IGF-II variants (Daughaday *et al.*, 1988).

In contrast to insulin, 99% of IGF-I and IGF-II is present in the form of a complex with specific binding proteins (IGF-BPs). Six different IGF-BPs (IGF-BP1 to IGF-BP6) have been identified to date (for a recent review, see Baxter (2000)). IGF-BP3 is the most abundant IGF-BP in serum. After binding to one molecule of IGF-I or IGF-II, IGF-BP3 also binds to an acid-labile subunit (ALS), thus forming a ternary complex of 150 kDa (Baxter and Martin, 1989). The human IGF-BPs range in size from 261 amino acids (IGF-BP6) to 289 amino acids (IGF-BP2) and all contain cysteine-rich N- and C-terminal domains, each constituting about a third of the mature peptide. The IGF-BP amino acid sequences are all well conserved between members, but the IGF-BPs differ among each other in their relative affinity for IGF-I and IGF-II; IGF-BPs 2, 5, and 6 have a higher affinity for IGF-II than for IGF-I, while IGF-BPs 1, 3, and 4 bind IGF-I and IGF-II with comparable affinity (for a review, see Jones and Clemmons (1995)). A number of different physiological roles have been postulated for the IGF-BPs (reviewed by Martin and Baxter (1999)). An important role may be to prolong the half-lives of the IGFs by preventing their degradation; they also inhibit the insulin-like effects of the IGFs in serum and they modulate the bioavailability of the IGFs. In addition, the IGF-BPs may have IGF- independent functions.

2. Expression and physiological role of the IGFs.

2.1. Receptors.

The actions of the IGFs are mediated through binding to specific membrane receptors (for recent reviews, see Adams *et al.* (2000); DaCosta *et al.* (2000)). The main receptors to which the IGFs can bind are the IGF type I and type II receptors. The IGFs can also bind to the insulin receptor, albeit with a much lower affinity (Ullrich *et al.*, 1986). Nevertheless, the insulin receptor can also mediate the growth promoting effects of IGF-II (Louvi *et al.*, 1997). As for the insulin receptor and many other growth factor receptors, the IGF type I receptor is a heterotetrameric receptor of the tyrosine kinase family. This receptor preferentially binds IGF-I but can also bind IGF-II with high affinity, implicating that both IGFs can function via the same receptor (Bondy *et al.*, 1993). Binding of IGF-I or IGF-II leads to autophosphorylation of the tyrosine residues in the cytoplasmic domains of the receptor (Jacobs *et al.*, 1983). The phosphotyrosines then serve as binding sites that interact with specific cytoplasmic signaling molecules, leading to a mitogenic signaling cascade through the activation of phosphatidylinositol 3'-kinase (PI₃K) (Dufourny *et al.*, 1997) as well as a second pathway involving the MAP-kinases (Jhun *et al.*, 1994).

The type II IGF receptor, which is identical to the cation-independent mannose-6-phosphate (M6P) receptor, binds IGF-II with much higher affinity than IGF-I (Nissley and Lopaczynski, 1991), although it cannot easily be explained what the structural basis for this difference in affinity is (Torres *et al.*, 1995). This receptor has a large extracellular domain consisting of 15 repeated subdomains, and it has been known to target lysosomal enzymes to the lysosomes. It was suggested that the type II receptor may also have a role in the clearance of IGF-II from extracellular fluid since upon IGF-II binding the receptor can be internalized leading to the degradation of IGF-II (Oka *et al.*, 1985). In addition, binding of IGF-II to this receptor activates a signaling pathway involving G-proteins (Nishimoto, 1993). Although the lysosomal enzymes and IGF-II bind to different sites in the type II/M6P receptor, they can influence each other's effects (Nissley and Lopaczynski, 1991).

In addition to the above mentioned receptors, another putative receptor that mediates IGF signaling in mouse placenta and embryos was postulated based on experiments with combined IGF/type I IGF receptor knockout mice (Baker *et al.*, 1993). Recently, this receptor was identified as the insulin receptor isoform A (Morrione *et al.*, 1997); overexpression of the insulin receptor in IGF type I-deficient cells restored the proliferative response of these cells to IGF-II. Frasca *et al.* (1999) established that one of the two isoforms (A and B) of the insulin receptor, isoform A binds IGF-II with an affinity close to that of insulin. Thus, it appears that there are two mitogenic receptors for IGF-II, the type I IGF receptor and the insulin receptor isoform A.

2.2. Expression and function during development.

Both IGF-I and IGF-II are expressed in a developmental stage-dependent manner, but their individual expression profiles differ significantly. IGF-I serum concentrations are low during pre- and neonatal periods and start to rise at the onset of growth hormone-dependent growth in man at 6-12 months of age. The IGF-I concentrations are highest during the pubertal stage and then decline with age (Hall *et al.*, 1980; Hall and Sara, 1984). IGF-II serum levels in humans are high during embryogenesis, decrease after birth and gradually rise again into adulthood (Bennett *et al.*, 1983; Gluckman and Ambler, 1993). In adult man, IGF-II serum levels are fourfold higher than those of IGF-I (Zapf *et al.*, 1981; Enberg and Hall, 1984). Targeted disruptions of the genes encoding IGF-I and IGF-II in knockout mice have generated considerable insight into the function of the IGFs during embryonic development. Mice with a complete disruption of IGF-I expression are viable but show a significant growth retardation such that birth weights are reduced by about 40% of that of normal mice (Baker *et al.*, 1993; Liu *et al.*, 1993), indicating that IGF-I plays a very important role in embryonic development. Due to respiratory insufficiency, a large number of the IGF-I deficient mice die immediately or shortly after birth (Powell-Braxton *et al.*, 1993). After birth, IGF-I knockout mice grow very poorly and adults are about 70% reduced in size compared to the wild type adult animals. Similar to mice carrying a disruption of IGF-I, mice in which the IGF-II gene was disrupted by gene targeting are reduced by about 40% in size compared to their wild type littermates but they are normally proportioned (DeChiara *et al.*, 1990). This indicates that IGF-II is indispensable for embryonic growth. In contrast with the mice carrying a disruption of the IGF-I gene, the size difference generated by the retarded fetal growth rate due to a loss of IGF-II remains the same throughout postnatal life, indicating that IGF-II in mice plays no significant role in growth after birth.

Clues about the mechanism by which IGF-II exerts its growth promoting function during development were revealed by double-knockout studies. Knockout of the IGF type I receptor results in mice with a body weight reduced by about 55% of the wild type animals and these mice do not survive to adulthood. The IGF-II/IGF type I receptor double-knockout further decreases the birth weight to 30%, indicating that not all IGF-II effects are mediated by the type I receptor (Liu *et al.*, 1993).

Knockout mice have also been generated for the IGF type II receptor. One might expect a growth defect if this receptor would be involved in transducing growth promoting signals, but such an effect was not observed (Lau *et al.*, 1994; Ludwig *et al.*, 1996). On the contrary, ablation of its expression results in a moderate fetal and placental overgrowth of 25 to 40% increase and cardiac hypertrophy, leading to neonatal lethality. This phenotype could be reversed to normal by combining the IGF type II receptor null-mutation with a knockout of IGF-II. These results suggest that the absence of a functional type II receptor leads to increased IGF-II levels, and therefore regulation of the IGF-II concentration was proposed as a major function of the IGF type II receptor in the developing mouse (Wang *et al.*, 1994).

2.3. Biological effects of the IGFs.

The biological effects of IGF-I and IGF-II have been studied extensively using both *in vitro* and *in vivo* experimental systems. Both IGF-I and IGF-II show *in vivo* and *in vitro* insulin-like metabolic effects (such as effects on glucose transport and on blood glucose levels), but only at relatively high concentrations. The presence of IGF-binding proteins in plasma prevents hypoglycemia under physiological conditions. The biologically relevant effects at the usual nanomolar concentrations are the stimulation of cell proliferation, and at least in certain tissues, cell differentiation.

For IGF-II, a wide range of biological activities in cells in culture have been observed: in addition to promoting cell proliferation, it can also induce differentiation *in vitro*, an effect which has been characterized in detail in myoblasts (Florini *et al.*, 1991). IGF-II profoundly affects cellular survival and counteracts apoptosis in various cell systems (Biddle *et al.*, 1988; Granerus *et al.*, 1995; Granerus and Engstrom, 1996), whereas in other cell lines an apoptosis-inducing effect by IGF-II has been observed (Granerus *et al.*, 1998). IGF-II can also induce a functional modulation in certain cell types without otherwise altering the differentiated phenotype; it stimulates hormone synthesis and secretion in ovarian granulosa and theca cells (Giudice, 1992). Furthermore, IGF-II can also potentiate the release of histamine from basophils in response to immunoglobulin E (Hirai *et al.*, 1993). Finally, it has been shown that IGF-II can stimulate motility in cultured rhabdomyosarcoma cells (Minniti *et al.*, 1992).

3. IGF-I gene structure and regulation of expression.

In humans, the IGF-I gene is present as a single copy which has been mapped to chromosome 12 (Brissenden *et al.*, 1984). The gene consists of six exons and 5 introns, and spans more than 80 kb of chromosomal DNA (Fig. 1). Alternative splicing, polyadenylation at three different sites, the usage of two different promoters and variable transcription initiation sites generates a diverse population of transcripts, which range in size from 1.1 to 7.6 kb.



Figure 1. Structure of the human IGF-I gene. The intron sequences are indicated by a line, the exons are indicated by boxes and are numbered. The coding exons are indicated in black and the untranslated regions are indicated in white. Promoters P1 and P2 are indicated by bent arrows. Polyadenylation sites are indicated by asterisks. The large central intron between exons 3 and 4 has been truncated, as indicated.

Exons 1 and 2 contain different 5'-UTR sequences, accompanied with different N-terminal parts of the signal peptide of the precursor IGF-I. The mature IGF-I protein is encoded by exons 3 and 4. Exons 5 and 6 encode different C-terminal parts of the E-domain of the precursor IGF-I protein as well as different 3'-UTR sequences (Rotwein, 1986; Steenbergh *et al.*, 1991). Thus, four different precursor IGF-I proteins can be formed by the alternative usage of two different N-terminal- and two different C-terminal peptide sequences, whereas the mature IGF-I sequence is invariant.

The IGF-I gene contains two promoters, P1 and P2, of which P1 is the major promoter. While P1 is active in all tissues in which IGF-I is expressed, P2 is active primarily in the liver, where it is responsible for transcription of approximately 20% of all IGF-I mRNAs (for a review, see Holthuisen *et al.* (1999)).

IGF-I gene expression can be regulated by a host of external stimuli; a very strong activator of IGF-I expression is growth hormone (GH) that has been found to have a strongly stimulating effect on IGF-I gene transcription in liver tissue and in primary hepatocytes (Norstedt and Moller, 1987; Bichell *et al.*, 1992). This is in agreement with the somatomedin hypothesis that GH exerts its growth promoting effect indirectly via increasing the serum concentrations of IGF-I.

4. IGF-II gene structure and regulation of expression.

4.1. Gene structure.

The human IGF-II gene has been mapped to chromosomal locus 11p15, directly downstream of the insulin gene and spans 30 kb of chromosomal DNA. The gene consists of 9 exons of which exons 7, 8, and the most 5' part of exon 9 encode the IGF-II precursor protein (De Pagter-Holthuisen *et al.*, 1988; Holthuisen *et al.*, 1990) (Fig. 2). Transcription of the gene is driven by four different promoters, P1 to P4, which precede exons 1, 4, 5, and 6, respectively. Transcription from these different promoters generates a family of mRNAs, which all share the IGF-II coding region, but differ in their 5'-UTR sequences. The transcripts from promoters P1 to P4 differ in size: 5.3 kb (P1), 5.0 kb (P2), 6.0 kb (P3), and 4.8 kb (P4). Further heterogeneity of the family of transcripts is accomplished by the differential usage of two alternative polyadenylation sites located in the 3' part of the gene. In most cases, a poly(A) site located approximately 4 kb downstream of the stopcodon is used, but a minor P3 derived mRNA species of 2.2 kb has been identified that is polyadenylated at an upstream poly(A) site leading to a shorter 3'-UTR (Sussenbach *et al.*, 1992). In addition to this population of mRNAs, a 1.8 kb RNA species has been identified that results from endonucleolytic cleavage, rather than from transcription from an additional promoter (De Pagter-Holthuisen *et al.*, 1988; Meinsma *et al.*, 1991) (see below).

As shown above, IGF-II is involved in many physiological processes, and interference with its expression causes a severe growth defect. Thus, a tight regulation of IGF-II gene expression is of crucial importance, which is reflected by the presence of a complex set of regulatory mechanisms operating to control IGF-II protein

production. A schematic overview of the different levels of control varying from genomic imprinting to translational regulation will be discussed below.

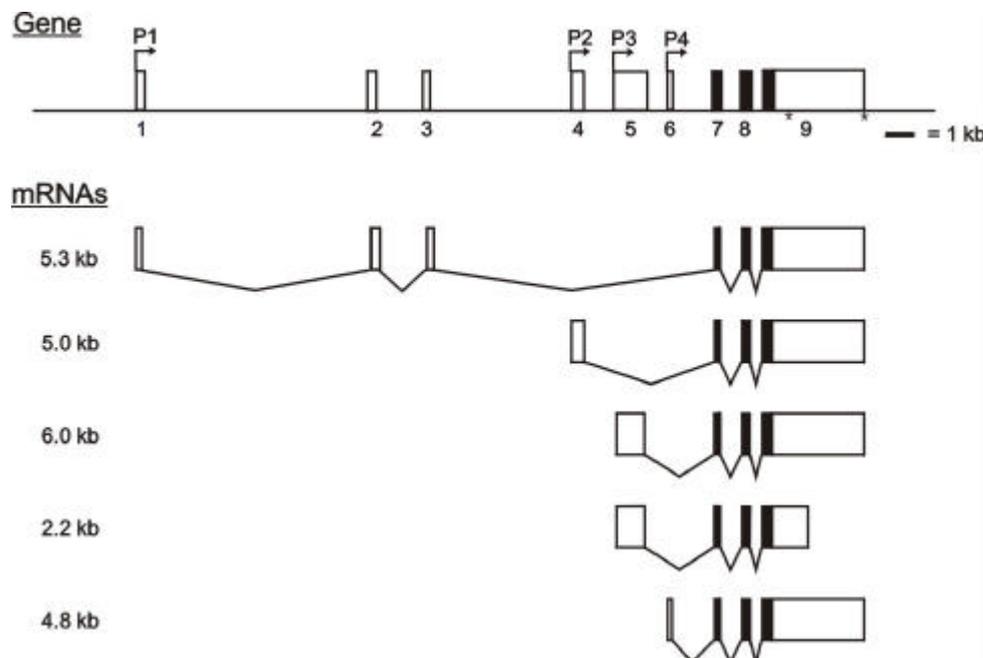


Figure 2. Structure of the human IGF-II gene with the different mRNAs generated by transcription from promoters P1 to P4, which are indicated by bent arrows. The sizes of the transcripts are indicated on the left. The exons are indicated by boxes and are numbered 1 to 9. Open boxes represent untranslated sequences; black boxes indicate the prepro-IGF-II encoding exons. The asterisks indicate the two alternative poly(A) sites in the 3'-UTR.

4.2. Effects of exogenous stimuli on IGF-II gene expression.

Regulation of IGF-II gene expression shows remarkable differences with regulation of the IGF-I gene. Whereas the IGF-I gene is stringently regulated by growth hormone (GH) (for a review, see Humbel (1990)), the expression of IGF-II is mainly regulated by other hormones and local environmental factors play an important role. For example, human ovarian cells in culture produce increased IGF-II mRNA levels in response to follicle stimulating hormone, chorionic gonadotropin, or prolactin (Voutilainen and Miller, 1987; Ramasharma and Li, 1987). One of the exogenous factors that influence IGF-II expression in a number of cell systems is glucose; in a rat pancreatic β -cell line, elevated glucose concentrations caused a threefold increase in IGF-II expression (Asfari *et al.*, 1995) and also in primary cultures of fetal rat hepatocytes, a glucose-dependent increase in IGF-II mRNA levels was observed (Goya *et al.*, 1999). Another local stimulus that can lead to increased IGF-II

expression is oxygen deprivation (hypoxia); exposure of the human hepatoma cell line HepG2 to hypoxia results in IGF-II overexpression which appears to be mediated via increased transcription from the fetal promoter P3 (Kim *et al.*, 1998; Bae *et al.*, 1999). These findings, and the fact that hepatocellular carcinoma is a typical hypervascular tumor (Mise *et al.*, 1996), raised the hypothesis that IGF-II may play an important role in the development of neovascularization of hepatocellular carcinoma.

IGF-II gene expression can also be controlled by glucocorticoids as evidenced by a number of studies. In some systems, IGF-II expression is downregulated by glucocorticoids (Beck *et al.*, 1988; Levinovitz and Norstedt, 1989; Backlin *et al.*, 1998), whereas in others an upregulation of IGF-II expression has been observed (Price *et al.*, 1992; Mouhieddine *et al.*, 1996). Dell *et al.* (1997) have reported that glucocorticoid treatment of mouse embryonic fibroblasts activates the murine fetal promoter P3, which is analogous to the human promoter P4.

4.3. Imprinting.

Genomic imprinting is a genetic mechanism by which genes are exclusively expressed from the maternal or paternal chromosome. It has been shown in human, rat and mouse that the IGF-II gene is subject to genomic imprinting as well as the H19 gene located at the same locus (chromosome 11p15). In most human tissues, the IGF-II gene is actively transcribed from the paternal allele, in contrast to the maternal allele, which is transcriptionally silent (DeChiara *et al.*, 1990; DeChiara *et al.*, 1991). Only in the adult human liver (in contrast to the fetal liver) and in the choroid plexus and the leptomeninges of the central nervous system, biallelic expression of IGF-II is observed (Weksberg *et al.*, 1993).

Clues of the mechanism by which imprinting of the IGF-II gene is controlled were obtained from studies on the H19 gene, which is located about 90 kb downstream of the IGF-II gene. The H19 and the IGF-II genes are reciprocally imprinted, and H19 RNA, which does not exhibit any conserved open reading frames, is expressed exclusively from the maternal allele (Bartolomei *et al.*, 1991). It was shown that deletion of the H19 gene and some flanking sequences results in loss of imprinting of the IGF-II gene (Leighton *et al.*, 1995). Based on these results, the 'enhancer-competition' model was proposed which states that the promoters of the IGF-II and H19 genes compete for two enhancers located downstream of H19; these enhancers normally stimulate H19 gene transcription, but upon deletion of the H19 region, the enhancers become available for activation of IGF-II transcription. In agreement with this model, the introduction of an extra set of enhancers between the IGF-II and the H19 gene resulted in a loss of transcription repression of the IGF-II gene on the maternal chromosome in fetal liver (Webber *et al.*, 1998). Contrary to the prediction of the enhancer competition model however, a smaller deletion encompassing only the H19 promoter has no effect on the IGF-II gene in liver and only a small effect in skeletal muscle (Schmidt *et al.*, 1999). Instead, deletion of an 1.6 kb sequence in the region between the IGF-II and H19 genes results in a complete loss of imprinting in mice with both genes co-expressed at reduced levels on both parental chromosomes

(Thorvaldsen *et al.*, 1998). Thus, these recent results suggest that a chromatin boundary between the IGF-II and the H19 genes may be responsible for the reciprocal imprinting rather than enhancer competition.

The physiological importance of imprinting of the IGF-II gene is evident, since loss of imprinting is associated with a variety of diseases and tumors. A major example is the Beckwith-Wiedemann syndrome (BWS), where in 80% of the cases IGF-II imprinting is lost and IGF-II is also expressed from the maternal allele (Weksberg *et al.*, 1993). Relaxation of IGF-II imprinting has also been implicated in the onset of several cancers including Wilms' tumor (Ogawa *et al.*, 1993), embryonic rhabdomyosarcoma (Zhan *et al.*, 1994), and non-small cell myosarcoma (Suzuki *et al.*, 1994).

4.4. Transcription regulation.

Transcription of the human IGF-II gene is driven by four promoters (P1 to P4), that are active in a tissue-specific and developmental stage-dependent fashion. During fetal development, P3 is the most active promoter in many fetal tissues including fetal liver, and is the main promoter in many tumor tissues as well. P4 is also active in many fetal tissues, while P2 activity has only been detected in certain tumor cell lines (Ikejiri *et al.*, 1991). Promoter P1 is inactive during fetal stages, but is switched on after birth whereas P3 and P4 are downregulated in the adult liver. Thus, P1 is the main activator of IGF-II gene expression in the adult liver, which is the source for circulating endocrine IGF-II. Although the molecular mechanism for this promoter switch is still unknown, extensive studies have revealed a number of important factors involved in the regulation of the different promoters (reviewed by Holthuisen *et al.* (1999)). Promoter P1 comprises a region of approximately 1 kb. A single binding site for the general transcription factor Sp1 is present in the proximal part of the promoter and is essential for basal P1 activity (Rodenburg *et al.*, 1997). In addition, a number of liver-enriched transcription factors, including C/EBP, HNF4, and HNF3 β were found to play a role in the regulation of P1 (Rodenburg, 1996). Major contributors to the postnatal liver-specific activation of P1 are the postnatally expressed C/EBP transcription factors. Two of the C/EBP family members are able to activate P1; C/EBP α activates the promoter six-fold and C/EBP β leads to an even stronger activation of the promoter of fifteen-fold. HNF-3 β stimulates P1 activity ten-fold, whereas HNF-4 strongly suppresses P1. Interestingly however, this suppressing effect is abolished by the presence of the C/EBP transcription factors. This provides a clue to the mechanism of P1 activation after birth: during the prenatal stage, HNF-4 suppresses P1, while around birth, the C/EBP transcription factors appear and counteract the suppressive effect of HNF-4, causing the full induction of P1 activity.

Little information is available about the regulation of promoter P2, which precedes exon 4. P2 is generally very weak, although some human tumors have been described that show elevated P2 activity (Ikejiri *et al.*, 1991). In contrast to promoters P1, P3, and P4, no Sp1 recognition sequences were found in P2 (Holthuisen *et al.*, 1990), and no other enhancer elements have been identified to date. However, an alternatively spliced P2 transcript, which contains an additional exon downstream of

exon 4 has been described by Mineo *et al.* (2000). This exon is named exon 4b, and is proposed as a tenth exon in the IGF-II gene by Mineo *et al.* (2000).

The major fetal promoter, P3, spans 1300 bp upstream of exon 5 and is very GC-rich with 70-80% GC base pairs (van Dijk *et al.*, 1991; van Dijk *et al.*, 1992). The promoter can be subdivided into two separate regions; a proximal region up to position -289 relative to the transcription start site and an upstream region from positions -1231 to -1063. The proximal region supports basal activity in several cell types, while the upstream region is important for cell-type specific expression of P3 (van Dijk *et al.*, 1992; Raizis *et al.*, 1993; Rietveld *et al.*, 1997). Cell-specific factors that may be involved in the cell-type specific regulation of P3 activity have been found to bind to the upstream region -1231/-1063 (Schneid *et al.*, 1993).

Several elements can be distinguished in the proximal P3 region that bind specific transcription factors, including promoter elements PE3-1 to PE3-4 (van Dijk *et al.*, 1992; Raizis *et al.*, 1993). The ubiquitous transcription factor Sp1 activates P3 through binding to several different sites in the promoter. The Egr transcription factor is also able to recognize and bind to multiple sites in P3 (Rauscher, 1993).

Interestingly, these sites can also be bound by WT1 (Drummond *et al.*, 1992), and it has been postulated that the Egr proteins may play a role in stimulating expression of the IGF-II gene resulting in autocrine growth stimulation of specific tumors, whereas WT1 may act as a suppressor of P3 activity (Madden and Rauscher, 1993). An element near position -180 is subdivided into two protein binding sites, named box A and box B. Box A is bound by a still unidentified protein, and box B is bound by the transcription factor AP2 and another protein that awaits identification (van Dijk *et al.*, 1992; Rietveld *et al.*, 1997; Rietveld *et al.*, 1999).

Promoter P4 precedes exon 6 and exhibits a moderate activity in most fetal and nonhepatic tissues adult human tissues examined. The Sp1 transcription factor is the major regulator of P4 and can bind to four sites within the first 125 nucleotides upstream of the transcription start site (van Dijk *et al.*, 1991; Hyun *et al.*, 1993). In addition, two binding sites for the retinoblastoma (Rb) protein have been identified and Rb can act as a positive regulator on Sp1 mediated transcription of P4 (Kim *et al.*, 1992).

4.5. Translation regulation.

As indicated above, transcription from the four different promoters generates a population of mRNAs that differ in their 5'-UTRs (leaders) (Fig. 2). These leaders vary significantly in length and composition. Leader 1 consists of exons 1-3 and is 586 nt long and is GC rich; leader 2 consists of exon 4 sequence and is 408 nt long and moderately rich in G and C residues; leader 3 consists of exon 5 sequence, is rich in C-residues and is the longest 5'-UTR with 1171 nt; leader 4 consist of exon 6 sequence and is the shortest 5'-UTR with only 109 nt. Two interesting additional features of leader 1 are the presence of an upstream open reading frame and an internal ribosome entry site (IRES), allowing cap-independent translation initiation (Teerink *et al.*, 1995). On the basis of its minor length, leader 4 can be expected to allow efficient translation, and that is in fact what has been observed; mRNAs with leader 4

sequences are associated with the membrane-bound polysomes and are actively translated. Also leader 2-containing mRNAs were found to be completely in the polysomal fraction in proliferating tissue culture cells and in fetal liver, indicating that leader 2 does not inhibit translation (de Moor *et al.*, 1994). The high GC content of leader 1 (32%G, 35% C) and the presence of an upstream open reading frame make it likely that mRNAs starting with leader 1 will be poorly translated. However, since this mRNA species is the only IGF-II mRNA detectable in adult liver, it is supposed to direct the synthesis of most or all of the IGF-II protein in the circulation. Thus, surprisingly, mRNAs with leader 1 can be expected to be translated efficiently, and this is what actually has been observed (de Moor, 1994). In contrast to mRNAs with leaders 1, 2, and 4, the mRNAs carrying leader 3 are hardly found in the polyribosome fraction, indicating that this mRNA species is not efficiently translated (Nielsen *et al.*, 1990; de Moor *et al.*, 1994). However, also the leader 3-containing IGF-II transcripts can be efficiently translated depending on the growth conditions; in rhabdomyosarcoma cells, the transcripts are translationally silent when the cells are quiescent but become selectively mobilized and actively translated in exponentially growing cells (Nielsen *et al.*, 1995). Activation of translation is inhibited by rapamycin and mimicked by anisomycin, suggesting that the translation of leader 3 mRNAs is regulated by the p70^{S6k} signaling pathway. In contrast, the translation of P4-derived 4.8 kb mRNA does not appear to be dependent on the growth status of the cell. Thus, mRNAs containing leader 4 are constitutively translated, whereas translation of leader 3 mRNA is related to cell growth and is regulated by the p70^{S6k} signaling pathway.

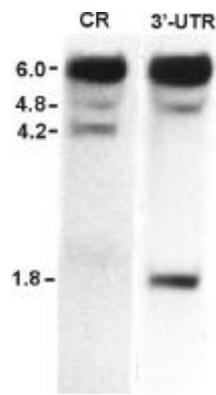
4.6. Endonucleolytic cleavage: scope of this thesis.

The subject of this thesis is site-specific endonucleolytic cleavage of the IGF-II mRNAs. In addition to the population of mRNAs transcribed from promoters P1 to P4, which differ in size from 6.0 to 2.2 kb, a shorter RNA species of 1.8 kb has been detected on Northern blots when a 3'-UTR-specific probe was used (Fig. 3A) (De Pagter-Holthuisen *et al.*, 1988). Further characterization of this 1.8 kb RNA revealed that it is produced by endonucleolytic cleavage of the full length IGF-II mRNAs and not by transcription from an additional promoter (Fig. 3B). Cleavage was found to occur at a unique site in the 3'-UTR, and the cleavage site was mapped to the single nucleotide resolution (De Pagter-Holthuisen *et al.*, 1988; Meinsma *et al.*, 1991). Northern analysis using a coding region-specific probe also detected a 5' cleavage product that contains the IGF-II coding region. This RNA, however, is much less abundant than the 1.8 kb 3' cleavage product, indicating that the 5' cleavage product is less stable than the 3' cleavage product (Fig. 3A). This observation raised the hypothesis that cleavage may act to destabilize the coding part of the IGF-II mRNAs and thus downregulate IGF-II protein synthesis.

Subsequent deletion analyses using an *in vivo* IGF-II minigene system mapped the sequences required for IGF-II mRNA cleavage to a region encompassing the cleavage site from position -173 to +150 relative to the cleavage site (element II; positions 3723 to 4046 in the 6.0 kb transcript) (Fig. 4). Two stable stem-loops are predicted in the upstream part of element II and the downstream part is very G-rich.

Surprisingly, a distantly located upstream region from positions -2116 to -2013 relative to the cleavage site also appeared to be necessary (element I; positions 1780 to 1883 in the 6.0 kb transcript). RNA folding algorithms and biochemical assays indicated that the C-rich element I can interact with the G-rich region in element II downstream of the cleavage site, forming a 83 nt long base paired duplex (Scheper *et al.*, 1995). In the absence of the duplex, the G-rich downstream region may fold into a compact G-quadruplex structure, thus accounting for the observed stability of the 1.8 kb RNA (Christiansen *et al.*, 1994).

(A)



(B)

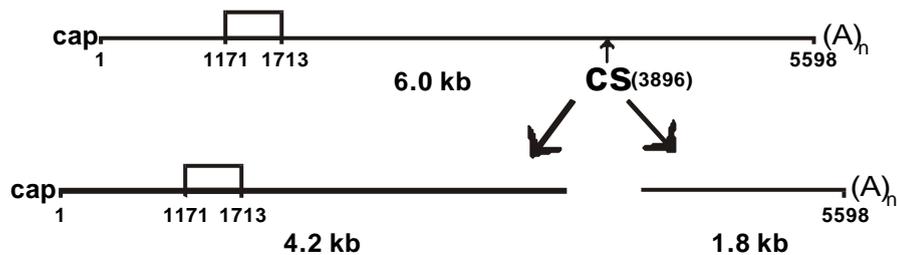


Figure 3. (A) Northern blot analysis of total RNA isolated from SHSY-5Y cells (adapted from De Pagter-Holthuisen *et al.* (1988)), that endogenously express IGF-II. When an IGF-II coding region (CR)-specific probe is used, the full-length IGF-II mRNAs of 6.0 kb and 4.8 kb are detected, and in addition the 5' cleavage product of 4.2 kb that contains the coding region. With an IGF-II 3'-UTR-specific probe, the full-length IGF-II mRNAs are detected as well as the 1.8 kb 3' cleavage product. The sizes of the different RNA species are indicated on the left. The probes used are indicated above the lanes. (B) Schematic representation of the endonucleolytic cleavage at a specific site in the 3'-UTR of the IGF-II mRNAs that generates an unstable 5' cleavage product containing the coding region and a stable 3' cleavage product that consists of 3'-UTR sequence. Shown is the 6.0 kb IGF-II mRNA as a target for cleavage, but the other IGF-II mRNAs are also cleaved. The cleavage site (CS) is indicated by an arrow, and the IGF-II coding region is indicated by an open box. The untranslated sequences are represented by lines, and the positions of the coding region and the cleavage site are indicated.

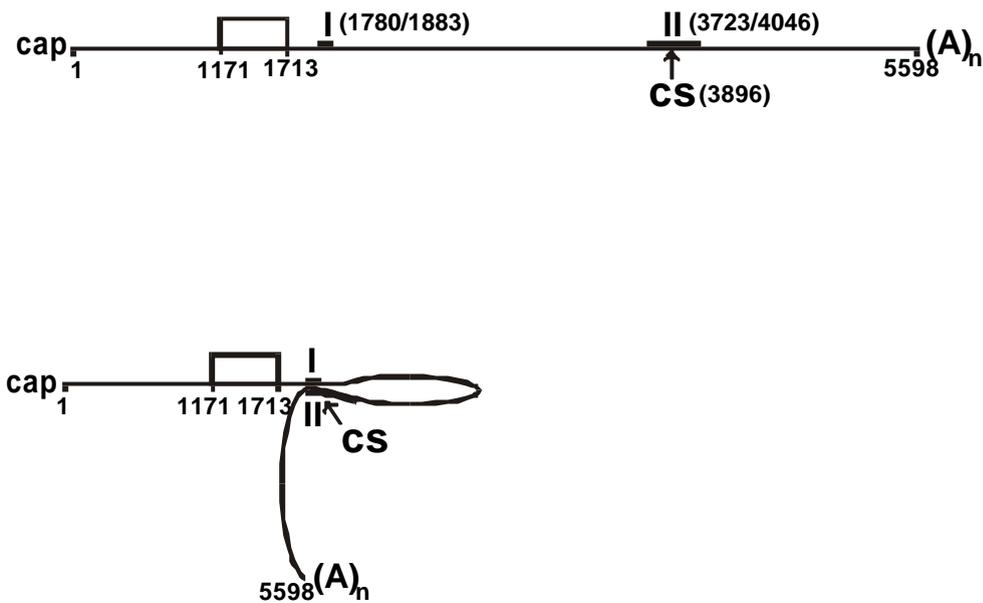


Figure 4. Schematic representation of the 6.0 kb IGF-II mRNA. Elements I and II can be brought into proximity by formation of a duplex of 83 nucleotides. Elements I and II (bars), the cleavage site (arrow), and the IGF-II coding region (open box), are indicated.

The work described in this thesis provides a follow-up study of the above-described studies. Chapter 3 describes the identification of a protein that specifically binds to a stem-loop in the proximity of the cleavage site and that may have a modulating effect on cleavage. In addition, an extensive mutational analysis is described that reveals the importance of the identity of specific nucleotides around the cleavage site for cleavage. In Chapter 4, RNA structural studies are described that provide insight into the importance of the various RNA folding domains in cleavage of the IGF-II mRNAs. In Chapter 5, clues about the physiological role of cleavage are presented.

CHAPTER 2

Introduction: RNA-protein interactions

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1. Introduction.

Many important events in the cell such as RNA processing, translation, RNA transport, and RNA degradation involve RNA-protein interactions. All post-transcriptional events in mRNA biogenesis require the formation of specific RNA-protein complexes. For example, in pre-mRNA splicing, a complex machinery built of RNA-protein particles (snRNPs) is required to correctly excise the introns and join the adjacent exons. Capping and polyadenylation require specific proteins that interact with the 5' end of the nascent transcript and the poly(A) signal, respectively. The coordinate actions of these complexes yield a mature mRNA that typically contains an N7-methylated G-residue (the cap structure) at the 5' end, and a poly(A) tail at the 3' end (Fig. 1).

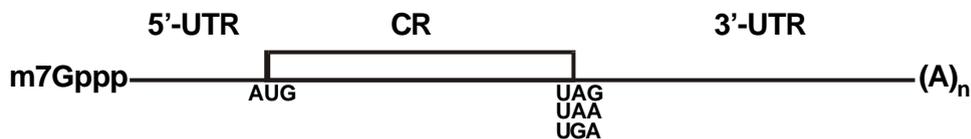


Figure 1. Structure of a typical mRNA. The cap structure at the 5' end, and the poly(A) tail at the 3' terminus of the messenger are shown. The 5'-UTR and the 3'-UTR are indicated by a line, the coding region (CR) is boxed. The AUG translation startcodon and the three possible translation stopcodons are indicated.

The sequence between the cap and the AUG translation start codon is designated the 5'-untranslated region (5'-UTR); the coding region of the transcript is flanked at its 3' end by a 3'-untranslated region (3'-UTR), which constitutes the sequence between the translation stopcodon and the poly(A) tail. The poly(A) tail generally consists of about 200 A-residues in mammalian cells. All these features of an mRNA can serve as specific binding sites for RNA-binding proteins. The cap and the poly(A) tail can be recognized by the translation initiation machinery and poly(A) binding proteins, respectively, which play an important role in translation and stability of the messenger. The 5'-UTR, the coding region, and especially the 3'-UTR commonly harbor *cis*-acting recognition sequences for specific proteins involved in regulation of translation, mRNA degradation, and mRNA localization.

Clearly, it is of vital importance that the RNA-protein interactions involved in the above mentioned processes occur in a highly specific manner. In order to form a specific RNA-protein complex, a specialized peptide motif must correctly recognize a specific target site in an RNA molecule. In this chapter, a brief summary of domains commonly found in RNA-binding proteins and several RNA motifs serving as specific binding sites for proteins will be described first. Secondly, proteins involved in endonucleolytic cleavage of RNA and their target sites in RNA will be discussed.

2. RNA binding proteins.

A wide range of RNA binding motifs has been found to date, and new RNA binding folds keep appearing. Since the topic has been extensively reviewed (Burd and Dreyfuss, 1994; Siomi and Dreyfuss, 1997; Cusack, 1999), only a few examples of the most common RNA binding motifs will be presented here.

The most widely found and best-characterized RNA-binding motif is the *ribonucleoprotein* (RNP) motif, also known as *RNA-binding domain* (RBD) or *RNA-recognition motif* (RRM), and it is present in many proteins involved in RNA processing and transport. Each RNP motif contains two conserved sequence motifs designated RNP1 and RNP2 that are part of the RNA-binding surface of this domain. The general pattern found in RNP motif structures is $\beta\alpha\beta\beta\alpha\beta$, which forms a large four-stranded antiparallel β -sheet packed against the two α -helices, which are perpendicularly oriented (Fig. 2). The conserved RNP1 and RNP2 sequences are located in the two central β -strands with the side chains of the conserved aromatic amino acids displayed on the surface. The crystal structure of a complex between the RNP domain of the U1A protein and a stem-loop structure from U1 snRNA was determined at a resolution of 1.92 Angstrom and provides a detailed view of the mode of specific RNA recognition by this motif (Oubridge *et al.*, 1994). The loop between the second and the third β strand is rich in basic residues and plays a critical role in specific binding by protruding into the RNA loop, while the β -sheet forms a non-sequence specific RNA binding platform. The nucleotides in the U1 snRNA loop, which can normally adopt multiple conformations through the formation of non-Watson-Crick base pairings, become ordered upon complex formation and are made available for specific interactions with amino acids from the β strands of the RNP domain. Thus, the observed structural adaptation of these nucleotides upon binding to the protein can be seen as an example of an induced-fit mechanism.

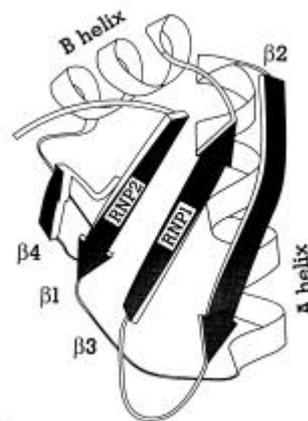


Figure 2. The typical fold of the ribonucleoprotein (RNP) motif. The two perpendicularly oriented α -helices A and B are indicated in white. The four β -strands are numbered and colored black; the conserved RNP1 and RNP2 sequences are indicated. Adapted with permission from Jessen *et al.* (1991).

Apparently, the RNP motif employs a β -sheet as a surface for RNA binding and stacking interactions between nucleotide bases and aromatic side chains on the β -sheet play an important role in stabilizing the complex. The use of β -sheets in RNA-protein complexes is a common theme seen in many RNA binding proteins. This is probably due to the fact that both the major and the minor grooves of helical RNA are too narrow to form a suitable surface for specific interactions with α -helices (a situation which is dramatically different from the smooth contact between α -helices and the major groove of DNA). Clearly, however, this is not a universal theme since in several RNA binding motifs α -helices also appear to have a major role in RNA binding.

One of these is the *double-stranded RNA binding domain* (dsRBD), a 65 amino acid motif that is formed in a variety of proteins that interact specifically with double-stranded RNA, but in a sequence-independent manner. Examples of dsRNA-binding proteins are the *Drosophila* Staufen protein (Bycroft *et al.*, 1995) and *Escherichia coli* RNase III (Kharrat *et al.*, 1995). How does this motif discriminate between dsRNA and DNA? Double-stranded RNA is distinguished from DNA by the presence of the ribose 2' hydroxyl group and by the fact that the extended RNA double helix is predominantly A-form, whereas DNA is generally B-form with significantly different helical parameters and groove dimensions. These general features permit nonspecific dsRNA binding proteins to recognize their correct nucleic acid substrate. The basic fold of the dsRBD is $\alpha\beta\beta\beta\alpha$, in which the three antiparallel β -strands pack to form two α -helices on one face (Fig. 3). Thus, the dsRBD shows some resemblance to the RNP domain. Its mode of binding to RNA is significantly different, however, since mutational analyses have indicated that the highly conserved basic residues at the amino terminus of the second α helix may directly interact with RNA and that dsRNA binds to one face of the domain, a cleft between the amino terminus of the second helix and one face of the second β strand.

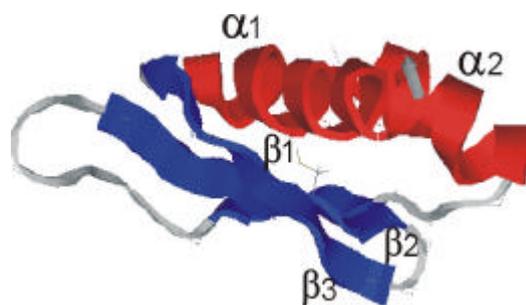


Figure 3. The typical structure of the double-stranded RNA-binding domain (dsRBD). The three antiparallel β -strands and the two α -helices are indicated. Adapted from the Protein Data Bank (www.rcsb.org/pdb/) (Berman *et al.*, 2000).

Another motif in which α -helices play an important role in RNA binding is the *KH domain* (for hnRNP K homology domain) that was first identified in the human hnRNP K protein (Siomi *et al.*, 1993). The KH domain is found in a wide variety of RNA-binding proteins, including ribosomal S3 proteins from divergent organisms such as archaeobacteria, the yeast alternative splicing factor Mer1p, and numerous human RNA-binding proteins, suggesting that it is an ancient protein structure with important cellular functions (Siomi *et al.*, 1993; Gibson *et al.*, 1993). The KH domain is comprised of about 60 amino acids and its typical pattern is $\beta\alpha\beta\beta\alpha$. Insight into the three-dimensional structure has come from NMR spectroscopy analysis of one of the KH domains of human vigilin (Musco *et al.*, 1996). The β -strands form a three-stranded antiparallel β -sheet which is packed against the three α -helices on one face of the β -sheet (Fig. 4). Sequence conservation and UV crosslinking experiments suggest that the helical side of the KH domain interacts with RNA. The loop between the first two α -helices contains the strongly conserved tetrapeptide Gly-X-X-Gly (in which X varies for different KH domains, but is often a positively charged amino acid) and plays an important role in RNA binding. This loop may penetrate the widened groove or a single-stranded loop of a target site in RNA and, because of steric hindrance, the glycines could therefore not be substituted by residues that contain large side chains.

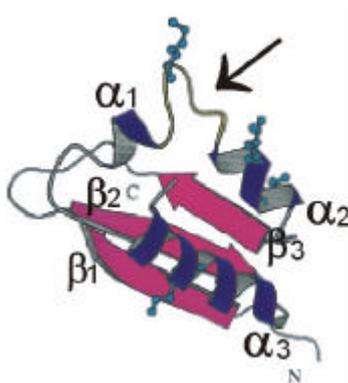


Figure 4. The solution structure of one of the human vigilin KH domains. The strongly conserved Gly-X-X-Gly loop between helices α_1 and α_2 is indicated by an arrow, and the conserved positively charged residues which are likely to interact with RNA are drawn as ball-and stick models. The three α -helices and the three β -sheets are indicated. Adapted with permission from Musco *et al.* (1996).

The *arginine-rich motif* (ARM) is a short module of 10 to 20 amino acids which is common in viral, bacteriophage and ribosomal proteins. The ARM is sufficient for specific binding to RNA target sites, but amino acids located outside the motif also contribute to binding (Tan *et al.*, 1993). Other than the abundance of arginine, there is little resemblance between different ARM sequences. Detailed structural information is available for the ARM regions of two HIV-encoded RNA-binding proteins, Rev and Tat. The structures of these regions appear to differ significantly, suggesting that most ARM proteins do not share a common structure (Fig. 5A and B). Rev is a regulatory RNA-binding protein that facilitates the export of unspliced HIV pre-mRNAs from the nucleus and mediates its function by binding to an internal loop designated the Rev responsive element (RRE) (Zapp *et al.*, 1991). Peptides encompassing the Rev ARM of 17 amino acids specifically bind the RRE as

an α -helix and at least six amino acids, including four arginines, are essential for specificity (Tan *et al.*, 1993). Battiste *et al.* (1996) solved the solution structure of the Rev-RRE complex by NMR spectroscopy (Fig. 5A). Several arginine side chains were found to make base-specific contacts, and an asparagine residue contacts an unusual G-A base-pair in the loop. Interestingly, a non-Watson-Crick G-G base-pair in the loop, which is not in direct contact with the peptide through hydrogen bonds, plays a critical role in the interaction. This base-pair, which can be replaced by an isosteric A-A pair (Bartel *et al.*, 1991), creates an important structural feature for specific binding. Thus, RNA structure plays an important role in the Rev-RRE interaction.

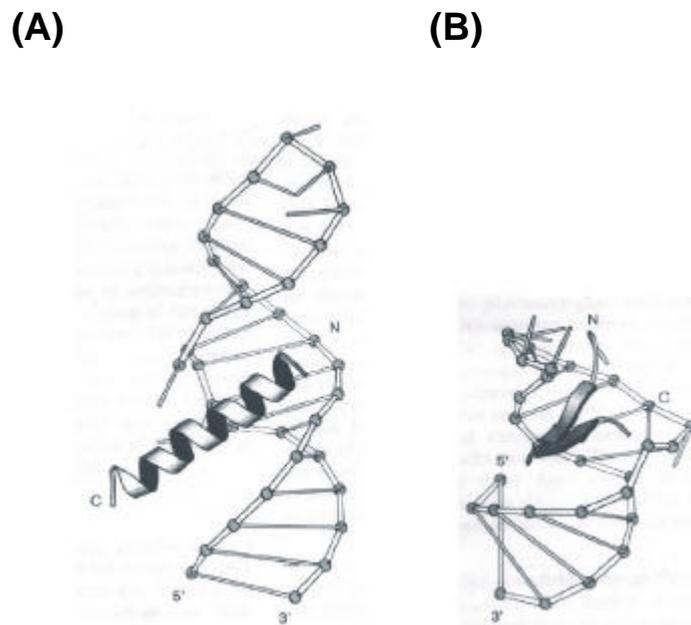


Figure 5. (A) A peptide derived from the HIV-1 Rev protein in complex with the HIV-1 Rev-responsive element (RRE) RNA. (B) A peptide derived from the BIV Tat protein in complex with the BIV TAR RNA. Adapted with permission from Ramos *et al.* (1997).

In contrast to the Rev ARM, the Tat ARM peptides are unstructured but adopt a stable conformation upon binding to TAR (transacting region) (Calnan *et al.*, 1991). This conformation appears to be a two-stranded antiparallel β -sheet, in contrast to the α -helical structure of the Rev ARM (Fig. 5B) (Puglisi *et al.*, 1995). The RNA target site of Tat contains a three nucleotide 5'-UCU-3' bulge that is part of the binding site for the Tat protein. Upon binding of Tat, a conformational change in the bulge region of TAR occurs, which allows the formation of a specific binding pocket. This indicates that also in the case of the Tat-TAR interaction, the structure of the RNA binding site rather than its particular sequence may be the major binding determinant.

The above examples, of the Rev-RRE and the Tat-TAR interaction, illustrate the importance of RNA structure to provide specific recognition surfaces for RNA-binding motifs. In the next paragraph, a brief overview of how RNA molecules create specific recognition sites through folding will be presented.

3. RNA structure: the formation of specific recognition sites.

Unlike DNA, all RNAs (except in double-stranded RNA viruses) are synthesized as single strands without a complementary second strand. However, RNAs do not exist as unstructured single-stranded molecules, but fold into secondary structures in which regions of Watson-Crick base-pairing are interrupted by single-stranded regions forming loops, mismatches, bulges, and helix junctions (Fig. 6). It is common for an RNA sequence to be presented in the form of a possible secondary structure, usually predicted by computer algorithms that are based on the assumption that RNA molecules adopt their thermodynamically most stable structure out of a number of alternatives that are energetically less favorable. However, since the structure adopted by an RNA molecule *in vivo* may be altered by the binding of proteins or other ligands, more recent algorithms also take the suboptimal structures into account (Zuker, 1989).

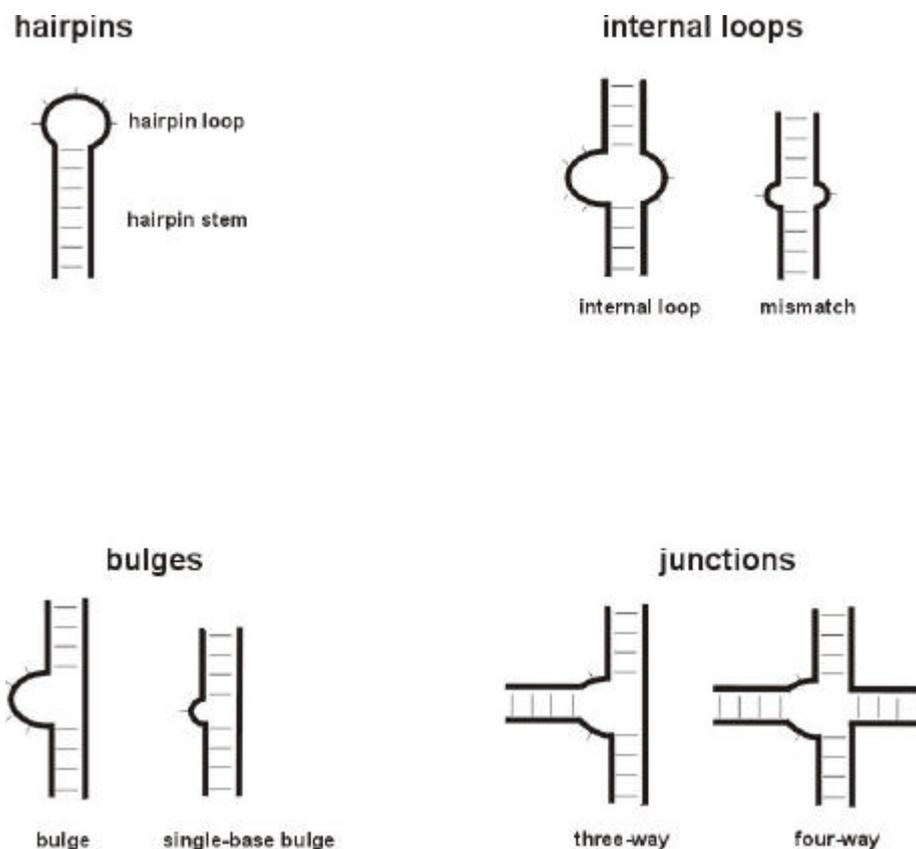


Figure 6. The various secondary structural elements of RNA.

How do RNA molecules form specific binding sites for proteins? As mentioned above, dsRNA differs from DNA in its helical parameters and groove dimensions. Undistorted A-form dsRNA is characterized by a deep and narrow major groove and a shallower minor groove, in contrast with B-form DNA, which has an easily accessible major groove (Fig. 7).

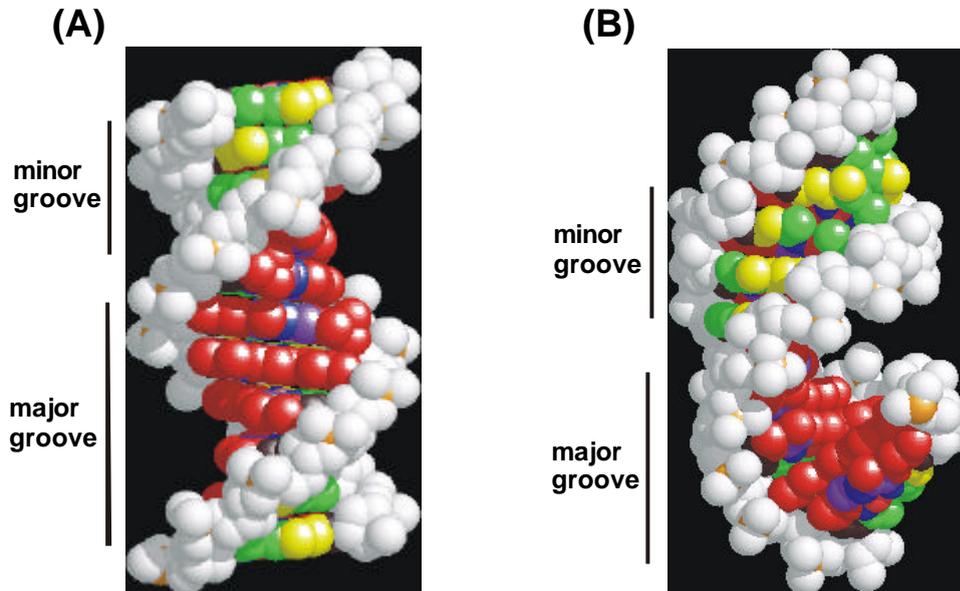


Figure 7. (A) Structure of a B-form DNA helix. The narrow minor groove and the easily accessible major groove are indicated by lines on the left. (B) Structure of an A-form RNA double helical fragment. The deep, narrow major groove and the wide, shallow minor groove are indicated. (adapted with permission from Dr. Kahn; http://www.chem.umd.edu/biochem/kahn/teach_res/dna_tutorial/)

As a consequence, the bases in the major groove of dsRNA are not well accessible for specific recognition by hydrogen bonding, and the readily accessible minor groove contains less information for base discrimination (varying only by the presence or absence of the exocyclic N2 of guanine). Thus, although the helix structure of dsRNA is the determinant that is used by non-sequence-specific dsRNA-binding proteins to distinguish it from DNA, it is not suitable for recognition by sequence-specific RNA-binding proteins. As a consequence, these proteins must use the single-stranded regions for specific binding. In fact, these ‘single-stranded’ regions are often structured through the formation of ‘non-Watson-Crick’ base-pairing interactions. In addition to the classical A-U and G-C Watson-Crick base-pairs, an extensive list of non-Watson-Crick base-pairs has been identified in NMR and crystallographic studies, and new types of base-pairs are still being discovered (reviewed by Schuster *et al.* (1997); Westhof and Fritsch (2000)). In fact, almost any type of base-pair can occur, including A-A, G-A, and U-U pairs. Such non-Watson-Crick base-pairs are commonly formed in RNA loops, creating a helical geometry. In contrast to a continuous Watson-Crick helix, however, in which the consecutive base-pairs are isosteric and form

independently of their neighboring base-pairs, these non-Watson-Crick helices have irregular shapes that depend on the combination of base-pairs. The specific irregular structures formed by these non-Watson-Crick base-pairs play an important role in the formation of specific recognition sites for proteins, as illustrated by the examples of the Rev-RRE and the Tat-TAR interaction (see above). Another classical example of how non-Watson-Crick base-pairs can produce a unique RNA shape suitable for recognition by proteins is the loop E of 5S ribosomal RNA, which is present both in prokaryotes as well as in eukaryotes. The loop E is a purine-rich internal loop that serves as a binding site for the ribosomal L25 protein (Douthwaite *et al.*, 1979). It was originally shown that the loop adopts a structured geometry in the presence of magnesium ions (Leontis *et al.*, 1986; Romby *et al.*, 1988). Later, NMR evidence indicated the presence of a non-Watson-Crick A-U and a G-A pair in the loop E of eukaryotic 5S rRNA (Wimberly *et al.*, 1993). Correll *et al.* (1997) determined the crystal structure of the *E. coli* loop E at 1.5 Angstrom resolution, and also observed the non-Watson-Crick A-U followed by G-A, and called this motif a ‘cross-strand A stack’ because the A residues, which come from opposite strands, stack on each other. This motif was found to occur three times in the loop E region, significantly altering the shape of both the major and minor groove; the minor groove obtains a unique hydrogen-bonding surface and the adjacent major groove is made wide enough to allow recognition by the ribosomal protein L25.

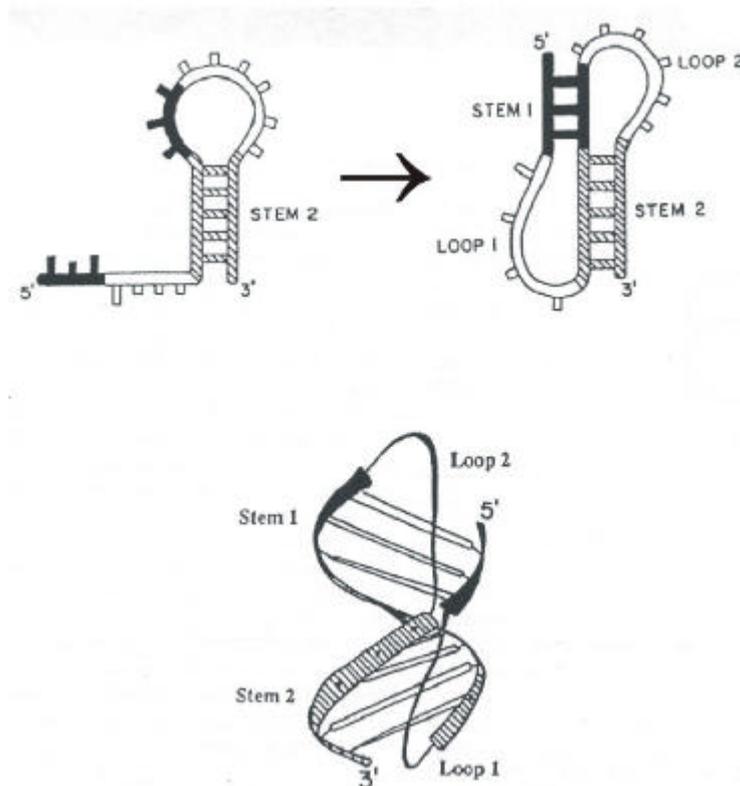


Figure 8. Folding of the classic RNA pseudoknot. Top: stems 1 and 2 are indicated by solid and hatched regions, respectively; loop 1 and 2 are indicated in white. Bottom: The three-dimensional structure of a pseudoknot with a continuous stacked structure for the two stems. Loop 1 crosses the major groove of stem 2, and loop 2 crosses the minor groove of stem 1.

Another important structural motif in RNA that can serve as a specific binding site for proteins is the pseudoknot (for a review, see Pleij (1995)). An RNA pseudoknot forms upon Watson-Crick base-pairing of a loop region with a complementary sequence outside the loop. That complementary sequence can be located in an unconstrained single-stranded region or in another loop. Pleij *et al.* (1985) proposed a model of the ‘classic’ pseudoknot which is characterized by two stem regions S1 and S2 and two loop regions L1 and L2 (Fig. 8). The two stem regions coaxially stack to form a continuous helical region, and the two loops L1 and L2 cross the deep major groove and the shallow minor groove in the helix, respectively. Several recent studies describe specific interactions between RNA pseudoknots and proteins. Detailed information is available of the interaction of the ribosomal protein S15 from *E. coli* and a pseudoknot in its own mRNA, an essential step in the translational autoregulation of S15 (Benard *et al.*, 1994). Two adjacent base-pairs, U-G/C-G, in the center of stem S1 of the pseudoknot are essential for S15 autoregulation and are presumably directly recognized by the protein (Benard *et al.*, 1998). A pseudoknot RNA aptamer was found to bind with high affinity to HIV-1 reverse transcriptase (Kensch *et al.*, 2000). The interaction was highly specific, since the closely related HIV-2 reverse transcriptase showed an almost four times lower binding affinity. Gilley and Blackburn (1999) identified an important biological role of a pseudoknot in the RNA component of telomerase in *Tetrahymena thermophila*. The pseudoknot was found to be critical for the stable association of the RNA with the catalytic reverse transcriptase component of telomerase.

In summary, it appears that RNA molecules can form specific three-dimensional interfaces for interaction with peptide motifs. The specific nucleotide composition of RNA loops determines their three dimensional configuration due to the interdependence of the individual nucleotide interactions. Thus, it is not so straightforward to distinguish between sequence- or structure recognition since there is a very close coupling between the presence of specific nucleotides in the RNA loop and the formation of a distinctive geometry that can be recognized by an RNA binding protein.

4. Endonucleolytic cleavage of RNA.

Endonucleolytic cleavage of RNA is a widespread phenomenon and plays a major role in RNA biogenesis and metabolism. Processes in which endonucleolytic cleavage occurs include pre-mRNA splicing and 3' end formation, maturation of small nucleolar (sno)RNAs, tRNAs and ribosomal RNAs, and mRNA turnover. A variety of endoribonucleases that are involved in these processes have been described. These enzymes differ among each other in their specificities and modes of substrate recognition.

4.1. Endoribonucleases.

A host of enzymes that hydrolyze phosphodiester bonds in RNA have been described to date. These enzymes can be classified into two large groups: (1) the exoribonucleases, which progressively cleave the bonds between consecutive nucleotides, starting at either the 5'- or the 3' end of an RNA chain, and (2) the endoribonucleases, which can cleave internal bonds in an RNA strand. In contrast to the exonucleases, which generally do not recognize specific RNA targets but degrade any RNA that is single-stranded, the endonucleases differ greatly among each other in their individual substrate specificities. Also the features recognized by these enzymes are different. The simplest modes of recognition are employed by the RNases T2 and V1, which cleave without any sequence specificity the bonds between nucleotides in a single- or double-stranded configuration, respectively. Examples of RNases that show a limited degree of sequence specificity are RNase A and RNase T1, which cleave 3' to single-stranded pyrimidines and G-residues, respectively. More complex recognition determinants are required for cleavage by the bacterial RNase E, which plays an important role in rRNA processing, mRNA degradation and regulation of plasmid replication. The consensus recognition site for this endoribonuclease is 5'-A/GAUUA/U-3' in a single-stranded environment usually flanked by a stem-loop structure (Ehretsmann *et al.*, 1992). Cleavage can occur at multiple different sites within this sequence, depending on the RNA molecule in which the sequence is located. A human homolog of RNase E was found that appeared to have a similar substrate specificity (Wennborg *et al.*, 1995). Later, Claverie-Martin *et al.* (1997) identified a similar activity and designated it ARD-1. These enzymes have the ability to cleave mRNAs in the well-described AU-rich instability elements, characterized by the occurrence of the 5'-AUUUA-3' motif.

An interesting and extensively studied group of endoribonucleases, exhibiting a very high degree of substrate specificity, is the group of endonucleases involved in eukaryotic tRNA maturation (a similar set of pre-tRNA processing endonucleases is present in prokaryotes). This group comprises three enzymes: (1) RNase P, which is responsible for the removal of the 5' leader of pre-tRNAs, (2) the 3' processing endonuclease which removes the 3' trailer of pre-tRNAs, and (3) the tRNA splicing endonuclease, which carries out the excision of an intron present in some (but not all) tRNA precursors. These three endonucleases, which differ significantly among each other (RNase P is a ribozyme in contrast to the other two enzymes), use a common tripartite set of substrate features for recognition. First, all three enzymes recognize determinants in the L-shaped mature tRNA domain, which is highly conserved and common for all tRNAs. This enables these enzymes to recognize all different tRNAs irrespective of their individual differences. Second, the enzymes 'measure' the distance from the binding site in the mature tRNA domain to their respective cleavage sites, determined by the length of the acceptor stem for RNase P, the anticodon stem for the intron endonuclease, and both the acceptor stem and the T stem for the 3' processing endonuclease (Altman, 2000; Kurz and Fierke, 2000; Phizicky and Greer, 1993; Nashimoto *et al.*, 1999a). Third, specific interactions with nucleotides in the proximity of the cleavage site provide an additional level of specificity for all three enzymes. The intron endonuclease requires a pyrimidine to be located 5' to the 5'

splice site and the 3' splice site to be located in a three nucleotide bulge loop (Di Nicola *et al.*, 1997). The 3' processing endonuclease interacts with the 3' trailer in which the identity of the nucleotide 3' to the cleavage site affects cleavage efficiency in the order (G~A>U>C) (Nashimoto *et al.*, 1999a; Nashimoto *et al.*, 1999b). A recent study by Ziehler *et al.* (2000) shows that the single-stranded 3' terminus also plays a substantial role in substrate binding by RNase P.

The above-described endoribonucleases are only a few examples of the extensive list of known endonucleases generally involved in RNA metabolism. As mentioned, the bacterial RNase E and its human homologs play a role in both RNA processing and mRNA turnover by cleaving AU-rich elements in mRNA molecules. The next paragraph will focus on specific endoribonucleases and their target sites in mRNAs involved in mRNA degradation.

4.2. Endonucleolytic cleavage of mRNA-regulation of mRNA stability

To date, a number of mRNAs have been described to be subject to endonucleolytic cleavage. In general, cleavage is thought to be the rate-limiting step in the degradation of a target message, although not necessarily the first step. A summary of the various systems described to date, and the differences and similarities among these different systems will be discussed in the next paragraph. First, two systems in which a group of mRNAs appears to be coordinately regulated by a common endoribonuclease activity will be described; second, a number of individual messengers that have been found to be targets for specific cleavage will be discussed.

4.2.1. Estrogen-regulated mRNAs.

An interesting example of selective stabilization or destabilization of mRNAs by environmental stimuli is the regulated turnover of specific mRNAs in response to estrogen stimulation. One system in which this occurs is the avian liver in which apolipoprotein (apo)II mRNA and vitellogenin II mRNA are relatively stable with a half-life of 13 hours in the presence of estrogen. After estrogen withdrawal, the half-life of these mRNAs is reduced to 1.5 hours. This suggests that estrogen either directly or indirectly represses an activity that selectively destabilizes apoII and vitellogenin II mRNAs upon hormone withdrawal (Gordon *et al.*, 1988). Subsequently, specific degradation intermediates were found for the apoII message that appeared to result from endonucleolytic cleavages at a number of sites flanked by 5'-AAU-3' or 5'-UAA-3' sequences in the 3'-UTR (Binder *et al.*, 1989). A correlation between destabilization of the apoII mRNA and an increase in the abundance of the cleavage products, but no difference in the poly(A) tail length was observed, indicating that endonucleolytic cleavage may be the first and rate-limiting step in the degradation of this message.

Also in the *Xenopus* liver, estrogen causes the stabilization of vitellogenin (Nielsen and Shapiro, 1990). In contrast, the mRNAs encoding the major serum proteins including albumin, transferrin and the fibrinogens are coordinately

destabilized by estrogen (Schoenberg *et al.*, 1989; Pastori *et al.*, 1990; Pastori *et al.*, 1991a). Pastori *et al.* (1991b) describe an estrogen-inducible endoribonuclease activity that may be responsible for this selective destabilization, since it degrades albumin mRNA 4 times faster *in vitro* than ferritin mRNA. Although the activity is present in a polysome extract, destabilization of albumin mRNA appears to be independent of translation (Moskaitis *et al.*, 1991). An RNase consisting of two isoforms of molecular mass 62 and 64 kDa was purified (Dompenciel *et al.*, 1995) which shares many of the features with the activity present in crude polysome extract, including selectivity for albumin versus ferritin mRNA, resistance to inhibition by EDTA or placental RNase inhibitor and an independence of divalent cations for activity. Subsequent cloning of the gene identified the protein as a novel member of the peroxidase family termed polysomal RNase 1, or PMR-1 (Chernokalskaya *et al.*, 1998).

Preferential cleavage sites for PMR-1 are located in the 5' half of the albumin mRNA in the sequence 5'-AU↓UGAC↓UGA-3'. This sequence can be seen as two overlapping copies of the pentamer ApyrUGA, which is present in multiple copies in mRNAs that are destabilized in response to estrogen stimulation; 14 copies are found in albumin mRNA, 9 in transferrin- and 7 in γ -fibrinogen mRNA. However, this element is absent from ferritin mRNA, which is not a target for PMR-1. Structural studies have indicated that cleavage requires the pentamer to be in a single-stranded configuration (Chernokalskaya *et al.*, 1997). Interestingly, the element is also present in the vitellogenin mRNA and is the specific binding site for an estrogen-inducible protein (Dodson and Shapiro, 1994). As a consequence, binding of this protein may protect the RNA from cleavage by PMR-1, resulting in the specific stabilization of this RNA in the presence of estrogen.

4.2.2. Interferon/ RNase L-regulated mRNAs.

In response to interferon stimulation, an RNA degradation pathway can be activated, which is responsible for many of the antiviral and antiproliferative/pro-apoptotic effects of interferons (Sen and Lengyel, 1992). This pathway is known as the 2-5A system, which is comprised of two major enzymatic components: (1) 2-5A synthetase which polymerizes ATP into 5'-phosphorylated, 2',5'-linked oligoadenylates in the presence of double-stranded RNA, and (2) RNase L which is activated upon binding to 2-5A (Dong and Silverman, 1995). Upon activation, RNase L cleaves RNA 3' of UpNp sequences (Floyd-Smith *et al.*, 1981). In addition to viral RNAs, which are targeted for degradation by RNase L in virus-infected cells, also cellular RNA substrates in the absence of virus infection have recently been identified. An mRNA encoding a 43 kDa ubiquitin-specific protease, designated ISG43 and a transcript encoding ISG15 were found to be selectively destabilized by RNase L in fibroblasts (Li *et al.*, 2000) and RNase L dependent degradation of MyoD mRNA has been observed in mouse myoblasts (Bisbal *et al.*, 2000).

Thus, both estrogen and interferon activate signaling pathways that lead to the selective destabilization or stabilization of a subset of mRNAs by regulating a specific endoribonuclease activity. These two systems represent examples of regulatory networks where groups of genes can be coordinately regulated at the post-

transcriptional level by a common endoribonuclease. In addition to the above-described systems, a number of individual mRNAs have been described to be targets for specific cleavage.

4.2.3. *c-myc* mRNA.

The human *c-myc* mRNA is a very labile messenger with a half-life of about 30 minutes and is an intriguing and a very complex example of modulation of mRNA turnover in response to physiological agents. It has been shown that two separated decay pathways exist for *c-myc* mRNA. In one pathway, 3'-UTR sequences containing two copies of the AUUUA motif stimulate poly(A) shortening followed by 3' to 5' exonucleolytic degradation of the message (Jones and Cole, 1987). In addition to the AUUUA motifs, other segments in the 3'-UTR may also contribute to the rapid decay (Bonnieu *et al.*, 1990). The second pathway involves destabilization through a region in the C-terminal part of the coding region from positions 1705 to 1886 encoding *c-myc* amino acids 335-439, referred to as the Coding Region mRNA stability Determinant or CRD (Swartwout and Kinniburgh, 1989; Wisdom and Lee, 1991). This region can confer instability on a heterologous mRNA independently of the 3'-UTR elements, indicating that it contains independently destabilizing sequences. The destabilizing effect of the CRD is coupled to translation, since cycloheximide or the introduction of an upstream stopcodon stabilizes mRNA that contains the CRD (Wisdom and Lee, 1991; Herrick and Ross, 1994). Ioannidis *et al.* (1996) provided direct evidence that the CRD is a target for endonucleolytic cleavage by the observation of truncated *c-myc* RNA species. The CRD is also a binding site for a protein that inhibits degradation, presumably by shielding the RNA from endonuclease attack (Bernstein *et al.*, 1992; Herrick and Ross, 1994; Prokipcak *et al.*, 1994). This binding protein is implicated in the developmental regulation of *c-myc* expression (Leeds *et al.*, 1997).

Recently, an endoribonuclease with the properties of the *c-myc* mRNAse has been partially purified (Lee *et al.*, 1998). The enzyme is associated with polysomes, consistent with the requirement of ongoing translation for cleavage. The estimated size of the protein is 39 kDa and the major cleavage sites reside within a 10 nt segment between nucleotides 1727 and 1736 with the sequence 5'-C↓AAUG↓AAAAG-3'. This sequence slightly resembles the ApyrUGA recognition motif for the albumin endonuclease PMR-1, which is also polysome-associated (see above). However, the endoribonuclease activity responsible for cleavage of *c-myc* mRNA is probably distinct from the albumin nuclease, since this enzyme is considerably larger (62-64 kDa) and is independent of divalent cations in contrast to the *c-myc* endonuclease, which is inactive in the absence of magnesium (Lee *et al.*, 1998). In addition, cleavage of the *c-myc* mRNA is dependent on translation, which is not the case for the albumin mRNA, a target of PMR-1 (Moskaitis *et al.*, 1991).

4.2.4. *Xlhbox2* mRNA.

Xlhbox2 is a homeobox gene of the *Antennapedia* class whose transcripts are found in *Xenopus* oocytes, eggs and embryos (Muller *et al.*, 1984). In contrast to most mRNAs, which are very stable in *Xenopus* oocytes, the transcripts from the homeobox gene *Xlhbox2* are specifically destabilized during development, suggesting that these mRNAs are targets for a specific nuclease (Wright *et al.*, 1987). Brown and Harland (1990) demonstrated that the *Xlhbox2* mRNA is endonucleolytically cleaved at 4 sites in a 90 nucleotides region in the 3'-UTR close to the translation stopcodon; the cleavage sites are flanked by 5'-ACCU-3' repeats and must be in a single-stranded configuration to allow cleavage. Subsequently, it was shown that a single copy of the sequence 5'-CCUACCUACCCACCUA-3' is sufficient for cleavage of a heterologous β -globin mRNA and reduces its half-life from 57 to 15 minutes (Brown *et al.*, 1993). Furthermore, it was established that cleavage is independent of translation since global inhibition of translation using cycloheximide or message-specific translation inhibition by injecting uncapped RNAs in the oocytes did not affect cleavage.

Brown *et al.* (1993) also showed that *Xenopus* oocytes contain a specific RNA-binding protein that protects the *Xlhbox2* mRNA from cleavage by binding to the cleavage site. The protein is regulated in a development-specific manner and may thus account for the developmental regulation of *Xlhbox2* mRNA stability. This provides a striking similarity with the *c-myc* system, where a protective binding protein is also implicated in the development-specific expression of *c-myc* by modulating cleavage of its mRNA (Leeds *et al.*, 1997). Brown *et al.* (1993) also identified another *Xenopus* gene named *Xool* of which the transcript can be cleaved in a similar target sequence as *Xlhbox2* mRNA in oocytes, suggesting that at least these two (and potentially more) different transcripts can be controlled by the same endoribonuclease in this system. Partial purification of the endonuclease activity revealed that the protein is about 123 kDa in size and is resistant to pancreatic RNase inhibitors (Brown *et al.*, 1993).

4.2.5. Interleukin-2 (IL-2) mRNA.

The cytokine interleukin-2 (IL-2) is expressed in human T lymphocytes, and its mRNA normally has a relatively short half-life of about 30 minutes. Stimulation of the CD28 surface antigen, however, leads to the stabilization of IL-2 mRNA (Lindstein *et al.*, 1989). Hua *et al.* (1993) have identified an endonuclease activity in cytosolic extract from the T cell line Jurkat that selectively cleaves IL-2 RNA at several sites in the C-terminal part of the coding region. Initial characterization revealed that the enzyme is 60 to 70 kDa in size, requires magnesium for optimal activity, and is insensitive to placental RNase inhibitor. More recently, a specific binding protein of 50–60 kDa was identified that protects IL-2 mRNA against degradation (Hua and Paetkau, 1996). Thus, also the IL-2 mRNA is degraded through endonucleolytic cleavage, which may be regulated by a protective binding protein.

4.2.6. 9E3 mRNA.

The 9E3 (or CEF-4) gene is expressed in chicken embryo fibroblasts (CEF) and encodes a secreted 6 kDa protein that is related to platelet growth factor 4 and to a number of other inducible proteins that are thought to be inflammatory mediators (Kawahara and Deuel, 1989). In CEF infected with a temperature-sensitive strain of Rous sarcoma virus (RSV), 9E3 mRNA is more stable at a permissive temperature than at a nonpermissive temperature, suggesting that the 9E3 mRNA is stabilized in RSV-transformed CEF (Bedard *et al.*, 1987). Evidence that 9E3 mRNA stability may be regulated by endonucleolytic cleavage in the 3'-UTR was provided by Stoeckle and Hanafusa (1989) who observed small RNA species that appeared to be degradation intermediates of 9E3 mRNA. The relative amount of the small RNAs was increased under conditions in which 9E3 mRNA was less stable, suggesting that increased cleavage activity may account for the destabilization of the 9E3 messenger. The cleavage site was roughly mapped to a region in the 3'-UTR 360 nt downstream of the stopcodon from positions 760 to 781 within the sequence 5'-GUAAUCCUCC UGCUCCCUGG-3'. The determined half-life of the 9E3 transcript was 8 hours in RSV-transformed CEF and 1 to 2 hours in untransformed cells.

For some mRNAs that are related to 9E3, degradation intermediates have been described, suggesting that these RNAs may also be subject to endonucleolytic cleavage. It was reported that several minor species of MONAP mRNA, another member of the platelet growth factor 4 family, exist; most of these small RNAs lack a poly(A) tail and were proposed to be degradation intermediates (Kowalski and Denhardt, 1989). However, for MONAP mRNA, cleavage was suggested to occur at AUUUA sites, which is not the case for 9E3 mRNA. Also for the *gro* gene, a mammalian homolog of 9E3, minor RNA species lacking 3' terminal sequences have been detected (Anisowicz *et al.*, 1987; Anisowicz *et al.*, 1988).

4.2.7. *Groa* mRNA.

Groa is an inflammatory cytokine gene that is also known as melanoma growth stimulatory activity (MGSA). It is a member of a large superfamily of inducible cytokine genes which encode secreted peptides structurally related to platelet factor 4 and β -thromboglobulin (Arraiano *et al.*, 1997).

It has been shown that interleukin-1 (IL-1) regulates *groa* and related genes (*grob*, *grog*, and IL-8) at the level of mRNA stability (Arnold *et al.*, 1998). The *gro* mRNAs were stable in the presence of IL-1, with a half-life of 8 hours, and were destabilized to a half-life of 1 hour in the absence of IL-1. Withdrawal of interleukin-1 was found to be associated with the increased abundance of a smaller RNA species of 0.9 kb derived from the full-length 1.3 kb *groa* mRNA that lacks a poly(A) tail (Stoeckle, 1992). It was shown that this shorter RNA species is formed by removal of a 130 nt sequence from the 3'-UTR. The 3' end of the 0.9 kb *groa* RNA maps to a region around position 970 in the 3'-UTR, 130 nt upstream of the poly(A) site with the sequence 5'-AAUAU-3'. This sequence is presumably a target site for an endonuclease, although exonuclease trimming cannot be excluded since no 3' cleavage

product has been detected. Furthermore, it was shown that degradation of *groa* RNA is associated with poly(A) tail shortening and requires ongoing translation. A coupling between endonucleolytic cleavage and deadenylation has been observed for two other mRNAs: the yeast *PGK1* mRNA, and the human α -globin mRNA.

4.2.8. *PGK1* mRNA.

The half-life of the yeast phosphoglycerate kinase (*PGK1*) mRNA can be increased about twofold by insertion of an 18 nucleotides long poly(G) stretch in the 3'-UTR (Vreken *et al.*, 1991). This insertion also caused the accumulation of a degradation intermediate extending from the poly(G) stretch to the transcription termination site. In a later report, Vreken and Raue (1992) showed that the poly(G) stretch stabilizes the RNA by interfering with endonucleolytic cleavage of the *PGK1* mRNA at two upstream sites between positions 1100 and 1200 near the 3' end (position 1251) of the coding region. Presumably, the poly(G) stretch inhibits cleavage by altering the RNA structure around the cleavage site and causes the accumulation of the 3' cleavage product by protecting it against 5' to 3' exonucleolytic degradation. Cleavage was found to occur in the sequence 5'-GGU↓G-3', and is the rate-limiting step in the degradation of *PGK1* mRNA, although not the first step; cleavage occurs after removal of the poly(A) tail.

4.2.9. α -globin mRNA.

The α -globin mRNA is very stable, which is conferred by a cytosine-rich element (CRE) in the 3'-UTR that forms an mRNP complex including the poly(C) binding proteins α CP1 and α CP2 (α -complex) (Wang *et al.*, 1995; Weiss and Liebhaber, 1995). The α -complex was found to stabilize the mRNA by an interaction with the poly(A) binding protein to slow the rate of deadenylation (Wang *et al.*, 1999). In addition to impeding deadenylation, the α -complex also protects the α -globin mRNA from cleavage by a specific endoribonuclease activity (Wang and Kiledjian, 2000a) that is enriched in erythroid cells. In agreement with the observation that the α -complex inhibits cleavage, the cleavage site was mapped to a position 63 nucleotides downstream of the translation stopcodon and 47 nucleotides upstream of the poly(A) site in a region protected by the complex. Endonucleolytic cleavage occurs in the sequence 5'-UCC↓UUG-3', and these specific nucleotides around the cleavage site are important for recognition, since cleavage is abolished when this sequence is mutated to 5'-GAGAGA-3'. A fragment of 45 nucleotides containing the cleavage site is sufficient for cleavage of a heterologous mRNA, indicating that all information required for cleavage is contained within this region. Thus, the α -complex stabilizes the mRNA by at least two mechanisms: one is to lower the rate of deadenylation by a stabilizing interaction with PABP, and the other is to protect the RNA from endonucleolytic cleavage. In a subsequent study, Wang and Kiledjian (2000b) demonstrated that both mechanisms are linked to each other, since the interaction between the α -complex and PABP also appears to stabilize the binding of the α -

complex to the CRE, and as a consequence inhibits cleavage. Thus, the α -globin mRNA becomes a more efficient substrate for cleavage upon removal of the poly(A) tail. This provides a mechanistic explanation of the link between deadenylation and endonucleolytic cleavage in the α -globin mRNA, and it is tempting to speculate that similar mechanisms might be operating in the *groa* and the yeast *PGK1* mRNAs described above.

4.2.10. Transferrin receptor mRNA.

The transferrin receptor (TfR) plays an important role in cellular iron uptake and its expression is tightly regulated by iron. Regulation of expression occurs primarily at the post-transcriptional level through modulation of the stability of the TfR mRNA, which displays a relatively short half-life of about 45 minutes when iron is abundant and a relatively long half-life of more than three hours when iron is scarce (Koeller *et al.*, 1991). The modulation of TfR mRNA stability is mediated via a region in the 3'-UTR. This region contains a rapid turnover determinant (Mullner and Kuhn, 1988; Casey *et al.*, 1989) and 5 RNA motifs named iron responsive elements (IREs) which are binding sites for a cytoplasmic IRE binding protein (IRE-BP). A region of 250 nucleotides is sufficient for iron regulation (Casey *et al.*, 1989). Binding of the IRE-BP to the IREs inhibits degradation of the transcript, suggesting that the function of the rapid turnover determinant is masked (Casey *et al.*, 1989). Evidence that the rapid turnover determinant is a target site for endonucleolytic cleavage was presented by Binder *et al.* (1994), who observed the appearance of shorter TfR RNA species upon treatment of cells with an iron source. The cleavage site was mapped to a single-stranded region near one of the iron-responsive elements in the 3'-UTR with the sequence 5'-AUAAG↓ACAAG-3'. Nucleotide substitutions around the cleavage site render the RNA refractory to cleavage, indicating that the identity of specific nucleotides adjacent to the cleavage site is important for recognition by an endoribonuclease. Furthermore, cleavage was found to be independent of poly(A) tail shortening since the 3' cleavage product appears to be polyadenylated to the same extent as the full-length RNA. Interestingly, cleavage of the TfR mRNA requires rather large regions of RNA structure, including a segment located about 300 nucleotides upstream of the cleavage site. This suggests that the endonuclease recognition site in the TfR mRNA is relatively complex in comparison with various other systems where short sequences are sufficient for recognition. Degradation of the TfR transcript was found to require ongoing translation since the translation inhibitors cycloheximide and puromycin led to a stabilization of the RNA. However, this does not reflect a requirement for translation of the TfR mRNA *per se* because the introduction of stable secondary structures in the 5'-UTR did not affect the stability of the message (Koeller *et al.*, 1991). Thus, the ongoing synthesis of a labile endoribonuclease may be required rather than translation of the TfR mRNA itself.

In summary, a growing number of mRNAs are found to be targets for specific endoribonucleases. The various systems differ among each other in their dependence on translation, either because a labile component is involved in degradation or the target message itself must be translated, and poly(A) tail removal. Differences with

respect to the cleavage site requirements are also evident. Populations of mRNAs exist that can be coordinately regulated by a common endoribonuclease, in which cleavage of individual messengers can be modulated by 'protective factors' that mask the endonuclease cleavage site. In fact, regulation of cleavage through protective binding proteins appears to be a common mechanism for most of the systems described. This allows the specific regulation of a large number of mRNAs by a limited set of endoribonucleolytic enzymes.

Table 1

Overview of endonucleolytic cleavage sites in eukaryotic mRNAs

mRNA	number of cleavage sites	positions of cleavage sites	target sequence(s)	regulation by (a) protective factor(s)	references
apoll (human)	~14 sites	region from 472 to 638 in 3'-UTR (stopcodon at position 401)	5'-AAU-3', and 5'-UAA-3'	not determined	(Binder <i>et al.</i> , 1989)
albumin (<i>Xenopus</i> oocytes)	3 major sites, and 2 minor sites	5' region of the mRNA	5'-ApyrUGA-3'	not determined (circumstantial evidence suggests yes)	(Pastori <i>et al.</i> , 1991a; Pastori <i>et al.</i> , 1991b; Chernokalskaya <i>et al.</i> , 1997)
c-myc (human)	2 major sites	3' part of the coding region between nt 1727 and 1736	5'-C↓AAUG↓A AAAG-3'	Yes, CDR-BP	(Herrick and Ross, 1994; Ioannidis <i>et al.</i> , 1996; Lee <i>et al.</i> , 1998; Doyle <i>et al.</i> , 1998)
Xlhxbox2 (<i>Xenopus</i> oocytes)	4 sites	90 nt long region in 3'-UTR close to stopcodon	5'-CACCUACC UACCCAAC/AUACU-3'	yes, unknown protein	(Brown and Harland, 1990; Brown <i>et al.</i> , 1993)
IL-2 (human)	3-4 major sites	Region from positions 400-430 (position stopcodon is 536)	not determined	yes, unknown protein	(Hua <i>et al.</i> , 1993; Hua and Paetkau, 1996)
9E3 (chicken embryo)	1 site	3'-UTR, 360 nt downstream of stopcodon	5'-GUAAUUC UCCUGCUC CCUGG-3'	not determined	(Stoeckle and Hanafusa, 1989)
PGK-1 (yeast)	2 sites	3' part of the coding region between 1100 and 1200 (stopcodon: 1251)	5'-GGU↓G-3'	not determined	(Vreken and Raue, 1992)
groα (human)	1 site	3'-UTR, 130 nt upstream of poly(A) site	5'-AAUUAU-3'	not determined	(Stoeckle, 1991; Stoeckle, 1992)
α-globin (human)	1 site	3'-UTR, 63 nt downstream of stopcodon	5'-UCC↓UUG-3'	yes, αCP1+αCP2	(Wang and Kiledjian, 2000a)
transferrin receptor (TfR) (human)	1 site	3'-UTR, 184 nt downstream of stopcodon	5'-AAG↓AAC-3'	Yes, IRE-BP	(Casey <i>et al.</i> , 1989; Binder <i>et al.</i> , 1994)

CHAPTER 3

Identification of RNA sequences and structures involved in site-specific cleavage of IGF-II mRNAs

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SUMMARY

Insulin-like growth factor-II (IGF-II) mRNAs are subject to site-specific endonucleolytic cleavage in the 3' untranslated region (UTR) rendering an unstable 5' cleavage product containing the coding region and a very stable 3' cleavage product of 1.8 kb consisting of the 3'-UTR sequence and the poly(A) tail. Previously, it was established that two widely separated elements in the 3'-UTR (elements I and II), that can form a duplex structure, are necessary and sufficient for cleavage. To further investigate the sequence and secondary structure requirements for cleavage, we have introduced a number of mutations around the cleavage site and assayed their effects on cleavage. Several recognition determinants involved in the endonucleolytic cleavage of IGF-II mRNAs were identified. Mutational analysis around the cleavage site revealed that cleavage is sequence-specific and that the cleavage site must be in a single-stranded conformation to allow efficient cleavage. In addition, we have identified an accessory protein that specifically interacts with a stem-loop structure located 133 to 73 nucleotides upstream of the cleavage site.

INTRODUCTION

The regulation of mRNA stability plays a very important role in the control of gene expression, and an increasing number of genes appears to be regulated at the post-transcriptional level (for reviews, see Beelman and Parker (1995); Ross (1995); Jacobson and Peltz (1996); Wickens *et al.* (1997)). The elements required for regulation of stability are often located in the 3' untranslated region (UTR) of mRNAs. These elements can be recognized by *trans*-acting factors serving as mediators of RNA degradation. In addition to poly(A) shortening or arrest of translation at a premature stopcodon, the initial and rate-limiting step in RNA degradation often appears to be an endonucleolytic attack on the mRNA resulting in efficient exonucleolytic processing of the mRNA. Due to the instability of the cleavage products, intermediate RNA fragments are usually difficult to detect. To date, a number of mRNAs has been found to be degraded through endonucleolytic cleavage, including the mRNAs encoding the transferrin receptor (Binder *et al.*, 1994), the cytokine *gro α* (Stoeckle, 1992), avian apo-very low density lipoprotein II (Binder *et al.*, 1989), maternal homeodomain-proteins (Brown and Harland, 1990; Brown *et al.*, 1993), and albumin (Dompenciel *et al.*, 1995; Chernokalskaya *et al.*, 1997). The *c-myc* mRNA can be cleaved endonucleolytically in its coding region, but can also be destabilized through AU-rich elements in the 3'-UTR (Swartwout and Kinniburgh, 1989; Ioannidis *et al.*, 1996).

Human insulin-like growth factor II (IGF-II) is a mitogenic polypeptide consisting of 67 amino acids showing strong structural and functional homology to insulin (Daughaday and Rotwein, 1989). It is essential for embryonic growth and development (DeChiara *et al.*, 1990). The human IGF-II gene comprises nine exons, of which exons 7, 8, and the first 237 nucleotides of exon 9 provide the coding region for pre-pro-IGF-II. The remaining part of exon 9 consists of a 4 kb long 3'-UTR. Human

IGF-II is expressed in a developmental stage dependent and tissue-specific manner by differential activation of four promoters (P1- P4) yielding multiple mature transcripts with different 5'-UTRs derived from exons 1-6. In addition, IGF-II expression is regulated at the post-transcriptional, translational, and the post-translational levels (for a recent review, see Holthuizen *et al.* (1999)).

In previous studies, we have shown that human IGF-II mRNAs are subject to site-specific endonucleolytic cleavage in the 3'-UTR, rendering an unstable 5' cleavage product and an exceptionally stable 3' cleavage product of 1.8 kb (De Pagter-Holthuizen *et al.*, 1988; Meinsma *et al.*, 1991; Meinsma *et al.*, 1992). It was established that for *in vivo* cleavage of IGF-II mRNAs two widely separated sequence elements in the 3'-UTR (elements I and II) are necessary and sufficient (Meinsma *et al.*, 1992). Element II spans a region of 323 nucleotides long running from positions -173 to +150 relative to the cleavage site. It comprises two domains that are highly conserved among human, rat, and mouse: a domain from positions -133 to -7 containing two stable stem-loop structures and a G-rich domain from positions -5 to +59 (Fig. 2A). In fact, the region from -14 to +60 exhibits the highest sequence homology within the entire IGF-II mRNA between human, rat and mouse (Nielsen and Christiansen, 1992). Element I (103 nucleotides long) is located approximately 2 kb upstream of the cleavage site at positions -2116 to -2013 (Fig. 1, 2A) and forms an RNA: RNA duplex structure with the downstream region in element II from positions +18 to +101 (Scheper *et al.*, 1995).

To investigate the structural requirements for cleavage of IGF-II mRNAs in more detail, we have altered the nucleotide sequence and/or secondary structures in the proximity of the cleavage site and assessed the consequences of the changes on cleavage efficiency and cleavage specificity. In addition, we have identified a protein that specifically binds to a stem-loop structure in element II upstream of the cleavage site and that may have a modulating effect on cleavage.

RESULTS

Experimental strategy

In order to study *in vivo* cleavage of IGF-II mRNAs, we previously constructed a human IGF-II minigene construct. In this construct, designated EP7-9, the immediate-early cytomegalovirus (CMV) enhancer-promoter region (Boshart *et al.*, 1985) is fused to the IGF-II protein-encoding exons 7, 8, and 9 (Fig. 1), giving rise to an IGF-II transcript of 4.8 kb (Meinsma *et al.*, 1992). Transient transfection assays of this construct into human 293 cells that do not express IGF-II endogenously, and subsequent Northern blotting analysis of the IGF-II mRNAs showed that in addition to the 4.8 kb transcript also a specific 1.8 kb cleavage product of IGF-II is formed as a consequence of site-specific endonucleolytic cleavage in the 3'-UTR of IGF-II mRNAs (Meinsma *et al.*, 1991). A NotI site was introduced in EP7-9 at position +83 for several cloning purposes (Fig. 1B), and it was shown that the cleavage efficiency

of this construct (EP7-9/Not) is comparable to that of EP7-9 (Meinsma *et al.*, 1992). In our studies we have used EP7-9/Not for the introduction of mutations disrupting a specific RNA-protein complex (see below). Analysis of deletion derivatives from EP7-9 revealed that a region in the 3'-UTR between elements I and II from positions -1955 to -174 relative to the cleavage site could be deleted without affecting cleavage efficiency (Fig. 1B). In this study, construct EP7-9 Δ -1955/-174 (in short Δ WT) giving rise to a 3.0 kb IGF-II transcript was used for the introduction of several point mutations in the proximity of the cleavage site. The cleavage efficiencies of Δ WT and EP7-9/Not were set at 100% in the separate experiments. All mutants are named after the positions of the altered residues relative to the cleavage site.

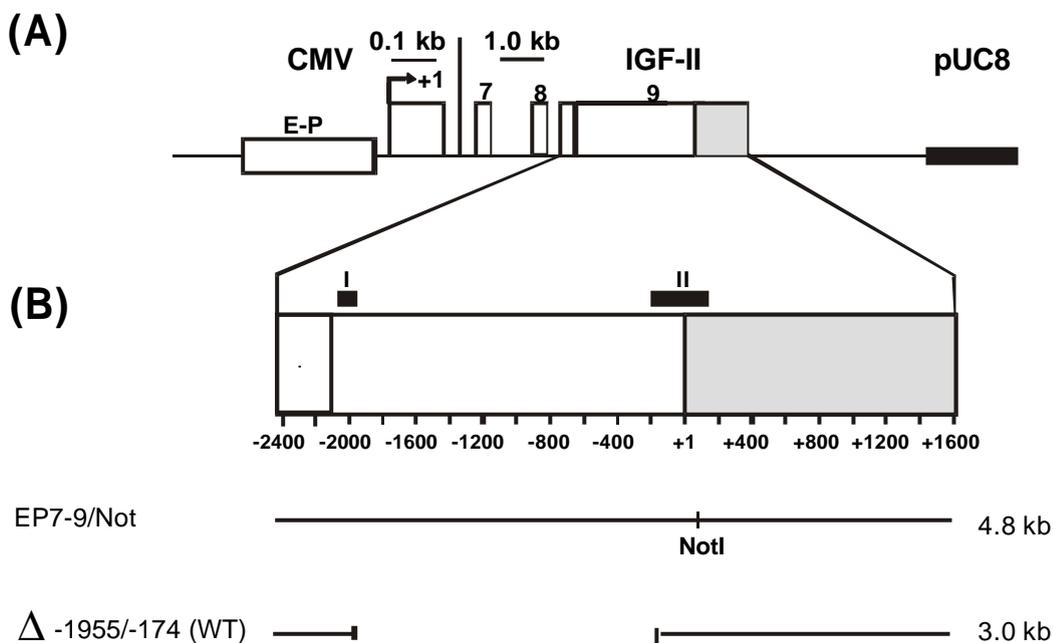


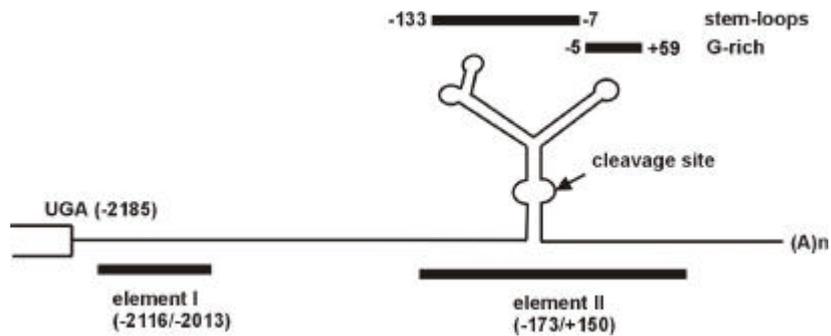
Figure 1. Schematic representation of the CMV-IGF-II minigenes. (A) Construct EP7-9 contains the enhancer/promoter region and exon 1 of cytomegalovirus (CMV) fused to a 10.9 kb genomic fragment of the human IGF-II gene containing exons 7, 8, and 9 cloned in pUC8 (bold line). The CMV regions and the IGF-II and pUC regions are drawn at different scales. The arrow indicates the transcription start site. Translated and untranslated IGF-II regions are depicted by filled and open boxes, respectively. The stippled area in exon 9 represents the 1.8 kb RNA region. (B) Enlarged representation of human IGF-II exon 9. Positions (bp) in exon 9 are relative to the cleavage site (+1). The two elements required for cleavage, Element I (-2116 to -2013) and Element II (-174 to +150) are indicated by bars (I, II). The EP7-9 derivatives, EP7-9/Not that contains an additional NotI site at position +83, and Δ -1955/-174 (WT) in which the region between elements I and II (-1955 to -174) is deleted, are indicated. The transcripts of EP7-9/Not (4.8 kb) and Δ -1955/-174 (3.0 kb) are endonucleolytically cleaved with wild type cleavage efficiency (Meinsma *et al.*, 1992).

Nucleotide sequence requirements around the cleavage site

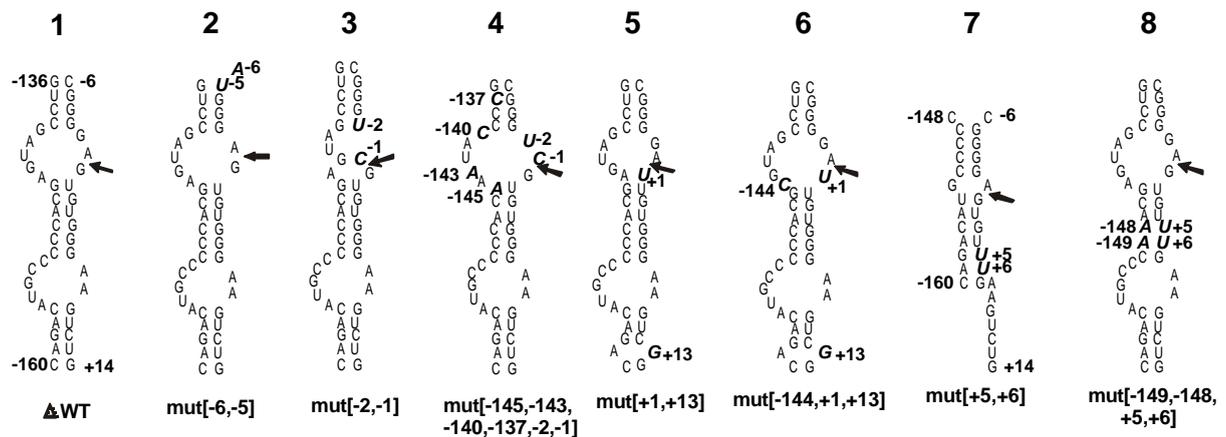
Previous experiments have shown that cleavage of IGF-II mRNAs occurs at a unique site in the cleavage unit consisting of elements I and II (De Pagter-Holthuizen *et al.*, 1988). The secondary structure of the region around the cleavage site was predicted by computer folding using the MFOLD computer program from the GCG package based on the algorithm of Zuker and Stiegler (Zuker and Stiegler, 1981) (Fig. 2A). The position of the cleavage site is indicated in the figure. To investigate whether specific nucleotides around the cleavage site are important for cleavage, several point mutations were introduced in the IGF-II minigene construct Δ WT in the highly conserved region surrounding the cleavage site. Mutations were introduced at positions -6,-5; -2,-1; +1,+13, and +5,+6, relative to the cleavage site. The effects of the mutations on the secondary structure of the corresponding RNA were predicted by computer folding using MFOLD (Fig. 2B).

Human 293 cells, which do not express IGF-II endogenously, but are able to perform cleavage of the various IGF-II minigene RNAs (Meinsma *et al.*, 1991), were transiently transfected with the minigene constructs. Subsequently, RNA was isolated from the transfected cells and subjected to Northern blot analysis using a probe that detects the 3' cleavage product as well as the full-length IGF-II RNA (Fig. 2C). As previously shown (Meinsma *et al.*, 1992), transcripts from Δ WT are efficiently cleaved (Fig. 2C, lane 1). Mutations at positions -2,-1 and +5,+6 relative to the cleavage site, that affect both the primary nucleotide sequence and the secondary structure around the cleavage site, completely abolish cleavage (lanes 3 and 7, respectively). Mutations at positions -6,-5 and +1,+13, only slightly affecting the secondary structure, have a less severe effect, reducing the cleavage efficiency to 25-30 % of the wild type (Fig. 2B and C, lanes 2 and 5). In order to discriminate between effects at the level of the primary nucleotide sequence and the secondary structure, we introduced compensatory mutations in the opposite strand for mutants [-2,-1], [+1,+13], and [+5,+6] (Fig. 2B). Compensatory mutations in the opposite strand restoring the wild type structure, but not the sequence (mut[-145,-143,-140,-137,-2,-1] and mut[-149,-148,+5,+6]) cannot restore cleavage (lanes 4 and 8). This indicates that the wild type structure alone is not sufficient for recognition and that the identity of the nucleotides at these positions around the cleavage site is important as well. Introduction of a compensatory mutation at position -144 in mut[+1,+13], restoring the predicted wild type structure, does not yield the wild type cleavage efficiency, but even further reduces cleavage to about 5% (lane 6). This indicates that the reduced cleavage efficiency observed with mut[+1,+13] is not solely due to the effect of mutation +1 on the secondary structure, but that the identity of the nucleotide at position +1 as well as the opposing nucleotide in the complementary strand are important.

(A)



(B)



(C)

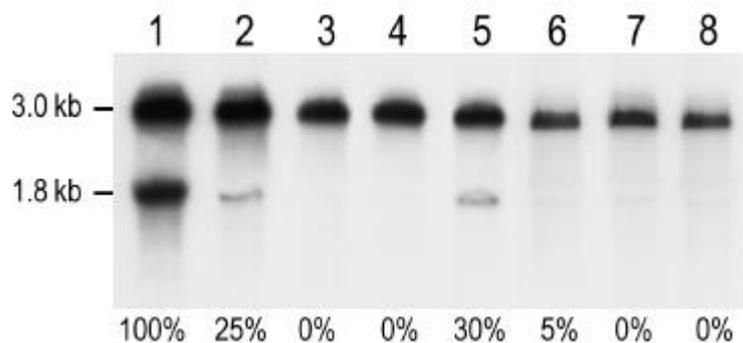
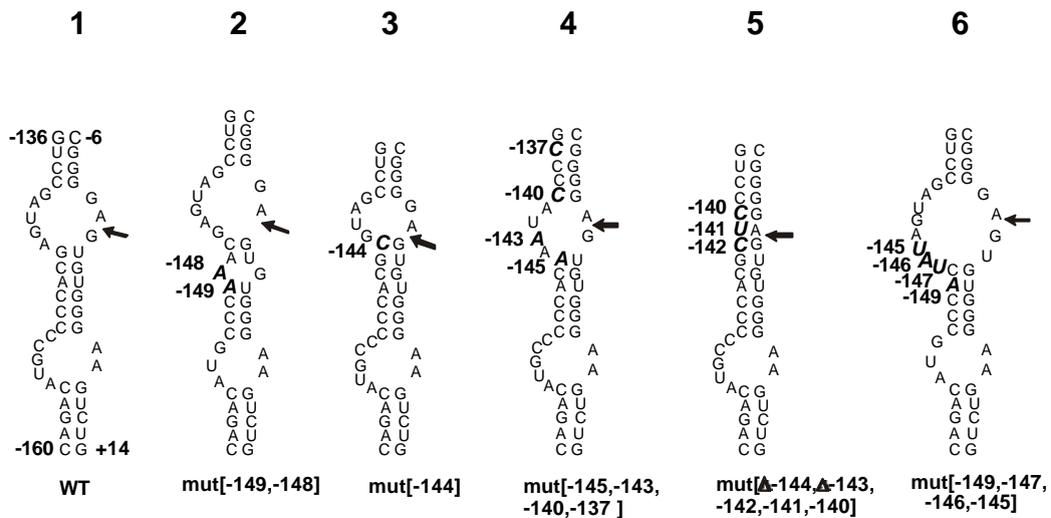


Figure 2. (A) Schematic representation of the 3'-UTR of IGF-II mRNAs. Elements I and II are indicated by bars, the secondary structure of the RNA region around the cleavage site (arrow) is shown. The positions of the conserved stem-loop region and the G-rich domain in element II are indicated. (B) Predicted local secondary structures around the cleavage site in ΔWT and the various mutant minigene constructs. In each structure, the cleavage site (arrow), the altered nucleotides (bold italics), and their positions are indicated. (C) Northern blot analysis of total RNA isolated from human 293 cells transiently transfected with the constructs indicated in (B). The blot was probed with a radiolabeled DNA fragment (+84 to +1096) that detects both full-length IGF-II mRNA as well as the 3' cleavage product of 1.8 kb. Sizes of the RNA species are indicated in kb. The numbers of the lanes correspond to the numbers of the constructs shown in (B). Mean cleavage efficiencies (determined as described in Materials and Methods) of 2-3 separate experiments are given as a percentage relative to the efficiency of cleavage of ΔWT, which was set at 100%. Variation between the calculated cleavage efficiencies in independent experiments was less than 10%.

Structural requirements around the cleavage site

The results described above suggest that the identity of specific nucleotides around the cleavage site is important for cleavage. To gain more insight into the role of secondary structures in this region, we introduced mutations in WT in the strand opposite to the strand in which the cleavage site is located (positions -160 to -136). These mutations were predicted to affect local secondary structure formation, but the nucleotide sequence surrounding the cleavage site was left intact (Fig 3A).

(A)



(B)

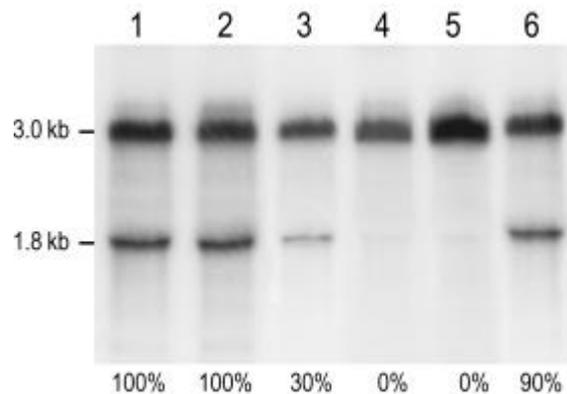


Figure 3. (A) Predicted local secondary structures around the cleavage site in WT and the various mutant minigene constructs. In each structure, the cleavage site (arrow), the altered nucleotides (bold italics), and their positions are indicated. (B) Northern blot analysis of total RNA from 293 cells transfected with the CMV-IGF-II minigene constructs. The lanes are numbered according to the numbers of the constructs shown in (A). The blot was hybridized to the 3'-end specific probe that detects both the full-length IGF-II RNA as well as the 3' cleavage product. The calculated mean cleavage efficiencies of 2-3 separate experiments are indicated (see Materials and Methods for calculation method). Variation between the calculated cleavage efficiencies in independent experiments was less than 10%.

The effects of these mutations were studied in transient transfection assays followed by Northern blot analysis of the RNAs isolated from the transfected cells (Fig. 3B). Mutations at positions -149,-148 in mut[-149,-148] cause a mismatch in the stem below the cleavage site and decrease the size of the single-stranded bubble from 3 to 2 nucleotides (Fig 3A). This mutant is cleaved with wild type efficiency (Fig. 3B, lane 2), in contrast to mut[-144], which is cleaved with a reduced efficiency of 30% (Fig. 3B, lane 3). In mut[-144], the size of the bubble is reduced from 3 to 2 nucleotides due to base-pairing of the G-residue downstream of the cleavage site, as in mut[-149,-148] (Fig. 3A). The difference with mut[-149,-148] is, however, that the stem below the cleavage site is uninterrupted. Thus, in mut[-149,-148] the structure shown in Fig. 3A may be in a dynamic equilibrium with a more open conformation, whereas in mut[-144] the cleavage site is less exposed. Cleavage is abolished when the bubble is completely distorted as in mut [-144, -143,-142,-141,-140], leaving the cleavage site in a perfect duplex (Fig. 3A,B, lane 5). This suggests that for cleavage, the cleavage site must be sufficiently exposed in a single-stranded conformation. To study the effect of a more open conformation around the cleavage site, we introduced mutations creating a bubble of increased size (mut[-149,-147,-146,-145], Fig. 3A). This mutant was cleaved with wild type efficiency, indicating that changing the structure towards a larger bubble is tolerated (Fig. 3B, lane 6). Surprisingly, no cleavage product was detected with mut[-145,-143,-140,-137], though the sequence surrounding the cleavage site is left intact and the predicted secondary structure is only slightly changed (Fig. 3A and B, lane 4) . This indicates that the identity of certain nucleotides in the opposite strand may also be important for efficient cleavage. In summary, these results suggest that secondary structure requirements are limited to a single-stranded conformation around the cleavage site of at least three nucleotides. In addition, the identity of certain nucleotides in the cleaved strand as well as in the opposite strand are important for efficient cleavage.

Primer extension analysis

The cleavage site has been determined previously to the nucleotide resolution (De Pagter-Holthuisen *et al.*, 1988). In order to investigate whether the mutations that still allowed cleavage, although with a reduced efficiency, affect the specificity of the site of cleavage, we performed primer extension analysis of poly(A)⁺ RNA isolated from 293 cells transiently transfected with the IGF-II minigene constructs bearing mutations which affect cleavage efficiency to a different extent. For mut[+1,+13] and mut[-6,-5], having a reduced cleavage efficiency of 30% and 25%, respectively (Figs. 2B, 2C, lanes 5 and 2), a single extension product of the expected length is observed (Fig. 4, lanes 2 and 3, respectively), as is the case with the wild type control (lane 1). This indicates that cleavage specificity was not affected in these mutants. No extension product was observed for mut[-2,-1], where cleavage is completely abolished (lane 4). Thus, although the cleavage efficiency may be decreased for some constructs, specificity of cleavage remains unaltered.

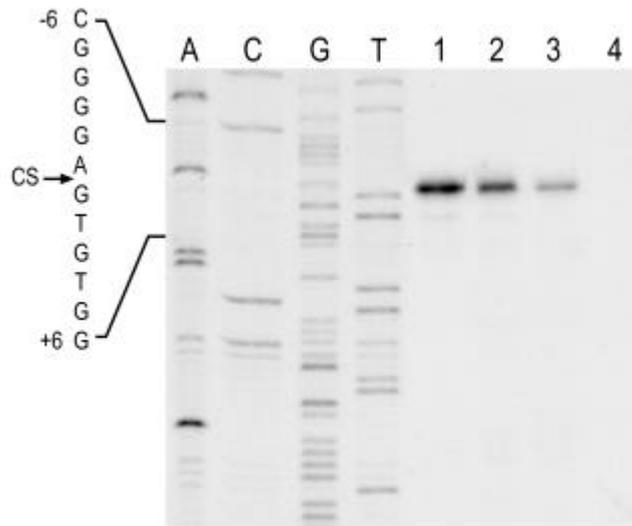
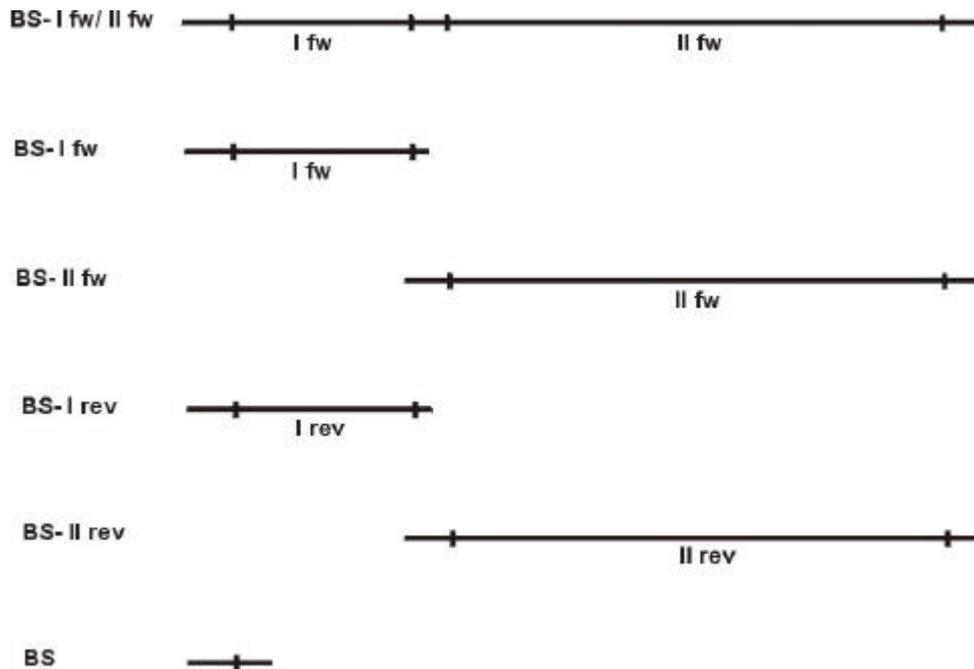


Figure 4. Primer extension analysis of IGF-II transcripts. A 5' end-labeled primer complementary to positions +99 to +120 was annealed to poly(A)⁺ RNA from 293 cells transiently transfected with CMV-IGF-II minigene construct Δ WT (lane 1), mut[+1,+13] (lane 2), mut[-6,-5] (lane 3), or mut[-2,-1] (lane 4). A sequencing reaction carried out with the same primer was used as a marker. The cleavage site (CS) as determined previously (De Pagter-Holthuisen *et al.*, 1988) is indicated by an arrow on the left.

Specific RNA-protein interaction in element II

In a search for *trans*-acting factors that specifically bind to the elements required for cleavage, we performed electrophoretic mobility shift assays (EMSAs) using *in vitro* synthesized RNAs containing element I and/or element II in forward or reverse orientation (Fig. 5A) and incubated these with cytoplasmic extracts from human Hep3B cells, that endogenously express and cleave IGF-II mRNAs. Due to the length of the RNA probes, it was necessary to digest the unbound RNA and the RNA-protein complexes with RNase T1 and RNase A prior to gel electrophoresis. Two RNA-protein complexes appeared with all probes tested, even with a control probe containing only pBluescript polylinker sequences (Fig. 5B, lane 11). Thus, these two complexes were nonspecific. A distinct complex, specific for IGF-II mRNA was observed when the synthesized RNAs contained element II in the forward orientation (lanes 1 and 5).

(A)



(B)

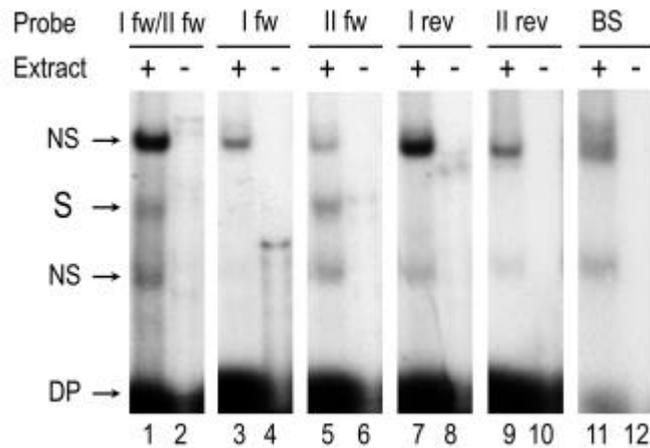


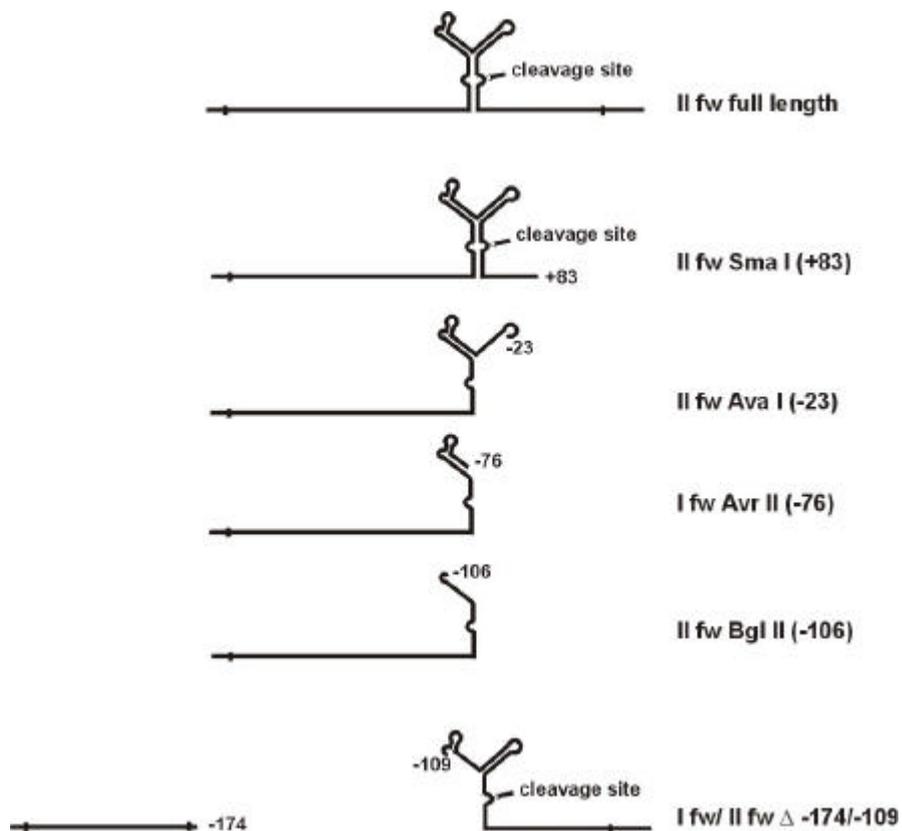
Figure 5. A specific RNA-protein complex is formed with element II. (A) Schematic representation of *in vitro* synthesized RNAs used for protein binding experiments. Elements I and II were cloned in forward (fw) or reverse (rev) orientation in plasmid pBluescript (BS). (B) Electrophoretic mobility shift assay of RNA-protein complexes separated by native polyacrylamide gel electrophoresis. Indicated ^{32}P -labeled RNAs were incubated in the presence (+) or absence (-) of cytoplasmic extract (10 μg of protein) from human Hep3B cells. RNA containing only pBluescript (BS) polylinker sequences was used as a control. IGF-II specific (S) and nonspecific (NS) RNA-protein complexes are indicated by arrows as well as the degraded probe (DP).

To determine the binding site of the protein more precisely, we used RNAs truncated in element II at positions +83 (SmaI), -23 (AvaI), -76 (AvrII), and -106 (BglII) (Fig. 6A). Truncations up to position -76 had no effect on complex formation (Fig. 6B, lanes 2, 3, and 4), but further truncation to position -106 abolished binding of the protein (lane 5). In addition, a mutant construct lacking the 5' part of element II from position -174 to -109 was tested for protein binding, but no specific complex formation was observed (lane 6). Based on these data, we anticipated that the protein binds to a region overlapping position -106, for which the formation of a stem-structure with a side-loop (from positions -133 to -73) is predicted (Fig. 7A). To test this, we introduced mutations in this stem structure as well as in the side-loop and investigated these mutants for binding of the protein (Fig. 7A and B). In contrast to deletion of the side-loop (positions -103 to -92), which has no effect on protein binding (Fig. 7B, lane 2), mutation of a part of the stem from positions -89 to -85 disrupts complex formation (lane 3). Even more subtle mutations of only 2 nucleotides in the region from -88 to -81 completely abolish binding of the protein (lanes 4-7). This confirms that the protein specifically binds to the stem, and not to the loop. Specificity of binding was further assessed in a competition experiment (Fig. 8). Several unlabeled competitor RNAs were added to the radiolabeled probe containing element II in forward orientation in a 100- and 250-fold molar excess, respectively. Effective competition was only observed with unlabeled wild type element II RNA and mutant Iifw Δ -103/-92 (which efficiently binds to the protein when used as a probe; Fig. 7B, lane 2, Fig. 8, lanes 3-6); the other mutated RNAs poorly competed for protein binding, confirming the results obtained with these RNAs as radiolabeled probes (Fig. 8, lanes 7 to 10; and data not shown).

In summary, we have identified a protein that specifically interacts with an RNA region from positions -89 to -81 pairing with nucleotides -126 to -117 in a stem-structure 133 to 73 nucleotides upstream of the cleavage site.

To examine whether this RNA-protein complex actually plays a role in cleavage, the mutations shown in Fig. 7 were introduced in the IGF-II minigene EP7-9/Not (Fig 1B) and the *in vivo* cleavage efficiency of the different mutants was studied. Mutants that lacked RNA-protein complex formation *in vitro* showed a 15 to 30% reduction in cleavage efficiency. Mutant Δ -103/-92, that still allowed efficient binding of the protein, could be cleaved *in vivo* with wild type efficiency (data not shown). This indicates that although the RNA-protein complex is not essential for cleavage, it may have a modulating effect on cleavage efficiency.

(A)



(B)

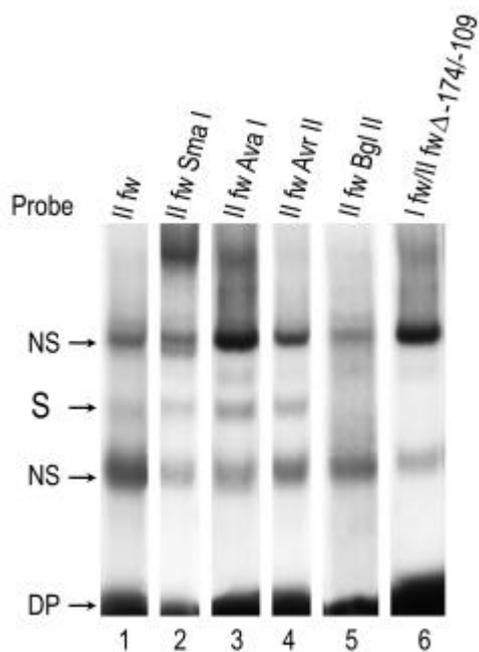
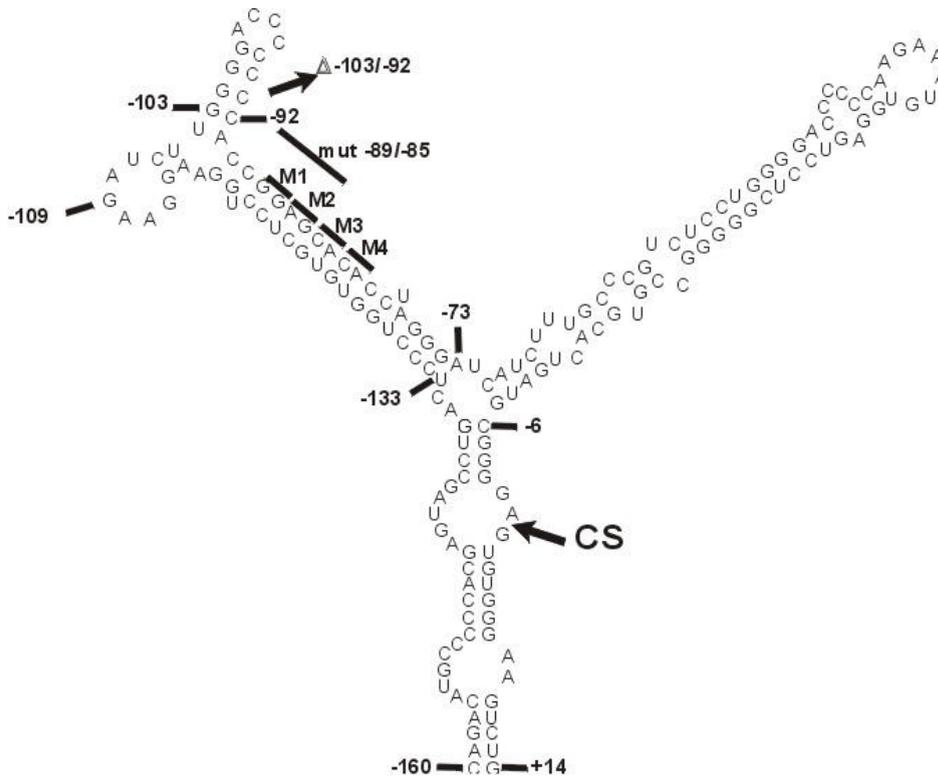


Figure 6. Determination of the binding site of the specific RNA-protein complex. (A) Schematic representation of a series of 3'-end truncations of element II in forward orientation (fw), at positions +83, -23, -76, and -106, respectively. Ifw/IIfw Δ -174/-109 is a deletion mutant containing element I, but lacking the 5' end of element II. (B) Electrophoretic mobility shift assay of 32 P-labeled RNAs with 10 μ g of Hep3B cytoplasmic extract. IGF-II specific (S) and nonspecific (NS) RNA-protein complexes are indicated by arrows as well as the degraded probe (DP).

(A)



(B)

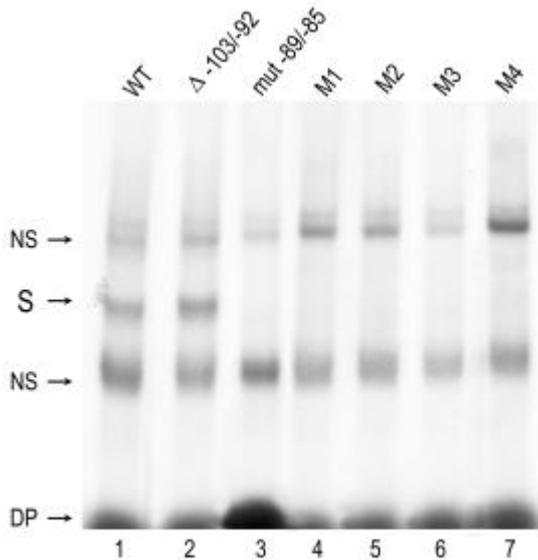


Figure 7. Fine-mapping of the protein binding site. (A) Various mutations were introduced in the stem-loop structure from positions -133 to -73 to determine the binding site of the protein in detail. WT: construct BS-lfw/llfw that contains the wild type configuration. The side-loop from positions -103 to -92 was deleted (Δ -103/-92), and nucleotides -89 to -85 in the stem-structure were mutated (5'-CGGAG-3' changed into 5'-AUAUA-3') (mut -89/-85). In addition, a set of four subtle mutations of two nucleotides each were introduced in the stem-structure: (-88G;-87G) were changed into (-88A;-87A) (**M1**), (-86A;-85G) were changed into (-86U;-85A) (**M2**), (-84C;-83A) were changed into (-84G;-83U) (**M3**), and (-82C;-81A) were changed into (-82G;-81U) (**M4**). The cleavage site (CS) is indicated by an arrow. (B) Electrophoretic mobility shift assay of 32 P-labeled RNAs with 10 μ g of Hep3B cytoplasmic extract. IGF-II specific (S) and nonspecific (NS) RNA-protein complexes are indicated by arrows as well as the degraded probe (DP).

DISCUSSION

A key to understanding the mechanism by which IGF-II mRNAs are cleaved lies in the determination how a specific site in the mRNAs is recognized by a putative endoribonuclease, and in the identification of factors involved in this process. Here we describe experiments addressing both issues. Mutational analyses indicate that for proper cleavage, both the primary nucleotide sequence as well as the local secondary structure of the IGF-II mRNAs are of relevance; recognition may occur mainly at the level of the primary nucleotide sequence, but the cleavage site must also be in an open conformation to allow cleavage. The effects of the mutations on secondary structure formation were predicted by computer folding using the MFOLD computer program from the GCG package based on the algorithm of Zuker and Stiegler (Zuker and Stiegler, 1981). Although it is not certain whether the structures shown here will exactly represent the RNA structures present in the cell, the effects of the mutations on the predicted structures are all in good agreement with the observed effects on cleavage efficiency. In addition to the RNA sequences and structures involved in cleavage, we have identified a protein that specifically interacts with a stem-loop structure in the 3'-UTR of the mRNA located 133 to 73 nucleotides upstream of the cleavage site.

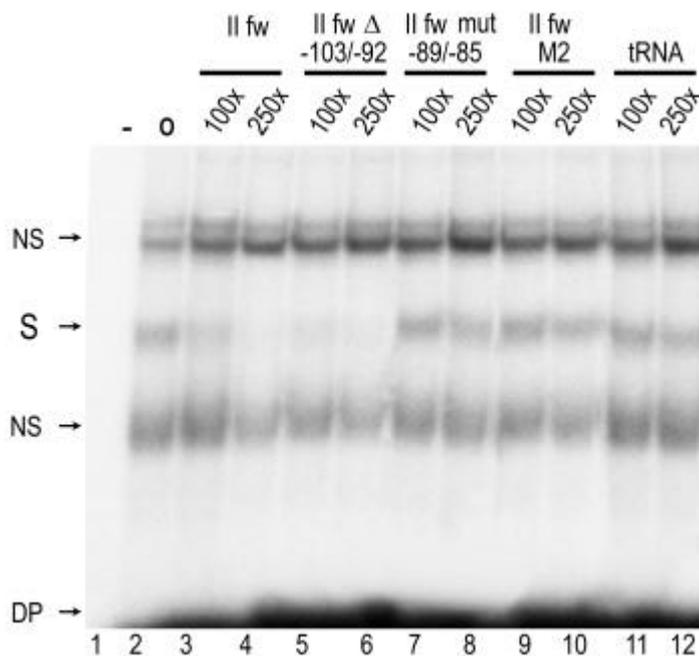


Figure 8. Electrophoretic mobility competition shift assay. Radiolabeled *in vitro* synthesized RNA containing element II in forward orientation (II fw) was incubated without (-) or with (0) cytoplasmic extract from Hep3B cells. II fw RNA as well as several of the mutated RNAs (indicated in Figure 7) were added in 100- or 250-fold molar excess as unlabeled competitor RNAs. As a control for nonspecific competition, yeast tRNA was added.

To gain more insight in the sequence requirements for cleavage, a series of mutants was generated with alterations at positions -6,-5; -2,-1; +1,+13; and +5,+6 relative to the cleavage site (Fig. 2B). Mutations at positions -6,-5 and at positions +1,+13 reduce the cleavage efficiency to 25-30% of the wild type, and mutations at positions -2,-1 and at positions +5,+6 abolish cleavage completely. As each mutation

is predicted to affect the local secondary structure, it is difficult to determine whether the effect should be ascribed to the change in primary nucleotide sequence, the altered secondary structure, or both. To solve this problem, we introduced compensatory mutations in the complementary sequence upstream of the cleavage site from positions -160 to -136, restoring the predicted wild type secondary structure of mut[-2,-1], mut[+1,+13], and mut[+5,+6] (see Fig. 2B). Cleavage was not restored in any of these mutants, indicating that a predicted wild type structure is not sufficient for proper cleavage. Surprisingly, in the case of mut[-144,+1,+13] cleavage efficiency was even further reduced. This might indicate that certain nucleotides in the complementary strand are also important for recognition. Alternatively, this mutation may cause structural alterations at the tertiary structure level, thus hampering cleavage. An additional set of mutations was introduced to assess the secondary structure requirements. Mutations at positions -149 and -148, which were used to restore the wild type secondary structure in mut[+5,+6], cause a mismatch in the stem below the cleavage site in the wild type context (Fig. 3A). This mutant is cleaved with wild type efficiency, indicating that the full integrity of this stem is not necessary for cleavage. Also, when the single-stranded bubble in which the cleavage site is located is enlarged and the lower stem is shortened by one base-pair, as in mut[-149,-147,-146,-145], no major effects are observed. This shows that a small increase in the size of the bubble is allowed, but leaves open the question whether decreasing the size of the bubble affects cleavage efficiency. Therefore, we introduced nucleotides able to base-pair with nucleotides adjacent to the cleavage site. In mut[\ddot{A} -144, \ddot{A} -143,-142,-141,-140], the cleavage site is in a perfect duplex, in mut[-144] the nucleotide 3' to the cleavage site is base-paired (as in the case of mut[+1,+13], Fig. 2B), and in mut[-145,-143,-140,-137], the second nucleotide 5' to the cleavage site is base-paired (Fig. 3A), as in the case of mut[-6,-5] (Fig. 2B). Mut[-144] is cleaved with a moderately reduced efficiency of about 40%, and surprisingly, mut[-145,-143,-140,-137] is not cleaved, though its predicted secondary structure is similar to that of mut[-6,-5], which is cleaved with 40% efficiency. Mut[\ddot{A} -144, \ddot{A} -143,-142,-141,-140] is not cleaved, indicating that the cleavage site must be in a single-stranded conformation to allow cleavage. This result is not unexpected, since in several other systems endonucleolytic cleavage appears to occur in single-stranded regions of the target RNA (Binder *et al.*, 1989; Binder *et al.*, 1994; Kim *et al.*, 1996).

The results obtained with mut[-144,+1,+13] and mut[-145,-143,-140,-137] indicate that nucleotides in the complementary strand are also important for recognition, either because they are specifically contacted by the endonuclease, or because they are involved in non-Watson-Crick base-pairings in the bubble which are not revealed by computer folding algorithms. The ability of RNA molecules to adopt unusual structures is now commonly recognized, as structural models have revealed the existence of a number of different non-Watson-Crick arrangements occurring as single, tandem, or consecutive base-pairs, among which A-A, U-U, G-G, G-A, and G-U are often found (Schuster *et al.*, 1997). Our finding that in contrast to mutations in the opposite strand, all mutations around the cleavage site affect cleavage shows that the sequence surrounding the cleavage site is less tolerant to mutations than the complementary sequence. This observation is in agreement with the fact that the nucleotide sequence surrounding the cleavage site is more conserved among human,

rat and mouse IGF-II mRNAs than the upstream sequence (Meinsma *et al.*, 1991; Nielsen and Christiansen, 1992). The reduced conservation of the upstream sequences results in differences at the secondary structure level, though in all three species the cleavage site is located in a single-stranded bubble. Interestingly, the mutated nucleotides in the upstream sequence affecting cleavage efficiency are conserved, whereas most of the residues which can be mutated without effect on cleavage efficiency are not conserved. These data strengthen the hypothesis that the identity of specific nucleotides, especially around the cleavage site, and to a lesser extent also in the opposite strand is important. In addition, the severe effects of mutations affecting the secondary structure formation around the cleavage site reveal a requirement for an open structure around the cleavage site.

In summary, we have identified three major determinants involved in recognition of the cleavage site; (a) the sequence surrounding the cleavage site, (b) the sequence in the opposite strand, and (c) secondary (and perhaps tertiary) structure formation of the cleavage site region.

Addition of these new data to our previous findings that for cleavage of IGF-II mRNAs two elements (I and II; 103 and 323 nucleotides long, respectively) which can form a stable duplex structure, are necessary and sufficient (Meinsma *et al.*, 1992; Scheper *et al.*, 1995), reveals that we are dealing with an unusually complex system. For comparison, short sequence elements resembling the AUUUA-motif, present in a number of short-lived human oncogene and growth factor mRNAs, appear to be sufficient for recognition by human RNase E-like endoribonucleases (Wennborg *et al.*, 1995; Claverie-Martin *et al.*, 1997). For sequence-specific cleavage of *Drosophila* and *Xenopus* maternal homeobox mRNAs, a 17 nt element is sufficient, and secondary structure formation does not seem to play a role (Brown and Harland, 1990; Brown *et al.*, 1993). Albumin mRNA is preferentially cleaved at the pentamer A(C/U)UGA, but can also be cleaved at other sites (Chernokalskaya *et al.*, 1997). To our knowledge, the only other described system where endonucleolytic cleavage of an mRNA involves recognition of specific sequences at the cleavage site as well as complex structures, is the transferrin receptor mRNA (Casey *et al.*, 1989; Binder *et al.*, 1994).

The highly structured nature of the cleavage unit formed by elements I and II raises the question whether cleavage could occur through a self-cleavage mechanism catalyzed by the RNA itself. However, incubation of IGF-II transcripts in the absence of cellular extract did not result in cleavage, whereas addition of extract led to the formation of the 1.8 kb 3' cleavage product (Nielsen and Christiansen, 1992); our unpublished results). Thus, it is clear that *trans*-acting factors are required for cleavage.

In addition to studying RNA features involved in recognition of the cleavage site, we have also searched for proteins binding specifically to RNA sequences/structures necessary for cleavage. To this end, we performed electrophoretic mobility shift assays and identified a protein exhibiting specific binding to the first stem-loop structure located 133 to 73 nucleotides upstream of the cleavage site (Figs. 5 and 6). We determined the binding site of the protein in more detail by introducing subtle point mutations and assessing the consequences on RNA-protein complex formation. Mutations in a region of the stem from positions -88 to -81 disrupted protein binding completely, but deletion of a side-loop (positions -103 to -92) had no effect (Fig. 7).

We conclude from these results that the protein interacts specifically with the stem-structure, and not with the loop. Computer prediction of secondary structure formation indicated that the 2 nt mutations in the stem-structure only cause local mismatches in the stem, but that the overall structure is unaffected. Thus, it cannot be determined from these data whether the altered bases or the mismatches in the stem are responsible for the effect on binding of the protein. To examine a possible role in cleavage for the identified protein, the mutations described in Fig. 7 were introduced in the EP7-9 minigene and the effect on cleavage efficiency *in vivo* was studied. We observed a reduction in cleavage efficiency for the mutations that abolish protein binding *in vitro*, but the effects were modest and varied among each other (data not shown). Thus, it is clear that additional experiments will be required to elucidate the function of the protein; our results suggest that the identified protein is not an essential factor in cleavage, but may have a modulating effect on the process.

Our results, obtained studying RNA features involved in site-specific endonucleolytic cleavage of IGF-II mRNAs and obtained with the search for protein factors involved in the process, shed new light on the mechanism of cleavage. It appears to be a highly specific process where multiple RNA features coordinately serve as a recognition site for an endoribonuclease. A proximally binding protein may have a modulating effect on cleavage.

ACKNOWLEDGMENTS

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MATERIALS AND METHODS

Materials. Plasmid pBluescript II (KS⁺) was obtained from Stratagene (La Jolla, CA, USA). Restriction enzymes, T4 DNA ligase, and T7 RNA polymerase were purchased from Boehringer, Mannheim, Germany. Pfu DNA polymerase was purchased from Stratagene, RNase-free DNase was from Kabi-Pharmacia (Uppsala, Sweden). RNase A and RNase T1 were purchased from Calbiochem (La Jolla CA, USA). Enzymes were used as specified by the manufacturers. Nucleoside triphosphates (NTPs) and dNTPs were obtained from Kabi-Pharmacia, BES [*N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] was purchased from Sigma (St. Louis, MO, USA). A random primer labeling kit was purchased from Amersham, United Kingdom, and a DNA sequencing kit was purchased from Kabi-Pharmacia. RNAzol reagent was purchased from Biotecx Laboratories, Inc. (Houston, Texas, USA), and Genescreen membranes were purchased from Du Pont de Nemours (Dreieich, Germany). [α -³²P]dCTP (3,000 Ci/mmol) and [α -³²P]CTP (760 Ci/mmol) were purchased from Amersham.

Construction of plasmids. Molecular cloning was performed according to established protocols (Sambrook *et al.*, 1989). Where necessary, 3' recessed ends were filled in with Klenow polymerase and 3' protruding ends were removed with T4 DNA polymerase. All positions within exon 9 of the IGF-II gene are indicated relative to the cleavage site; the nucleotide upstream of the cleavage site is -1, the nucleotide downstream is +1 (Fig. 1). When exon 9 sequences are deleted or additional restriction sites are introduced, the numbers indicated still refer to their original position in exon 9 relative to the cleavage site. IGF-II minigene constructs were based on the construct EP7-9 and its deletion derivative EP7-9Δ-1957/-174 (ΔWT), both described previously in (Meinsma *et al.*, 1992). EP7-9 contains a human genomic IGF-II fragment that starts 373 bp upstream of exon 7 and extends to 4.7 kb downstream of the gene. ΔWT lacks the sequence between elements I and II, which is dispensable for cleavage (Meinsma *et al.*, 1992). Expression of EP7-9 in transfected cells was driven by the immediate-early cytomegalovirus enhancer/promoter. Introduction of point mutations in the proximity of the cleavage site (the region from positions -6 to +14) was done in ΔWT, and was performed via site-directed mutagenesis using the pAlter system according to the manufacturer's instructions (Promega). The mutants are named after the positions of the mutated nucleotides. Oligonucleotides used are shown below; WS9502 was used to construct mut[-6,-5], WS9504 was used for mut[-2,-1], WS9505 was used for mut[+1,+13], and WS9507 was used for mut[+5,+6]. Mutations in the upstream region (from positions -160 to -136) complementary to the region surrounding the cleavage site were introduced via the substitution of a ClaI/BglII fragment (positions -174/-109) in ΔWT by double-stranded oligonucleotides carrying the desired mutations. Again, the names of the mutants are after the positions of the altered residues. Oligonucleotides used are described below. Oligonucleotides ED9726 and ED9727 were introduced into ΔWT to construct mut[Δ-144,Δ-143,-142,-141,-140]; ED9728 and ED9729 to construct mut[-149,-148], ED9830 and ED9831 were inserted to generate mut[-144], ED9832 and ED9833 to generate mut[-149,-147,-146,-145] and ED9834 and ED9835 to generate mut[-145,-143,-140,-137]. To construct mut[-149,-148,+5,+6], oligonucleotides ED9828 and ED9829 were inserted in the above described mut[+5,+6]. To create mut[-144,+1,+13], oligonucleotides ED9830 and ED9831 were inserted in mut[+1,+13], and oligonucleotides ED9834 and ED9835 were inserted in mut[-2,-1] to generate mut[-145,-143,-140,-137,-2,-1].

To generate constructs for *in vitro* transcription, elements I and II were cloned in pBluescript II KS⁺ in the forward (fw) and reverse (rev) orientation behind the T7 promoter as follows. First, elements I and II were PCR amplified using oligonucleotides ED9601 (5' XbaI -2119/-2100 3') and ED9602 (5' XbaI-1964/-1983 3') for element I, and oligonucleotides ED9603 (5' BamHI -204/-185 3') and ED9604 (5' BamHI +181/+202 3') for element II. The PCR products containing element I and element II were digested with XbaI and BamHI, respectively, and subsequently cloned in the corresponding sites in pBluescript II KS⁺. This resulted in 5 plasmids: BS-I_{fw}, BS-I_{rev}, BS-II_{fw}, BS-II_{rev}, BS-I_{fw}/II_{fw}.

Introduction of mutations into the region 130-80 nucleotides upstream of the cleavage site in element II was performed as follows. Plasmid BS-II_{fw} was digested with BglII and AvrII (positions -110 and -80, respectively) and religated to create BS-II_{fw}Δ-110/-80. Double-stranded oligonucleotides containing the desired mutations were inserted in the BglII/Avr II sites to generate the other mutants (for sequences of the oligonucleotides, see list below). A deletion mutant lacking the 5' part of element II from positions -174 to -110 was constructed by digesting plasmid BS-I_{fw}/II_{fw} with BglII (position -110) and SpeI (in the 10 nt long polylinker fragment of pBluescript between the 3' XbaI site of element I and the 5' BamHI site of element II) and religation of the plasmid.

Several of the mutations originally introduced into BS-II_{fw} were transferred to the expression plasmid EP7-9/NotI (plasmid EP7-9 with a unique NotI site constructed at position

+83, Meinsma *et al.*, 1992) via PCR amplification of the region in element II containing the mutations using the upstream primer ED9723 hybridizing to nucleotides -154/-128 (upstream of the BglII site at position -110) and the downstream primer ED9722 hybridizing to nucleotides +60/+93 (introducing a NotI site at position +80). Subsequent digestion of the PCR products with BglII and NotI resulted in BglII/NotI fragments that were cloned in the corresponding sites in EP7-9/NotI. Sequences of the oligonucleotides used for the introduction of (point) mutations or PCR amplification are listed below. Altered nucleotides that were introduced in order to construct restriction sites or point mutations are underlined.

Oligonucleotide	Sequence
WS9502	5'-CACACTCCC <u>AT</u> CATCAGTGCAC-3'
WS9504	5'-TTCCACAC <u>CGA</u> CCCGCATCAGT-3'
WS9505	5'-CGACTTCCC <u>ACCA</u> TCCCCGCAT-3'
WS9507	5'-GCCAGACTT <u>CAA</u> AACTCCCCG-3'
DM6	5'-CTGTCAATCCTCCTGACTTTTC-3'
ED9601	5'-GGCTGGT <u>CTAGA</u> GTTTCCATCAGGTTCCATCC-3'
ED9602	5'-GAGGCCT <u>CTAGA</u> TTGTACATGTTTGAAGATGC-3'
ED9603	5'-TGCCGC <u>GGATCCT</u> GAGGAAGGAGTTTGGCCAC-3'
ED9604	5'-GTCCGC <u>GGATCCA</u> AAGAAACAAAGAGGGGGAAT-3'
ED9706	5'-GATCTTACCGGAGCACAC-3'
ED9707	3'-AATGGCCTCGTGTGGATC-5'
ED9708	5'-GATCTTGGGGACCCCCACCGGC-3'
ED9709	3'-AACCCCTGGGGGGGTGGCCGGATC-5'
ED9710	5'-GATCTTGGGGACCCCCCA <u>TATA</u> CACAC-3'
ED9711	3'-AACCCCTGGGGGGGT <u>GATAT</u> GTGTGGATC-5'
ED9714	5'-GATCTTGGGGACCCCCCA <u>AG</u> CACAC-3'
ED9715	3'-AACCCCTGGGGGGG <u>TT</u> TCGTGTGGATC-5'
ED9716	5'-GATCTTGGGGACCCCCACCGG <u>TA</u> CACAC-3'
ED9717	3'-AACCCCTGGGGGGGTGGCC <u>AT</u> GCGTGGATC-5'
ED9718	5'-GATCTTGGGGACCCCCACCGGAGG <u>TC</u> CAC-3'
ED9719	3'-AACCCCTGGGGGGGTGGCC <u>TCA</u> GTGGATC-3'
ED9720	5'-GATCTTGGGGACCCCCACCGGAGCA <u>GTC</u> -3'
ED9721	3'-AACCCCTGGGGGGGTGGCCTCGT <u>CA</u> GGATC-3'
ED9722	5'-AAAATCTCCCG <u>CGGCC</u> GCTTCCTACCCAGAAC-3'
ED9723	5'-TGCCCCACGAGTAGCCTGACTCCCTG-3'
ED9726	5'-CGATGCCGGTAGCTGAGCAGACATGCCCCACG <u>CTCC</u> - TGACTCCCTGGTGTGCTCCTGGAAGGAA-3'
ED9727	3'-TACGGCCATCGACTCGTCTGTACGGGGGTGC <u>GAGGG</u> AC- TGAGGGACCACACGAGGACCTTCCTTCTAG-5'
ED9728	5'-CGATGCCGGTAGCTGAGCAGACATGCCCCA <u>AA</u> ACGAGT- AGCCTGACTCCCTGGTGTGCTCCTGGAAGGAA-3'
ED9729	3'-TACGGCCATCGACTCGTCTGTACGGG <u>TTT</u> GCTCATCG- GACTGAGGGACCACACGAGGACCTTCCTTCTAG-5'
ED9830	5'-CGATGCCGGTAGCTGAGCAGACATGCCCCACG <u>CGTAGC</u> - CTGACTCCCTGGTGTGCTCCTGGAAGGAA-3'
ED9831	3'-ACGGCCATCGACTCGTCTGTACGGGGGTGC <u>GCATCGGA</u> - CTGAGGGACCACACGAGGACCTTCCTTCTAG-5'
ED9832	5'-CGATGCCGGTAGCTGAGCAGACATGCCCCA <u>CTATAGTA</u> - GCCTGACTCCCTGGTGTGCTCCTGGAAGGAA-3'
ED9833	3'-TACGGCCATCGACTCGTCTGTACGGG <u>TGATA</u> TTCATCGGA- CTGAGGGACCACACGAGGACCTTCCTTCTAG-5'
ED9834	5'-CGATGCCGGTAGCTGAGCAGACATGCCCCACA <u>AA</u> TACC- CCGACTCCCTGGTGTGCTCCTGGAAGGAA-3'
ED9835	3'-TACGGCCATCGACTCGTCTGTACGGGGGT <u>TTTATGGG</u> - <u>GCTGAGGG</u> ACCACACGAGGACCTTCCTTCTAG-5'

Cell culture and transfection. Human 293 cells were grown in Dulbecco's modified Eagle's medium, and human Hep3B cells were grown in α -modified minimum essential medium. Both media were supplemented with 10% fetal calf serum, 100 IU of penicillin per ml, 100 μ g of streptomycin per ml, and 300 μ g of glutamine per ml. Human 293 cells were transfected in 75 cm² flasks at a confluence of about 50% by the calcium phosphate coprecipitation method (Sambrook *et al.*, 1989; Graham and van der Eb, 1973). Precipitates were prepared with BES-buffered saline (BBS) (Sambrook *et al.*, 1989) and contained 10 μ g of CMV/IGF-II constructs and 1.0 μ g of RSV-Luciferase (de Wet *et al.*, 1987), and were added to the cells. After 4 h, the medium was aspirated and the cells were treated for 1 min with medium containing 10% dimethylsulfoxide. Subsequently, the cells were refed with serum-containing medium. After 30 to 48 h, the cells were washed with PBS and harvested in PBS with 0.025 % trypsin-0.02 % EDTA. To compare transfection efficiencies of the various IGF-II expression constructs, 10% of the cells were used for a luciferase assay (de Wet *et al.*, 1987). RNA was isolated from the remaining 90% of the cells.

RNA isolation and analysis. Total RNA was isolated using the RNazol reagent according to the manufacturer's instructions. Cells isolated from a 75 cm² flask were lysed in 1.5 ml of RNazol reagent and RNA was isolated from the water phase by isopropanol precipitation and resuspended in water. RNA was glyoxalated, size-separated on a 1% agarose-10 mM sodium phosphate gel, and transferred to a Genescreen membrane. The RNA was fixed on the membrane by irradiation with long-wavelength UV light for 2.5 min. Northern blots were hybridized in the presence of 50% formamide in glass cylinders with continuous rotation at 42 °C according to the Genescreen protocols. As a probe, a 1.0 kb SmaI fragment from the human IGF-II exon 9 (positions +84 to +1096), hybridizing to both the full-length IGF-II RNAs and the 3' cleavage product, was used. The DNA fragments were labeled by random priming with [α -³²P]dCTP following the Amersham protocols. After a 2-h prehybridization, probe was added at a final concentration of 10⁶ cpm/ml. Blots were washed after overnight hybridization, to a final stringency of 0.5 x SSC- 1% SDS at 65 °C (1xSSC is 0.15 M NaCl plus 0.015 M sodium citrate) and exposed to Fuji RX X-ray film. The amounts of cleavage products were determined by densitometric scanning of the autoradiographs of at least 2 independent experiments. Cleavage efficiencies were determined by calculating the amount of 3' cleavage product divided by the total amount of IGF-II RNA. Variation between the calculated cleavage efficiencies in separate experiments was less than 10%.

In vitro transcription. Radiolabeled RNA probes were synthesized using T7 RNA polymerase on linearized DNA templates according to instructions of the manufacturer in the presence of 1 mM ATP, GTP, and UTP each and 20 μ Ci [α -³²P]-CTP and 0.1 mM CTP. pBluescript plasmids containing IGF-II inserts (described above) were linearized with EcoRV to generate full-length RNA. To obtain RNAs harboring progressive truncations in element II, pBluescript-IGF-II plasmids were linearized with SmaI, AvaI, AvrII or BglII, resulting in RNAs truncated at positions +83, -23, -76, and -106, respectively. After RNA synthesis (30 min.), the template was removed by DNase I treatment (1 U in 20 μ l reaction mixture for 10 min. at 37 °C) and the RNA was phenol/chloroform extracted, ethanol precipitated, washed in 70% ethanol and dissolved in DEPC-treated water. Specific activity of the RNA was determined by scintillation counting.

Electrophoretic mobility shift assays. For electrophoretic mobility shift assays (EMSAs), ³²P-labeled RNA probes (10⁵ cpm, 2-3 ng RNA) were incubated in cytoplasmic extracts from Hep3B cells, with a protein content of 10 μ g in a 10 μ l reaction mixture containing 20 mM HEPES-KOH pH 7.5, 100 mM KCl, 1 mM MgCl₂, 1 mM DTT, and 0.01 % NP40 for 45 min on

ice. After the binding reaction, the samples were incubated at room temperature for 20 min with a mixture of 12.5 μ g/ml RNase A and 2 U/ml RNase T1. Samples were subsequently electrophoresed on non-denaturing 6% polyacrylamide gels. Gels were dried and exposed to Fuji RX X-ray film. In competition assays, a 100- to 500-fold molar excess of unlabeled competitor RNA was added to the reaction mixture 10 min prior to addition of the probe.

Primer extension analysis. Primer extension was carried out as described by (McKnight and Kingsbury, 1982). A 5' [α ³²P]ATP end-labeled oligonucleotide (DM 6), complementary to nucleotides +99/+120 downstream of the cleavage site, was annealed to 1 μ g of poly(A)⁺ RNA (from cells expressing the EP7-9 Δ -1957/-1110 construct, 8 μ g of poly(A)⁺ RNA was used instead of 1 μ g, because of the low abundance of the 3' cleavage product) in 1 M NaCl, 0.17 M Hepes-KOH pH 7.5, and 0.33 mM EDTA pH 8.0. Subsequently, the primer was extended towards the cleavage site in 0.55 mM dATP/dCTP/dGTP/dTTP, 50 mM Tris-HCl pH 8.2, 5 mM MgCl₂, 50 mM KCl, 0.05 mg/ml BSA, and 5 mM DTT. The extension products were electrophoresed on sequencing gels along with a sequencing reaction carried out with the same primer as a marker. The gels were exposed to Fuji RX X-ray films or to phosphorimager screens (Molecular Dynamics, USA).

Preparation of extracts. Cells were washed in PBS and harvested in 0.025 % trypsin/0.02% EDTA, followed by washing in PBS twice. Then the cells were spun down quickly and the pellet was dissolved in 20 mM Hepes-KOH pH 7.5, 5 mM KCl, 0.1% NP40, 10 mM sodium metabisulfite and left on ice for 20 min. Subsequently, the cells were Dounce homogenized and centrifuged for 5 min at 4 °C 3500rpm. After additional centrifugation for 40 min at 4 °C, 30,000 rpm in a Beckman SW 50.1 rotor, the supernatant was supplemented with 20% glycerol and 1 mM DTT and stored at -80°C.

CHAPTER 4

Distinct RNA structural domains cooperate to maintain a specific cleavage site in the 3'-UTR of IGF-II mRNAs

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SUMMARY

The insulin-like growth factor II (IGF-II) mRNAs are targets for site-specific endonucleolytic cleavage in the 3'-UTR, which results in a very stable 3' cleavage product of 1.8 kb that consists of 3'-UTR sequences and a poly(A) tail. The 5' cleavage product contains the coding region and is rapidly degraded. Thus, cleavage is thought to provide an additional way to control IGF-II protein synthesis. We have established previously that cleavage requires two widely separated sequence elements (I and II) in the 3'-UTR that form a stable duplex of 83 nucleotides. The cleavage site itself is located in an internal loop preceded by two stable stem-loop structures. Furthermore, in a study that was based on RNA folding algorithms, we have shown that there are specific sequence- and structural requirements for the cleavage reaction. In this study, the functions of the different structural domains in cleavage were assessed by deletion/mutational analyses, and biochemical structure probing assays were performed to better characterize the RNA structures formed and to verify the computer folding predictions. The data suggest that the stem-loop domain contributes to maintain a highly specific cleavage site by preventing the formation of alternative structures in the cleavage site domain. Involvement of the nucleotides in the cleavage site loop itself in non-Watson-Crick interactions may be important to provide a specific recognition surface for an endoribonuclease activity.

INTRODUCTION

Human insulin-like growth factor II (IGF-II) is a mitogenic polypeptide consisting of 67 amino acids showing strong structural and functional homology to insulin (Daughaday and Rotwein, 1989). IGF-II is essential for embryonic growth and development as was demonstrated by targeted gene disruption (DeChiara *et al.*, 1990). IGF-II also exerts a wide range of biological activities in cultured cells: it can promote either cell proliferation or differentiation, depending on the cell type in which it is expressed. IGF-II profoundly affects cellular survival and counteracts apoptosis in some cell systems, whereas in other cell lines an apoptosis-inducing effect by IGF-II is observed (Engstrom *et al.*, 1998, and references therein). Thus, as IGF-II is involved in many physiological processes, a proper regulation of IGF-II gene expression is of crucial importance. Therefore, an elaborate set of regulatory mechanisms is available for this gene, ranging from developmental stage-dependent and tissue-specific expression of four different promoters to post-translational processing of the IGF-II precursor protein (for a review, see Holthuisen *et al.* (1999)).

Previously, we established that the human IGF-II gene can also be regulated at the level of mRNA stability. It was shown that the protein-coding part of the IGF-II mRNAs is targeted for rapid degradation through site-specific endonucleolytic cleavage in the 3'-UTR. In contrast to the unstable 5' cleavage product, a very stable 3' cleavage product of 1.8 kb is formed (De Pagter-Holthuisen *et al.*, 1988; Meinsma *et al.*, 1991; Meinsma *et al.*, 1992). Similar observations were made for the rat IGF-II

gene (Nielsen and Christiansen, 1992), and also for the mouse IGF-II gene (Holthuisen *et al.*, 1993).

Regulation of gene expression at the level of mRNA stability is now becoming a common theme, as a rapidly increasing number of genes appears to be regulated at this post-transcriptional level (for reviews, see Beelman and Parker (1995); Ross (1995); Jacobson and Peltz (1996); Wickens *et al.* (1997)). Initiation of mRNA degradation can occur through poly(A) shortening, arrest of translation at a premature stopcodon (nonsense-mediated decay, or NMD), or through endonucleolytic cleavage. To date, for a number of mRNAs endonucleolytic cleavage has been found to be the first step of degradation. Examples are the mRNAs encoding albumin (Dompenciel *et al.*, 1995; Chernokalskaya *et al.*, 1997), the cytokine *groa* (Stoeckle, 1992), avian apo-very-low density lipoprotein II (Binder *et al.*, 1989), the transferrin receptor (Binder *et al.*, 1994), and maternal homeodomain proteins (Brown and Harland, 1990; Brown *et al.*, 1993). The *c-myc* mRNA can be cleaved in the coding region, but can also be destabilized through AU-rich elements in the 3'-UTR (Swartwout and Kinniburgh, 1989; Ioannidis *et al.*, 1996).

We have established that for *in vivo* cleavage of IGF-II mRNAs, two widely separated sequence elements in the 3'-UTR are necessary and sufficient (Fig. 1A) (Meinsma *et al.*, 1992). Element II encompasses the cleavage site, and is located from positions -173 to +150 relative to the cleavage site. It contains two domains that are highly conserved among human, rat and mouse: an upstream domain from positions -133 to -7 containing two stable stem-loop structures, and a downstream G-rich domain from positions -5 to +59. Element I is a very C-rich element of 103 nucleotides long and it is located approximately 2 kb upstream of the cleavage site from positions -2116 to -2013 (Fig. 1A).

Previous RNA folding studies have shown that element I can form a stable RNA:RNA duplex with the downstream region of element II from positions +18 to +101 (Scheper *et al.*, 1995). Thus, the RNA regions found to be required for cleavage can be subdivided into three separate folding domains: (1) the upstream stem-loop domain consisting of a 5'- and a 3' stem-loop; (2) the duplex domain consisting of the downstream region of element II that folds into an extended duplex of 83 nt with element I, and (3) the cleavage site itself, located in an internal loop flanked by two helical regions and a second internal loop (Fig. 1B). We will refer to this latter region as the 'cleavage site domain'. In a previous study, we have found that there are both sequence- and structural requirements for efficient cleavage (van Dijk *et al.*, 1998). Here, we assess the roles of the various RNA folding domains in cleavage. Structure probing experiments were performed to obtain biochemical information on the RNA structures formed and to evaluate the computer predictions. Our data indicate that the stem-loop domain serves to stabilize a proper conformation in the cleavage site domain. Non-Watson-Crick interactions in the cleavage site loop may be important for recognition by an endoribonuclease.

RESULTS

Experimental strategy

In order to study *in vivo* cleavage of IGF-II mRNAs, we previously constructed a human IGF-II minigene construct. In this construct, designated EP7-9, the immediate-early cytomegalovirus (CMV) enhancer-promoter region (Boshart *et al.*, 1985) is fused to the IGF-II protein-encoding exons 7, 8, and 9 (Fig. 2A), giving rise to an IGF-II transcript of 4.8 kb (Meinsma *et al.*, 1992). Transient transfection assays of this construct into human 293 cells that do not express IGF-II endogenously, and subsequent Northern blotting analysis of the IGF-II mRNAs showed that in addition to the 4.8 kb transcript also a specific 1.8 kb cleavage product is formed as a consequence of site-specific endonucleolytic cleavage in the 3'-UTR of IGF-II mRNAs (Meinsma *et al.*, 1991). Analysis of deletion derivatives of EP7-9 revealed that a region in the 3'-UTR between elements I and II from positions -1955 to -174 relative to the cleavage site could be deleted without affecting cleavage efficiency (Meinsma *et al.*, 1992). This construct, named Δ WT in (van Dijk *et al.*, 1998) (Fig. 2B), gives rise to a full-length 3.0 kb IGF-II transcript and a 1.8 kb 3' cleavage product (Fig. 3B, lane 1).

In the present study, we have generated a number of constructs that are similar to Δ WT, but contain in addition deletions in the stem-loop region upstream of the cleavage site (-133/-7). Since Δ WT RNA is cleaved as efficiently as the full-length wild type RNA, the cleavage efficiency of Δ WT was set at 100%. All mutants are named after the positions of the deletions relative to the cleavage site. The minimal free energy foldings of the various RNAs were predicted using the MFOLD version 2.3 Internet server (<http://mfold2.wustl.edu/~mfold/rna/form1-2.3.cgi>) based on the computer algorithm by (Zuker, 1989). For each mutant, the energetically most favorable structure out of a maximum of five alternative foldings is shown.

Major deletions in the stem-loop domain.

To gain insight into the importance of the RNA region from positions -133 to -7, which is predicted to fold into two stem-loop structures, a set of several major deletions was introduced using the *Bal31* exonuclease system (see Materials and Methods). All four resulting deletion mutants lacked a major part of the stem-loop domain, and in two mutants ([Δ -145/-22] and [Δ -170/-6]) also the residues opposite of the cleavage site were deleted (Fig. 3A). In all four mutants, the nucleotides directly adjacent to the cleavage site are still present, and in [Δ -116/-31] and [Δ -116/-24], all sequences constituting the cleavage site domain (Fig. 1B) are still intact. Computer folding however, predicted that formation of the internal loop around the cleavage site is distorted in all mutants, causing the cleavage site to be in a structurally different environment than in the wild type situation (Fig. 3A). The minimal free energy foldings of mutants [Δ -116/-31] and [Δ -116/-24], which bear similar deletions, might be expected to be similar, but are in fact significantly different.

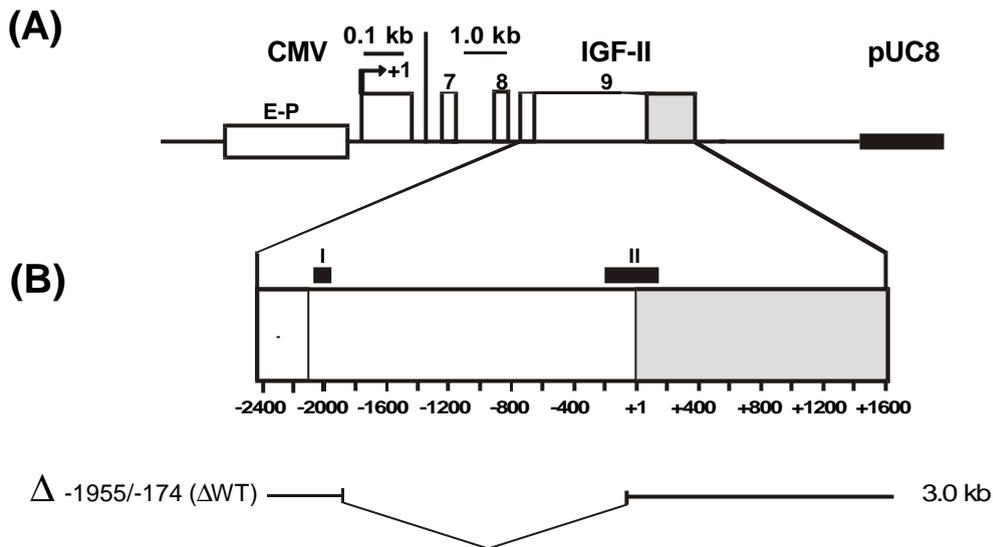


Figure 2. Schematic representation of the CMV-IGF-II minigenes. (A) Construct EP7-9 contains the enhancer/promoter region and exon 1 of cytomegalovirus (CMV) fused to a 10.9 kb genomic fragment of the human IGF-II gene containing exons 7, 8, and 9 cloned in pUC8 (bold line). The CMV regions and the IGF-II and pUC regions are drawn at different scales. The arrow indicates the transcription start site. Translated and untranslated IGF-II regions are depicted by filled and open boxes, respectively. The stippled area in exon 9 represents the 1.8 kb RNA region. (B) Enlarged representation of human IGF-II exon 9. Positions (bp) in exon 9 are relative to the cleavage site (+1). The two elements required for cleavage, element I (-2116 to -2013) and element II (-174 to +150) are indicated by bars (I, II). The EP7-9 derivative Δ WT, in which the region between elements I and II (-1955 to -174) is deleted, is shown. The transcript of Δ WT (3.0 kb) is endonucleolytically cleaved with wild type cleavage efficiency (Meinsma *et al.*, 1992).

A suboptimal folding of mutant [Δ -116/-24] with only a slight increase in Δ G (from -185.7 to -184.1), however, appeared similar to the folding shown for [Δ -116/-31], indicating that both mutants can fold into the same structure.

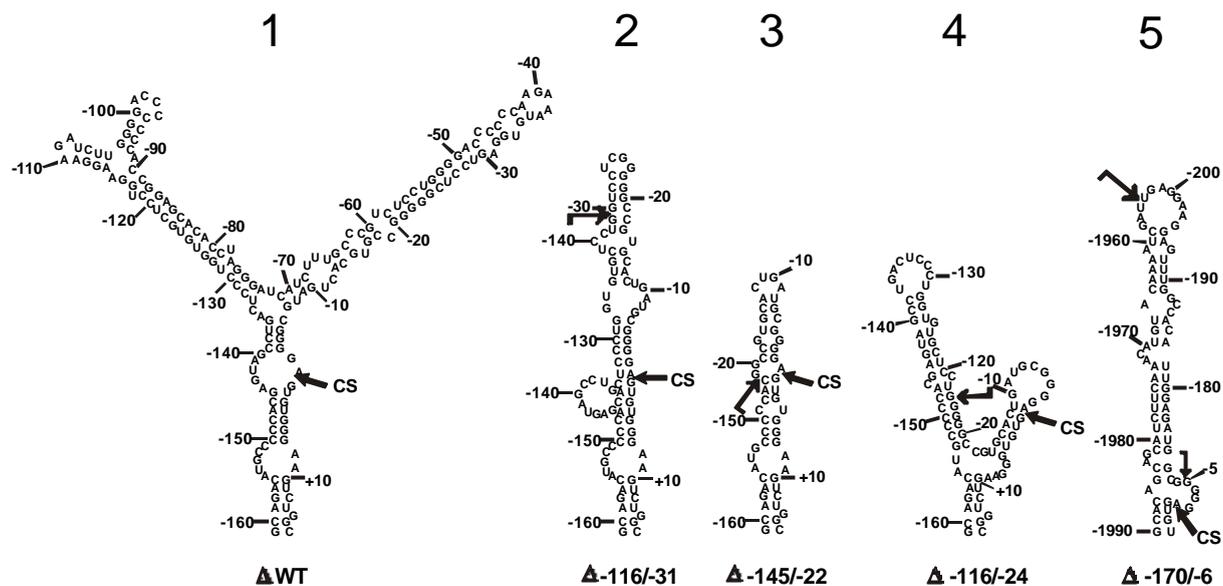
The effects of the deletions on cleavage efficiency were tested in transient transfection experiments of human 293 cells, which do not express IGF-II endogenously. After transfection, RNAs were isolated from the cells and subjected to Northern blot analysis using a probe that detects the 1.8 kb 3' cleavage product and the full-length IGF-II RNA (Fig. 3B). Cleavage of the wild type RNA (Δ WT) was very efficient as shown by the amount of 1.8 kb RNA relative to full-length IGF-II mRNAs (lane 1). This cleavage efficiency was set to 100%. Compared to the wild type RNA, all mutants showed a severe reduction in cleavage activity, or were not cleaved at all (lanes 2-5). These results indicate that large deletions of the stem-loop region upstream of the cleavage site strongly interfere with cleavage. Although the nucleotide sequence around the cleavage site itself is intact, the folding of the RNA is severely affected. Absence of IGF-II mRNA cleavage could be caused either because one or both of the stem-loops has/have a specific function in cleavage or because this RNA region is involved in maintaining a proper fold around the cleavage site.

Subdomain deletions in the stem-loop domain.

To distinguish between these two possibilities, several specific deletions were introduced in the stem-loop region while maintaining the predicted wild type secondary structure around the cleavage site (Fig 4A).

It should be noted that in all these constructs, the duplex domain (positions -2108 to -2029 and +18 to +101) was left intact, since this region is absolutely required for cleavage; absence of the duplex domain was shown to abolish cleavage completely (Scheper *et al.*, 1995). Computer folding and structure probing studies indicate that absence of the duplex interaction results in structural changes in the cleavage site domain (see Discussion). The cleavage efficiency of the various mutants was tested in transient transfection assays of human 293 cells followed by Northern blot analysis of the isolated RNAs. A region from positions -104 to -78 was deleted in mutant [Δ -104/-78], leading to a truncated 5' stem-loop (Fig 4A, structure 2). This mutant shows a moderate reduction in cleavage efficiency to 57% of the wild type, as shown in Fig. 4B, lane 2. In mutant [Δ -135/-72], the entire 5' stem-loop is deleted while the 3' stem-loop is intact (Fig. 4A, structure 3). This mutant structure can also still be cleaved with a reasonable efficiency (51%), indicating that the 5' stem-loop is not absolutely required for cleavage. In mutant [Δ -135/-72, Δ -60/-18], the 5' stem-loop is deleted and the 3' stem-loop is truncated, leading to the predicted structure shown in Fig. 4A, structure 4. Although the cleavage site loop is still predicted to be in the wild type configuration in this mutant, cleavage is almost absent (Fig. 4B, lane 4). Surprisingly, however, when the deletion is extended with another 11 nucleotides, as in mutant [Δ -135/-18] (Fig. 4A, structure 5), thus practically removing both the 5' and the 3' stem-loops, the cleavage efficiency is restored again to 47% of the wild type level (Fig. 4B, lane 5). These data indicate that although the deletions in the stem-loop region reduce the cleavage efficiency about twofold, cleavage is not completely abolished. The result with mutant [Δ -135/-18] shows that cleavage can occur even in the complete absence of both stem-loops. Thus, it appears that the stem-loop domain *per se* is not required for cleavage, but rather that the maintenance of a native conformation around the cleavage site is important. The observation that mutant [Δ -135/-72, Δ -60/-18] is cleaved with a markedly reduced efficiency while the predicted cleavage site domain is intact might be explained by conformational changes around the cleavage site, which are not predicted by the computer program. To identify such conformational changes, we have performed RNA structure probing experiments (see below), and primer extension analysis was performed to check the integrity of the cleavage site position in the various mutants.

(A)



(B)

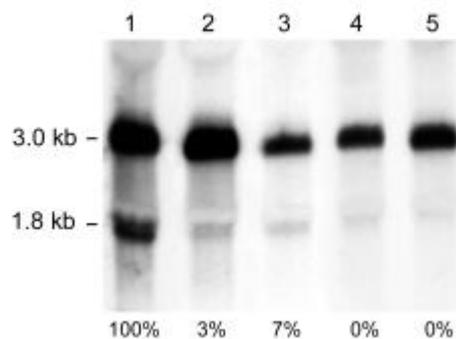


Figure 3. (A) MFOLD predicted secondary structures of the RNA regions encompassing the cleavage site in Δ WT and the various deletion mutants generated with the *Ba*/31 exonuclease system (see Materials and Methods). Shown are the energetically most favorable structures. In all constructs, nucleotides are numbered according to their original position relative to the cleavage site (straight arrow) in the wild type RNA, irrespective of position changes due to the introduced deletions. The deletions are indicated by bent arrows. (B) Northern blot analysis of total RNA isolated from human 293 cells transiently transfected with the CMV-IGF-II minigene constructs. The lanes are numbered according to the numbers of the constructs shown in (A). The blot was hybridized to a radiolabeled DNA fragment (+84 to +1096) that detects both full-length IGF-II mRNA (3.0 kb) and the 3' cleavage product of 1.8 kb (indicated on the left). For each mutant, the cleavage efficiency relative to Δ WT as determined in at least 3 independent experiments (see Materials and Methods) is shown under the lanes.

(A)

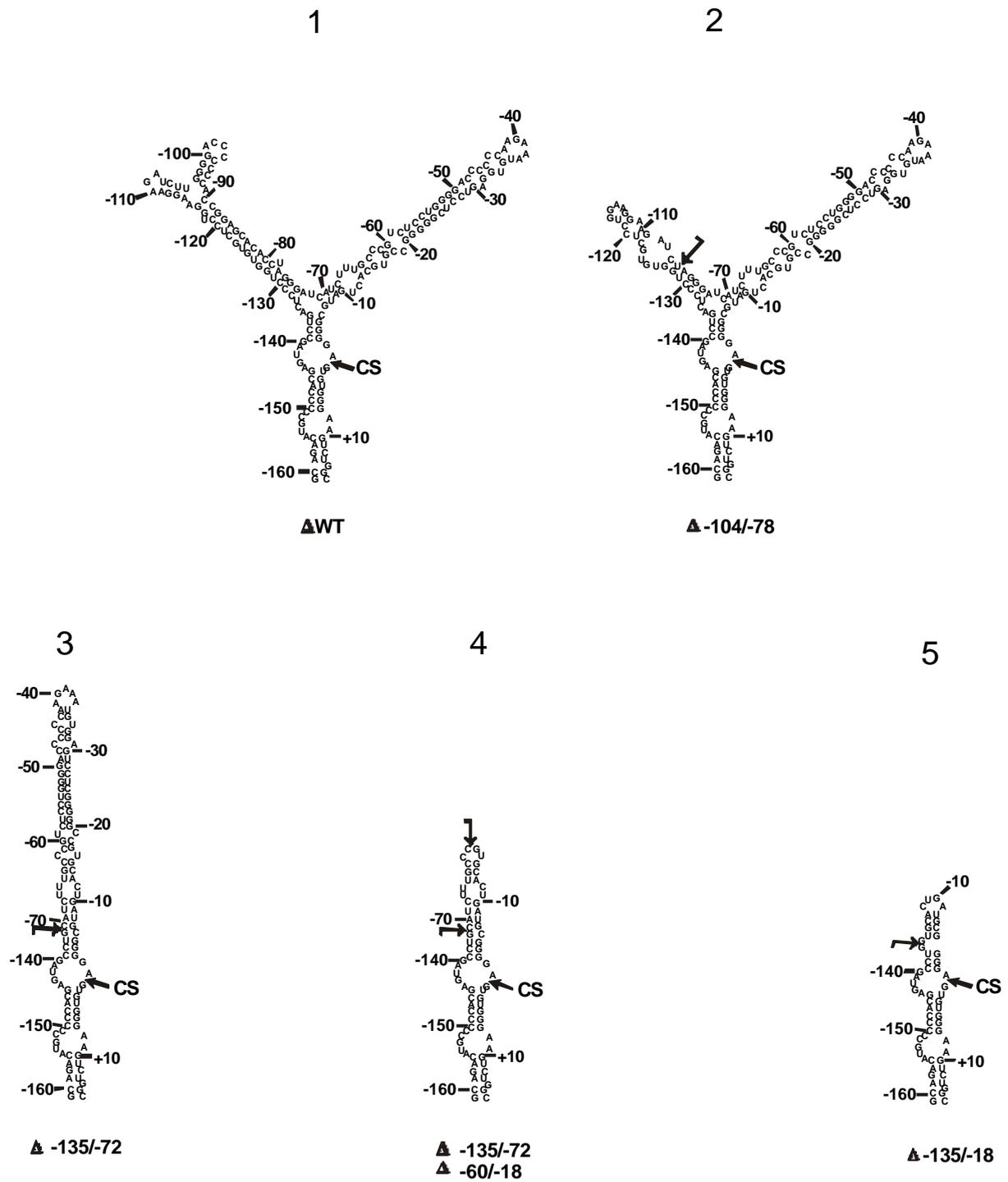


Figure 4. (legend shown on next page)

(B)

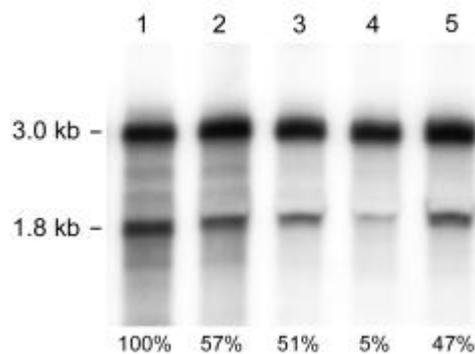


Figure 4. (A) Predicted minimal free energy structures of Δ WT and the various mutants harboring increasing deletions. Numbering of the nucleotides is according to their original positions relative to the cleavage site (straight arrow) in the wild type. The deletions are indicated by bent arrows. (B) Northern blot analysis of total RNA from 293 cells transfected with the various constructs shown in (A). The lanes are numbered according to the numbers of the constructs. Full-length IGF-II mRNA as well as the 3' cleavage product of 1.8 kb was detected using a 3' specific probe hybridizing to nucleotides +84/+1096. Sizes of the RNA species are indicated on the left in kb. The cleavage efficiency of each mutant (relative to Δ WT) as determined in at least three independent experiments is shown under the lanes.

Primer extension analysis.

In order to determine whether the cleavage site specificity is affected by the various deletions in the stem-loop region, primer extension analysis was performed of poly(A)⁺ RNA isolated from 293 cells transiently transfected with the different constructs. Oligonucleotide DM6, complementary to nucleotides +99 to +120, was used for the extension reactions. With all mutants, a single extension product of the expected size was observed, indicating that if cleavage occurs, it is at the correct site (Fig. 5). Even with the *Bal31*-generated deletion mutants [Δ -116/-31] and [Δ -145/-22], which have a very low cleavage efficiency of 3 and 7% of the wild type (Fig. 3B), respectively, an extension product of the expected size could be detected. Thus, neither the absence of the stem-loop region nor the distortion of a native conformation around the cleavage site appears to affect cleavage site specificity. This indicates that the residues adjacent to the cleavage site suffice for specific recognition, although it is clear that structural requirements must be met to allow efficient cleavage.

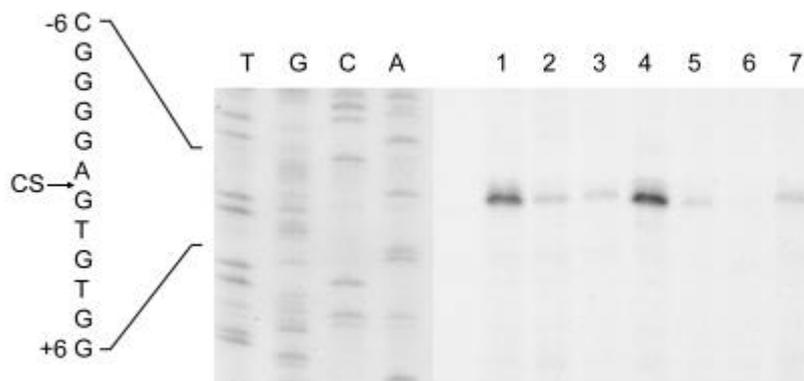


Figure 5. Primer extension analysis of IGF-II RNAs. A 5' end-labeled primer (DM6) complementary to nucleotides +99 to +120 was hybridized to 1 or 3 μg (see Materials and Methods) of poly(A)⁺ RNA from 293 cells transiently transfected with the various IGF-II minigene constructs. A sequencing reaction carried out with the same primer was run along with the extension reactions as a size marker. The position of the cleavage site is shown on the left by an arrow. Contents of the lanes is as follows: lane 1: ΔWT , lane 2: [Δ -116/-31], lane 3: [Δ -145/-22], lane 4: [Δ -104/-78], lane 5: [Δ -135/-72], lane 6: [Δ -135/-72, Δ -60/-18], lane 7: [Δ -135/-18].

Structure probing of the RNA region comprising the stem-loop domain and the cleavage site domain.

In order to evaluate the computer predictions of the secondary structures around the cleavage site, RNA structure probing experiments were performed using the primer extension approach (see Materials and Methods). *In vitro* synthesized RNA was subjected to limited RNase hydrolysis or chemical modification using structure-specific reagents. After stopping the reactions, the RNAs were purified and hybridized with oligonucleotides and primer extension reactions were carried out. Modified nucleotides or cleaved bonds were detected as reverse transcriptase stops in the primer extension reactions.

First, to detect nucleotides not involved in Watson-Crick base-pairing, the following reagents were used: RNase T2, which cleaves 3' to any unpaired nucleotide; RNase T1, which cleaves 3' to unpaired G residues, dimethylsulphate (DMS), which methylates unpaired A's at N-1 and unpaired C's at N-3, and 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulphonate (CMCT), which modifies the N-3 group of unpaired U's and weakly, the N-1 group of unpaired G's. Second, to detect double-stranded RNA regions, RNase V1 was used, which cleaves RNA that is in helical conformations, whether base-paired or single-stranded but stacked. For the primer extension reactions, two different primers were used: WS9502, hybridizing to nucleotides -1/+21 and ED9954, hybridizing to nucleotides +30/+60.

Typical experiments are shown in Fig. 6A and B; a summary of the probing data, based on at least 5 independent experiments is shown in Fig. 6C.

The chemical modification analysis combined with the enzymatic probing allowed a characterization of a substantial part of the RNA region around the cleavage site. In general, the reactivity pattern confirms the predicted secondary structure obtained with the computer algorithm, although also some discrepancies were observed.

The tops of both stem-loops upstream of the cleavage site were especially reactive, indicating that these regions are very well accessible to the reagents (Fig. 6C). At the top of the 5' stem-loop, nucleotides that were predicted to be located in short helices, reacted with single-strand specific reagents, indicating that under our assay conditions these base-pairs were rather unstable, if present at all. Also at the top of the 3' stem, some residues predicted to be base-paired, were reactive to single-strand specific probes, indicating that also here the conformation is more open than predicted.

At the base of the 3' stem, a U-rich region from positions -69 to -63 showed reactivity to RNase T2, suggesting that these nucleotides are not stably base-paired (Fig. 6A and C). This may be due to the formation of an internal loop and two mismatches in this region causing breathing of the helix.

Thus, the stem-loop domain contains regions of flexibility at the tops of both stems and at the base of the 3' stem. However, RNase V1 reactivity was seen in predicted base-paired regions in the stem-loop domain at positions -122 to -119, -82 to -79 and -49 to -45. This indicates that stable helices were formed in these regions. Strong RNase V1 reactivity was also observed in the cleavage site domain at positions -139, -138, confirming the formation of a stable helix above the cleavage site loop. Under the assay conditions used, RNase V1 showed a preference for C-residues, whereas it has been described to attack any nucleotide in helical conformation.

The residues at positions -144 to -140 and -2 to +1, forming the internal loop around the cleavage site, showed no clear reactivity to single-strand specific probes, except weakly for the G residue at position -143. This indicates that these bases are not free in solution under the native conditions used. The U-residue at position +2, which is predicted to be base-paired with the G at position -145 reacted with CMCT, but in contrast, the nucleotides 5'-GA9G-3' at the cleavage site were not reactive under native conditions. These data show that the reactive sites of the nucleotides in the cleavage site loop are protected from the reagents, suggesting that they are not exposed, as would be expected for an open loop conformation.

In contrast to the residues of the cleavage site loop, nucleotides in the second internal loop in the cleavage site domain (positions -155 to -151 and +8, +9) clearly showed reactivity under native conditions (Figure 6A, B, and C). The A at position +8 and to a lesser extent the A at +9 were reactive to DMS and RNase T2; the G at position -153 was strongly reactive to RNase T1. These results indicate that this loop has a more open conformation than the loop of the cleavage site.

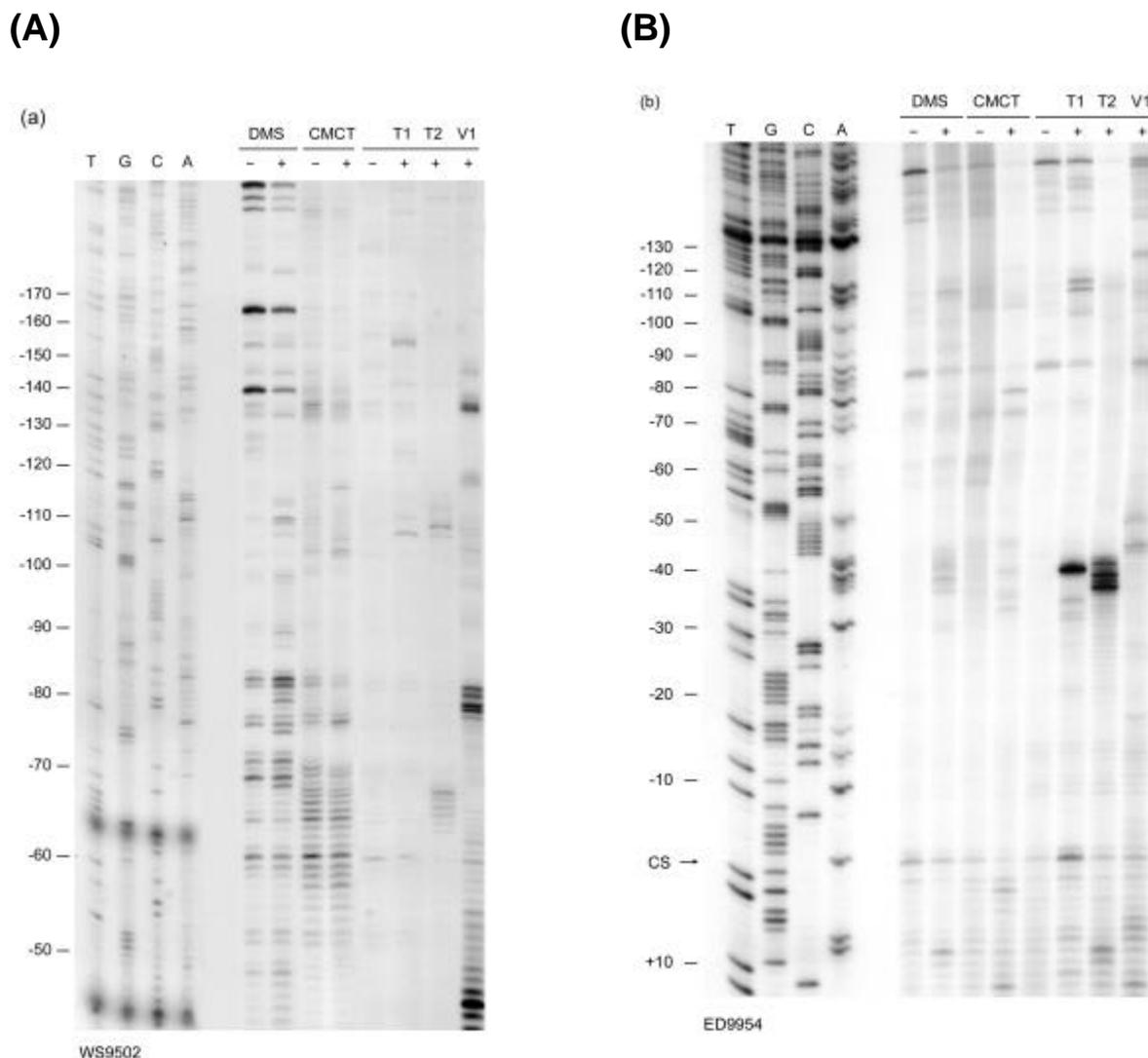


Figure 6. (legend shown on next page)

Structure probing of deletion mutants [Δ -135/-18] and [Δ -135/-72, Δ -60/-18] under native and semi-denaturing conditions.

In order to obtain a better insight into functionally important structural aspects of the cleavage site domain, structure probing of deletion mutants [Δ -135/-18] and [Δ -135/-72, Δ -60/-18] was carried out. Mutant [Δ -135/-72, Δ -60/-18] has a much lower cleavage efficiency than [Δ -135/-18] (Fig. 4A and B), suggesting that important recognition determinants may be lost.

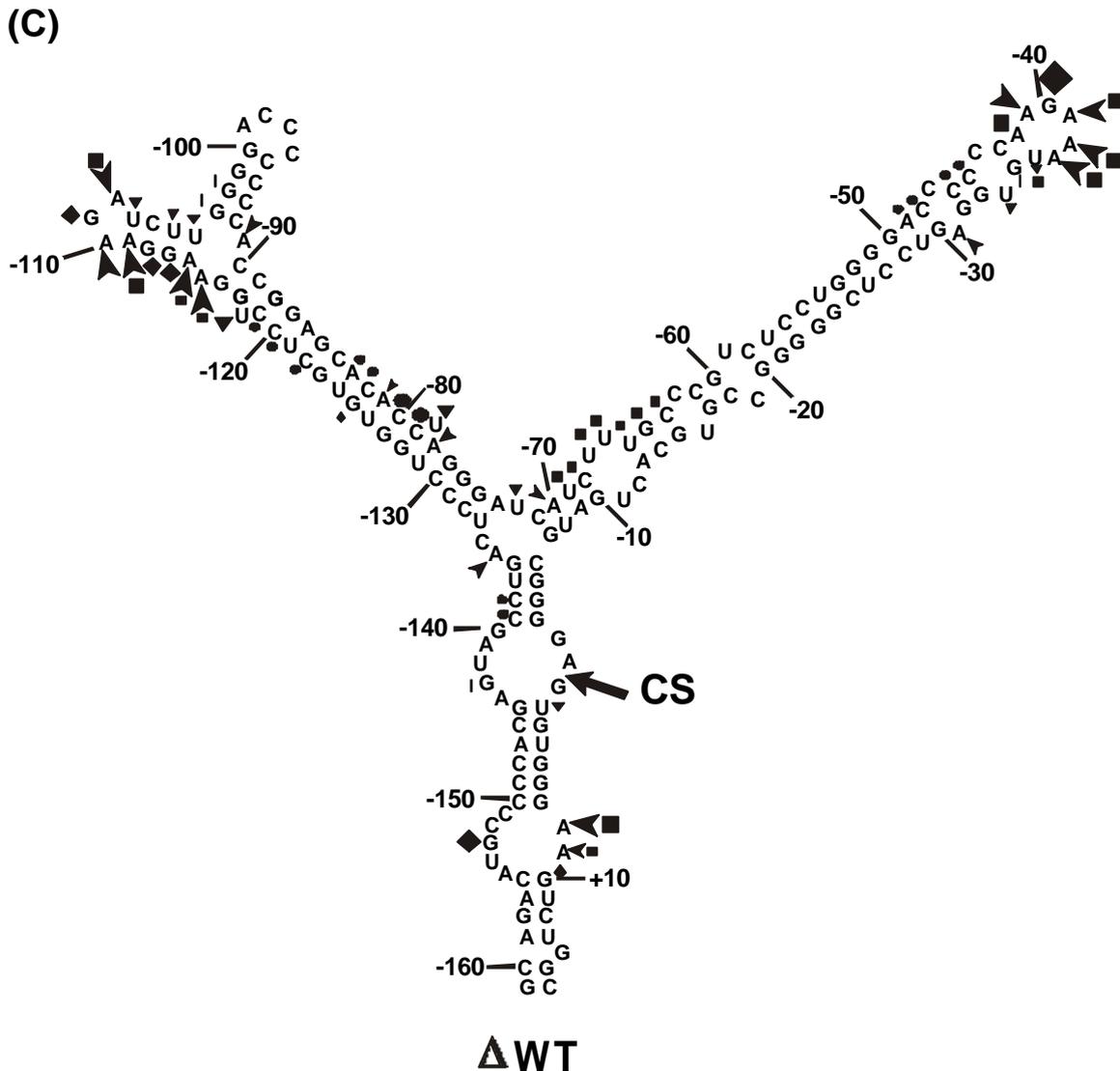


Figure 6. Chemical modification analysis with DMS and CMCT, and enzymatic digestion with RNases T1, T2, and V1. Modifications or digestions were performed in native buffer at 30 °C or room temperature, respectively. Reverse transcription was carried out with (A) primer WS9502, complementary to nucleotides -1 to +21 and (B) primer ED9954, complementary to nucleotides +30 to +60. Control reactions without reagents, or sequencing reactions performed with the same primer were run in parallel. The position of the cleavage site is indicated in (B). Numbering of the nucleotides is as in (C). (C) Summary of the probing results. Residues that were reactive to DMS are indicated by an arrow ('), residues reactive to CMCT are indicated by a triangle (–), and residues showing reactivity to RNases T1, -T2, or -V1 are indicated by a diamond (–), a square (•), or a dot (Ž), respectively. Weak or strong reactivity is represented by smaller or larger symbols.

Structure probing was performed in the presence (native conditions) and in the absence (semi-denaturing conditions) of Mg^{2+} ions. Under the latter conditions, non-Watson-Crick interactions are expected to be weakened. DMS was used to detect single-stranded A's and C's, and CMCT for single-stranded U's and G's. Kethoxal

was also used to detect single-stranded G-residues, instead of RNase T1, because this compound might have a higher resolution due to its smaller size. Primer extensions were done using primer DM6 that is complementary to nucleotides +99 to +120.

With mutant [Δ -135/-18], DMS modification was observed of the A residues at positions +8 and +9 under native conditions, similar to Δ WT (Figs. 6B, 7A and 7B). The reactivity of these nucleotides was not significantly enhanced under semi-denaturing conditions. Similarly, the C at -12 and the A at -9, predicted to be located in the terminal loop were reactive under both native and semi-denaturing conditions, confirming their single-strandedness. Strong reactivity was observed with the A-residues at positions -13 and -147, although these nucleotides are predicted to be located in Watson-Crick helices. Also with CMCT and kethoxal, reactivity of nucleotides in the predicted helices was seen. Thus, these helices may be rather unstable. However, strong RNase V1 reactivity was observed with the C residues at -139 and -138, similar to Δ WT (Fig. 6A, C, and data not shown). This indicates that a short but stable helix is formed right above the cleavage site. The two bulged G residues at positions -136 and -17, bordering the deletion site, are reactive to kethoxal only under semi-denaturing conditions, suggesting that these nucleotides exist in a stacking conformation.

Most significantly, nucleotides -144 to -140 and -2 to +1 show little or no reactivity under native conditions. Under semi-denaturing conditions, however, the A's at -144, -140, and -1 become reactive to DMS, the U at -142 becomes reactive to CMCT, and the G at -140 becomes reactive to kethoxal. The G's at -143, -2 and +1 remain nonreactive. These results indicate that the nucleotides constituting the cleavage site loop are not free in solution, but may be involved in non-Watson-Crick base-pairings.

In mutant [Δ -135/-72, Δ -60/-18], the situation is significantly different. In general, a lower reactivity in the cleavage site domain was observed for this mutant, but the nucleotides in the cleavage site loop were much more reactive (Fig. 8A and B). Their reactivity was similar to the reactivity of the residues predicted to be located in the terminal loop, which were also readily accessible by the reagents.

The reactivity of the A at position -1 towards DMS was also tested in deletion mutant [Δ -135/-72] (Fig. 4A) and a number of previously constructed mutants containing point mutations in or near the cleavage site loop (van Dijk *et al.*, 1998). In mutant [Δ -135/-72] and in the point mutants that are still cleaved, the same reactivity pattern was observed as in [Δ -135/-18], whereas in the inactive mutants the A residue was already reactive under native conditions (data not shown).

Thus, although an exact characterization of the structures formed in the cleavage site domain will require additional experiments, there is correlative evidence that an involvement of the cleavage site loop residues in non-Watson-Crick base pairings may be important for cleavage.

What may cause the altered structure of the cleavage site loop in mutant [Δ -135/-72, Δ -60/-18]? The computer algorithm predicts a wild type conformation in the minimal free energy folding, and also the predicted suboptimal structures of this mutant are rather similar to those of mutant [Δ -135/-18] (data not shown). Thus, it is conceivable that the explanation could lie in the RNA folding pathway and/or higher order interactions that are not considered by the algorithm. In summary, the structure

probing data suggest that a specific structure maintained by a set of non-Watson-Crick interactions in the cleavage site loop may be important for efficient recognition by an endoribonuclease.

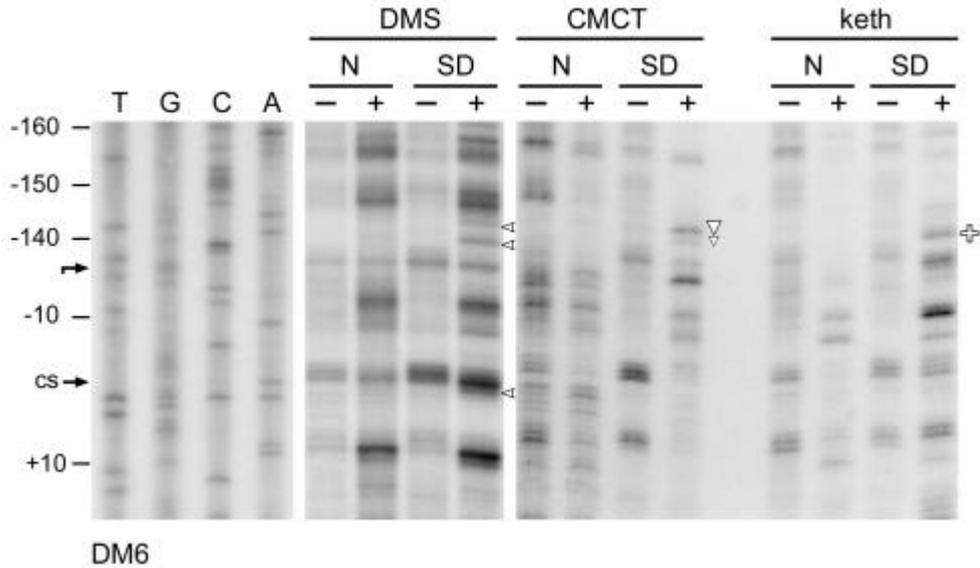
DISCUSSION.

In previous studies, it was established that for site-specific cleavage of full-length IGF-II mRNAs, a 323 nucleotides long sequence element encompassing the cleavage site (element II, positions -173 to +150) as well as a 2 kb upstream located element of 103 nucleotides long (element I, running from positions -2116 to -2013) are necessary. These two elements form a stable duplex, and the resulting RNA structure can be subdivided into three separate folding domains: (1) a domain upstream of the cleavage site that folds into two stable stem-loops, (2) an 83 nt duplex domain formed by element I and the region downstream of the cleavage site, and (3) the RNA domain in the direct proximity of the cleavage site, where the cleavage site itself is located in an internal loop flanked by two helical regions and a second internal loop. In this study, we set out to gain an understanding of the function of the different RNA domains in cleavage by deletion analyses and the consequences of the deletions on RNA folding were predicted by the MFOLD computer algorithm (Zuker, 1989). RNA structure probing studies were employed to verify these computer predictions.

Assessing the importance of the stem-loop domain.

In order to gain insight into the importance of the conserved stem-loop region upstream of the cleavage site, we initially introduced major deletions in this RNA region using the *Bal31* exonuclease system, resulting in four deletion mutants that were predicted to have an altered configuration around the cleavage site (Fig. 3A). Cleavage efficiency was severely reduced in all mutants, indicating that the deleted sequences are important for cleavage. In other deletion mutants that bear increasing deletions in the stem-loop domain but still have a predicted wild type configuration in the cleavage site domain (Fig. 4A), the effects on cleavage efficiency were much less severe. Even in mutant [Δ -135/-18], where the complete stem-loop domain was deleted, cleavage efficiency was still 47% of the wild type. This indicates that the stem-loop domain is not necessary for cleavage *per se*, but rather that the cleavage site domain must be kept in a proper fold to allow cleavage. The observed reductions in cleavage efficiency to about half of the wild type may be due to a destabilization of the RNA structure or to the loss of the binding site for a specific protein which we have identified previously and was found to have a moderately stimulating effect on cleavage (van Dijk *et al.*, 1998). Alternatively, determinants in the stem-loops may interact directly with the endoribonuclease to stabilize nuclease binding and enhance cleavage efficiency.

(A)



(B)

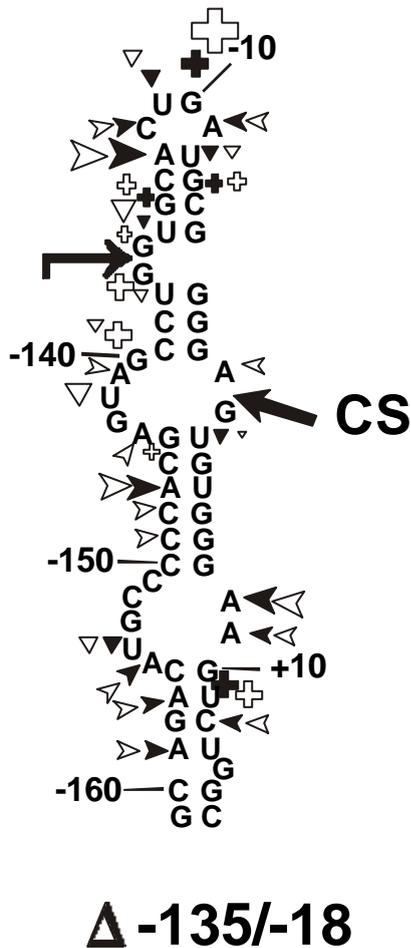


Figure 7. Secondary structure analysis of deletion mutant [Δ-135/-18] under native and semi-denaturing conditions. (A) Autoradiograms resulting from the chemical probing using DMS, CMCT, and kethoxal. Modifications were performed under native (N) or semi-denaturing (SD) conditions at 30 °C. Reverse transcription reactions were done using primer DM6, hybridizing to nucleotides +99 to +120. Control reactions without reagents and a sequencing reaction carried out with the same primer were run in parallel. The positions of the cleavage site (straight arrow) and the deletion site (bent arrow) are indicated. The nucleotides in the cleavage site loop reactive under semi-denaturing conditions to DMS, CMCT, and kethoxal are marked by open arrows, triangles and crosses, respectively. (B) Summary of the probing data. Reactivity to DMS is indicated by an arrow ('), reactivity to CMCT is indicated by a triangle (-), and reactivity to kethoxal is indicated by a cross ([). Reactivity under native conditions is indicated by filled symbols, and reactivity under semi-denaturing conditions is indicated by open symbols. Stronger or weaker reactivity is accompanied by larger or smaller symbols, respectively.

In mutant [Δ -135/-72, Δ -60/-18], cleavage was almost absent. This was surprising, because it has a predicted wild type configuration around the cleavage site. RNA structure probing data however, indicate the presence of conformational changes in the cleavage site loop of this particular construct that may be responsible for this effect (see below).

(A)

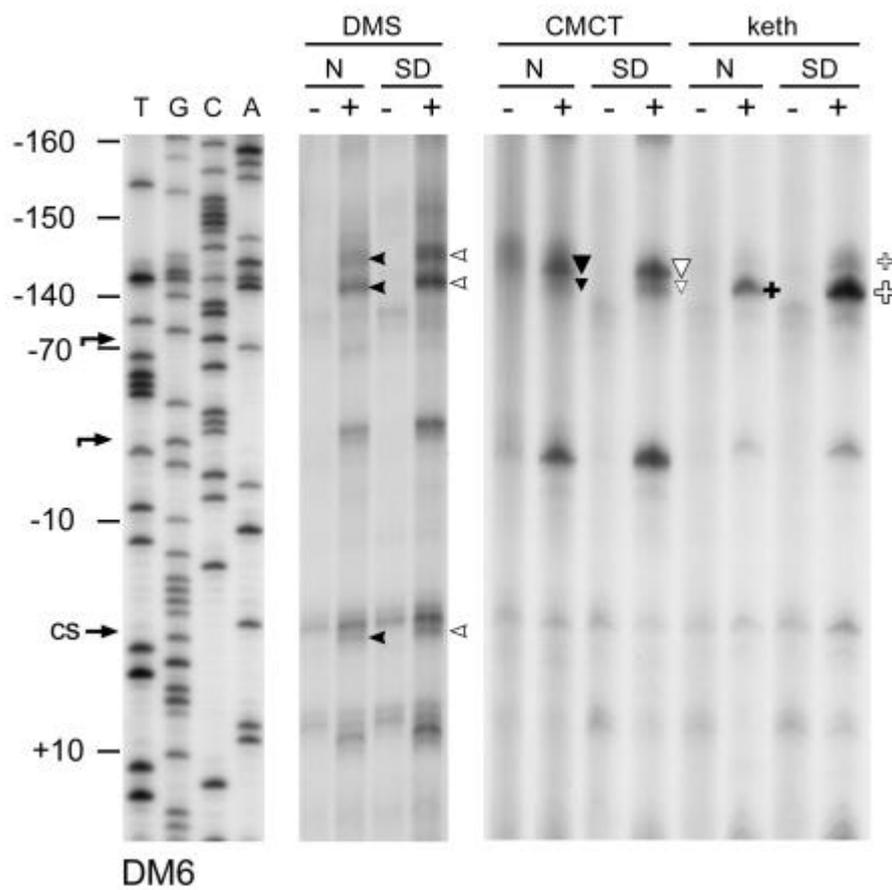


Figure 8. (legend opposite)

(B)

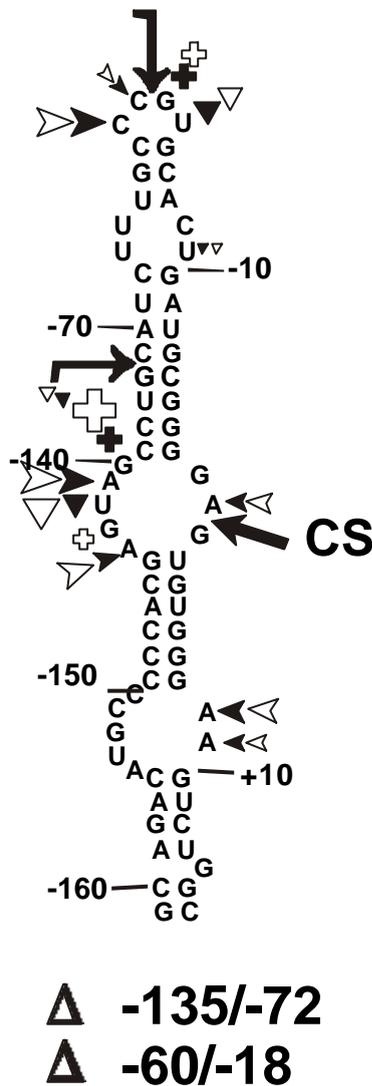


Figure 8. Secondary structure analysis of deletion mutant [Δ -135/-72, Δ -60/-18] under native (N) and semi-denaturing (SD) conditions. (A) Autoradiograms of chemical probing using DMS, CMCT, and kethoxal. Reverse transcription reactions were done using primer DM6. Control reactions without reagents and a sequencing reaction performed with the same primer were run in parallel. The positions of the cleavage site (straight arrow) and the deletion sites (bent arrows) are indicated. The nucleotides in the cleavage site loop reactive to DMS, CMCT and kethoxal under native conditions are shown by filled arrows, triangles and crosses, respectively. Open marks indicate reactive nucleotides under semi-denaturing conditions. (B) Summary of the probing data. Reactivity to DMS is indicated by an arrow ('), reactivity to CMCT is indicated by a triangle (—), and reactivity to kethoxal is indicated by a cross (⊕). Reactivity under native conditions is indicated by filled symbols, and reactivity under semi-denaturing conditions is indicated by open symbols. Stronger or weaker reactivity is accompanied by larger or smaller symbols, respectively.

In order to determine whether the deletions that influence cleavage efficiency to varying extents would also affect cleavage specificity, primer extension mapping of the cleavage site was done in the different mutants. The results show that in all mutants, if cleavage occurs, it is always at the correct cleavage site, since an extension product at the expected position is observed for all mutants (Fig. 5). Thus, none of the deletions affect cleavage site selection, not even in the case of the *Bal31*-generated deletion mutants and mutant [Δ -135/-72, Δ -60/-18], where the efficiency of cleavage is very low.

Our results thus indicate that deletions in the stem-loop domain that affect the structure around the cleavage site severely reduce cleavage efficiency, but nevertheless

specificity is not lost. This suggests that the most important recognition determinants lie in the direct proximity of the cleavage site, and the major role of the flanking stem-loop domain may be to stabilize the structure of this RNA region. Similarly, in an archaeal precursor rRNA, main recognition of three cleavage sites appears to occur at the sequences encompassing each site but adjacent structural elements enhance the efficiency of cleavage (Russell *et al.*, 1999).

Possible function of the duplex domain.

What might be the role of the duplex domain? We have previously shown that the duplex domain is necessary for cleavage as its disruption, either by deletion or inversion of element I abolishes cleavage completely (Scheper *et al.*, 1995). Computer folding predicts that in the absence of the duplex interaction between elements I and II, the region of element II around the cleavage site from positions -20 to +20 can fold back on itself to form a hairpin loop, thus disrupting the structure of the cleavage site domain. In addition, a G-rich region of element II from positions +24 to +56 can adopt a compact G-quadruplex structure in the absence of the duplex (Christiansen *et al.*, 1994), which might also interfere with structure formation around the cleavage site. Preliminary structure probing data confirm that in the absence of the duplex interaction structural rearrangements occur around the cleavage site (data not shown). Although additional experiments will be required to get a clearer picture of the function(s) of the duplex domain, we have strong indications that it may serve to stabilize the cleavage site domain. This would be a similar function to that of the stem-loop domain. Supporting this interpretation, (Christiansen *et al.*, 1994) proposed a secondary structure model for the RNA region around the cleavage site where the cleavage site is also located in a hairpin loop formed by the flanking sequences. These authors based their model on structural analyses of RNAs lacking element I, suggesting that in the absence of the duplex, the structure around the cleavage site may indeed be different. Therefore, element I may act in a manner similar as U3 snoRNA, which is proposed to function as a chaperone to mold pre-rRNA into the correct higher order structure resulting in the exposure of specific cleavage sites (Borovjagin and Gerbi, 1999).

Structure probing of the RNA region comprising the cleavage site domain and the stem-loop domain.

In order to obtain biochemical information on the RNA structures formed in the cleavage site domain and the stem-loop domain, chemical and enzymatic structure probing experiments were performed. In general, the probing data fit well with the predicted structure, although reactivity to single-strand specific probes was observed in predicted base-paired regions at the tops of both stem-loops in the stem-loop domain (Fig. 6A, B, and C). This indicates that in these regions the structure is less compact than predicted, presumably due to the presence of numerous loops and mismatches. In addition, computer predictions suggest the possibility of alternative structure formation in these regions since an older version of MFOLD predicts a

slightly different folding at the tops of the stems (van Dijk *et al.*, 1998). Reactivity to RNase V1, which is specific for helical regions, was observed in predicted double-stranded regions in the stem-loop domain and the cleavage site domain, supporting the computer model.

Strikingly, the nucleotides in the internal loop of the cleavage site (positions -144 to -140 and -2 to +1) show little or no reactivity under native conditions. In contrast, residues in the second loop (positions -155 to -151 and +8, +9) are readily detected (Fig. 6A, B and C). Under semi-denaturing conditions however, the nucleotides of the cleavage site loop also become reactive, except the G residues at positions -2 and +1 (Fig. 7A and B). This suggests that the nucleotides constituting the cleavage site loop are not free in solution, but are involved in non-Watson-Crick interactions, causing the loop to adopt a structured geometry. In this respect, it is relevant to note that the cleavage site loop is very rich in purines, which are known to have a strong tendency to undergo stacking interactions (Saenger, 1984). An alternative possibility is that the cleavage site loop is involved in a long-range tertiary interaction. However, this possibility seems less likely since deletion of the complete stem-loop domain in mutant [Δ -135/-18] did not alter the reactivity pattern of the nucleotides in the loop (see below). In addition, no sequences were found in the duplex domain that are expected on the basis of complementarity to be involved in tertiary contacts with the cleavage site loop.

Comparison of the wild type structure with mutants.

Additional evidence that a specific conformation stabilized by non-Watson-Crick pairings in the cleavage site loop may be functionally important was provided by structure probing assays of deletion mutants [Δ -135/-72, Δ -60/-18], and [Δ -135/-18]. In our deletion experiments, we did the surprising observation that the intermediate deletion mutant [Δ -135/-72, Δ -60/-18] was cleaved with a much lower efficiency (5% of the wild type) than mutants [Δ -104/-78], [Δ -135/-72], and [Δ -135/-18] which are cleaved with a two-fold reduced efficiency compared to the wild type level (Fig. 4B). In the structure probing experiments, the residues in the cleavage site loop of mutant [Δ -135/-18] showed a reactivity pattern that resembles Δ WT; the nucleotides become reactive only under semi-denaturing conditions (Fig. 7A and B). In mutant [Δ -135/-72, Δ -60/-18], however, these nucleotides are already highly reactive under native conditions, indicating that the structure of the loop is different (Fig. 8A and B). Such a conformational change in the loop may explain the reduced cleavage efficiency observed in mutant [Δ -135/-72, Δ -60/-18]. A similar relation between changes in the structure of the cleavage site loop and effects on cleavage efficiency was observed in previously constructed mutants (van Dijk *et al.*, 1998) that contain point mutations in or near the cleavage site loop. Mutations that reduce cleavage efficiency caused the A residue at position -1 to become reactive under native conditions, whereas mutants that are efficiently cleaved, showed a wild type reactivity pattern (data not shown). These results support the idea that a specific geometry is important for cleavage. Stacking interactions and/or non-Watson-Crick base-pairs in the cleavage site loop may allow functional groups to be presented for specific interaction with an endoribonuclease.

Similarly, in a number of other, comparable, purine-rich internal loops, non-Watson-Crick base-pairs were found to facilitate specific interactions with proteins. For instance, in the HIV-1 Rev-responsive element (RRE), a G-G pair in the loop causes a widening of the major groove, allowing base-specific contacts of the Rev protein to an adjacent G-A pair (Battiste *et al.*, 1996). Likewise, in the loop E and α -sarcin loop from rRNA, non-Watson-Crick G-A and A-U pairs provide specific recognition surfaces for proteins (Wimberly *et al.*, 1993; Szewczak and Moore, 1995).

A model of the functions of the various RNA regions in cleavage.

On the basis of the above mentioned data, we suggest that the main function of the stem-loop domain and also of the duplex domain is to stabilize the cleavage site domain, since deletions in both regions interfere with local structure formation around the cleavage site, accompanied by a loss of, or severe decrease in cleavage efficiency. Thus, these two RNA folding domains may act in a cooperative manner to maintain a correct structure in the cleavage site domain. Alternatively, determinants in the stem-loops may directly interact with the nuclease to stabilize its binding and to enhance the cleavage efficiency. The cleavage site loop adopts a compact structure through the formation of unusual base-pairs and stacking interactions. Such a specific geometry in the loop may be important for cleavage, since all mutants with an altered loop configuration as judged by an increased sensitivity to single-strand specific reagents showed a marked reduction in cleavage efficiency. A general implication of our results is that a specific RNA recognition site can be heavily dependent on rather large surrounding RNA regions for the maintenance of an appropriate structural context.

ACKNOWLEDGMENTS

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MATERIALS AND METHODS

Materials. Plasmid pBluescript II (KS⁺) was obtained from Stratagene (La Jolla, CA, USA). Restriction enzymes, T4 DNA ligase, and T7 RNA polymerase were purchased from Boehringer, Mannheim, Germany. *Pfu* DNA polymerase was purchased from Stratagene, RNase-free DNase was from Kabi-Pharmacia (Uppsala, Sweden). AMV reverse transcriptase was from Pharmacia, RNase A and RNase T1 were purchased from Calbiochem (La Jolla CA, USA). RNase T2 and RNase V1 were purchased from Gibco BRL Life Technologies and Pharmacia Biotech, respectively. *Bal31* exonuclease was purchased from BioLabs. Enzymes were used as specified by the manufacturers. Nucleoside triphosphates (NTPs) and dNTPs were obtained from Kabi-Pharmacia; BES [*N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] was purchased from Sigma (St. Louis, MO, USA). A random primer labeling kit was purchased from Amersham (UK), and a DNA sequencing kit was purchased from Kabi-Pharmacia. An *in vitro* transcription kit and a poly(A)⁺ RNA isolation kit were purchased

from Promega, RNazol reagent was purchased from Biotech Laboratories, Inc. (Houston, Texas, USA), and Genescreen membranes were purchased from Du Pont de Nemours (Dreieich, Germany). [α - 32 P]dCTP (3,000 Ci/mmol) and [α - 32 P]CTP (760 Ci/mmol) were purchased from Amersham. Dimethylsulphate (DMS) was purchased from Sigma (St. Louis, MO, USA); 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide (CMCT) and kethoxal were purchased from ICN Biomedicals Inc. *E. coli* tRNA was purchased from Boehringer Mannheim (Germany).

Construction of plasmids. Molecular cloning was performed according to established protocols (Sambrook *et al.*, 1989). Where necessary, 3' recessed ends were filled in with Klenow polymerase and 3' protruding ends were removed with T4 DNA polymerase. All positions within exon 9 of the IGF-II gene are indicated relative to the cleavage site; the nucleotide upstream of the cleavage site is -1; the nucleotide downstream is +1 (Fig. 2B). When exon 9 sequences were deleted or additional restriction sites were introduced, the numbers indicated still refer to their original position in exon 9 relative to the cleavage site. IGF-II minigene constructs were based on the construct EP7-9 and its derivatives EP7-9/Not and EP7-9 Δ -1955/-174 (Δ WT), all three described previously in (Meinsma *et al.*, 1992). EP7-9 contains a human genomic IGF-II fragment that starts 373 bp upstream of exon 7 and extends to 4.7 kb downstream of the gene. In EP7-9/Not, a NotI site was constructed at position +80. Δ WT lacks the sequence between elements I and II. All three constructs are cleaved with a similar efficiency, indicating that neither the NotI site at position +80 nor deletion of the sequence from -1955 to -174 interferes with cleavage (Meinsma *et al.*, 1992). Expression of the constructs in transfected cells was driven by the immediate-early cytomegalovirus enhancer/promoter (Boshart *et al.*, 1985).

Major deletions were introduced in the upstream stem-loop domain (nt -7/-133) using the Bal31 exonuclease as follows. Plasmid BS-Ifw/Iifw, which contains both sequence elements I and II in the forward orientation (van Dijk *et al.*, 1998) was digested with AvrII (position -80). The digested DNA was then incubated with 0.5 U Bal31 and samples were taken from the reaction mixture at various time points. Subsequently, the trimmed DNAs were blunted and ligated. After transformation to *E. coli*, the resulting new clones were checked by digestion analysis and sequencing, and four mutants, harboring deletions from positions [-116/-31]; [-145/-22]; [-116/-24]; and [-170/-6] were selected for PCR amplification of the region encompassing the deletions. PCR was done using oligonucleotides ED9843 (5' ClaI -204/-185 3') and ED9722 (5' NotI +60/+93). The resulting PCR products were digested with ClaI and NotI, and cloned in the corresponding sites of plasmid EP7-9/Not. The derived plasmids were checked by digestion analysis and sequencing.

In addition to the Bal31 mutants, another set of deletion mutants was generated using oligonucleotides. Mutant [Δ -104/-78] was described previously in van Dijk *et al.* (1998). Mutant [Δ -135/-72] was constructed by ligation of oligonucleotides ED9848 and ED9849 (containing a ClaI and a DraII site at the ends) with a DraII- NotI (positions -52 and +80, respectively) fragment of EP7-9/Not. The resulting fragment was cloned into the ClaI/NotI sites of EP7-9/Not. Mutant [Δ -135/-72, Δ -60/-18] was generated by ligation of oligonucleotides ED9844 and ED9845 (ending in a ClaI and an Alw44I site) with an Alw44I-NotI (positions -17/+80) fragment of EP7-9/Not. The product was cloned into the ClaI/NotI sites of EP7-9/Not. For the construction of mutant [Δ -135/-18], the same procedure was followed as for mutant [Δ -135/-72, Δ -60/-18], but now oligonucleotides ED9846 and ED9847 were used. Mutants [Δ -104/-78], [Δ -135/-72], [Δ -135/-72, Δ -60/-18], and [Δ -135/-18] were recloned into pBluescript II (KS⁺) for *in vitro* transcription purposes. The region encompassing elements I and II was PCR-amplified using oligonucleotides ED9601

(5' XbaI -2119/-2100 3') and ED9604 (5' BamHI +181/+202 3'). The PCR products were digested with XbaI and BamHI and cloned into the corresponding sites in pBluescript II (KS⁺). Sequences of the oligonucleotides used for the introduction of deletions, PCR amplification, or primer extension are listed below. Altered nucleotides that were introduced in order to construct restriction sites are underlined.

Oligonucleotide	Sequence
DM6	5'-CTGTCAATCCTCCTGACTTTTC-3'
WS9502	5'-CACACTCCC <u>AT</u> CATCAGTGAC-3'
ED9601	5'-GGCTGG <u>TCTAGA</u> GTTTCCATCAGGTTCCATCC-3'
ED9604	5'-GTCCGC <u>GGATCCA</u> AAGAAACAAAGAGGGGGAAT-3'
ED9721	5'-AACCCCTGGGGGGTGGCCTCGT <u>CA</u> GGATC-3'
ED9722	5'-AAAATCTCCC <u>GCGGCCG</u> CTTCTACCCCAGAAC-3'
ED9843	5'-TGCCGC <u>ATCGA</u> TTGAGGAAGGAGTTTGCCAC-3'
ED9844	5'-CGATGCCGGTAGCTGAGCAGACATGCCCCACGAGTAGCCTGCATCTTTGCCCG-3'
ED9845	5'-TGCACGGGCAAAGATGCAGGCTACTCGTGGGGGCATGTCTGCTCAGCTACCGGCAT-3'
ED9846	5'-CGATGCCGGTAGCTGAGCAGACATGCCCCACGAGTAGCCTGG-3'
ED9847	5'-TGCACCAGGCTACTCGTGGGGGCATGTCTGCTCAGCTACCGGCAT-3'
ED9848	5'-CGATGCCGGTAGCTGAGCAGACATGCCCCACGAGTAGCCTGCATC- TTTGCCCGTCTCCTGGG-3'
ED9849	5'-GTCCCCAGGAGACGGGCAAAGATGCAGGCTACTCGTGGGGGCATG- TCTGCTCAGCTACCGGCAT-3'
ED9954	5'-CTCCCCCGCCCAGCCCCACTGCCCCCACCCACCCC-3'

Cell culture and transfection. Human 293 cells were grown in Dulbecco's modified Eagle's medium. The medium was supplemented with 10% fetal calf serum, 100 IU of penicillin per ml, 100 µg of streptomycin per ml, and 300 µg of glutamine per ml. The 293 cells were transfected in 75 cm² flasks at a confluence of about 50% by the calcium phosphate coprecipitation method (Sambrook *et al.*, 1989; Graham and van der Eb, 1973). Precipitates were prepared with BES-buffered saline (BBS) (Sambrook *et al.*, 1989) and contained 10 µg of CMV/IGF-II constructs and 1.0 µg of RSV-Luciferase (de Wet *et al.*, 1987), and were added to the cells. After 4 hours, the medium was aspirated and the cells were supplied with fresh serum-containing medium. After 30 to 48 hours the cells were washed with PBS and harvested in PBS with 0.025 % trypsin-0.02 % EDTA. To compare transfection efficiencies of the various IGF-II expression constructs, 10% of the cells were used for a luciferase assay (de Wet *et al.*, 1987). RNA was isolated from the remaining 90% of the cells.

RNA isolation and analysis. Total RNA was isolated using the RNeasy reagent according to the manufacturer's instructions. Cells isolated from a 75 cm² flask were lysed in 1.5 ml of RNeasy reagent and RNA was isolated from the water phase by isopropanol precipitation and resuspended in water. RNA was glyoxalated, size-separated on a 1% agarose-10 mM sodium phosphate gel, and transferred to a GeneScreen membrane. The RNA was fixed on the membrane by irradiation with long-wavelength UV light for 2.5 minutes. Northern blots were hybridized in the presence of 50% formamide in glass cylinders with continuous rotation at 42 °C according to the GeneScreen protocols. As a probe, a 1.0 kb SmaI fragment from the human IGF-II exon 9 (positions +84 to +1096), hybridizing to both the full-length IGF-II RNAs and the 3' cleavage product, was used. The DNA fragments were labeled by random

priming with [α - 32 P]dCTP following the Amersham protocols. After 2 hours of prehybridization, the probe was added at a final concentration of 10^6 cpm/ml. Blots were washed after overnight hybridization, to a final stringency of 0.5 x SSC- 1% SDS at 65 °C (1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and exposed to Fuji RX X-ray film. The amounts of cleavage products were determined by densitometric scanning of the autoradiographs of at least 3 independent experiments. Cleavage efficiencies were determined by calculating the amount of 3' cleavage product divided by the total amount of IGF-II RNA. Variation between the calculated cleavage efficiencies in separate experiments was less than 10%.

***In vitro* transcription.** *In vitro* RNAs were synthesized using T7 RNA polymerase on linearized DNA templates according to the instructions of the manufacturer in the presence of 1 mM of each NTP. pBluescript II (KS⁺) plasmids containing IGF-II inserts (described above) were linearized with EcoRV to generate full-length RNA. After RNA synthesis (30 minutes), the template was degraded by DNaseI treatment (1 U in 25 μ l reaction mixture for 10 minutes at 37 °C) and the RNA was phenol/chloroform extracted, ethanol precipitated, washed in 70% ethanol and dissolved in DEPC-treated water.

Primer extension mapping of the cleavage site. Primer extensions were carried out as described by (McKnight and Kingsbury, 1982). Poly(A)⁺ RNA was isolated using the Promega Poly(A)⁺ RNA isolation kit according to the manufacturer's instructions. A 5' [α - 32 P]ATP end-labeled oligonucleotide (DM6), complementary to nucleotides +99/+120 downstream of the cleavage site, was annealed to 1-3 μ g of poly(A)⁺ RNA (from cells expressing the *Bal31*-generated constructs and constructs [Δ -135/-72, Δ -60/-18] and [Δ -135/-18], 3 μ g of poly(A)⁺ RNA was used instead of 1 μ g) in 1 M NaCl, 0.17 M HEPES-KOH pH 7.5, and 0.33 mM EDTA pH 8.0. Subsequently, the primer was extended towards the cleavage site in 0.55 mM dATP/dCTP/dGTP/dTTP, 50 mM Tris-HCl pH 8.2, 5 mM MgCl₂, 50 mM KCl, 0.05 mg/ml BSA, and 5 mM DTT. The extension products were electrophoresed on sequencing gels along with a sequencing reaction carried out with the same primer as a marker. The gels were exposed to Fuji RX X-ray films or to Phosphorimager screens (Molecular Dynamics, USA).

Chemical and enzymatic probing. The method for structure probing was adapted from Christiansen *et al.* (1994). Prior to modification of *in vitro* transcribed RNAs with DMS, 4 μ g (10 pmol) RNA was denatured/renatured in 20 μ l modification buffer (70 mM HEPES-KOH pH 7.8, 10 mM MgCl₂, 270 mM KCl for native conditions; 70 mM HEPES-KOH pH 7.8, 1 mM EDTA for semi-denaturing conditions). The RNAs were heated to 95 °C for 1 minute, followed by a 10 minutes incubation at 56 °C. Then, the samples were allowed to cool slowly to room temperature. An additional 180 μ l of modification buffer was added, and reactions were initiated by the addition of 2 μ l of DMS. Untreated control reactions were treated similarly, except for the fact that DMS was omitted. Reactions were incubated for 5 minutes at 30 °C, and terminated by the addition of DMS termination buffer (1.0 M Tris-acetate pH 7.5, 1.0 M mercaptoethanol, 1.5 M sodium-acetate, 0.1 mM EDTA).

For modification with CMCT, the same denaturation/renaturation procedure was followed as for DMS modification, but now the modification buffer contained 16.7 mM sodium-borate, 10 mM MgCl₂, and 270 mM KCl for native conditions, and 16.7 mM sodium-borate, 1 mM EDTA for semi-denaturing conditions. Subsequently, 20 μ l of a 42 mg/ml solution of CMCT in modification buffer was added, and the samples were incubated for 20 minutes at 30 °C. The reactions were stopped by placing the samples on ice.

For modification with kethoxal, the same denaturation/renaturation procedure as for DMS modification was followed. Then samples were incubated with 200 μg kethoxal for 10 minutes at 30 $^{\circ}\text{C}$. The reactions were terminated by adding 20 μl kethoxal precipitation buffer (0.3 M NaAc, pH 6.0; 0.25 M boric acid).

The DMS- or CMCT modified RNAs were precipitated with 3 volumes ethanol in the presence of 10 μg *E. coli* tRNA as a carrier. Then, the RNAs were reprecipitated together with a [γ - ^{32}P] end-labeled oligonucleotide (DM6 or ED9722) followed by annealing and primer extension analysis as described above. For the kethoxal-modified RNAs, the procedure was the same, except that precipitation was done in the presence of kethoxal precipitation buffer.

For enzymatic probing, 2 μg (10 pmol) of *in vitro* synthesized RNA and 10 μg *E. coli* tRNA was denatured/renatured in 80 μl reaction buffer (10 mM Tris-HCl pH 7.5, 10 mM MgCl_2 , 50 mM KCl for native conditions, and 10 mM Tris-HCl, 1 mM EDTA for semi-denaturing conditions) for 1 minute at 95 $^{\circ}\text{C}$, 10 minutes at 56 $^{\circ}\text{C}$, and slow cooling to room temperature. Then, 0.1 U RNase T1, 0.1 U RNase T2, or 0.2 U RNase V1 was added, followed by 10 minutes incubation at room temperature. The reactions were terminated by phenol-chloroform extraction of the RNAs. Subsequently, the RNAs were precipitated by 3 volumes ethanol in the presence of an extra 10 μg of *E. coli* tRNA, and reprecipitated with a [^{32}P] end-labeled oligonucleotide (DM6, ED9954, or WS9502). Then, the RNAs were subjected to primer extension analysis, as described above.

CHAPTER 5

Kinetics and regulation of site-specific endonucleolytic cleavage of human IGF-II mRNAs

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SUMMARY

Human insulin-like growth factor II (IGF-II) mRNA can be cleaved at a specific site in the 4 kb long 3'-UTR. This yields a stable 3' cleavage product of 1.8 kb consisting of 3'-UTR sequences and a poly(A) tail, and an unstable 5' cleavage product containing the coding region. Obviously, after cleavage the coding region of a particular IGF-II mRNA is targeted to rapid degradation and it is not involved in protein synthesis anymore. Cleavage is therefore thought to provide an additional way to control IGF-II gene expression. In this study, we have investigated the cleavage efficiency of the various IGF-II mRNA species, which are generated by transcription from four promoters in the IGF-II gene, and differ among each other in their 5'-UTR (leader) sequences. In addition, we have examined the kinetics of cleavage and its role in destabilization and translation of the IGF-II mRNAs using IGF-II constructs that are under the control of a tetracycline-responsive promoter. The data indicate that under standard cell culture conditions, cleavage is a slow process and has little effect on the mRNA decay rate and protein production. In studies with human Hep3B cells that endogenously express IGF-II, no effect on the cleavage efficiency is observed when the cells are grown in the presence of trichostatin A (an inhibitor of IGF-II gene expression), dexamethasone (a stimulator of IGF-II gene expression), or in the presence or absence of serum. However, when the cells reach confluence, cleavage is upregulated leading to 2-3 fold higher levels of the 3' cleavage product, indicating that cleavage is regulated in a cell density-dependent manner. Our results indicate that site-specific endonucleolytic cleavage of the IGF-II mRNAs may have a limited role in the degradation of the IGF-II mRNAs and the production of IGF-II protein. Possibly, the 3' cleavage product itself may have an intrinsic cellular function.

INTRODUCTION

Human insulin-like growth factor II (IGF-II) is a mitogenic polypeptide of 67 amino acids with important functions in cell growth and development (Jones and Clemmons, 1995). Depending on the cell type in which it is expressed, IGF-II can exhibit remarkably different activities. IGF-II can promote either cell proliferation or differentiation; it strongly affects cellular survival and counteracts apoptosis in some cell systems, whereas in other cell lines an apoptosis-inducing effect by IGF-II is observed (Engstrom *et al.*, 1998, and references therein). Thus, as IGF-II is involved in many physiological processes, a proper regulation of its expression is of crucial importance. Indeed, IGF-II gene expression is regulated at virtually all levels, ranging from developmental stage-dependent and tissue-specific activity of four different promoters to post-translational processing of the IGF-II precursor protein (for a review, see Holthuisen *et al.* (1999)).

Previously, we have observed that, in addition to the different mRNAs that are transcribed from the four promoters P1 to P4 of the human IGF-II gene and range in size from 6.0 to 2.2 kb, also a smaller RNA species of 1.8 kb is formed (De Pagter-

Holthuisen *et al.*, 1988). This RNA is the product of endonucleolytic cleavage at a specific site in the 3'-UTR of the full-length IGF-II mRNAs (Meinsma *et al.*, 1991). In addition to this 1.8 kb RNA, which represents the 3' cleavage product, also a 5' cleavage product is formed that contains the coding region of the mRNA. Similar observations were made for the rat IGF-II gene (Nielsen and Christiansen, 1992), and the mouse IGF-II gene (Holthuisen *et al.*, 1993). Because the 5' cleavage product lacks a poly(A) tail, it is very unstable and not translated (Nielsen and Christiansen, 1992). Thus, as cleavage abolishes the protein-coding potential of a particular mRNA, it provides the cell with a putative additional mechanism to control IGF-II protein production, acting at the level of mRNA stability.

In recent years, it has become clear that gene regulation at the level of mRNA stability is rather common (Beelman and Parker, 1995; Ross, 1995; Jacobson and Peltz, 1996; Wickens *et al.*, 1997). Initiation of mRNA degradation can occur through poly(A) shortening, arrest of translation at a premature stopcodon (nonsense-mediated decay, NMD), or through endonucleolytic cleavage. To date, specific endonucleolytic cleavage has been observed for a number of mRNAs. Examples are the mRNAs encoding albumin (Dompenciel *et al.*, 1995; Chernokalskaya *et al.*, 1997), the cytokine *groa* (Stoeckle, 1992), avian apo-very-low density lipoprotein II (Binder *et al.*, 1989), the transferrin receptor (Binder *et al.*, 1994), and the *Xenopus* homeodomain protein Xlhbox2 (Brown and Harland, 1990; Brown *et al.*, 1993). The *c-myc* mRNA can be cleaved in the coding region, but can also be destabilized through AU-rich elements in the 3'-UTR (Swartwout and Kinniburgh, 1989; Ioannidis *et al.*, 1996). Recently, it has been reported that the human α -globin mRNA can also be cleaved at a specific site in the 3'-UTR (Wang and Kiledjian, 2000a).

Our previous studies aimed at elucidating the determinants for recognition of the cleavage site by an endoribonuclease. We have found that extensive RNA secondary structures formed by two widely separated sequence elements are required for cleavage (Scheper *et al.*, 1995). These RNA structures may act cooperatively to maintain a sequence- and structure-specific cleavage site located in an RNA internal loop (van Dijk *et al.*, 1998; van Dijk *et al.*, 2000).

The aim of the present study is to obtain a better insight into the kinetics of specific endonucleolytic cleavage of the IGF-II mRNAs and the impact on the overall mRNA decay rate and protein production using a tetracycline-inducible system. In addition, we have studied the effects of various exogenous factors on cleavage in human Hep3B cells that endogenously express IGF-II.

RESULTS

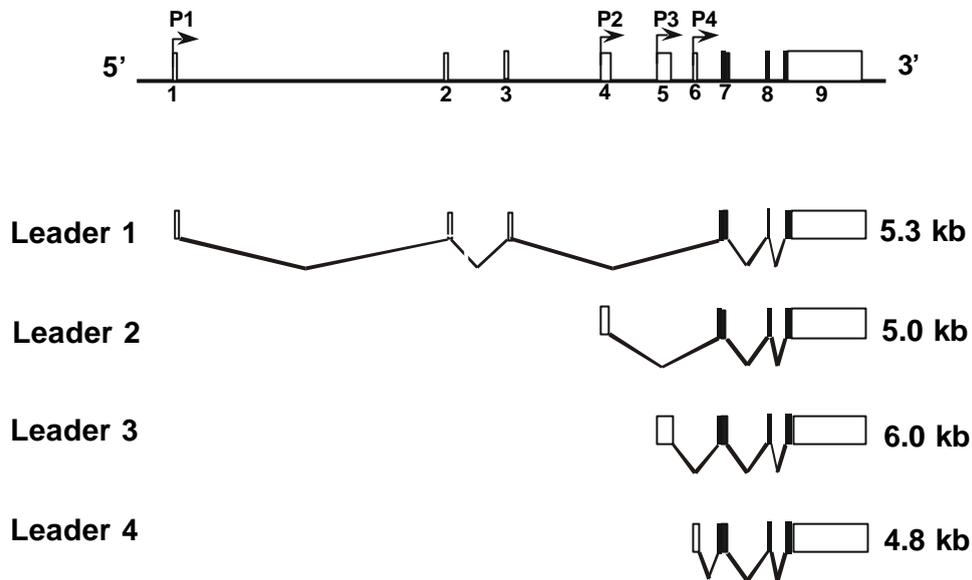
Influence of the 5' leader sequences on the efficiency of specific endonucleolytic cleavage.

The human IGF-II gene contains four promoters (P1-P4), which are differentially active during development and in different tissues. Transcription from these four promoters results in transcripts that differ in their 5'-UTRs (leaders), but have the IGF-II coding region and 3'-UTR in common (Fig. 1A). The leaders vary significantly in their length and composition. Leader L1 consists of exons 1-3 and is 586 nt long and very GC rich. Leader L2 (408 nt) consists of exon 4 sequence and is moderately rich in G and C residues; leader L3 (1171 nt) consists of exon 5 sequence, is rich in C-residues and is the longest 5'-UTR; leader L4 consists of exon 6 sequence and is the shortest 5'-UTR with only 109 nt. Computer-assisted RNA secondary structure prediction suggests that leaders L1 and L3 contain stable secondary structures; leader L2 is less structured, and leader L4 does not contain any stable secondary structure at all (not shown). It was established that transcripts with leaders 1, 2, and 4 are efficiently translated, whereas leader 3 has a strongly repressive effect on translation (de Moor *et al.*, 1994). Thus, it appears that the leaders have significant effects on translation of the messengers.

In order to test the possibility that the nature of the individual leaders also affects cleavage, we set out to determine the cleavage efficiency of IGF-II mRNAs carrying the different leaders. For this purpose, we generated constructs in which the four different leaders (L1 to L4) are connected to the IGF-II open reading frame and the 4 kb long 3'-UTR (Fig. 1A). These constructs were transiently transfected to human 293 cells, which do not express IGF-II endogenously, but are able to perform the cleavage reaction. After transfection, total RNA was isolated from the cells and subjected to Northern blot analysis. Unfortunately, especially for the 5.0 and the 5.3 kb mRNA species, considerable interference of the full-length IGF-II RNA signals with the 28S ribosomal RNA was observed, due to comigration of this rRNA species with the IGF-II mRNAs (data not shown). Therefore, poly(A)⁺ RNA was isolated and analyzed by Northern blotting using the 3'-UTR specific probe (Fig. 1B). The result shows that all four IGF-II transcripts are cleaved, since the 1.8 kb 3' cleavage product is observed with all mRNAs. The IGF-II RNA signals on a number of Northern blots were quantified by Phosphorimager scanning, and the efficiency of cleavage was calculated by dividing the amount of 3' cleavage product by the total amount of IGF-II RNA, i.e. the sum of the full-length RNA and the cleavage product. Small, but distinct differences in cleavage efficiency were observed; the cleavage efficiency of the transcript with the shortest and least structured 5'-UTR, L4, was set at 100%. The L1 and L3 RNAs are cleaved rather efficiently, with 140 and 141%, respectively, relative to L4 RNA; L2 is cleaved less efficiently than L1 and L3, but still more efficiently than L4, with 111%. Thus, although the 5'-UTR sequences do not have all-or-none effects on cleavage, they do affect the efficiency of cleavage to a certain extent. The results suggest a positive correlation between complex structure formation in the

5'-UTR and cleavage, since the highly structured leaders L1 and L3 are cleaved with a higher efficiency than the unstructured leader L4.

(A)



(B)

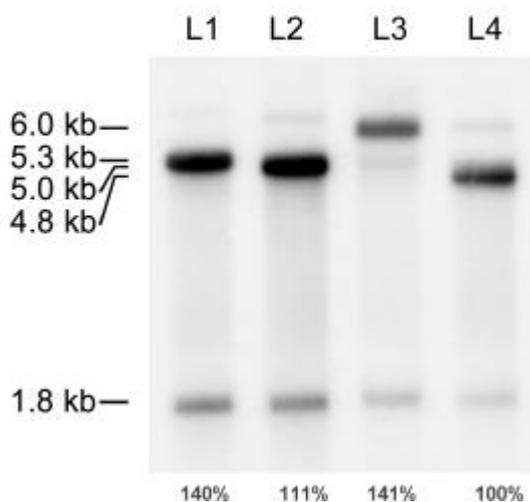
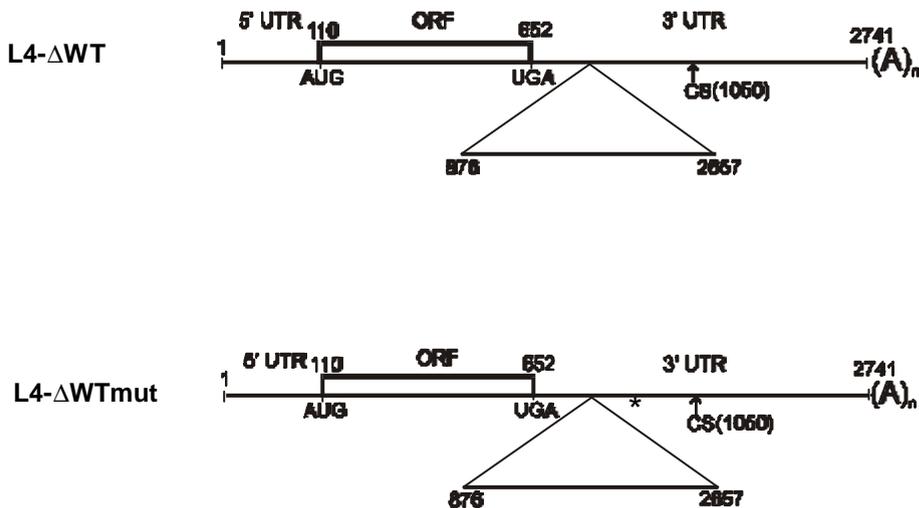


Figure 1. Effect of the 5' leaders on cleavage efficiency. (A) Structure of the human IGF-II gene and the mRNAs generated by transcription from promoters P1 to P4; the transcripts differ among each other in their 5' leader sequences, but have their coding region and 3'-UTR in common. Promoters P1 to P4 are indicated by bent arrows. Exons 1 to 9 are indicated by boxes; open boxes represent untranslated sequences, filled boxes represent the IGF-II coding region. The sizes of the transcripts with leaders L1 to L4 are indicated in kb on the right. (B) Northern blot analysis of poly(A)⁺ RNAs isolated from 293 cells transfected with plasmids L1 to L4; these plasmids express the IGF-II transcripts with leaders L1 to L4 shown in (A). The blot was hybridized with the IGF-II 3'-UTR specific probe; the detected RNA species are indicated in kb on the left. The calculated cleavage efficiencies of the different transcripts are indicated below the lanes.

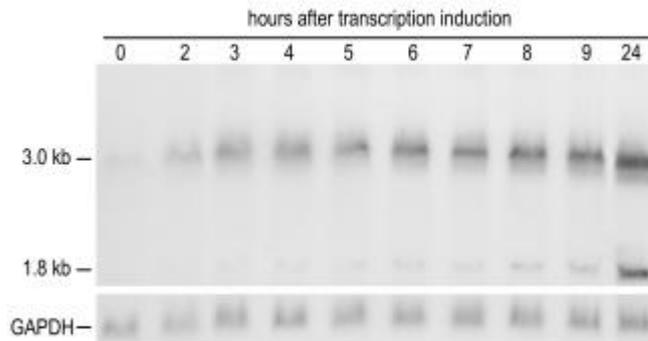
Effect of cleavage on the rate of mRNA decay and protein production.

Subsequently, we have investigated the consequence of cleavage on overall mRNA degradation. Initial studies were performed in human Hep3B cells that endogenously express IGF-II and murine Ltk⁻ cells stably transfected with IGF-II minigene expression plasmids. Actinomycin D was used to inhibit transcription and the IGF-II RNA levels were measured at various time points after administration of this drug. No significant changes in the IGF-II RNA levels were observed within the first 24 hours after transcription arrest, whereas the unstable *c-myc* transcript quickly vanished under the same conditions, indicating that transcription inhibition was complete (data not shown). These results suggested that the IGF-II transcripts are very stable, although it is generally known that actinomycin D can cause artificial stabilization of some RNAs. Since actinomycin D non-specifically inhibits transcription of all genes, especially after longer time-points, non-physiological conditions may result from extended actinomycin D incubations. Therefore, we favored to use a tetracycline-inducible system, in which the expression of a specific gene of interest can be induced or repressed with minimal interference with the cellular physiology.

(A)



(B)



(C)

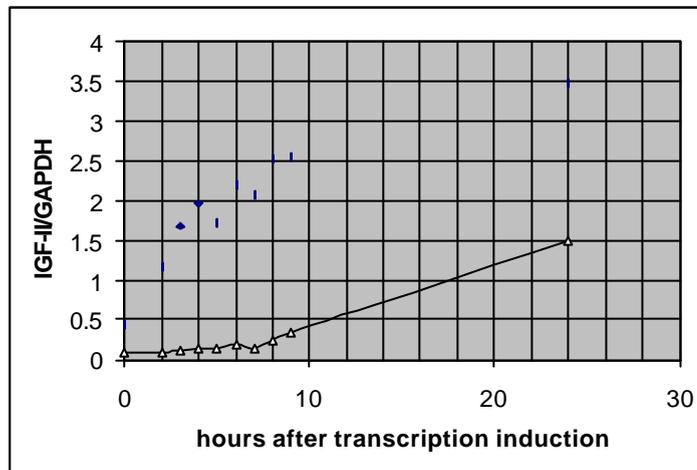


Figure 2. (A) Schematic representation of the transcripts from plasmids pTet-L4- Δ WT and pTet-L4- Δ WTmut. The nucleotide positions bordering the 5'-UTR (leader L4), the IGF-II open reading frame (ORF), and the 3'-UTR are indicated. The deleted region of the 3'-UTR as in Δ WT (van Dijk *et al.*, 1998) is indicated by a triangle. The cleavage site (CS) is marked by an arrow. The asterisk indicates the positions of the point mutations in pTet-L4- Δ WTmut. (B) Northern blot analysis of total RNA isolated from human 293 cells transiently transfected with pTet-L4- Δ WT in the presence of 100 ng/ml tetracycline. After 24 hours, the tetracycline was removed and the cells were harvested for RNA isolation at the indicated time points. The blot was probed with an IGF-II 3'-UTR specific probe hybridizing to nucleotides 84 to 1096 downstream of the cleavage site and a GAPDH coding region probe. (C) Kinetics of the appearance of the full-length L4- WT RNA (\blacklozenge) and the 3' cleavage product (\blacktriangle) after transcription induction. The IGF-II RNA signals were quantified by Phosphorimager scanning and were normalized against the GAPDH RNA signals.

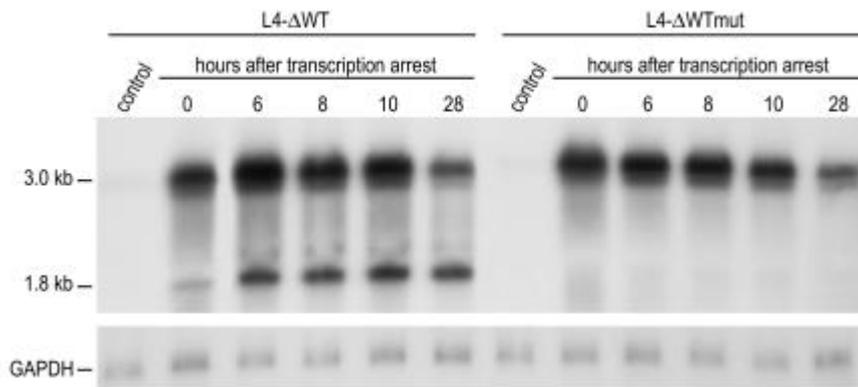
We have constructed two IGF-II expression plasmids that contain a promoter that can be repressed by tetracycline (Fig. 2A). In plasmid pTet-L4- Δ WT, the 5'-UTR of the 4.8 kb IGF-II mRNA (leader 4), which is the shortest leader and has no significant secondary structure, is fused to the IGF-II open reading frame and the

3'-UTR of IGF-II mRNA. The 3'-UTR lacks the region from positions 876 to 2657 in the transcript which was designated Δ WT in van Dijk *et al.* (1998) because it was previously shown to be dispensable for cleavage (Meinsma *et al.*, 1992). To generate plasmid pTet-L4- Δ WTmut, point mutations that completely abolish cleavage were introduced (Fig. 2A) (three nucleotide substitutions at positions -142, -141, and -140 and two nucleotide deletions at positions -144 and -143; see Materials and Methods) (van Dijk *et al.*, 1998). The plasmids were used for transient transfection assays in human 293 cells, which are a suitable model system to study *in vivo* cleavage, since these cells do not express IGF-II endogenously, but are able to perform the cleavage reaction.

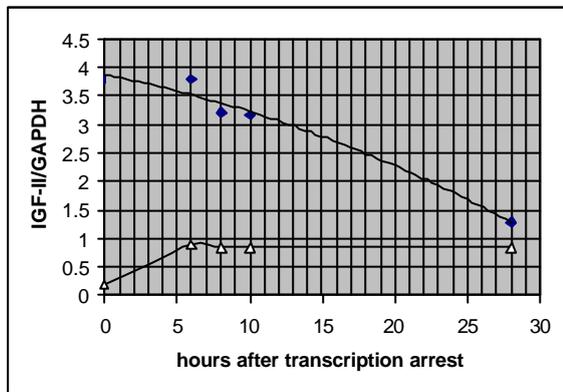
First, to examine the rate of cleavage, the 293 cells were transfected with plasmid pTet-L4- Δ WT in the presence of 100 ng/ml tetracycline to repress the expression of the construct. After 24 hours, tetracycline was removed and subsequently, RNA was isolated at various time points and analyzed by Northern blotting using an IGF-II 3'-UTR-specific probe that detects both the full-length IGF-II mRNAs as well as the 3' cleavage product (Fig. 2B). The blot was also probed for endogenous GAPDH mRNA levels as a control for loading differences. The full-length IGF-II RNA levels increased rapidly up to 6 hours after tetracycline removal, followed by a slower increase up to 24 hours (Fig. 2B, C). Accumulation of the 1.8 kb 3' cleavage product, however, was very slow up to 9 hours after transcription induction. The relative amount of the 3' cleavage product was only 7.7% of the total IGF-II RNA at 6 hours after transcription induction as determined by Phosphorimager scanning and quantification; at 9 hours after transcription induction, the relative amount of the 3' cleavage product was slightly increased to 12% of the total IGF-II RNA. From 9 to 24 hours after transcription induction, a more rapid increase was seen up to 30% at 24 hours. Thus, there appears to be a significant time delay between formation of the full-length RNA and the cleavage product. These results indicate that the accumulation of the 3' cleavage product proceeds rather slowly.

In order to directly test the effect of cleavage on the rate of mRNA degradation, the reverse approach was taken in a subsequent experiment. The 293 cells were transiently transfected with plasmids pTet-L4- Δ WT and pTet-L4- Δ WTmut in the absence of tetracycline to allow expression of the constructs. At 24 hours after transfection, tetracycline was added to a concentration of 500 ng/ml to ensure complete inhibition of transcription. Subsequently, RNA was isolated at various time points, and subjected to Northern blot analysis using the IGF-II 3'-UTR-specific probe and the GAPDH probe (Fig. 3A). Control transfections carried out in the presence of 500 ng/ml tetracycline did not yield any expression of the constructs, showing that the inhibition of transcription was complete. For both constructs, no significant decrease in the full-length RNA levels was observed up to 6 hours after transcription inhibition, indicating that the RNAs are rather stable. After 8 and 10 hours, however, the RNA levels were decreased to about 85% of the initial levels at time-point 0, and after 28 hours, a reduction to ~33% was found. Similar decay kinetics were observed for both the L4- Δ WT and the L4- Δ WTmut RNAs, irrespective of the presence or absence of cleavage, indicating that cleavage did not accelerate the decay rate of the L4- Δ WT RNA (Fig. 3B, C).

(A)



(B)



(C)

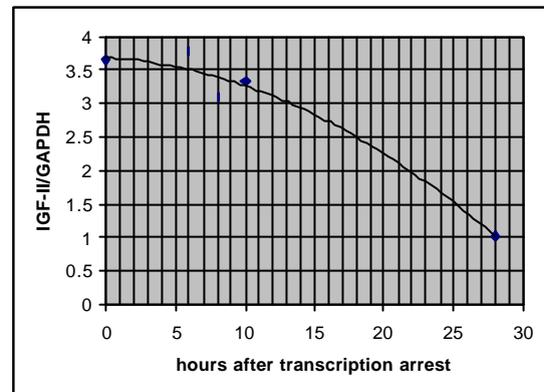


Figure 3. (A) Northern blot analysis of total RNA isolated from human 293 cells transiently transfected with pTet-L4- Δ WT or pTet-L4- Δ WTmut in the absence of tetracycline or, as a control, in the presence of 500 ng/ml tetracycline. At 24 hours after transfection, tetracycline was added to a concentration of 500 ng/ml to repress expression of the constructs and cells were harvested at the indicated time points. The blot was hybridized with the IGF-II 3'-UTR specific probe and a GAPDH coding region probe. (B) Kinetics of the full-length L4- Δ WT RNA levels (◆) and the levels of the 3' cleavage product (▲) after transcription arrest. (C) Kinetics of the L4- Δ WTmut RNA levels (◆) after transcription arrest.

While the L4- Δ WT full-length RNA levels declined, the abundance of the 3' cleavage product did not decrease throughout the course of the experiment (Fig. 3B). Although the formation of the 3' cleavage product continues during the experiment due to ongoing cleavage, this will be with a diminished rate since the substrate full-length RNA levels decline. This result confirms previous observations that the 3' cleavage product is a very stable RNA species. Notably, the level of the 3' cleavage product at time-point 0 was about fourfold lower than that at 6 hours after transcription inhibition. This suggests that at time-point 0 (24 hours after transfection), the levels of the 3' cleavage product had not yet reached a steady-state level, reflecting the slow

rate of cleavage. Thus, both the formation and the degradation of the 1.8 kb 3' cleavage product appear to be very slow.

In order to examine whether protein synthesis is affected by cleavage of the IGF-II mRNAs, reporter plasmids pTet-L4-luc- Δ WT and pTet-L4-luc- Δ WTmut were constructed. In these plasmids, the IGF-II open reading frame was replaced by the luciferase open reading frame. This was necessary, since it is impossible to measure IGF-II production by the cells due to the large amounts of IGF-II present in the medium. The 293 cells were transiently transfected with these plasmids; suboptimal amounts of DNA were used for transfection to ensure that the expression levels of the constructs are in the linear range. After transfection, RNA was isolated from the cells and analyzed on Northern blots. The transcript derived from pTet-L4-luc- Δ WT is efficiently cleaved, indicating that cleavage can still occur when the IGF-II open reading frame is replaced by the luciferase open reading frame (Fig. 4A). This is in agreement with our previous results showing that a region from the IGF-II 3'-UTR containing sequence elements I and II can confer cleavage of a heterologous mRNA (Meinsma *et al.*, 1992). In contrast to L4-luc- Δ WT, the transcript from pTet-L4-luc- Δ WTmut is not cleaved at all, confirming our previous observation that the introduced mutations completely abolish cleavage (Fig. 4A) (van Dijk *et al.*, 1998). At 30 hours after transfection, the cells were tested for their luciferase activity. No difference was observed in luciferase activity of the cells transfected with either pTet-L4- Δ WT or pTet-L4- Δ WTmut, indicating that cleavage has no effect on luciferase expression (Fig. 4B).

In conclusion, the results indicate that under the standard cell culture conditions used in our transient transfection assays, cleavage is a slow process and has little effect on the mRNA decay rate and protein production.

Influence of TSA, dexamethasone, and serum on IGF-II gene expression and mRNA cleavage in Hep3B cells.

To study the effects on regulation of cleavage by exogenous factors, we have used the human hepatoma cell line Hep3B as a model system. These cells endogenously express IGF-II and have the ability to grow continuously in the absence of serum, suggesting that the endogenously synthesized IGF-II stimulates the sustained growth of these cells. Hep3B cells express high amounts of the 6.0 kb mRNA, derived from promoter P3 and lower quantities of the smaller 4.8 kb mRNA species, derived from promoter P4. Several exogenous factors have been described to influence IGF-II expression, such as glucose levels, hormones, glucocorticoids and growth conditions (Asfari *et al.*, 1995; Ramasharma and Li, 1987; Backlin *et al.*, 1998; Dell *et al.*, 1997; Corkins *et al.*, 1999). To investigate whether any of these factors would affect cleavage, Hep3B cells were grown in the absence of serum (with serum replacement medium (TCH) lacking IGF-II, see Materials and Methods) to allow measurements of secreted IGF-II, or in the presence of 10% fetal calf serum. The cells were exposed to a variety of conditions and subsequently, media were collected and RNA was isolated from the cells and analyzed on Northern blots.

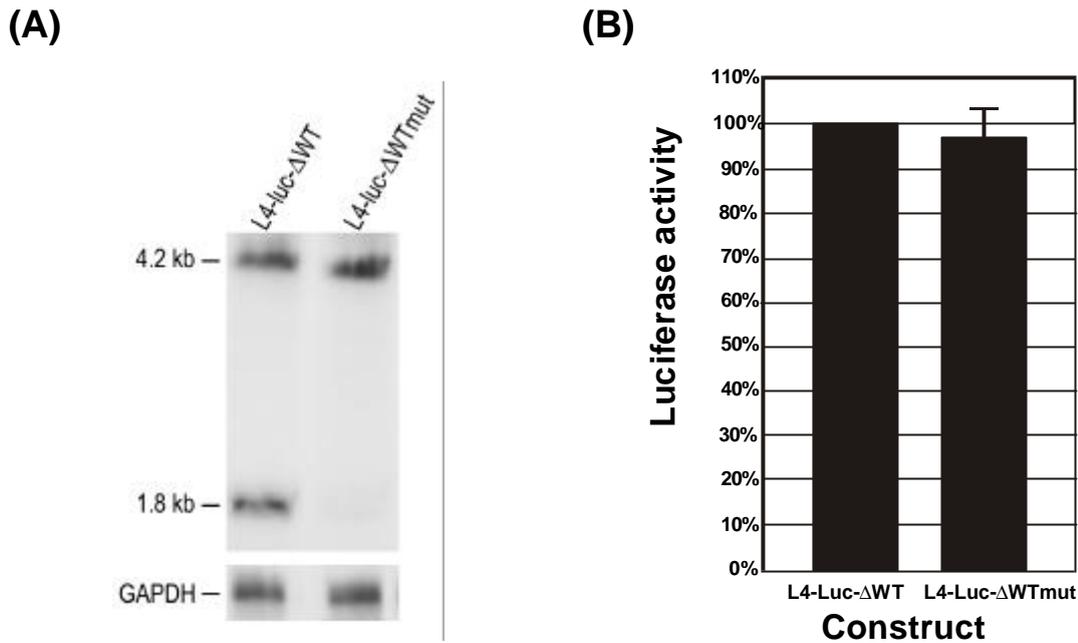


Figure 4. Effect of cleavage on luciferase expression. (A) Northern blot analysis of RNA isolated from 293 cells transiently transfected with pTet-L4-luc-ΔWT or pTet-L4-luc-ΔWTmut. The blot was hybridized with the IGF-II 3'-UTR specific probe. The sizes of the full-length RNAs and the 3' cleavage product are indicated in kb on the left. As a control for loading differences, the blot was also probed with a GAPDH DNA fragment. (B) The luciferase activity of cells transiently transfected with pTet-L4-luc-ΔWTmut relative to the activity of cells transfected with pTet-L4-luc-ΔWT, which was set at 100%. The result shown represents the mean value of 5 independent experiments, and the error bar shows the calculated standard deviation.

First, the histone deacetylase inhibitor trichostatin A (TSA), which has been described to inhibit IGF-II gene expression (Gray *et al.*, 1999), was examined for its effect on the cleavage efficiency in Hep3B cells growing in serum replacement medium. The cells were grown to a density of 50% confluence, and TSA was added to concentrations of 100 and 240 ng/ml, respectively. After 18 hours of incubation, cells were harvested for RNA isolation, and the isolated RNAs were analyzed by Northern blotting (Fig. 5A). The results show that upon TSA treatment, the 6.0 kb full-length IGF-II mRNA levels decline, but the levels of the 3' cleavage product remain constant. Phosphorimager quantification of the signals after normalization with the 28S rRNA and the GAPDH mRNA levels in a number of independent experiments indicates that the decline of the 6.0 kb full-length mRNA is about twofold. However, this decline of the full-length RNA is probably not due to increased cleavage efficiency, since no increase in the level of the 1.8 kb 3' cleavage product is observed. These data suggest that the TSA treatment leads to an inhibition at the level of transcription and does not affect cleavage. In fact, the kinetics after TSA administration resembles that observed after transcription inhibition using tetracycline in the tetracycline-inducible system (Fig. 3A).

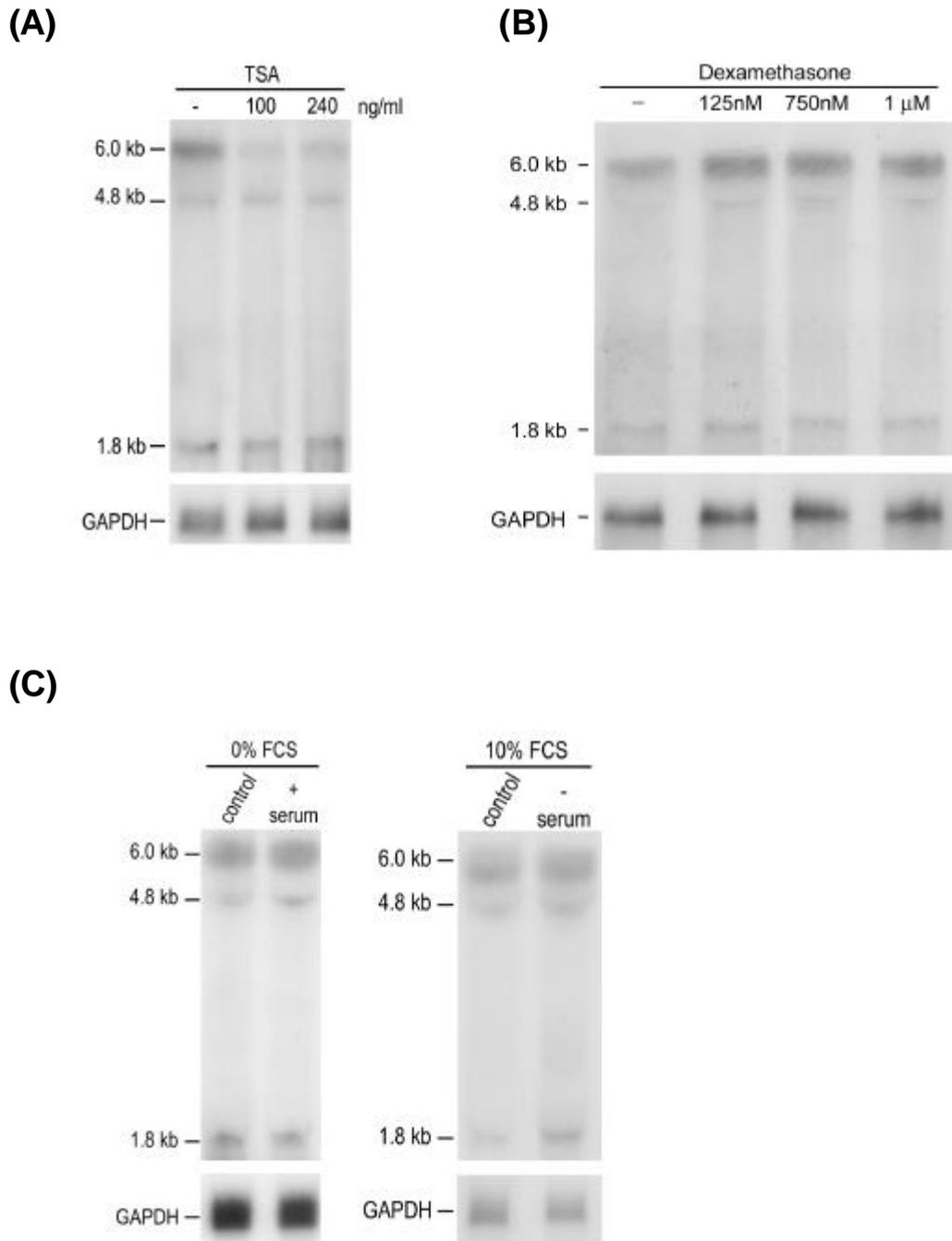


Figure 5. Effects of various exogenous factors on IGF-II mRNA levels and cleavage in Hep3B cells. (A-C) Northern blot analyses of RNAs isolated from Hep3B cells, using the IGF-II 3'-UTR specific probe. As a control for loading differences, the blots were also hybridized with a GAPDH probe. (A) Cells were treated with either DMSO alone (-) or with the indicated amounts of TSA dissolved in DMSO. Cells were harvested for total RNA isolation after 18 hours. (B) Cells were treated with the indicated concentrations of dexamethasone and grown for a further 18 hours before harvesting for RNA isolation. (C) Cells growing in the serum replacement medium TCH without fetal calf serum were supplied with 10% fetal calf serum and harvested after 24 hours. Cells growing on 10% fetal calf serum were deprived from serum for 24 hours and harvested for RNA isolation.

In contrast to the decline of the 6.0 kb mRNA levels, the levels of the 4.8 kb mRNA species are not decreased, which is in agreement with observations by Gray *et al.* (1999), and suggests that the decrease in the 6.0 kb mRNA abundance is due to specific effects of TSA on human promoter P3, which drives the formation of the 6.0 kb mRNA species. We also observed that the TSA treatment inhibits the growth of the Hep3B cells (not shown), which might at least partly be due to the decrease in IGF-II expression.

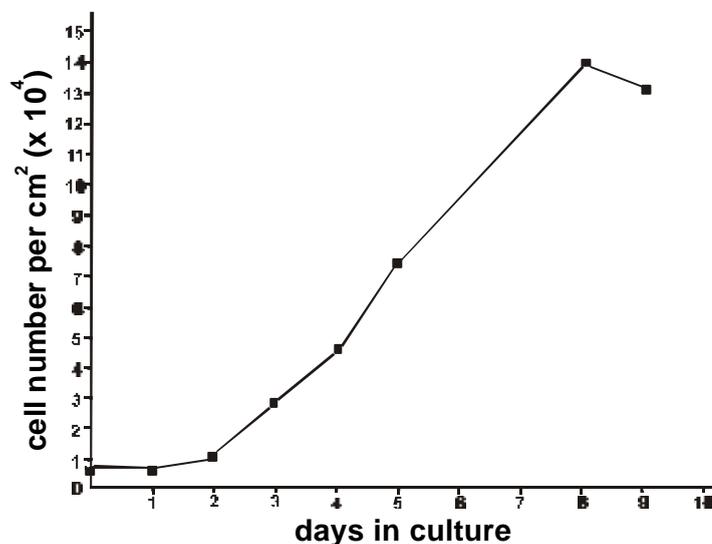
Second, the effect of dexamethasone addition on cleavage efficiency was tested. Dexamethasone is a synthetic glucocorticoid, which has been reported to stimulate IGF-II expression in a number of systems (Price *et al.*, 1992; Dell *et al.*, 1997; Cheng *et al.*, 1998; Yoshiko *et al.*, 1998), although also downregulation of IGF-II expression has been observed (Senior *et al.*, 1996; Backlin *et al.*, 1998). In order to test whether cleavage of the IGF-II mRNAs is influenced by dexamethasone, Hep3B cells growing in the presence of 10% fetal calf serum were treated with increasing amounts of this drug up to 1 μ M. After 18 hours of incubation, the cells were harvested for RNA isolation, and the isolated RNAs were subjected to Northern blot analysis (Fig. 5B). Dexamethasone treatment leads to an increase of about twofold in both the 6.0 kb and the 4.8 kb full-length IGF-II mRNA levels at all concentrations used, indicating that IGF-II gene expression in Hep3B cells is upregulated by dexamethasone. Similar results were obtained with cells growing in the serum replacement medium (not shown). The abundance of the 3' cleavage product also slightly increased, although less than the full-length mRNAs. These results suggest that the increased levels of the full-length IGF-II mRNAs are not due to a significant reduction in cleavage efficiency, since no absolute decrease in the levels of the 3' cleavage product was seen. However, the lower relative abundance of the 3' cleavage product compared to the full-length IGF-II mRNAs may be due to a slight decrease in cleavage efficiency, although it is also possible that the levels of the 3' cleavage product had not yet reached a steady-state level. Thus, the increase in the full-length IGF-II mRNA levels upon dexamethasone treatment may be mainly due to an increased transcriptional activity. This indicates that dexamethasone, like TSA, acts at the level of transcription.

Third, we have tested to what extent serum addition or deprivation affects cleavage. Hep3B cells growing in the serum replacement medium were supplied with 10% fetal calf serum, and at 24 hours after addition of the serum, the cells were harvested for RNA isolation. The reverse experiment was also performed: cells growing in the presence of 10% fetal calf serum were transferred to serum-free medium and harvested after 24 hours for RNA isolation. The isolated RNAs were analyzed by Northern blotting. Fig. 5C shows that no significant changes in the full-length IGF-II mRNA levels or the levels of the 3' cleavage product were observed after 24 hours of addition or deprivation of serum. Similar results were obtained after 4, 8, and 32 hours of incubation (data not shown). These data indicate that in Hep3B cells, neither IGF-II gene transcription nor cleavage are significantly affected by the presence or absence of serum.

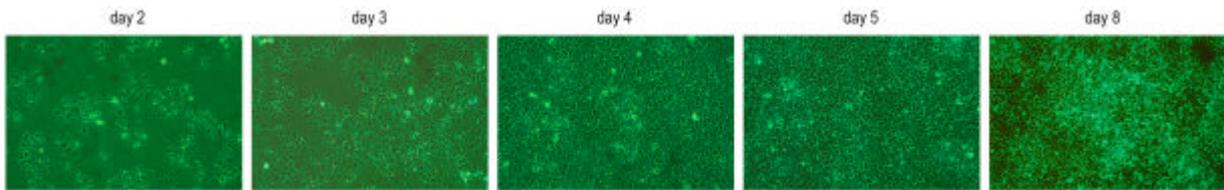
Cleavage is upregulated at high cell density.

Another factor that has been described to affect IGF-II expression in several systems is cell density. For this reason, we examined the effect of the growth status of the Hep3B cells on cleavage efficiency. Cells were seeded at a density of 0.8×10^4 cells /cm² (day 0), and were grown in serum replacement medium, which was refreshed every day. Starting day 1, cells were counted each day and were harvested for RNA isolation; media were collected for IGF-II protein measurements. From day 1 to day 4, the cells proliferated rapidly till 100% confluence was reached at a density of $\sim 4.5 \times 10^4$ cells /cm² (day 4) (Fig. 6A and B). Instead of arresting growth at this point, however, the cells continued to proliferate at a high rate, followed by growth arrest at day 8 at a density of $\sim 15 \times 10^4$ cells /cm² (Fig. 6A). At this point, multiple layers of cells had formed (Fig. 6B). On the subsequent day after growth arrest, a slight decrease in cell number was seen due to cell death. RNA was isolated from cells in the subsequent phases of the growth curve and subjected to Northern blot analysis (Fig. 6C) using the IGF-II 3'-UTR specific probe. When the cells reached confluence, a significant 2-fold elevation of the amount of the 3' cleavage product was observed and this elevation further continued to about 3-fold. At day 5, a peak level of the 3' cleavage product was observed, after which the levels declined again. No significant changes in the full-length IGF-II mRNA levels were seen, however.

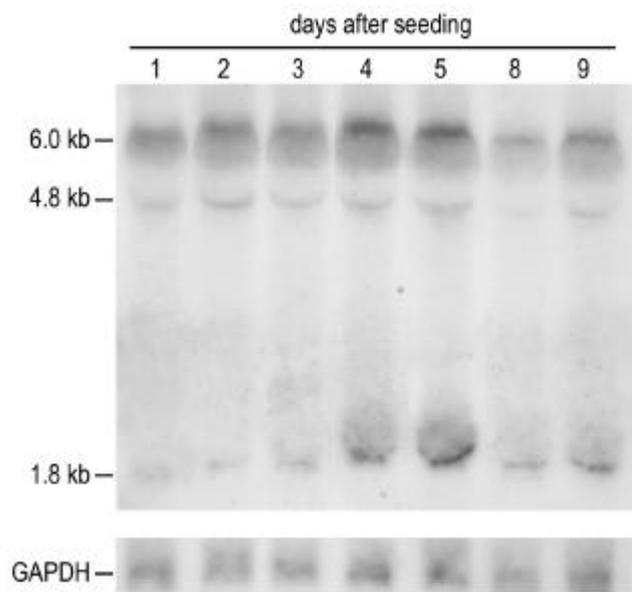
The IGF-II protein levels in the medium during the growth of the Hep3B cells were also examined; medium samples taken from cells at 2, 4, 5, 8, and 9 days after seeding were analyzed by Western blotting (Fig. 6D). At 2 days after seeding, no IGF-II protein could be detected in the medium. From 4 to 9 days after seeding, a marked increase in the IGF-II levels was observed, up to high amounts of IGF-II at day 9. These observations indicate that high cell density conditions do not lead to a decrease in IGF-II production in Hep3B cells. Rather, the strong increase in the IGF-II levels up to day 9 suggests that the production of IGF-II is even stimulated at high density, although the number of cells also increases.

(A)

(B)



(C)



(D)

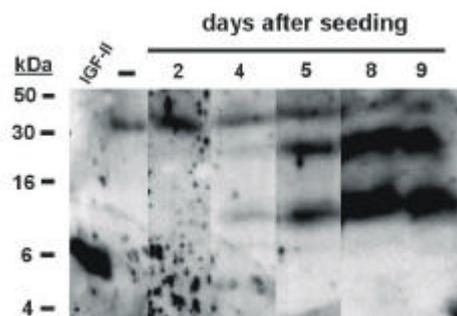


Figure 6. IGF-II gene expression and mRNA cleavage efficiency as a function of Hep3B cell density. (A) Growth curve of Hep3B cells growing on the TCH serum replacement medium. Cells were seeded at 0.8×10^4 cells /cm² (day 0). Medium was refreshed daily. (B) Photomicrographs of Hep3B cells at day 2, 3, 4, 5, and 8. (C) Northern blot analysis of RNAs isolated from the cells harvested at days 1 to 9 after seeding, and grown to the densities indicated in (A). The blot was first hybridized with the IGF-II 3'-UTR specific probe, and subsequently re-probed with the GAPDH probe to control for loading differences. (D) Western blot analysis of medium samples taken from the cells at days 2, 4, 5, 8, and 9, simultaneously while harvesting them for RNA isolation. A protein size marker, recombinant IGF-II (20 ng), and a control medium sample (-) in which no cells had grown, were run in parallel.

Notably, the Hep3B IGF-II proteins migrate with two distinct rates through the gel, both slower than the 7.5 kDa recombinant IGF-II used as a control; one migrates as a protein of ~10 kDa, and the other as a protein of ~25 kDa. Presumably, these signals represent improperly processed precursor IGF-II; it is rather common that tumor cell lines express so-called “big IGF-II” (Zapf, 1994).

The observations described above suggest that IGF-II gene transcription and protein production per cell do not change drastically at high cell densities. The levels of the 3' cleavage product, however, are significantly upregulated.

In SHSY-5Y cells, it was originally observed that cleavage of IGF-II mRNA results in the formation of an abundant 3' cleavage product of 1.8 kb and much lower levels of the 4.2 kb 5' cleavage product, indicating that this RNA is less stable than the 3' cleavage product (Holthuizen *et al.*, 1993). In order to confirm that this is also the case in Hep3B cells, RNA isolated from Hep3B cells grown in the serum replacement medium to 50% confluence was subjected to Northern blot analysis using an IGF-II coding-region specific probe and the 3'-UTR-specific probe (Fig 7A). Hybridization with the 3'-UTR specific probe readily detects the 3' cleavage product in addition to the full-length IGF-II mRNA species of 6.0 and 4.8 kb. Using the coding region-specific probe, the full-length IGF-II mRNAs are also detected, but the 5' cleavage product is hardly visible. The same experiment was performed with RNA isolated from cell grown to 100% confluence (Fig 7B). Again, a significant increase in the level of the 3' cleavage product was seen. Under these conditions, the 5' cleavage product of 4.2 kb also becomes visible, but the levels are much lower than for the 3' cleavage product. This result confirms that also in Hep3B cells, cleavage of the IGF-II mRNAs results in the formation of a stable 3' cleavage product and an unstable 5' cleavage product. In addition, the observation that the levels of both cleavage products increase, indicates that the elevated levels of the 3' cleavage product observed at high cell density in Hep3B are due to an increased cleavage efficiency cells, rather than a stabilization of the 3' cleavage product.

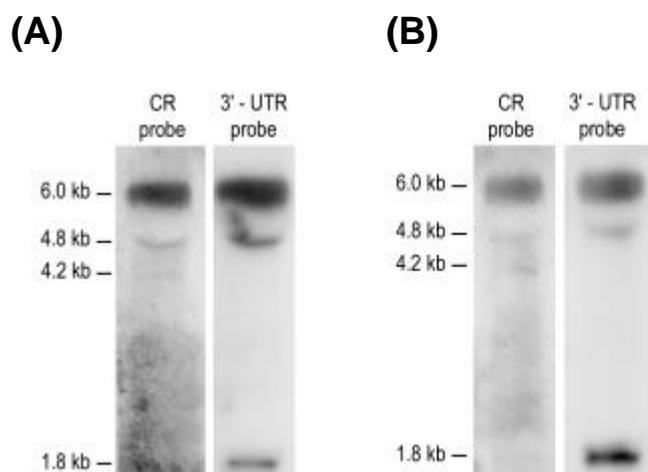


Figure 7. Northern blot analyses of RNA isolated from Hep3B cells at (A) 50% and (B) 100% confluence. The blots were probed with an IGF-II coding region (CR) specific probe and the IGF-II 3'-UTR specific probe as indicated above the lanes. The sizes of the detected RNA species are indicated in kb on the left.

Having established that Hep3B cells show increased cleavage efficiency at high cell densities, we determined whether this phenomenon was specific for Hep3B cells or could occur in other cell types as well. Murine Ltk⁻ cells, which do not produce endogenous IGF-II, have been stably transfected with the human IGF-II minigene construct Δ WT, and it was shown that these cells express and cleave the transcript from this IGF-II minigene (Meinsma *et al.*, 1992; van Dijk *et al.*, 1998). These stably transfected Ltk⁻ cells were grown to 50 and 100% confluence. Subsequent Northern blot analysis of total RNA isolated from the cells using the IGF-II 3'-UTR specific probe shows that no elevation of the levels of the 3' cleavage product occurs in these cells at high densities (Fig. 8). This indicates that the increase in cleavage efficiency under high cell density conditions is not a general phenomenon that occurs in any cell type but may be specific for a restricted set of cell types including Hep3B cells.

In conclusion, our data indicate that cleavage is upregulated at high cell density in Hep3B cells. This phenomenon may be specific for cells that endogenously express IGF-II.

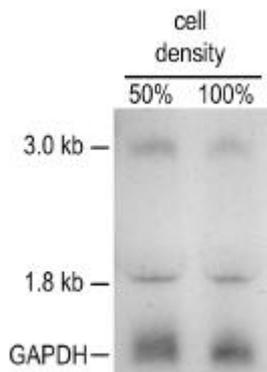


Figure 8. Northern blot analysis of RNA isolated from murine Ltk⁻ cells expressing the human IGF-II minigene Δ WT, carrying a deletion in the 3'-UTR from position -1955 to -174 relative to the cleavage site (van Dijk *et al.*, 1998). The blot was probed with the IGF-II 3'-UTR specific probe. The cells were grown to the % confluence indicated above the lanes; the sizes of the detected RNA species are indicated in kb on the left.

DISCUSSION.

In the present study, we have investigated the effects on cleavage efficiency of the different 5' leader sequences present in the various IGF-II transcripts in transient transfection assays. Subsequently, the kinetics of IGF-II mRNA cleavage and its effect on mRNA decay and protein production was studied, again in transient transfection assays. In addition, we have examined regulation of IGF-II mRNA cleavage in human Hep3B cells, which endogenously express IGF-II.

The human IGF-II gene contains four promoters, which are differentially active during development and in different tissues. The 5' leaders (L1- L4) of the different transcripts derived from these promoters differ significantly in their length and composition; while L2 and L4 do not show significant secondary structure, L1 and L3 are rather long and highly structured. It was established previously that the leaders have significant effects on translation efficiency (de Moor *et al.*, 1994); while IGF-II

mRNAs carrying leaders 1, 2, and 4 are efficiently translated, leader 3 strongly represses translation. In this report, we show that the leaders also differentially influence cleavage efficiency. The efficiency of cleavage of the L4-containing transcript was set at 100%; the mRNAs with L1, L2, and L3 were found to be cleaved with efficiencies of 140, 111, and 141% relative to L4, respectively. These results suggest a positive correlation between secondary structure formation in the leader and cleavage efficiency, with the extensively structured leaders L1 and L3 being cleaved with the highest efficiencies. Thus, the leaders appear to affect both translation and cleavage, but in a different manner. Whereas the L3-transcript deviates strongly from the other transcripts in its translation efficiency, it is cleaved with an intermediate efficiency. This suggests that there is no direct link between translation and cleavage, in contrast to several other systems; cleavage of the 9E3, the *c-myc*, and the *groa* transcripts have been described to be dependent on translation (Stoeckle and Hanafusa, 1989; Wisdom and Lee, 1990; Stoeckle, 1992). Similar to IGF-II mRNA cleavage, however, endonucleolytic cleavage of the *Xenopus* homeobox mRNA *Xlhbox2* was also found to be independent of translation (Brown and Harland, 1990). The lack of a connection with translation allows cleavage to be regulated in response to exogenous stimuli independently of translation regulation.

Another important issue to be addressed is the rate of cleavage and its role in IGF-II mRNA degradation. For this reason, we have performed kinetic studies using a tetracycline-inducible system. This provides a way to manipulate the expression of a specific gene of interest without interfering with the expression of the other genes. We have made the following observations. (1) There is a significant time delay between the appearance of the full-length IGF-II mRNA and the formation of the 3' cleavage product after transcription induction. After the lag phase, the accumulation of the cleavage product accelerates. The delay in formation of the cleavage product may indicate that cleavage only occurs when there is a sufficient amount of IGF-II mRNA. Alternatively, cleavage may require the formation of suitable higher order structures in the IGF-II mRNAs, which occurs slowly. Previously, we have already shown that complex secondary structures are required for cleavage (Scheper *et al.*, 1995). A third possibility is that specific RNA-protein complexes must be formed before cleavage can occur. (2) Point mutations that inactivate cleavage do not lead to a slower degradation rate of the RNAs after transcription inhibition; both RNAs that are cleaved and RNAs that are not cleaved were reduced to 33% of the initial levels after 28 hours. This indicates that specific endonucleolytic cleavage does not contribute significantly to the general decay of the IGF-II mRNAs. In contrast to the full-length RNAs, no decline in the levels of the 3' cleavage product was seen throughout the experiment indicating that this RNA species is very stable. (3) No difference in luciferase expression was observed between cleaved and non-cleaved RNA, in agreement with the lack of effects of specific endonucleolytic cleavage on IGF-II mRNA turnover.

From these data, we conclude that under the standard cell culture conditions used in our transient transfection assays, cleavage is slow and does not play a significant role in overall IGF-II mRNA degradation or protein production. Cleavage leads to the production of a very stable 1.8 kb 3' cleavage product, which remains present in the cells for a long time after its formation.

Hep3B cells, which endogenously express high amounts of the 6.0 kb IGF-II transcript and lower quantities of the 4.8 kb mRNA species, were used to examine whether under certain circumstances the efficiency of specific endonucleolytic cleavage might be altered. Hep3B cells were grown in the presence of different exogenous factors: (1) the histone deacetylase inhibitor trichostatin A (TSA), which was previously described to have a negative effect on IGF-II expression in Hep3B cells (Gray *et al.*, 1999), (2) the synthetic glucocorticoid dexamethasone, which appears to have a stimulating effect on IGF-II expression (Price *et al.*, 1992; Dell *et al.*, 1997; Cheng *et al.*, 1998; Yoshiko *et al.*, 1998), although downregulation of IGF-II as a result of dexamethasone treatment has also been observed (Senior *et al.*, 1996; Backlin *et al.*, 1998); (3) also the effect of addition or deprivation of serum on IGF-II expression and cleavage was tested.

TSA and dexamethasone oppositely affect the full-length IGF-II mRNA levels (Fig. 5A and B); TSA causes a decline in the 6.0 kb full-length IGF-II mRNA levels (but not the 4.8 kb mRNA levels), whereas dexamethasone leads to a 2-fold increased abundance of both full-length mRNA species. Neither of the two compounds, however, influences the levels of the 3' cleavage product. This suggests that the observed effects on the full-length mRNA levels are not caused by changes in cleavage efficiency, but by changes in transcriptional activity. No significant effects were seen on the full-length IGF-II mRNA levels or on the levels of the 3' cleavage product upon addition or deprivation of serum (Fig. 5C), suggesting that neither IGF-II gene transcription nor endonucleolytic cleavage of the mRNAs is responsive to serum factors.

Several studies have shown that cell density affects IGF-II expression in different cell types. Both in the rat IEC-6 and the rat BRL-3A cell lines an upregulation of IGF-II has been observed at increasing cell densities, leading to the hypothesis that IGF-II may act as a survival factor at high cell densities in these cells (Kutoh *et al.*, 1995; Corkins *et al.*, 1999). These reports prompted us to investigate whether IGF-II gene expression and IGF-II mRNA cleavage in Hep3B cells are also regulated in a cell density-dependent manner. The cells were grown to different densities and isolated RNAs were analyzed on Northern blots using an IGF-II 3'-UTR-specific probe. Interestingly, a consistent increase of 2-3 fold in the abundance of the 3' cleavage product was observed when the cells reached confluence, indicating that cleavage is upregulated with increasing cell densities (Fig. 6C). It can be excluded that the increase in the levels of the 3' cleavage product is an indirect consequence of changes in the rate of IGF-II mRNA synthesis, because no changes in the levels of the cleavage product were seen upon treatment of the cells with TSA or dexamethasone. No cell density-dependent increase in cleavage efficiency was observed in stably transfected murine Ltk⁻ cells expressing the human IGF-II minigene Δ WT. This suggests that the phenomenon is cell-type specific, rather than a general effect of growth inhibition.

What might be the physiological relevance of this increased cleavage efficiency? It is unlikely that in Hep3B cells, even at high cell densities, the specific endonucleolytic cleavage significantly affects the expression of IGF-II; under standard conditions, the amount of cleavage product is about 15% of the total IGF-II mRNA, i.e. the sum of the 6.0 and the 4.8 kb full-length mRNAs and the 3' cleavage product.

This is raised to about 45% at high cell densities. This level of cleavage efficiency is similar to that observed with the transcript L4-luc- Δ WT (Fig. 4A). This RNA is also cleaved relatively efficiently, but still shows no decrease in luciferase expression in comparison with the mutant RNA that is not cleaved. This suggests that with the endogenous IGF-II mRNAs in Hep3B cells even under high cell density conditions and with the L4-luc- Δ WT transcripts under standard conditions in the 293 cells, the rate of cleavage is still rather low. In that case, the 1.8 kb 3' cleavage product must be exceptionally stable to explain the high relative abundance of this RNA species. Indeed, the 1.8 kb 3' cleavage product appears to be very stable, since the level of the 3' cleavage product did not decline after transcription arrest in the transient transfection experiment shown in Fig. 3A and B, in contrast to the full-length mRNAs, which were reduced to ~33% of the initial levels after 28 hours. The unusual stability of the 3' cleavage product is probably conferred by a highly conserved G-quadruplex structure at its 5' end that may protect the RNA from exonucleolytic degradation (Christiansen *et al.*, 1994). These observations also imply that cleavage with an efficiency that is sufficiently high to accelerate IGF-II mRNA decay should lead to very high cellular levels of the 1.8 kb 3' cleavage product.

Although it is possible that in other cell types and/or under the influence of other exogenous stimuli, cleavage efficiency is sufficient to accelerate the decay of the IGF-II mRNAs, our data do not support a significant role for cleavage in regulation of IGF-II mRNA stability. Rather, the 3' cleavage product may have an intrinsic cellular function. The unusual stability of this RNA is in favor of this hypothesis since stable non-coding RNAs have been shown to carry out diverse functions in both prokaryotes and eukaryotes (reviewed by Wagner and Simons (1994); Delihias (1995); Kelley and Kuroda (2000)). Computer-assisted secondary structure prediction suggests that the 1.8 kb 3' cleavage product is highly base-paired. Interestingly, several regions in this RNA appear to be rich in structural motifs that are common to functional non-coding RNAs as determined with a specialized computer algorithm available on the World Wide Web at <http://rnagene.lbl.gov/>.

How does cleavage of the IGF-II mRNAs relate to other systems? For a number of mRNAs, endonucleolytic cleavage has been described. Cleavage of the IGF-II mRNAs is distinct from these systems in a number of respects. For example, the determinants for recognition of the cleavage site are very complex in the case of IGF-II mRNA cleavage; while for cleavage of the apoII, the albumin, and the *Xenopus* Xlhbox2B mRNAs short sequences in a single-stranded environment appear to be sufficient (Binder *et al.*, 1989; Chernokalskaya *et al.*, 1997; Brown *et al.*, 1993), cleavage of the IGF-II mRNAs requires extensive RNA structures that may act in a cooperative manner to stabilize a highly specific cleavage site (van Dijk *et al.*, 1998; van Dijk *et al.*, 2000). To our knowledge, the transferrin receptor mRNA is the only other system that has been described to require large regions of RNA structure for specific cleavage in its 3'-UTR. Interestingly, an upstream sequence element was found to be required for cleavage of this mRNA as well (Binder *et al.*, 1994), similar to the requirement for the upstream element I in the IGF-II mRNAs. Cleavage of the IGF-II mRNAs occurs independently of deadenylation, similar to most, but not all systems; it was shown that cleavage of *groa* mRNA is associated with poly(A) tail shortening (Stoeckle, 1992), and also cleavage of the yeast *PGK1* mRNA and the

human α -globin mRNA have been described to be coupled with poly(A) tail shortening, (Vreken and Raue, 1992; Wang and Kiledjian, 2000a). In addition to its complex structural requirements and its independence of deadenylation, also its independence of translation distinguishes IGF-II mRNA cleavage from a number of other systems, as mentioned above.

In summary, experiments have been performed to gain insight into the kinetics and physiological role of the site-specific endonucleolytic cleavage of the IGF-II mRNAs, which appears to be rather unique. Unfortunately, we have not been able to define its exact physiological function, but we have established that it is regulated in a cell density-dependent manner. The unusually stable 1.8 kb 3' cleavage product shows interesting structural features, such as the G-quadruplex structure and the motifs common to functional RNAs. This makes it tempting to speculate that this RNA might have an intrinsic physiological function.

ACKNOWLEDGEMENTS

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MATERIALS AND METHODS

Materials. Expression plasmids pTet-Splice and pTet-tTak were purchased from Gibco BRL Life Technologies. Plasmid pGL3 was from Promega. Tetracycline was purchased from Sigma (St. Louis, MO, USA). Restriction enzymes and T4 DNA ligase were purchased from Roche Molecular Biochemicals, Mannheim, Germany. Enzymes were used as specified by the manufacturers. dNTPs were obtained from Kabi-Pharmacia, BES [*N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] was from Sigma (St. Louis, MO, USA). A random primer labeling kit was purchased from Amersham (UK), and a DNA sequencing kit was purchased from Kabi-Pharmacia. A poly(A)⁺ RNA isolation kit were purchased from Promega, RNazol reagent was purchased from Biotecx Laboratories, Inc. (Houston, Texas, USA), and Genescreen membranes were purchased from Du Pont de Nemours (Dreieich, Germany). [³²P]dCTP (3,000 Ci/mmol) was purchased from Amersham. Trichostatin A and dexamethasone were purchased from ICN Biomedicals Inc. (Costa Mesa, CA, USA) and Sigma (St. Louis, MO, USA), respectively. TCH defined serum replacement was purchased from ICN Biomedicals Inc.

Construction of plasmids. Molecular cloning was performed according to established protocols (Sambrook *et al.*, 1989). Where necessary, 3' recessed ends were filled in with Klenow polymerase and 3' protruding ends were removed with T4 DNA polymerase. All positions within exon 9 of the IGF-II gene are indicated relative to the cleavage site; the nucleotide upstream of the cleavage site is -1; the nucleotide downstream is +1. When exon 9 sequences were deleted or additional restriction sites were introduced, the numbers indicated still refer to their original position in exon 9 relative to the cleavage site.

The leader plasmids were constructed as follows. The IGF-II expression plasmid EP7-9/Not (Meinsma *et al.*, 1992) was digested with Alw44I to generate a fragment that contains the

IGF-II 3'-UTR sequence from position -17 to 210 nucleotides downstream of the polyadenylation site; this fragment was inserted in the Xba site of expression plasmid CMV-0. The resulting plasmid, designated CMV-1.8, was digested with BamHI and NotI (position +80). DNAs containing PCR-generated leader sequences L1, -L2, -L3, and -L4 and the IGF-II open reading frame (de Moor, 1994) were digested with BamHI and XhoI to generate fragments that contain the 5'-UTR and the IGF-II coding region. Plasmid EP7-9/Not was digested with XhoI and NotI (positions -2291 and +80). The resulting fragment was ligated with the leader-fragments and subsequently, the BamHI/NotI digested plasmid CMV-1.8 was added to the ligation reactions, resulting in plasmids CMV-L1, CMV-L2, CMV-L3, and CMV-L4.

Plasmid pTet-L4- Δ WT was constructed as follows. Plasmid pTet-Splice was digested with XhoI and BamHI to generate a fragment that contains the tetracycline-responsive minimal CMV promoter. This fragment was inserted in the AatII and BamHI sites of plasmid CMV-L4 that encompass the CMV promoter, resulting in plasmid pTet-L4. Plasmids Δ WT and Δ WTmut were digested with ClaI and XbaI (positions -1961 and +1051 in the 3'-UTR) and the resulting fragments were inserted in the ClaI and XbaI sites of plasmid pTet-L4. Construct Δ WTmut contains the following mutations: A(-144) and G(-143) have been deleted, and U(-142), A(-141), G(-140) have been changed to C, U, and C, respectively (van Dijk *et al.*, 1998).

To generate plasmids pTet-L4-luc- Δ WT and pTet-L4-luc- Δ WTmut, the following procedure was followed. First, an XhoI-XbaI fragment of the IGF-II 3'-UTR (from positions -2292 to +1047) was inserted in the XbaI site of the luciferase expression plasmid pGL3 (directly downstream of the luciferase coding region). The resulting plasmid was digested with HindIII and ClaI, yielding a fragment that contains the luciferase open reading frame and the IGF-II sequence from positions -2292 to -1961. This fragment was inserted in the HindIII/ClaI sites of plasmids pTet-L4- Δ WT and pTet-L4- Δ WTmut.

Culture and transient transfection of 239 cells. Human 293 cells were grown in Dulbecco's modified Eagle's medium. The medium was supplemented with 10% fetal calf serum, 100 IU of penicillin per ml, 100 μ g of streptomycin per ml, and 300 μ g of glutamine per ml. The cells were transfected in 25 cm² flasks at a confluence of 50% by the calcium phosphate coprecipitation method (Graham and van der Eb, 1973). Two hours before transfection, tetracycline was added to the medium to a concentration of 100 ng/ml to repress the expression of the constructs. Precipitates were prepared with BES-buffered saline (BBS) (Sambrook *et al.*, 1989) and contained 3.3 μ g of pTet-IGF-II constructs and 3.3 μ g of pTet-tTak (de Wet *et al.*, 1987), and were added to the cells. After 4 hours, the medium was aspirated and the cells were supplied with fresh serum-containing medium, again with 100 ng/ml tetracycline. After 24 hours, the tetracycline was removed to induce expression of the constructs. At various time-point after transcription induction, the cells were washed with PBS and harvested in PBS with 0.025 % trypsin-0.02 % EDTA. For the reverse experiment, to follow the degradation of the transcripts, the cells were transfected in the absence of tetracycline. After 24 hours, tetracycline was added to a concentration of 0.5 μ g/ml to repress transcription of the constructs. At various time-point thereafter, the cells were washed with PBS and harvested in PBS with 0.025 % trypsin-0.02 % EDTA. For the experiments to determine the effect of cleavage on protein production, 3.3 μ g of pTet-L4-luc- Δ WT or pTet-L4- Δ WTmut were cotransfected with pTet-tTak in the absence of tetracycline. After 4 hours, medium was refreshed and 30 hours later, cell extracts were prepared for luciferase assays (Sambrook *et al.*, 1989; de Wet *et al.*, 1987).

Culture of Hep3B cells. Human Hep3B cells were grown in α -Minimal Essential Medium (α -MEM) supplemented with 100 IU of penicillin per ml, 100 : g of streptomycin per ml, 300 μ g of glutamine per ml and 2% of the defined serum replacement TCH (ICN Biomedicals Inc.) or 10% fetal calf serum.

To study the effect of trichostatin A (TSA) on cleavage efficiency, Hep3B cells growing on serum replacement medium or on 10% fetal calf serum were seeded at a density of 3×10^3 cells/ cm^2 in 25 cm^2 flasks, grown to a density of 1.6×10^4 cells/ cm^2 (~50% confluence) and supplied with fresh medium containing 0-240 ng/ml TSA. After 18 hours, the cells were harvested in PBS with 0.025% trypsin-0.02% EDTA. Media were collected for IGF-II measurements and RNA was isolated and subjected to Northern blot analysis.

To study the effect of dexamethasone on cleavage efficiency, the same procedure was followed except that the cells were given fresh medium containing 0-1000 ng/ml dexamethasone.

To study the effect of serum/ growth factors, cells growing on 10% fetal calf serum at a density of 50% confluence were deprived from serum by replacing the serum-containing medium with serum-free medium. At various time points ranging from 4 to 32 hours, the cells were harvested for RNA isolation. The reverse experiment was also performed; cells growing on TCH were supplied with 10% fetal calf serum and harvested at various time points ranging from 4 to 32 hours thereafter.

The effect of the growth conditions was examined as follows. Cells were seeded at a density of 0.8×10^4 in 25 cm^2 flasks and grown to a variety of different densities ranging from 0.8×10^4 to 15×10^4 cells/ cm^2 (~25% to >>100% confluence). Medium was refreshed daily. Cells were counted each day using a counting chamber after suspending them in PBS with 0.025% trypsin-0.02% EDTA. After harvesting the cells, RNA was isolated and subjected to Northern blot analysis.

RNA isolation and analysis. Total RNA was isolated using the RNazol reagent according to the manufacturer's instructions. Cells isolated from a 25 cm^2 flask were lysed in 0.75 ml of RNazol reagent and RNA was isolated from the water phase by isopropanol precipitation and resuspended in water. RNA was glyoxalated, size-separated on a 1% agarose-10 mM sodium phosphate gel, and transferred to a GeneScreen membrane. The RNA was fixed on the membrane by irradiation with long-wavelength UV light for 2.5 minutes. Northern blots were hybridized in the presence of 50% formamide in glass cylinders with continuous rotation at 42 °C according to the GeneScreen protocols. As a probe, a 1.0 kb SmaI fragment from the human IGF-II exon 9 (positions +84 to +1096), hybridizing to both the full-length IGF-II RNAs and the 3' cleavage product, was used. The DNA fragments were labeled by random priming with [α - 32 P]dCTP following the Amersham protocols. After 2 hours of pre-hybridization, the probe was added at a final concentration of 10^6 cpm/ml. Blots were washed after overnight hybridization, to a final stringency of 0.5 x SSC- 1% SDS at 65 °C (1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and exposed to Fuji RX X-ray film. The amounts of cleavage products were determined by Phosphorimager scanning of the blots of at least 3 independent experiments. Cleavage efficiencies were determined by calculating the amount of 3' cleavage product divided by the total amount of IGF-II RNA. Variation between the calculated cleavage efficiencies in separate experiments was less than 10%.

Poly(A)⁺ RNA was isolated from total RNA preparations using the Poly(A)⁺ RNA isolation kit from Promega according to the manufacturer's instructions.

Western blot analysis. IGF-II protein levels in media taken from Hep3B cells growing on serum replacement medium TCH were measured as follows: All protein present in 400 μ l medium was precipitated by adding 1000 μ l of ice-cold acetone and pelleted by centrifugation

in a microfuge at 14,000 rpm. Pellets were dissolved in 25 μ l of SDS sample buffer, and loaded onto a 10-20% gradient SDS polyacrylamide gel. After electrophoresis, protein was transferred to a nitrocellulose membrane by electroblotting. Subsequently, the membrane was stained with Ponceau S (Sigma) to check for equal loading and blocked in 2% milk powder (Protivar) for 1 h. Incubation with rabbit-anti-human anti IGF-II polyclonal antiserum (GroPep Ltd. Adelaide, Australia) was performed overnight at 4 °C (1:1000 in 0.1% milk powder in TBS-Tween (150 mM NaCl, 10 mM Tris-HCl pH 8.0, 0.2% Tween-20)). The membrane was washed in TBS-Tween, and exposed to biotinylated donkey-anti-rabbit Ig (Amersham)(1:400 in TBS-Tween) for 1 h. The membrane was again washed in TBS-Tween, followed by incubation with horseradish-coupled streptavidin (Amersham) (1:1500 in TBS-Tween). For detection, the Enhanced Chemiluminescence (ECL) system (NEN Life Science Products Inc., Boston, MA) was used.

CHAPTER 6

Summary and conclusions

6.1. Introduction.

The human IGF-II gene is a complex transcription unit containing four promoters that are active in a developmental stage-dependent and tissue-specific fashion. Transcription from these different promoters yields a population of transcripts ranging in size from 2.2 kb to 6.0 kb. The various transcripts differ in their 5'-UTR sequences and are translated with different efficiencies. All transcripts share the 552 nt long IGF-II coding region, and, apart from the minor 2.2 kb transcript which is polyadenylated at an alternative upstream site, the 4 kb long 3'-UTR is also common to all transcripts. In addition to these different transcripts, a smaller, polyadenylated RNA species of 1.8 kb was detected on Northern blots when an IGF-II 3'-UTR specific probe was used. Subsequent experiments indicated that this RNA is not transcribed from another promoter, but is formed by endonucleolytic cleavage of the full-length IGF-II mRNAs. The cleavage site was mapped to the single nucleotide resolution to a specific site located 2183 nt downstream of the translation stopcodon in the 3875 nt long 3'-UTR.

6.2. Mechanistic aspects of cleavage.

Having mapped the specific endonucleolytic cleavage site, experiments were initiated to identify the RNA regions necessary for cleavage. Various deletions were introduced in an IGF-II minigene construct and subsequently tested for their effects on cleavage efficiency in an *in vivo* system (Meinsma *et al.*, 1992). This revealed that for *in vivo* cleavage, a region from positions -173 to +150 relative to the cleavage site is necessary (element II). Unexpectedly, deletion of a region 2 kb upstream of the cleavage site abolished cleavage, demonstrating that an additional sequence is required for cleavage. Further analysis mapped this sequence to be located between positions -2116 and -2013 (element I). Thus, two regions, the 104 nucleotides long element I and the 323 nucleotides long element II, are required for endonucleolytic cleavage of the IGF-II mRNAs. Subsequent RNA folding studies indicated that a major part of element I, which is rich in C-residues can form a 83 nt long duplex with the G-rich downstream region of element II from positions +18 to +101. The upstream part of element II is predicted to fold into two stem-loops and the cleavage site itself is predicted to be located in an internal loop. Thus, three separate RNA folding domains can be distinguished in the cleavage unit: (1) the stem-loop domain from positions -133 to -7, (2) the duplex domain from -2108 to -2029 and +18 to +101, and (3) the cleavage site domain from -161 to -136 and -6 to +16.

Several experiments have been performed to gain a better understanding of the role of these different RNA folding domains in cleavage. First, to gain insight into the recognition determinants in the direct proximity of the cleavage site within the cleavage site domain, a number of point mutations were introduced in the loop of the cleavage site and in its flanking sequences that were predicted to be base-paired (Chapter 3). Apart from a few exceptions, all of the introduced mutations reduced the efficiency of cleavage to 0-30% of the wild type cleavage efficiency, indicating that the presence of specific nucleotides around the cleavage site is of critical importance.

Only a few mutations in the region from -161 to -136, opposite to the cleavage site, allow wild type cleavage efficiency, suggesting that the requirement for specific nucleotide identity in this region is less stringent than in the region adjacent to the cleavage site. In agreement with this view, the -161 to -136 region is also less conserved among related species than the region around the cleavage site. In fact, the region from positions -14 to +52 is 100% conserved between human, rat and mouse. Interestingly, the nucleotides in the region from -161 to -136 that could not be mutated without interfering with cleavage, are also invariantly conserved between human, rat and mouse, suggesting an important physiological function of cleavage. The dramatic reduction in cleavage efficiency upon two G-C base pair substitutions into two A-U base pairs in a predicted 6 bp helix near the cleavage site (mutant [-149,-148,+5,+6]) is somewhat surprising. For it is known that in a continuous A-form Watson-Crick RNA double helix, the bases are not well accessible for specific interactions with proteins and one would not expect the requirement for specific residues in such an environment. This result thus suggests that the region around the cleavage site may adopt another structure than the one predicted by the computer algorithm.

In order to gain more insight into the RNA structures formed around the cleavage site in solution and to verify the computer predictions, biochemical structure probing assays were performed using *in vitro* synthesized RNAs that contain both elements I and II (Chapter 4). Generally, the probing results confirm the computer predictions; we obtained strong evidence that the upstream stem-loops are indeed formed. Importantly however, the reactivities of the nucleotides around the cleavage site are not in agreement with the computer prediction. These nucleotides do not react with single-strand-specific reagents, suggesting that they are not easily accessible in solution. In the absence of magnesium, however, they do react with the reagents, indicating that the residues around the cleavage site are involved in non-Watson-Crick base pairings. A functional significance of these non-Watson-Crick pairings is suggested by the observation that point mutations that interfere with cleavage also affect the reactivity of the nucleotides around the cleavage site (unpublished data), whereas mutations that allow efficient cleavage do not. Thus, the data suggest that specific nucleotides around the cleavage site are necessary, and these nucleotides must be present in a specific configuration maintained by non-Watson-Crick interactions. However, more detailed structural analyses using chemical reagents or NMR structure determination will be required to gain further detailed information on the RNA structures formed around the cleavage site.

What are the roles of the flanking stem-loop domain and of the duplex domain? It was previously shown that formation of the duplex is absolutely required for cleavage, since deletion or inversion of element I completely abolishes cleavage (Scheper *et al.*, 1995). Computer folding suggests that the absence of the duplex interaction leads to dramatic structural changes around the cleavage site. One main role of the duplex domain may be the maintenance of a correct folding around the cleavage site. A similar role may be played by the stem-loop domain, since deletions in this region are predicted to cause different foldings around the cleavage site and indeed such deletions completely disrupt cleavage. Specific deletions in the stem-loop domain, designed to maintain the proper folding around the cleavage site, however, do not result in a strong decrease in cleavage efficiency. Even in the absence of the

complete stem-loop domain, cleavage occurs relatively efficiently provided that the structure around the cleavage site remains intact. Combining these results leads to a model where the cleavage site is located between two large highly structured RNA domains that mainly serve to keep the local structure intact. Furthermore, it can be implied that the loop of the cleavage site consists of irregular RNA structures that must be formed by non-Watson-Crick interactions.

Cleavage of the IGF-II mRNAs requires much more complex RNA structures than cleavage of a number of other mRNAs known to date, such as the transcript of the *Xenopus* maternal homeobox gene *Xlhbox2*, which only requires a short sequence in a single-stranded environment (Brown and Harland, 1990; Brown *et al.*, 1993). This does not necessarily indicate that the recognition of the cleavage site in IGF-II mRNAs *per se* is more complex than that of the cleavage site in the *Xlhbox2* message. Rather, the complex requirements for cleavage of the IGF-II mRNAs may reflect the fact that the nucleotides constituting the internal loop of the cleavage site are distant in the primary sequence; nucleotides -144 to -140 must be placed opposite to nucleotides -2 to +1 by RNA folding. In contrast, the sequence recognized by the *Xlhbox2* endonuclease is a continuous stretch of 16 nucleotides that may be located in a hairpin loop. This requires only one stable helix below it to prevent it from being captured in alternative foldings. It will be interesting to determine whether nucleotides -144 to -140 and -1 to +2 on top of a hairpin loop can be sufficient to provide a specific cleavage site. Thus, while cleavage is possible in the absence of the flanking stem-loop domain and possibly also of the duplex domain, these regions may have a regulatory function in cleavage, possibly through binding specific proteins that influence cleavage efficiency.

In a search for such proteins, EMSA studies were carried out using *in vitro* synthesized IGF-II RNAs and protein extracts from human Hep3B cells that endogenously express and cleave IGF-II mRNA. These studies yielded the identification of a protein that specifically binds to the 5' stem-loop in the proximity of the cleavage site (Chapter 3). Point mutations in the protein binding site that disrupt binding of the protein lead to a moderate reduction in cleavage efficiency of 15 to 35%, suggesting that this protein is not an essential factor, but that it has a modulating effect on cleavage. Thus, the stem-loop domain harbors a binding site for a specific protein that may have a regulatory role in cleavage.

Another potential target for cleavage regulation and an interesting structural feature of the cleavage unit is the G-rich sequence in element II downstream of the cleavage site from positions +24 to +56. This region was found to adopt a stable G-quadruplex structure by (Christiansen *et al.*, 1994) based on studies with RNAs that lack element I. In the presence of element I, however, this region can form a duplex structure with element II. Thus, a competition may exist between formation of the duplex between elements I and II or formation of the G-quadruplex structure in element II. In the 'duplex state' cleavage can occur because the cleavage site is in the correct structure, whereas in the 'G-quadruplex state', cleavage cannot occur. An interesting analogy comes from the telomerase system. Telomerase is a specialized reverse transcriptase that contains its own RNA template for synthesis of telomeric DNA, and plays an important role in tumor growth (Greider and Blackburn, 1989; Kim *et al.*, 1994). The sequence of the telomerase RNA template is

5'-CUAACCCUAAC-3' and reverse transcription results in the DNA sequence 5'-GGTTAGGGTTAG-3' (Feng *et al.*, 1995). This DNA sequence can adopt a G-quadruplex structure, and it has been hypothesized that formation of this G-quadruplex may facilitate unwinding of the telomeric RNA:DNA duplex (Zahler *et al.*, 1991). Subsequently, a new duplex must be formed to allow a next extension. On this basis, compounds have been designed that interfere with this process to inhibit telomerase activity. Several compounds that inhibit the G-quadruplex to duplex transition by stabilizing the quadruplex have been described (Hurley *et al.*, 2000). Also specific proteins that stabilize quadruplex structures have been identified (Fang and Cech, 1993a; Fang and Cech, 1993b). In a similar manner, factors that stabilize or destabilize the G-quadruplex in the IGF-II mRNA could inhibit or stimulate cleavage, respectively. After cleavage of the IGF-II mRNAs, the G-quadruplex is thought to protect the 3' cleavage product from exonucleolytic degradation.

6.3. Functional aspects of cleavage.

Several observations argue in favor of an important biological function of endonucleolytic cleavage of the IGF-II mRNAs. (1) The 100% conservation between human, rat and mouse of the sequence around the cleavage site between positions -14 to +52. In fact, this region is the most conserved part of the entire IGF-II gene (Nielsen and Christiansen, 1992). (2) Also the nucleotides in the opposite strand that by mutagenesis studies were found to be important for cleavage are invariantly conserved, whereas those that are not important for cleavage are not conserved. (3) The specificity of cleavage; single point mutations around the cleavage site completely disrupt cleavage. (4) The regulated manner of cleavage in Hep3B cells; a 2-3 fold upregulation of cleavage is consistently observed when the cells reach confluence (Chapter 5).

What may be the function of cleavage? Cleavage results in the destabilization of the coding part of an mRNA, since an unstable 5' cleavage product is formed. Thus, it is obvious that cleavage could serve to accelerate the degradation of the IGF-II mRNAs, provided that it occurs efficiently enough. The experiments described in Chapter 5, however, indicate that at least under standard cell culture conditions in 293 cells, this is not the case. Cleavage has no significant effect on protein production, despite the fact that about 43% of the RNA exists as a cleavage product in the case of the reporter L4-luc- Δ WT RNA that contains the luciferase open reading frame and the IGF-II 3'-UTR. In Hep3B cells, which endogenously express IGF-II, a lower level of cleavage efficiency is observed (only 15% of the mRNAs are cleaved). Under high cell density conditions, an upregulation of IGF-II mRNA cleavage of 2-3 fold was seen. But even this level of cleavage efficiency is unlikely to affect the overall decay rate of the full-length mRNAs since a similar level was also observed for the L4-luc- Δ WT RNA but did not affect the steady-state levels of this RNA. Similar steady-state levels were observed for the L4-luc- Δ WT RNA and the L4-luc- Δ WTmut RNA, which is not cleaved. In addition, no decrease in the full-length IGF-II mRNA levels was seen at high cell density, provided that the cells were frequently given fresh medium containing growth components. Thus, although it is possible that under different

conditions and/or in a different cell type, cleavage efficiency is sufficient to accelerate mRNA decay, our data do not support a significant role for cleavage in mRNA degradation.

An interesting possibility is that the 3' cleavage product itself performs an intrinsic cellular function. Two major observations point into this direction. (1) The unusual stability of the 1.8 kb IGF-II RNA species. Numerous stable non-coding RNAs have been shown to carry out diverse functions in both prokaryotes and eukaryotes (reviewed by Wagner and Simons (1994); Delihias (1995); Kelley and Kuroda (2000)). Computer-assisted secondary structure prediction suggests that the 1.8 kb 3' cleavage product is highly base-paired. Interestingly, several regions in this RNA appear to be rich in structural motifs that are common to functional non-coding RNAs as determined with a specialized computer algorithm available on the World Wide Web at <http://rnagene.lbl.gov/>. (2) The G-quadruplex formed by the sequence from positions +24 to +56, which is likely to play an important role in the stability of the 3' cleavage product. The sequence that forms this G-quadruplex is, except from one nucleotide at position +53, 100% conserved between human rat and mouse. It will be interesting to determine the possible intrinsic biological function of the IGF-II 3' cleavage product.

REFERENCES

- Adams,T.E., Epa,V.C., Garrett,T.P., and Ward,C.W. (2000). Structure and function of the type 1 insulin-like growth factor receptor. *Cell Mol. Life Sci.* 57, 1050-1093.
- Altman,S. (2000). The road to RNase P. *Nat. Struct. Biol.* 7, 827-828.
- Anisowicz,A., Bardwell,L., and Sager,R. (1987). Constitutive overexpression of a growth-regulated gene in transformed Chinese hamster and human cells. *Proc. Natl. Acad. Sci. U. S. A* 84, 7188-7192.
- Anisowicz,A., Zajchowski,D., Stenman,G., and Sager,R. (1988). Functional diversity of gro gene expression in human fibroblasts and mammary epithelial cells. *Proc. Natl. Acad. Sci. U. S. A* 85, 9645-9649.
- Arnold,T.E., Yu,J., and Belasco,J.G. (1998). mRNA stabilization by the *ompA* 5' untranslated region: Two protective elements hinder distinct pathways for mRNA degradation. *Exp. Cell Res.* 4, 319-330.
- Arraiano,C.M., Cruz,A.A., and Kushner,S.R. (1997). Analysis of the *in vivo* decay of the *Escherichia coli* dicistronic *pyrF-orfF* transcript: Evidence for multiple degradation pathways. *J. Mol. Biol.* 268, 261-272.
- Asfari,M., De,W., Noël,M., Holthuisen,P.E., and Czernichow,P. (1995). Insulin-like growth factor-II gene expression in a rat insulin- producing beta-cell line (INS-1) is regulated by glucose. *Diabetologia* 38, 927-935.
- Backlin,B.M., Gessbo,A., Forsberg,M., Shokrai,A., Rozell,B., and Engstrom,W. (1998). Expression of the insulin-like growth factor II gene in polychlorinated biphenyl exposed female mink (*Mustela vison*) and their fetuses. *Mol. Pathol.* 51, 43-47.
- Bae,S.K., Bae,M.H., Ahn,M.Y., Son,M.J., Lee,Y.M., Bae,M.K., Lee,O.H., Park,B.C., and Kim,K.W. (1999). Egr-1 mediates transcriptional activation of IGF-II gene in response to hypoxia. *Cancer Res.* 59, 5989-5994.
- Baker,J., Liu,J.P., Robertson,E.J., and Efstratiadis,A. (1993). Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* 75, 73-82.
- Barnes,C.A. (1998). Upf1 and Upf2 proteins mediate normal yeast mRNA degradation when translation initiation is limited. *Nucleic Acids Res.* 26, 2433-2441.
- Bartel,D.P., Zapp,M.L., Green,M.R., and Szostak,J.W. (1991). HIV-1 Rev regulation involves recognition of non-Watson-Crick base pairs in viral RNA. *Cell* 67, 529-536.
- Bartolomei,M.S., Zemel,S., and Tilghman,S.M. (1991). Parental imprinting of the mouse H19 gene. *Nature* 351, 153-155.
- Batey,R.T. and Doudna,J.A. (1998). The parallel universe of RNA folding. *Nature Struct. Biol.* 5, 337-340.

- Battiste, J.L., Mao, H., Rao, N.S., Tan, R., Muhandiram, D.R., Kay, L.E., Frankel, A.D., and Williamson, J.R. (1996). Alpha helix-RNA major groove recognition in an HIV-1 rev peptide-RRE RNA complex. *Science* 273, 1547-1551.
- Baxter, R.C. (2000). Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. *Am. J Physiol Endocrinol. Metab* 278, E967-E976.
- Baxter, R.C. and Martin, J.L. (1989). Structure of the Mr 140,000 growth hormone-dependent insulin-like growth factor binding protein complex: determination by reconstitution and affinity-labeling. *Proc. Natl. Acad. Sci. U. S. A* 86, 6898-6902.
- Beck, F., Samani, N.J., Senior, P., Byrne, S., Morgan, K., Gebhard, R., and Brammar, W.J. (1988). Control of IGF-II mRNA levels by glucocorticoids in the neonatal rat. *J Mol. Endocrinol.* 1, R5-R8.
- Bedard, P.A., Alcorta, D., Simmons, D.L., Luk, K.C., and Erikson, R.L. (1987). Constitutive expression of a gene encoding a polypeptide homologous to biologically active human platelet protein in Rous sarcoma virus-transformed fibroblasts. *Proc. Natl. Acad. Sci. U. S. A* 84, 6715-6719.
- Beelman, C.A. and Parker, R. (1995). Degradation of mRNA in eukaryotes. *Cell* 81, 179-183.
- Bein, K., Ware, J.A., and Simons, M. (1998). Myb-dependent regulation of thrombospondin 2 expression - Role of mRNA stability. *J. Biol. Chem.* 273, 21423-21429.
- Bellaoui, M., Pelletier, G., and Budar, F. (1997). The steady-state level of mRNA from the Ogura cytoplasmic male sterility locus in *Brassica* hybrids is determined post-transcriptionally by its 3' region. *EMBO J.* 16, 5057-5068.
- Benard, L., Mathy, N., Grunberg-Manago, M., Ehresmann, B., Ehresmann, C., and Portier, C. (1998). Identification in a pseudoknot of a U.G motif essential for the regulation of the expression of ribosomal protein S15. *Proc. Natl. Acad. Sci. U. S. A* 95, 2564-2567.
- Benard, L., Philippe, C., Dondon, L., Grunberg-Manago, M., Ehresmann, B., Ehresmann, C., and Portier, C. (1994). Mutational analysis of the pseudoknot structure of the S15 translational operator from *Escherichia coli*. *Mol. Microbiol.* 14, 31-40.
- Bennett, A.L., Wilson, D.M., and Liu, F. (1983). Levels of insulin-like growth factors I and II in human cord blood. *J. Clin. Endocrinol. Metab.* 57(3), 609-612.
- Berman, H.M., Bhat, T.N., Bourne, P.E., Feng, Z., Gilliland, G., Weissig, H., and Westbrook, J. (2000). The Protein Data Bank and the challenge of structural genomics [In Process Citation]. *Nat. Struct. Biol.* 7 Suppl:957-9, 957-959.
- Bernstein, P.L., Herrick, D.J., Prokipcak, R.D., and Ross, J. (1992). Control of c-myc mRNA half-life in vitro by a protein capable of binding to a coding region stability determinant. *Genes Dev.* 6, 642-654.
- Bichell, D.P., Kikuchi, K., and Rotwein, P. (1992). Growth hormone rapidly activates insulin-like growth factor I gene transcription *in vivo*. *Mol. Endocrinol.* 6, 1899-1908.

Biddle,C., Li,C.H., Schofield,P.N., Tate,V.E., Hopkins,B., Engstrom,W., Huskisson,N.S., and Graham,C.F. (1988). Insulin-like growth factors and the multiplication of Tera-2, a human teratoma-derived cell line. *J. Cell Sci.* 90, 475-484.

Binder,R., Horowitz,J.A., Basilion,J.P., Koeller,D.M., Klausner,R.D., and Harford,J.B. (1994). Evidence that the pathway of transferrin receptor mRNA degradation involves an endonucleolytic cleavage within the 3' UTR and does not involve poly(A) tail shortening. *EMBO J.* 13, 1969-1980.

Binder,R., Hwang,S.P., Ratnasabapathy,R., and Williams,D.L. (1989). Degradation of apolipoprotein II mRNA occurs via endonucleolytic cleavage at 5'-AAU-3'/5'-UAA-3' elements in single-stranded loop domains of the 3'-noncoding region. *J. Biol. Chem.* 264, 16910-16918.

Bisbal,C., Silhol,M., Laubenthal,H., Kaluza,T., Carnac,G., Milligan,L., Le Roy,F., and Salehzada,T. (2000). The 2'-5' oligoadenylate/RNase L/RNase L inhibitor pathway regulates both MyoD mRNA stability and muscle cell differentiation. *Mol. Cell Biol.* 20, 4959-4969.

Biskobing,D.M., Fan,D., and Rubin,J. (1997). *c-fms* mRNA is regulated posttranscriptionally by 1,25(OH)₂D₃ in HL-60 cells. *Calcif. Tissue Int.* 61, 205-209.

Bondy,C.A., Chin,E., and Zhou,J. (1993). Significant species differences in local IGF-I and -II gene expression. *Adv. Exp. Med. Biol.* 343:73-7, 73-77.

Bonnieu,A., Roux,P., Marty,L., Jeanteur,P., and Piechaczyk,M. (1990). AUUUA motifs are dispensable for rapid degradation of the mouse *c-myc* RNA. *Oncogene* 5, 1585-1588.

Borovjagin,A.V. and Gerbi,S.A. (1999). U3 small nucleolar RNA is essential for cleavage at sites 1, 2 and 3 in pre-rRNA and determines which rRNA processing pathway is taken in *Xenopus* oocytes. *J Mol. Biol.* 286, 1347-1363.

Boshart,M., Weber,F., Jahn,G., Dorsch-Hasler,K., Fleckenstein,B., and Schaffner,W. (1985). A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* 41, 521-530.

Brissenden,J.E., Ullrich,A., and Francke,U. (1984). Human chromosomal mapping of genes for insulin-like growth factors I and II and epidermal growth factor. *Nature* 310, 781-784.

Brown,B.D. and Harland,R.M. (1990). Endonucleolytic cleavage of a maternal homeo box mRNA in *Xenopus* oocytes. *Genes Dev.* 4, 1925-1935.

Brown,B.D., Zipkin,I.D., and Harland,R.M. (1993). Sequence-specific endonucleolytic cleavage and protection of mRNA in *Xenopus* and *Drosophila*. *Genes Dev.* 7, 1620-1631.

Brown,E.J. and Schreiber,S.L. (1996). A signaling pathway to translational control. *Cell* 86, 517-520.

Burd,C.G. and Dreyfuss,G. (1994). Conserved structures and diversity of functions of RNA-binding proteins. *Science* 265, 615-621.

- Butcher,S.E., Dieckmann,T., and Feigon,J. (1997). Solution structure of a GAAA tetraloop receptor RNA. *EMBO J.* 16, 7490-7499.
- Bycroft,M., Grunert,S., Murzin,A.G., Proctor,M., and St Johnston,D. (1995). NMR solution structure of a dsRNA binding domain from *Drosophila* staufer protein reveals homology to the N-terminal domain of ribosomal protein S5. *EMBO J.* 14, 3563-3571 [published erratum appeared in *EMBO J* 1995 Sep 1;14(17):4385].
- Calnan,B.J., Biancalana,S., Hudson,D., and Frankel,A.D. (1991). Analysis of arginine-rich peptides from the HIV Tat protein reveals unusual features of RNA-protein recognition. *Genes Dev.* 5, 201-210.
- Campbell,S., McLaren,E.H., and Danesh,B.J. (1996). Rapidly reversible increase in insulin requirement with interferon. *BMJ* 313, 92.
- Cannistraro,V.J. and Kennell,D. (1997). RNase YI* and RNA structure studies. *Nucleic Acids Res.* 25, 1405-1411.
- Cao,G.J., Kalapos,M.P., and Sarkar,N. (1997). Polyadenylated mRNA in *Escherichia coli*: Modulation of poly(A) RNA levels by polynucleotide phosphorylase and ribonuclease II. *Biochimie* 79, 211-220.
- Casey,J.L., Koeller,D.M., Ramin,V.C., Klausner,R.D., and Harford,J.B. (1989). Iron regulation of transferrin receptor mRNA levels requires iron-responsive elements and a rapid turnover determinant in the 3' untranslated region of the mRNA. *EMBO J* 8, 3693-3699.
- Chagnovich,D. and Cohn,S.L. (1997). Activity of a 40 kDa RNA-binding protein correlates with *MYCN* and *c-fos* mRNA stability in human neuroblastoma. *Eur. J. Cancer [A]* 33A, 2064-2067.
- Chagnovich,D., Fayos,B.E., and Cohn,S.L. (1996). Differential activity of ELAV-like RNA-binding proteins in human neuroblastoma. *J. Biol. Chem.* 271, 33587-33591.
- Chan,M.T. and Yu,S.M. (1998). The 3' untranslated region of a rice α -amylase gene functions as a sugar-dependent mRNA stability determinant. *Proc. Natl. Acad. Sci. USA* 95, 6543-6547.
- Chanfreau,G. and Jacquier,A. (1996). An RNA conformational change between the two chemical steps of group II self-splicing. *EMBO J.* 15, 3466-3476.
- Chen,J.L., Nolan,J.M., Harris,M.E., and Pace,N.R. (1998). Comparative photocross-linking analysis of the tertiary structures of *Escherichia coli* and *Bacillus subtilis* RNase P RNAs. *EMBO J.* 17, 1515-1525.
- Cheng,S.L., Zhang,S.F., Mohan,S., Lecanda,F., Fausto,A., Hunt,A.H., Canalis,E., and Avioli,L.V. (1998). Regulation of insulin-like growth factors I and II and their binding proteins in human bone marrow stromal cells by dexamethasone. *J Cell Biochem.* 71, 449-458.
- Chernokalskaya,E., Dompenciel,R., and Schoenberg,D.R. (1997). Cleavage properties of an estrogen-regulated polysomal ribonuclease involved in the destabilization of albumin mRNA. *Nucleic Acids Res.* 25, 735-742.

Chernokalskaya,E., Dubell,A.N., Cunningham,K.S., Hanson,M.N., Dompenciel,R.E., and Schoenberg,D.R. (1998). A polysomal ribonuclease involved in the destabilization of albumin mRNA is a novel member of the peroxidase gene family. *RNA*. 4, 1537-1548.

Christiansen,J., Kofod,M., and Nielsen,F.C. (1994). A guanosine quadruplex and two stable hairpins flank a major cleavage site in insulin-like growth factor II mRNA. *Nucleic Acids Res.* 22, 5709-5716.

Ciesiolka,J., Michalowski,D., Wrzesinski,J., Krajewski,J., and Krzyzosiak,W.J. (1998). Patterns of cleavages induced by lead ions in defined RNA secondary structure motifs. *J. Mol. Biol.* 275, 211-220.

Claverie-Martin,F., Wang,M., and Cohen,S.N. (1997). ARD-1 cDNA from human cells encodes a site-specific single-strand endoribonuclease that functionally resembles *Escherichia coli* RNase E. *J Biol. Chem.* 272, 13823-13828.

Collins,M.K.L., Furlong,I.J., Malde,P., Ascaso,R., Oliver,J., and Rivas,A.L. (1996). An apoptotic endonuclease activated either by decreasing pH or by increasing calcium. *J. Cell Sci.* 109, 2393-2399.

Cooke,H.J. and Elliott,D.J. (1997). RNA-binding proteins and human male infertility. *Trends Genet.* 13, 87-89.

Corkins,M.R., Park,J.H., Davis,D.V., Slentz,D.H., and MacDonald,R.G. (1999). Regulation of the insulin-like growth factor axis by increasing cell number in intestinal epithelial (IEC-6) cells. *Growth Horm. IGF. Res.* 9, 414-424.

Correll,C.C., Freeborn,B., Moore,P.B., and Steitz,T.A. (1997). Metals, motifs, and recognition in the crystal structure of a 5S rRNA domain. *Cell* 91, 705-712.

Cusack,S. (1999). RNA-protein complexes. *Curr. Opin. Struct. Biol.* 9, 66-73.

Czyzyk-Krzeska,M.F., Paulding,W.R., Lipski,J., Beresh,J.E., and Kroll,S.L. (1996). Regulation of tyrosine hydroxylase mRNA stability by oxygen in PC12 cells. *Adv. Exp. Med. Biol.* 410, 143-150.

DaCosta,S.A., Schumaker,L.M., and Ellis,M.J. (2000). Mannose 6-phosphate/insulin-like growth factor 2 receptor, a bona fide tumor suppressor gene or just a promising candidate? *J Mammary. Gland. Biol. Neoplasia.* 5, 85-94.

Danner,S., Frank,M., and Lohse,M.J. (1998). Agonist regulation of human β_2 -adrenergic receptor mRNA stability occurs via a specific AU-rich element. *J. Biol. Chem.* 273, 3223-3229.

Daughaday,W.H., Emanuele,M.A., Brooks,M.H., Barbato,A.L., Kapadia,M., and Rotwein,P. (1988). Synthesis and secretion of insulin-like growth factor II by a leiomyosarcoma with associated hypoglycemia. *N. Engl. J. Med.* 319, 1434-1440.

Daughaday,W.H., Hall,K., Raben,M.S., Salmon,W.D., Jr., Brande JL,V.d., and Wyk,J.V. (1972). Somatomedin: proposed designation for sulphation factor. *Nature* 235, 107.

- Daughaday, W.H. and Rotwein, P. (1989). Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. *Endocr. Rev.* *10*, 68-91.
- De la Cruz, J., Kressler, D., Tollervey, D., and Linder, P. (1998). Dob1p (Mtr4p) is a putative ATP-dependent RNA helicase required for the 3' end formation of 5.8S rRNA in *Saccharomyces cerevisiae*. *EMBO J.* *17*, 1128-1140.
- de Moor, C. H. Translation of Insulin-like Growth Factor 2 mRNAs. 1994. Thesis. Laboratory for Physiological Chemistry, Utrecht University, Utrecht, The Netherlands.
- de Moor, C.H., Jansen, M., Sussenbach, J.S., and Van den Brande, J.L. (1994). Differential polysomal localization of human insulin-like growth factor 2 mRNAs, both in cell lines and foetal liver. *Eur. J. Biochem.* *222*, 1017-1022.
- De Pagter-Holthuizen, P., Jansen, M., van der Kammen, R.A., Van Schaik, F.M.A., and Sussenbach, J.S. (1988). Differential expression of the human insulin-like growth factor II gene. Characterization of the IGF-II mRNAs and an mRNA encoding a putative IGF-II-associated protein. *Biochim. Biophys. Acta* *950*, 282-295.
- de Wet, J.R., Wood, K.V., DeLuca, M., Helinski, D.R., and Subramani, S. (1987). Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell Biol.* *7*, 725-737.
- DeChiara, T.M., Efstratiadis, A., and Robertson, E.J. (1990). A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature* *345*, 78-80.
- DeChiara, T.M., Robertson, E.J., and Efstratiadis, A. (1991). Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* *64*, 849-859.
- Delihias, N. (1995). Regulation of gene expression by trans-encoded antisense RNAs. *Mol. Microbiol.* *15*, 411-414.
- Dell, G., Ward, A., and Engstrom, W. (1997). Regulation of a promoter from the mouse insulin like growth factor II gene by glucocorticoids. *FEBS Lett.* *419*, 161-165.
- Di Nicola, N.E., Fabbri, S., Bufardecchi, E., Baldi, M.I., Gandini, A.D., Mattoccia, E., and Tocchini-Valentini, G.P. (1997). The eucaryal tRNA splicing endonuclease recognizes a tripartite set of RNA elements. *Cell* *89*, 859-866.
- Dodson, R.E. and Shapiro, D.J. (1994). An estrogen-inducible protein binds specifically to a sequence in the 3' untranslated region of estrogen-stabilized vitellogenin mRNA. *Mol. Cell Biol.* *14*, 3130-3138.
- Dompenciel, R.E., Garnepudi, V.R., and Schoenberg, D.R. (1995). Purification and characterization of an estrogen-regulated *Xenopus* liver polysomal nuclease involved in the selective destabilization of albumin mRNA. *J. Biol. Chem.* *270*, 6108-6118.
- Dong, B. and Silverman, R.H. (1995). 2-5A-dependent RNase molecules dimerize during activation by 2-5A. *J. Biol. Chem.* *270*, 4133-4137.

- Douthwaite, S., Garrett, R.A., Wagner, R., and Feunteun, J. (1979). A ribonuclease-resistant region of 5S RNA and its relation to the RNA binding sites of proteins L18 and L25. *Nucleic Acids Res.* 6, 2453-2470.
- Doyle, G.A.R., Betz, N.A., Leeds, P.F., Fleisig, A.J., Prokipcak, R.D., and Ross, J. (1998). The *c-myc* coding region determinant-binding protein: a member of a family of KH domain RNA-binding proteins. *Nucleic Acids Res.* 26, 5036-5044.
- Drummond, I.A., Madden, S.L., Rohwer-Nutter, P., Bell, G.I., Sukhatme, V.P., and Rauscher, F.J., III (1992). Repression of the insulin-like growth factor II gene by the Wilms tumor suppressor WT1. *Science* 257, 674-678.
- Dufourny, B., Alblas, J., van Teeffelen, H.A., van Schaik, F.M., van der, B.B., Steenbergh, P.H., and Sussenbach, J.S. (1997). Mitogenic signaling of insulin-like growth factor I in MCF-7 human breast cancer cells requires phosphatidylinositol 3-kinase and is independent of mitogen-activated protein kinase. *J. Biol. Chem.* 272, 31163-31171.
- Ehretsmann, C.P., Carpousis, A.J., and Krisch, H.M. (1992). Specificity of Escherichia coli endoribonuclease RNase E: in vivo and in vitro analysis of mutants in a bacteriophage T4 mRNA processing site. *Genes Dev.* 6, 149-159.
- Enberg, G. and Hall, K. (1984). Immunoreactive IGF-II in serum of healthy subjects and patients with growth hormone disturbances and uraemia. *Acta Endocrinol. (Copenh)* 107, 164-170.
- Engstrom, W., Shokrai, A., Otte, K., Granerus, M., Gessbo, A., Bierke, P., Madej, A., Sjolund, M., and Ward, A. (1998). Transcriptional regulation and biological significance of the insulin like growth factor II gene. *Cell Prolif.* 31, 173-189.
- Fang, G. and Cech, T.R. (1993a). Characterization of a G-quartet formation reaction promoted by the beta-subunit of the Oxytricha telomere-binding protein. *Biochemistry* 32, 11646-11657.
- Fang, G. and Cech, T.R. (1993b). The beta subunit of Oxytricha telomere-binding protein promotes G-quartet formation by telomeric DNA. *Cell* 74, 875-885.
- Feng, J., Funk, W.D., Wang, S.S., Weinrich, S.L., Avilion, A.A., Chiu, C.P., Adams, R.R., Chang, E., Allsopp, R.C., and Yu, J. (1995). The RNA component of human telomerase. *Science* 269, 1236-1241.
- Florini, J.R., Magri, K.A., Ewton, D.Z., James, P.L., Grindstaff, K., and Rotwein, P.S. (1991). "Spontaneous" differentiation of skeletal myoblasts is dependent upon autocrine secretion of insulin-like growth factor-II. *J. Biol. Chem.* 266, 15917-15923.
- Floyd-Smith, G., Slattery, E., and Lengyel, P. (1981). Interferon action: RNA cleavage pattern of a (2'-5')oligoadenylate--dependent endonuclease. *Science* 212, 1030-1032.
- Frasca, F., Pandini, G., Scalia, P., Sciacca, L., Mineo, R., Costantino, A., Goldfine, I.D., Belfiore, A., and Vigneri, R. (1999). Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. *Mol. Cell Biol.* 19, 3278-3288.

Gibson,T.J., Thompson,J.D., and Heringa,J. (1993). The KH domain occurs in a diverse set of RNA-binding proteins that include the antiterminator NusA and is probably involved in binding to nucleic acid. *FEBS Lett.* 324, 361-366.

Gilley,D. and Blackburn,E.H. (1999). The telomerase RNA pseudoknot is critical for the stable assembly of a catalytically active ribonucleoprotein. *Proc. Natl. Acad. Sci. U. S. A* 96, 6621-6625.

Giudice,L.C. (1992). Insulin-like growth factors and ovarian follicular development. *Endocr. Rev.* 13, 641-669.

Gluckman,P.D. and Ambler,G.R. (1993). What is the function of circulating insulin-like growth factor-2 in postnatal life? *Mol. Cell Endocrinol.* 92, C1-C3.

Gordon,D.A., Shelness,G.S., Nicosia,M., and Williams,D.L. (1988). Estrogen-induced destabilization of yolk precursor protein mRNAs in avian liver. *J. Biol. Chem.* 263, 2625-2631.

Goya,L., de la,P.A., Ramos,S., Martin,M.A., Escriva,F., and Pascual-Leone,A.M. (1999). Regulation of insulin-like growth factor-I and -II by glucose in primary cultures of fetal rat hepatocytes. *J Biol. Chem.* 274, 24633-24640.

Graham FL and van der Eb AJ (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52, 456-467.

Granerus,G., Lonqvist,B., Nystrand,J., and Roupe,G. (1998). Serum tryptase measured with B12 and G5 antibody-based immunoassays in mastocytosis patients and its relation to histamine turnover. *Br. J. Dermatol.* 139, 858-861.

Granerus,M. and Engstrom,W. (1996). Growth factors and apoptosis. *Cell Prolif.* 29, 309-314.

Granerus,M., Schofield,P., Bierke,P., and Engstrom,W. (1995). Growth factors and apoptosis in development. The role of insulin like growth factor I and TGFbeta1 in regulating cell growth and cell death in a human teratocarcinoma derived cell line. *Int. J. Dev. Biol.* 39, 759-764.

Gray,S.G., Yakovleva,T., Hartmann,W., Tally,M., Bakalkin,G., and Ekström,T.J. (1999). IGF-II enhances Trichostatin A-induced *TGF 1* and p21^{Waf1, Cip1, Sdi1} expression in Hep3B cells. *Exp. Cell Res.* 253, 618-628.

Greider,C.W. and Blackburn,E.H. (1989). A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. *Nature* 337, 331-337.

Hall,K., Enberg,G., Ritzen,M., Svan,H., Fryklund,L., and Takano,K. (1980). Somatomedin A levels in serum from healthy children and from children with growth hormone deficiency or delayed puberty. *Acta Endocrinol. (Copenh)* 94, 155-165.

Hall,K. and Sara,V.R. (1984). Somatomedin levels in childhood, adolescence and adult life. *Clin. Endocrinol. Metab* 13, 91-112.

- Herrick,D.J. and Ross,J. (1994). The half-life of c-myc mRNA in growing and serum-stimulated cells: influence of the coding and 3' untranslated regions and role of ribosome translocation. *Mol. Cell Biol.* *14*, 2119-2128.
- Hirai,K., Miyamasu,M., Yamaguchi,M., Nakajima,K., Ohtoshi,T., Koshino,T., Takaishi,T., Morita,Y., and Ito,K. (1993). Modulation of human basophil histamine release by insulin-like growth factors. *J Immunol.* *150*, 1503-1508.
- Holthuizen,P., van der Lee,F.M., Ikejiri,K., Yamamoto,M., and Sussenbach,J.S. (1990). Identification and initial characterization of a fourth leader exon and promoter of the human IGF-II gene. *Biochim. Biophys. Acta* *1087*, 341-343.
- Holthuizen,P.E., Cleutjens,C.B.J.M., Veenstra,G.J.C., van der Lee,F.M., Koonen-Reemst,A.M.C.B., and Sussenbach,J.S. (1993). Differential expression of the human, mouse and rat IGF- II genes. *Regul. Pept.* *48*, 77-89.
- Holthuizen,P.E., Steenbergh,P.H., and Sussenbach,J.S. (1999). Regulation of IGF gene expression. In *The IGF System. Molecular Biology, Physiology, and Clinical Applications.*, R.G.Rosenfeld and C.T.Roberts, eds. (Totowa, New Jersey, USA.: Humana Press.), pp. 37-61.
- Hua,J., Garner,R., and Paetkau,V. (1993). An RNasin-resistant ribonuclease selective for interleukin 2 mRNA. *Nucleic Acids Res.* *21*, 155-162.
- Hua,J. and Paetkau,V. (1996). A binding factor for interleukin 2 mRNA. *Nucleic Acids Res.* *24*, 970-976.
- Humbel,R.E. (1990). Insulin-like growth factors I and II. *Eur. J Biochem.* *190*, 445-462.
- Hunter,S.J., Daughaday,W.H., Callender,M.E., McKnight,J.A., McIlrath,E.M., Teale,J.D., and Atkinson,A.B. (1994). A case of hepatoma associated with hypoglycaemia and overproduction of IGF-II (E-21): beneficial effects of treatment with growth hormone and intrahepatic adriamycin. *Clin. Endocrinol. (Oxf)* *41*, 397-401.
- Hurley,L.H., Wheelhouse,R.T., Sun,D., Kerwin,S.M., Salazar,M., Fedoroff,O.Y., Han,F.X., Han,H., Izbicka,E., and Von Hoff,D.D. (2000). G-quadruplexes as targets for drug design. *Pharmacol. Ther.* *85*, 141-158.
- Ikejiri,K., Wasada,T., Haruki,K., Hizuka,N., Hirata,Y., and Yamamoto,M. (1991). Identification of a novel transcription unit in the human insulin-like growth factor-II gene. *Biochem. J.* *280*, 439-444.
- Ioannidis,P., Havredaki,M., Curtis,N., and Trangas,T. (1996). In vivo generation of 3' and 5' truncated species in the process of c-myc mRNA decay. *Nucleic. Acids. Res* *24*, 4969-4977.
- Jacobs,S., Kull,F.C., Jr., Earp,H.S., Svoboda,M.E., Van Wyk,J.J., and Cuatrecasas,P. (1983). Somatomedin-C stimulates the phosphorylation of the beta-subunit of its own receptor. *J. Biol. Chem.* *258*, 9581-9584.
- Jacobson,A. and Peltz,S.W. (1996). Interrelationships of the pathways of mRNA decay and translation in eukaryotic cells. *Annu. Rev. Biochem.* *65*:693-739, 693-739.

Jessen, T.H., Oubridge, C., Teo, C.H., Pritchard, C., and Nagai, K. (1991). Identification of molecular contacts between the U1 A small nuclear ribonucleoprotein and U1 RNA. *EMBO J* 10, 3447-3456.

Jhun, B.H., Meinkoth, J.L., Leitner, J.W., Draznin, B., and Olefsky, J.M. (1994). Insulin and insulin-like growth factor-I signal transduction requires p21ras. *J. Biol. Chem.* 269, 5699-5704.

Jones, J.I. and Clemmons, D.R. (1995). Insulin-like growth factors and their binding proteins: biological actions. *Endocr. Rev.* 16, 3-34.

Jones, T.R. and Cole, M.D. (1987). Rapid cytoplasmic turnover of c-myc mRNA: requirement of the 3' untranslated sequences. *Mol Cell Biol* 7, 4513-4521.

Kawahara, R.S. and Deuel, T.F. (1989). Platelet-derived growth factor-inducible gene JE is a member of a family of small inducible genes related to platelet factor 4. *J. Biol. Chem.* 264, 679-682.

Kelley, R.L. and Kuroda, M.I. (2000). Noncoding RNA genes in dosage compensation and imprinting. *Cell* 103, 9-12.

Kensch, O., Connolly, B.A., Steinhoff, H.J., McGregor, A., Goody, R.S., and Restle, T. (2000). HIV-1 reverse transcriptase-pseudoknot RNA aptamer interaction has a binding affinity in the low picomolar range coupled with high specificity. *J Biol. Chem.* 275, 18271-18278.

Kharrat, A., Macias, M.J., Gibson, T.J., Nilges, M., and Pastore, A. (1995). Structure of the dsRNA binding domain of E. coli RNase III. *EMBO J.* 14, 3572-3584.

Kiess, W., Yang, Y., Kessler, U., and Hoeflich, A. (1994). Insulin-like growth factor II (IGF-II) and the IGF-II/mannose-6-phosphate receptor: the myth continues. *Horm. Res.* 41 Suppl 2:66-73, 66-73.

Kim, K.W., Bae, S.K., Lee, O.H., Bae, M.H., Lee, M.J., and Park, B.C. (1998). Insulin-like growth factor II induced by hypoxia may contribute to angiogenesis of human hepatocellular carcinoma. *Cancer Res.* 58, 348-351.

Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L., Coviello, G.M., Wright, W.E., Weinrich, S.L., and Shay, J.W. (1994). Specific association of human telomerase activity with immortal cells and cancer. *Science* 266, 2011-2015.

Kim, S., Kim, H., Park, I., and Lee, Y. (1996). Mutational analysis of RNA structures and sequences postulated to affect 3' processing of M1 RNA, the RNA component of Escherichia coli RNase P. *J Biol. Chem.* 271, 19330-19337.

Koeller, D.M., Horowitz, J.A., Casey, J.L., Klausner, R.D., and Harford, J.B. (1991). Translation and the stability of mRNAs encoding the transferrin receptor and c-fos. *Proc. Natl. Acad. Sci. U. S. A* 88, 7778-7782.

Kotani, K., Tsuji, M., Oki, A., Kashihara, T., Yamada, K., Kawakami, F., Tako, H., Okuno, G., Hizuka, N., and Aiba, M. (1993). IGF-II producing hepatic fibrosarcoma associated with hypoglycemia. *Intern. Med.* 32, 897-901.

- Kowalski,J. and Denhardt,D.T. (1989). Regulation of the mRNA for monocyte-derived neutrophil-activating peptide in differentiating HL60 promyelocytes. *Mol. Cell Biol.* 9, 1946-1957.
- Kurz,J.C. and Fierke,C.A. (2000). Ribonuclease P: a ribonucleoprotein enzyme. *Curr. Opin. Chem. Biol.* 4, 553-558.
- Kutoh,E., Schwander,J., and Margot,J.B. (1995). Cell-density-dependent modulation of the rat insulin-like-growth-factor-binding protein 2 and its gene. *Eur. J Biochem.* 234, 557-562.
- Lau,M.M., Stewart,C.E., Liu,Z., Bhatt,H., Rotwein,P., and Stewart,C.L. (1994). Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. *Genes Dev.* 8, 2953-2963.
- Lee,C.H., Leeds,P., and Ross,J. (1998). Purification and characterization of a polysome-associated endoribonuclease that degrades *c-myc* mRNA *in vitro*. *J. Biol. Chem.* 273, 25261-25271.
- Leeds,P., Kren,B.T., Boylan,J.M., Betz,N.A., Steer,C.J., Gruppuso,P.A., and Ross,J. (1997). Developmental regulation of CRD-BP, an RNA-binding protein that stabilizes *c-myc* mRNA *in vitro*. *Oncogene* 14, 1279-1286.
- Leighton,P.A., Ingram,R.S., Eggenschwiler,J., Efstratiadis,A., and Tilghman,S.M. (1995). Disruption of imprinting caused by deletion of the H19 gene region in mice [see comments]. *Nature* 375, 34-39.
- Leontis,N.B., Ghosh,P., and Moore,P.B. (1986). Effect of magnesium ion on the structure of the 5S RNA from *Escherichia coli*. An imino proton magnetic resonance study of the helix I, IV, and V regions of the molecule. *Biochemistry* 25, 7386-7392.
- Levinovitz,A. and Norstedt,G. (1989). Developmental and steroid hormonal regulation of insulin-like growth factor II expression. *Mol. Endocrinol.* 3, 797-804.
- Li,X.L., Blackford,J.A., Judge,C.S., Liu,M., Xiao,W., Kalvakolanu,D.V., and Hassel,B.A. (2000). RNase-L-dependent destabilization of interferon-induced mRNAs. A role for the 2-5A system in attenuation of the interferon response. *J. Biol. Chem.* 275, 8880-8888.
- Lindstein,T., June,C.H., Ledbetter,J.A., Stella,G., and Thompson,C.B. (1989). Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. *Science* 244, 339-343.
- Liu,J., Kahri,A.I., Heikkilä,P., Blum,W.F., and Voutilainen,R. (1994). Glucocorticoids increase insulin-like growth factor-II mRNA accumulation in cultured human pheochromocytoma cells. *J. Endocrinol.* 142, 29-35.
- Liu,J.P., Baker,J., Perkins,A.S., Robertson,E.J., and Efstratiadis,A. (1993). Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell* 75, 59-72.
- Louvi,A., Accili,D., and Efstratiadis,A. (1997). Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. *Dev. Biol.* 189, 33-48.

- Ludwig, T., Eggenschwiler, J., Fisher, P., D'Ercole, A.J., Davenport, M.L., and Efstratiadis, A. (1996). Mouse mutants lacking the type 2 IGF receptor (IGF2R) are rescued from perinatal lethality in *Igf2* and *Igf1r* null backgrounds. *Dev. Biol.* 177, 517-535.
- Madden, S.L. and Rauscher, F.J., III (1993). Positive and negative regulation of transcription and cell growth mediated by the EGR family of zinc-finger gene products. *Ann. N. Y. Acad. Sci.* 684:75-84, 75-84.
- Martin J.L and Baxter, R.C. (1999). IGF binding proteins as modulators of IGF action. In *The IGF System, Molecular Biology, Physiology and Clinical Applications*, Rosenfeld R. and Roberts C., eds. (Totowa, NJ: Humana), pp. 227-255.
- McKnight, S.L. and Kingsbury, R. (1982). Transcriptional control signals of a eukaryotic protein-coding gene. *Science* 217, 316-324.
- Meisma, D., Holthuisen, P., Van den Brande, J.L., and Sussenbach, J.S. (1991). Specific endonucleolytic cleavage of IGF-II mRNAs. *Biochem. Biophys. Res. Commun.* 179, 1509-1516.
- Meisma, D., Scheper, W., Holthuisen, P., Van den Brande, J.L., and Sussenbach, J.S. (1992). Site-specific cleavage of IGF-II mRNAs requires sequence elements from two distinct regions of the IGF-II gene. *Nucleic Acids Res.* 20, 5003-5009.
- Mineo, R., Fichera, E., Liang, S.J., and Fujita-Yamaguchi, Y. (2000). Promoter usage for insulin-like growth factor-II in cancerous and benign human breast, prostate, and bladder tissues, and confirmation of a 10th exon. *Biochem. Biophys. Res. Commun.* 268, 886-892.
- Minniti, C.P., Kohn, E.C., Grubb, J.H., Sly, W.S., Oh, Y., Muller, H.L., Rosenfeld, R.G., and Helman, L.J. (1992). The insulin-like growth factor II (IGF-II)/mannose 6-phosphate receptor mediates IGF-II-induced motility in human rhabdomyosarcoma cells. *J. Biol. Chem.* 267, 9000-9004.
- Mise, M., Arai, S., Higashitani, H., Furutani, M., Niwano, M., Harada, T., Ishigami, S., Toda, Y., Nakayama, H., Fukumoto, M., Fujita, J., and Imamura, M. (1996). Clinical significance of vascular endothelial growth factor and basic fibroblast growth factor gene expression in liver tumor. *Hepatology* 23, 455-464.
- Morrione, A., Valentinis, B., Xu, S.Q., Yumet, G., Louvi, A., Efstratiadis, A., and Baserga, R. (1997). Insulin-like growth factor II stimulates cell proliferation through the insulin receptor. *Proc. Natl. Acad. Sci. U. S. A* 94, 3777-3782.
- Moskaitis, J.E., Buzek, S.W., Pastori, R.L., and Schoenberg, D.R. (1991). The estrogen-regulated destabilization of *Xenopus* albumin mRNA is independent of translation. *Biochem. Biophys. Res. Commun.* 174, 825-830.
- Mouhieddine, O.B., Cazals, V., Kuto, E., Le Bouc, Y., and Clement, A. (1996). Glucocorticoid-induced growth arrest of lung alveolar epithelial cells is associated with increased production of insulin-like growth factor binding protein-2. *Endocrinology* 137, 287-295.
- Muller, M.M., Carrasco, A.E., and DeRobertis, E.M. (1984). A homeo-box-containing gene expressed during oogenesis in *Xenopus*. *Cell* 39, 157-162.

- Mullner,E.W. and Kuhn,L.C. (1988). A stem-loop in the 3' untranslated region mediates iron-dependent regulation of transferrin receptor mRNA stability in the cytoplasm. *Cell* 53, 815-825.
- Musco,G., Stier,G., Joseph,C., Castiglione Morelli,M.A., Nilges,M., Gibson,T.J., and Pastore,A. (1996). Three-dimensional structure and stability of the KH domain: molecular insights into the fragile X syndrome. *Cell* 19;85, 237-245.
- Nashimoto,M., Tamura,M., and Kaspar,R.L. (1999a). Selection of cleavage site by mammalian tRNA 3' processing endoribonuclease. *J. Mol. Biol.* 287, 727-740.
- Nashimoto,M., Wesemann,D.R., Geary,S., Tamura,M., and Kaspar,R.L. (1999b). Long 5' leaders inhibit removal of a 3' trailer from a precursor tRNA by mammalian tRNA 3' processing endoribonuclease. *Nucleic Acids Res.* 27, 2770-2776.
- Nielsen,D.A. and Shapiro,D.J. (1990). Estradiol and estrogen receptor-dependent stabilization of a minivitellogenin mRNA lacking 5,100 nucleotides of coding sequence. *Mol. Cell Biol.* 10, 371-376.
- Nielsen,F.C. and Christiansen,J. (1992). Endonucleolysis in the turnover of insulin-like growth factor II mRNA. *J. Biol. Chem.* 267, 19404-19411.
- Nielsen,F.C., Gammeltoft,S., and Christiansen,J. (1990). Translational discrimination of mRNAs coding for human insulin-like growth factor II. *J. Biol. Chem.* 265, 13431-13434.
- Nielsen,F.C., Ostergaard,L., Nielsen,J., and Christiansen,J. (1995). Growth-dependent translation of IGF-II mRNA by a rapamycin-sensitive pathway. *Nature* 377, 358-362.
- Nishimoto,I. (1993). The IGF-II receptor system: a G protein-linked mechanism. *Mol. Reprod. Dev.* 35, 398-406.
- Nissley,P. and Lopaczynski,W. (1991). Insulin-like growth factor receptors. *Growth Factors* 5, 29-43.
- Norstedt,G. and Moller,C. (1987). Growth hormone induction of insulin-like growth factor I messenger RNA in primary cultures of rat liver cells. *J. Endocrinol.* 115, 135-139.
- Ogawa,O., Eccles,M.R., Szeto,J., McNoe,L.A., Yun,K., Maw,M.A., Smith,P.J., and Reeve,A.E. (1993). Relaxation of insulin-like growth factor II gene imprinting implicated in Wilms' tumour. *Nature* 362, 749-751.
- Oka,Y., Rozek,L.M., and Czech,M.P. (1985). Direct demonstration of rapid insulin-like growth factor II Receptor internalization and recycling in rat adipocytes. Insulin stimulates 125I-insulin-like growth factor II degradation by modulating the IGF-II receptor recycling process. *J. Biol. Chem.* 260, 9435-9442.
- Oubridge,C., Ito,N., Evans,P.R., Teo,C.H., and Nagai,K. (1994). Crystal structure at 1.92 Å resolution of the RNA-binding domain of the U1A spliceosomal protein complexed with an RNA hairpin. *Nature* 372, 432-438.

- Pastori,R.L., Moskaitis,J.E., Buzek,S.W., and Schoenberg,D.R. (1991a). Coordinate estrogen-regulated instability of serum protein-coding messenger RNAs in *Xenopus laevis*. *Mol. Endocrinol.* 5, 461-468.
- Pastori,R.L., Moskaitis,J.E., and Schoenberg,D.R. (1991b). Estrogen-induced ribonuclease activity in *Xenopus* liver. *Biochemistry* 30, 10490-10498.
- Pastori,R.L., Moskaitis,J.E., Smith,L.H., Jr., and Schoenberg,D.R. (1990). Estrogen regulation of *Xenopus laevis* gamma-fibrinogen gene expression. *Biochemistry* 29, 2599-2605.
- Phizicky,E.M. and Greer,C.L. (1993). Pre-tRNA splicing: variation on a theme or exception to the rule? *Trends Biochem. Sci.* 18, 31-34.
- Pleij,C.W. (1995). Structure and function of RNA pseudoknots. *Genet. Eng (N. Y.)* 17:67-80, 67-80.
- Pleij,C.W., Rietveld,K., and Bosch,L. (1985). A new principle of RNA folding based on pseudoknotting. *Nucleic Acids Res.* 13, 1717-1731.
- Powell-Braxton,L., Hollingshead,P., Warburton,C., Dowd,M., Pitts-Meek,S., Dalton,D., Gillett,N., and Stewart,T.A. (1993). IGF-I is required for normal embryonic growth in mice. *Genes Dev.* 7, 2609-2617.
- Price,W.A., Stiles,A.D., Moats-Staats,B.M., and D'Ercole,A.J. (1992). Gene expression of insulin-like growth factors (IGFs), the type 1 IGF receptor, and IGF-binding proteins in dexamethasone-induced fetal growth retardation. *Endocrinology* 130, 1424-1432.
- Prokipcak,R.D., Herrick,D.J., and Ross,J. (1994). Purification and properties of a protein that binds to the C-terminal coding region of human c-myc mRNA. *J Biol Chem* 269, 9261-9269.
- Puglisi,J.D., Chen,L., Blanchard,S., and Frankel,A.D. (1995). Solution structure of a bovine immunodeficiency virus Tat-TAR peptide-RNA complex. *Science* 270, 1200-1203.
- Raizis,A.M., Eccles,M.R., and Reeve,A.E. (1993). Structural analysis of the human insulin-like growth factor-II P3 promoter. *Biochem. J.* 289, 133-139.
- Ramasharma,K. and Li,C.H. (1987). Human pituitary and placental hormones control human insulin-like growth factor II secretion in human granulosa cells. *Proc. Natl. Acad. Sci. U. S. A* 84, 2643-2647.
- Ramos,A., Gubser,C.C., Varani,G. (1997). Recent solution structures of RNA and its complexes with drugs, peptides and proteins. *Curr.Opin.Struct.Biol.* 7, 317-323
- Rauscher,F.J., III (1993). Tumor suppressor genes which encode transcriptional repressors: Studies on the EGR and Wilms' tumor (WT1) gene products. *Adv. Exp. Med. Biol.* 348, 23-29.
- Rietveld,L.E., Holthuizen,P.E., and Sussenbach,J.S. (1997). Identification of a key regulatory element for the basal activity of the human insulin-like growth factor II gene promoter P3. *Biochem. J.* 327, 689-697.

Rietveld,L.E., Koonen-Reemst,A.M., Sussenbach,J.S., and Holthuisen,P.E. (1999). Dual role for transcription factor AP-2 in the regulation of the major fetal promoter P3 of the gene for human insulin-like growth factor II. *Biochem. J.* 338, 799-806.

Rinderknecht,E. and Humbel,R.E. (1978a). Primary structure of human insulin-like growth factor II. *FEBS Lett.* 89, 283-286.

Rinderknecht,E. and Humbel,R.E. (1978b). The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *J Biol. Chem.* 253, 2769-2776.

Rodenburg, R. J. Transcriptional regulation of the liver-specific promoter of the human insulin-like growth factor II gene. 1996. Thesis. Laboratory for Physiological Chemistry, Utrecht University, Utrecht, The Netherlands.

Rodenburg,R.J., Holthuisen,P.E., and Sussenbach,J.S. (1997). A functional Sp1 binding site is essential for the activity of the adult liver-specific human insulin-like growth factor II promoter. *Mol. Endocrinol.* 11, 237-250.

Romby,P., Westhof,E., Toukifimpa,R., Mache,R., Ebel,J.P., Ehresmann,C., and Ehresmann,B. (1988). Higher order structure of chloroplastic 5S ribosomal RNA from spinach. *Biochemistry* 27, 4721-4730.

Ross,J. (1995). mRNA stability in mammalian cells. *Microbiol. Rev.* 59, 423-450.

Rotwein,P. (1986). Two insulin-like growth factor I messenger RNAs are expressed in human liver. *Proc. Natl. Acad. Sci. USA* 83, 77-81.

Russell,A.G., Ehardt,H., and Dennis,P.P. (1999). Substrate requirements for a novel archaeal endonuclease that cleaves within the 5' external transcribed spacer of *Sulfolobus acidocaldarius* precursor rRNA. *Genetics* 152, 1373-1385.

Saenger W (1984). *Principles of Nucleic Acid Structure.* (New York: Springer).

Salmon,W.D. and Daughaday,W.H. (1957). A hormonally controlled serumfactor which stimulates sulfate incorporation by cartilage in vitro. *J. Lab. Clin. Med.* 49, 825.

Sambrook J, Fritsch EF, and Maniatis T (1989). *Molecular cloning: A laboratory manual.* (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Scheper,W., Meinsma,D., Holthuisen,P.E., and Sussenbach,J.S. (1995). Long-range RNA interaction of two sequence elements required for endonucleolytic cleavage of human insulin-like growth factor II mRNAs. *Mol. Cell Biol.* 15, 235-245.

Schmidt,J.V., Levorse,J.M., and Tilghman,S.M. (1999). Enhancer competition between H19 and Igf2 does not mediate their imprinting. *Proc. Natl. Acad. Sci. U. S. A* 96, 9733-9738.

Schneid,H., Holthuisen,P.E., and Sussenbach,J.S. (1993). Differential promoter activation in two human insulin-like growth factor-II-producing tumor cell lines. *Endocrinology* 132, 1145-1150.

Schoenberg,D.R., Moskaitis,J.E., Smith,L.H., Jr., and Pastori,R.L. (1989). Extranuclear estrogen-regulated destabilization of *Xenopus laevis* serum albumin mRNA. *Mol. Endocrinol.* 3, 805-814.

Schuster,P., Stadler,P.F., and Renner,A. (1997). RNA structures and folding: From conventional to new issues in structure predictions. *Curr. Opin. Struct. Biol.* 7, 229-235.

Sen,G.C. and Lengyel,P. (1992). The interferon system. A bird's eye view of its biochemistry. *J. Biol. Chem.* 267, 5017-5020.

Senior,P.V., Tucci,J., Blythe,N.L., and Beck,F. (1996). Expression of IGF-II and H19 mRNA in the neonatal rat during normal maturation and after dexamethasone administration. *J. Mol. Endocrinol.* 17, 217-223.

Siomi,H. and Dreyfuss,G. (1997). RNA-binding proteins as regulators of gene expression. *Curr. Opin. Genet. Dev.* 7, 345-353.

Siomi,H., Matunis,M.J., Michael,W.M., and Dreyfuss,G. (1993). The pre-mRNA binding K protein contains a novel evolutionarily conserved motif. *Nucleic Acids Res.* 21, 1193-1198.

Steenbergh,P.H., Koonen-Reemst,A.M.C.B., Cleutjens,C.B.J.M., and Sussenbach,J.S. (1991). Complete nucleotide sequence of the high molecular weight human IGF-I mRNA. *Biochem. Biophys. Res. Commun.* 175, 507-514.

Stoeckle,M.Y. (1991). Post-transcriptional regulation of *gro* alpha, beta, gamma, and IL-8 mRNAs by IL-1 beta. *Nucleic Acids Res.* 19, 917-920.

Stoeckle,M.Y. (1992). Removal of a 3' non-coding sequence is an initial step in degradation of *gro* alpha mRNA and is regulated by interleukin-1. *Nucleic Acids Res.* 20, 1123-1127.

Stoeckle,M.Y. and Hanafusa,H. (1989). Processing of 9E3 mRNA and regulation of its stability in normal and Rous sarcoma virus-transformed cells. *Mol. Cell Biol.* 9, 4738-4745.

Sussenbach,J.S., Steenbergh,P.H., and Holthuisen,P. (1992). Structure and expression of the human insulin-like growth factor genes. *Growth Regul.* 2, 1-9.

Suzuki,H., Veda,R., Takahashi,T., and Takahashi,T. (1994). Altered imprinting in lung cancer. *Nat. Genet.* 6, 332-333.

Swartwout,S.G. and Kinniburgh,A.J. (1989). *c-myc* RNA degradation in growing and differentiating cells: possible alternate pathways. *Mol Cell Biol* 9, 288-295.

Szewczak,A.A. and Moore,P.B. (1995). The sarcin/ricin loop, a modular RNA. *J Mol. Biol.* 247, 81-98.

Tan,R., Chen,L., Buettner,J.A., Hudson,D., and Frankel,A.D. (1993). RNA recognition by an isolated alpha helix. *Cell* 73, 1031-1040.

Teerink,H., Voorma,H.O., and Thomas,A.A.M. (1995). The human insulin-like growth factor II leader 1 contains an internal ribosomal entry site. *Biochim. Biophys. Acta Gene Struct. Expression* 1264, 403-408.

- Thorvaldsen, J.L., Duran, K.L., and Bartolomei, M.S. (1998). Deletion of the H19 differentially methylated domain results in loss of imprinted expression of H19 and Igf2. *Genes Dev.* *12*, 3693-3702.
- Torres, A.M., Forbes, B.E., Aplin, S.E., Wallace, J.C., Francis, G.L., and Norton, R.S. (1995). Solution structure of human insulin-like growth factor II. Relationship to receptor and binding protein interactions. *J. Mol. Biol.* *248*, 385-401.
- Ullrich, A., Gray, A., Tam, A.W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., and Chen, E. (1986). Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. *EMBO J.* *5*, 2503-2512.
- van Dijk, E.L., Sussenbach, J.S., and Holthuisen, P.E. (1998). Identification of RNA sequences and structures involved in site-specific cleavage of IGF-II mRNAs. *RNA.* *4*, 1623-1635.
- van Dijk, E.L., Sussenbach, J.S., and Holthuisen, P.E. (2000). Distinct RNA structural domains cooperate to maintain a specific cleavage site in the 3'-UTR of IGF-II mRNAs. *J Mol Biol* *300*, 449-467.
- van Dijk, M.A., Rodenburg, R.J., Holthuisen, P., and Sussenbach, J.S. (1992). The liver-specific promoter of the human insulin-like growth factor II gene is activated by CCAAT/enhancer binding protein (C/EBP). *Nucleic Acids Res.* *20*, 3099-3104.
- van Dijk, M.A., van Schaik, F.M., Bootsma, H.J., Holthuisen, P., and Sussenbach, J.S. (1991). Initial characterization of the four promoters of the human insulin-like growth factor II gene. *Mol. Cell Endocrinol.* *81*, 81-94.
- Voutilainen, R. and Miller, W.L. (1987). Coordinate tropic hormone regulation of mRNAs for insulin-like growth factor II and the cholesterol side-chain-cleavage enzyme, P450scc [corrected], in human steroidogenic tissues [published erratum appears in *Proc Natl Acad Sci U S A* 1987 Sep;84(17):6194]. *Proc. Natl. Acad. Sci. U. S. A* *84*, 1590-1594.
- Vreken, P. and Raue, H.A. (1992). The rate-limiting step in yeast PGK1 mRNA degradation is an endonucleolytic cleavage in the 3'-terminal part of the coding region. *Mol. Cell Biol.* *12*, 2986-2996.
- Vreken, P., van, d., V, de Regt, V.C., de Maat, A.L., Planta, R.J., and Raue, H.A. (1991). Turnover rate of yeast PGK mRNA can be changed by specific alterations in its trailer structure. *Biochimie* *73*, 729-737.
- Wagner, E.G. and Simons, R.W. (1994). Antisense RNA control in bacteria, phages, and plasmids. *Annu. Rev. Microbiol.* *48*:713-42, 713-742.
- Wang, X., Kiledjian, M., Weiss, I.M., and Liebhaber, S.A. (1995). Detection and characterization of a 3' untranslated region ribonucleoprotein complex associated with human alpha-globin mRNA stability. *Mol. Cell Biol.* *15*, 1769-1777 [published erratum appeared in *Mol Cell Biol* 1995 Apr;15(4):2331].
- Wang, Z.Q., Fung, M.R., Barlow, D.P., and Wagner, E.F. (1994). Regulation of embryonic growth and lysosomal targeting by the imprinted Igf2/Mpr gene. *Nature* *372*, 464-467.

- Wang,Z.R., Day,N., Trifillis,P., and Kiledjian,M. (1999). An mRNA stability complex functions with poly(A)-binding protein to stabilize mRNA in vitro. *Mol. Cell. Biol.* *19*, 4552-4560.
- Wang,Z.R. and Kiledjian,M. (2000a). Identification of an erythroid-enriched endoribonuclease activity involved in specific mRNA cleavage. *EMBO J.* *19*, 295-305.
- Wang,Z.R. and Kiledjian,M. (2000b). The poly(A)-binding protein and an mRNA stability protein jointly regulate an endoribonuclease activity. *Mol. Cell Biol.* *20*, 6334-6341.
- Webber,A.L., Ingram,R.S., Levorse,J.M., and Tilghman,S.M. (1998). Location of enhancers is essential for the imprinting of H19 and Igf2 genes. *Nature* *391*, 711-715.
- Weiss,I.M. and Liebhaber,S.A. (1995). Erythroid cell-specific mRNA stability elements in the alpha 2-globin 3' nontranslated region. *Mol. Cell Biol.* *15*, 2457-2465.
- Weksberg,R., Shen,D.R., Fei,Y.L., Song,Q.L., and Squire,J. (1993). Disruption of insulin-like growth factor 2 imprinting in Beckwith-Wiedemann syndrome. *Nature Genet.* *5*, 143-150.
- Wennborg,A., Sohlberg,B., Angerer,D., Klein,G., and von Gabain,A. (1995). A human RNase E-like activity that cleaves RNA sequences involved in mRNA stability control. *Proc. Natl. Acad. Sci. U.S.A* *92*, 7322-7326.
- Westhof,E. and Fritsch,V. (2000). RNA folding: beyond Watson-Crick pairs. *Structure. Fold. Des* *8*, R55-R65.
- Wickens,M., Anderson,P., and Jackson,R.J. (1997). Life and death in the cytoplasm: messages from the 3' end. *Curr. Opin. Genet. Dev.* *7*, 220-232.
- Wimberly,B., Varani,G., and Tinoco,I., Jr. (1993). The conformation of loop E of eukaryotic 5S ribosomal RNA. *Biochemistry* *32*, 1078-1087.
- Wisdom,R. and Lee,W. (1990). Translation of c-myc mRNA is required for its post-transcriptional regulation during myogenesis. *J Biol Chem* *265*, 19015-19021.
- Wisdom,R. and Lee,W. (1991). The protein-coding region of c-myc mRNA contains a sequence that specifies rapid mRNA turnover and induction by protein synthesis inhibitors. *Genes Dev* *5*, 232-243.
- Wright,C.V., Cho,K.W., Fritz,A., Burglin,T.R., and De Robertis,E.M. (1987). A *Xenopus laevis* gene encodes both homeobox-containing and homeobox-less transcripts. *EMBO J.* *20*;6, 4083-4094.
- Yoshiko,Y., Hirao,K., and Maeda,N. (1998). Dexamethasone regulates the actions of endogenous insulin-like growth factor-II during myogenic differentiation. *Life Sci.* *63*, 77-85.
- Zahler,A.M., Williamson,J.R., Cech,T.R., and Prescott,D.M. (1991). Inhibition of telomerase by G-quartet DNA structures. *Nature* *350*, 718-720.
- Zapf,J. (1994). Role of insulin-like growth factor II and IGF binding proteins in extrapancreatic tumor hypoglycemia. *Horm. Res.* *42*, 20-26.

Zapf,J., Futo,E., Peter,M., and Froesch,E.R. (1992). Can "big" insulin-like growth factor II in serum of tumor patients account for the development of extrapancreatic tumor hypoglycemia? *J. Clin. Invest* 90, 2574-2584.

Zapf,J., Walter,H., and Froesch,E.R. (1981). Radioimmunological determination of insulinlike growth factors I and II in normal subjects and in patients with growth disorders and extrapancreatic tumor hypoglycemia. *J. Clin. Invest* 68, 1321-1330.

Zapp,M.L., Hope,T.J., Parslow,T.G., and Green,M.R. (1991). Oligomerization and RNA binding domains of the type 1 human immunodeficiency virus Rev protein: a dual function for an arginine-rich binding motif. *Proc. Natl. Acad. Sci. U.S.A* 88, 7734-7738.

Zhan,S., Shapiro,D.N., and Helman,L.J. (1994). Activation of an imprinted allele of the insulin-like growth factor II gene implicated in rhabdomyosarcoma. *J. Clin. Invest* 94, 445-448.

Ziehler,W.A., Day,J.J., Fierke,C.A., and Engelke,D.R. (2000). Effects of 5' leader and 3' trailer structures on pre-tRNA processing by nuclear RNase P. *Biochemistry* 39, 9909-9916.

Zuker,M. (1989). On finding all suboptimal foldings of an RNA molecule. *Science* 244, 48-52.

Zuker,M. and Stiegler,P. (1981). Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. *Nucleic Acids Res.* 9, 133-148.

SAMENVATTING

Het humane insuline-achtige groeifactor II (IGF-II) speelt een belangrijke rol bij de embryonale ontwikkeling en is geassocieerd met tumorgroei. In gekweekte cellen kan IGF-II diverse effecten veroorzaken, afhankelijk van het celtype waarin het tot expressie komt, zoals groeistimulatie, of juist remming van groei en stimulatie van celdifferentiatie. Terwijl IGF-II een overlevingsfactor is in sommige systemen, induceert het in andere celtypes juist geprogrammeerde celdood (apoptose). IGF-II is dus betrokken bij diverse fysiologische processen, en het is derhalve waarschijnlijk dat IGF-II genexpressie nauwkeurig gereguleerd wordt. Inderdaad vindt er op verscheidene niveaus regulatie plaats; op het niveau van transcriptie (i.e. synthese van het 'boodschapper' mRNA) door middel van ontwikkelings- en weefsel-specifieke activiteit van vier verschillende promotors (i.e. de startplaats van transcriptie)(P1-P4) in het IGF-II gen. Transcriptie vanaf deze promotors leidt tot de vorming van mRNA moleculen die onderling verschillen in de lengte en samenstelling van hun 5' onvertaalde deel (5'-UTR). Dit leidt vervolgens tot verschillen in de translatie (eiwitproductie)-efficiëntie van deze mRNAs en genereert de mogelijkheid om IGF-II expressie te reguleren op het niveau van translatie. Verder is nog bekend dat IGF-II expressie gereguleerd wordt op het niveau van post-translationele bewerking van het IGF-II eiwit.

Tijdens de karakterisering van het humane IGF-II gen en de IGF-II mRNAs werd een RNA met een grootte van 1.8 kb gedetecteerd dat co-lineair is met het 3' uiteinde van het IGF-II gen. Dit RNA bleek gevormd te worden door plaats-specifieke endonucleolytische klieving in het 3' onvertaalde deel (3'-UTR) van de IGF-II mRNAs en niet door transcriptie vanaf een extra promotor. Het 1.8 kb RNA vertegenwoordigt dus het 3' klievingsproduct; het bestaat uit 3'-UTR sequentie en een poly(A) staart, en is zeer stabiel. Klieving van de IGF-II mRNAs genereert ook een 5' klievingsproduct, hetgeen de IGF-II coderende sequentie bevat. Dit 5' klievingsproduct is in tegenstelling tot het 1.8 kb 3' klievingsproduct onstabiel, en wordt niet meer getransleerd. Deze observaties hebben geleid tot de hypothese dat klieving van de IGF-II mRNAs kan fungeren als een additioneel mechanisme om IGF-II genexpressie te reguleren.

Begonnen werd met het in kaart brengen van de RNA gebieden die nodig zijn voor *in vivo* klieving. Hiertoe werden deleties aangebracht in een IGF-II minigen in een expressieplasmide voor transfectie-studies. Deze experimenten toonden aan dat er twee gebieden in de IGF-II 3'-UTR nodig zijn voor klieving: een sequentie van 323 nucleotiden rondom de klievingsplaats (element II), en een sequentie van 104 nucleotiden lang dat 2013 nucleotiden stroomopwaards gelegen is (element I). RNA-vouwings-studies lieten vervolgens zien dat element I, dat zeer rijk is aan C-nucleotiden, een stabiele duplex kan vormen met een G-rijk stroomafwaards gelegen gebied in element II. Direct stroomopwaards van de klievingsplaats in element II kunnen twee 'haarspeldlusen' gevormd worden, en de klievingsplaats zelf wordt voorspeld in een interne lus gelegen te zijn. De RNA gebieden die nodig zijn voor klieving kunnen dus onderverdeeld worden in drie verschillende vouwingsdomeinen: (1) het duplex domein, gevormd door de interactie tussen nucleotiden -2108/-2029 (posities ten opzichte van de klievingsplaats) van element I en +18/+101 van element

II, (2) het haarspeldlussen domein in het stroomopwaards gelegen deel (posities -133 tot -7) van element II, en (3) het klievingsplaatsdomein, gevormd door nucleotiden -161/-136 en -6/+16, met de klievingsplaats gelegen in een interne lus.

De studies beschreven in dit proefschrift begonnen met pogingen om eiwitten te identificeren die specifiek aan de hierboven beschreven RNA gebieden binden, en zo een rol zouden kunnen spelen bij klieving (Hoofdstuk 3). Hiertoe werden *in vitro* gesynthetiseerde RNA moleculen die deze gebieden bevatten geïncubeerd met extracten van humane Hep3B cellen, die endogeen IGF-II tot expressie brengen. Dit leverde een specifiek RNA-eiwit complex op dat alleen gevormd wordt in de aanwezigheid van het haarspeldlussen domein. Met behulp van puntmutaties werd de bindingsplaats van het eiwit nauwkeurig in kaart gebracht. Het complex bleek gevormd te worden halverwege de eerste van de twee haarspeldlussen. Wanneer puntmutaties die de eiwitbinding verstoren in het IGF-II minigen werden geïntroduceerd, leverde dit een gematigde reductie in klievingsefficiëntie op, hetgeen suggereert dat het RNA-eiwitcomplex niet essentieel is voor klieving, maar een modulerende rol in klieving zou kunnen hebben.

Hoofdstuk 3 beschrijft ook experimenten waarin het belang van de nucleotiden rondom de klievingsplaats voor klieving werd onderzocht. Verscheidene puntmutaties werden geïntroduceerd rondom de klievingsplaats in het IGF-II minigen, en het effect hiervan op de *in vivo* klievingsefficiëntie werd onderzocht. De resultaten tonen duidelijk aan dat de identiteit van specifieke nucleotiden rondom de klievingsplaats zeer belangrijk is, omdat vrijwel alle puntmutaties de klieving ernstig verstoren. De nucleotiden in het gebied direct naast de klievingsplaats van posities -6 tot +16 lijken belangrijker te zijn dan de nucleotiden in het hier tegenover liggende deel van posities -161 tot -136. Interessant genoeg is dit gebied ook duidelijk meer geconserveerd in de evolutie, hetgeen het belang van deze nucleotiden voor klieving onderstreept.

Hoofdstuk 4 beschrijft verdere experimenten om meer inzicht te krijgen in de rol van de verschillende RNA vouwingsdomeinen in klieving. Verscheidene deleties werden geïntroduceerd in het haarspeldlussen domein en de effecten op de voorspelde RNA vouwing en *in vivo* klievingsefficiëntie werden bestudeerd. Hieruit bleek dat alle deleties die de voorspelde RNA vouwing veranderen ook de klieving vrijwel volledig verstoren. Deleties die de wild type RNA vouwing intact laten hebben echter een veel minder drastisch effect op klieving, hetgeen aangeeft dat een specifieke vouwing rondom de klievingsplaats belangrijk is. Het haarspeldlussen domein is op zichzelf niet nodig voor klieving, omdat het geheel verwijderd kan worden zonder de klieving drastisch te verstoren, zolang de vouwing rondom de klievingsplaats maar intact blijft. Dit blijkt uit de resultaten met een mutant (Δ -135/-18) waarbij het gehele haarspeldlussen domein verwijderd is; deze mutant laat een redelijke klievingsefficiëntie zien van 47% van de wild type. Een opvallend resultaat echter werd verkregen met een mutant waarbij op een deel van de tweede haarspeldlus na het gehele haarspeldlussendomein verwijderd was (Δ -135/-72, Δ -60/-18). Hoewel de klievingsplaats zich bij deze mutant in de voorspelde wild type configuratie bevindt, is de klievingsefficiëntie ernstig verstoord. Dit resultaat gaf aanleiding de computervoorspelling van de RNA-vouwing te verifiëren door middel van biochemische structuuranalyse van het RNA (Hoofdstuk 4). De resultaten van deze studies suggereren dat zowel in het wild type RNA als in mutant Δ -135/-18 de

nucleotiden in de lus rondom de klievingsplaats niet ongepaard zijn, maar betrokken bij niet-Watson-Crick interacties, hetgeen een specifieke conformatie genereert. In mutant Δ -135/-72, Δ -60/-18 echter, gedragen deze nucleotiden zich als vrij in oplossing. Deze resultaten suggereren dat een specifieke conformatie, gevormd door niet-Watson-Crick interacties, belangrijk is voor klieving. In overeenstemming met deze hypothese werd gevonden dat puntmutaties rondom de klievingsplaats die de klieving verstoren ook de conformatie rondom de klievingsplaats veranderen in tegenstelling tot mutaties die geen effect hebben op klieving.

Hoofdstuk 5 laat experimenten zien die uitgevoerd zijn om meer inzicht te verkrijgen in de fysiologische functie van klieving van IGF-II mRNA. De kinetiek van klieving en de rol van klieving in de afbraak van IGF-II mRNA en eiwitproductie werd getest met behulp van IGF-II constructen onder controle van een tetracycline-induceerbare promotor. De niveaus van het IGF-II mRNA en het 3' klievingsproduct werden bepaald door Northern blot analyse. Na inductie van transcriptie werd een snelle toename gezien van de niveaus van het IGF-II mRNA; de niveaus van het 3' klievingsproduct begonnen pas geruime tijd later toe te nemen, hetgeen aangeeft dat klieving een traag proces is. In het omgekeerde experiment waarbij de transcriptie werd geremd, werd de afbraaksnelheid van wild type IGF-II mRNA vergeleken met de afbraaksnelheid van mutant IGF-II mRNA dat niet meer gekliefd wordt. Beide mRNAs werden met dezelfde snelheid afgebroken. Ook op het niveau van eiwitsynthese werd geen verschil gezien tussen het wild type IGF-II mRNA en de gemuteerde versie. Deze resultaten laten zien dat in het gebruikte testsysteem klieving een gering effect heeft op mRNA afbraak en eiwitsynthese.

Regulatie van klieving onder invloed van externe factoren werd onderzocht in humane Hep3B cellen, die endogeen IGF-II tot expressie brengen en het mRNA klieven. Het effect van verscheiden externe factoren op de efficiëntie van klieving werd getest. De invloed van trichostatine A (TSA), een histon deacetylase remmer, dexamethason, een synthetisch glucocorticoïde, aan- of afwezigheid van serum in het medium, en celdichtheid werden onderzocht. De verkregen resultaten suggereren dat TSA, dexamethason, en serum geen invloed hebben op klieving. Celdichtheid echter, heeft wel effect op klieving; bij een toename van de cellen werd een toename van de niveaus van het 3' klievingsproduct waargenomen, terwijl de niveaus van het volle lengte IGF-II mRNA gelijkbleven of afnamen, afhankelijk van de frequentie van medium verversing. We hebben dus waargenomen dat klieving in Hep3B cellen gereguleerd wordt afhankelijk van de dichtheid van de cellen. De betekenis van de toename van klieving bij hoge celdichtheid is echter niet duidelijk. De ongebruikelijke stabiliteit van het 3' klievingsproduct, waarschijnlijk veroorzaakt door de geconserveerde G-rijke sequentie aan het 5' uiteinde maakt het aantrekkelijk te speculeren dat dit RNA wellicht een intrinsieke functie vervult in de cel.

LIST OF PUBLICATIONS

Meijer, A.H., van Dijk, E.L., and Hoge, J.H. (1996).

Novel members of a family of AT hook-containing DNA-binding proteins from rice are identified through their in vitro interaction with consensus target sites of plant and animal homeodomain proteins. *Plant Mol Biol* 31: 607-18.

Meijer, A.H., Scarpella, E., van Dijk, E.L., Qin, L., Taal, A.J., Rueb, S., Harrington, S.E., McCouch, S.R., Schilperoort, R.A., and Hoge, J.H. (1997).

Transcriptional repression by Oshox1, a novel homeodomain leucine zipper protein from rice. *Plant J.* 11: 263-76.

van Dijk, E.L., Sussenbach, J.S., and Holthuizen, P.E. (1998). Identification of RNA sequences and structures involved in site-specific cleavage of IGF-II mRNAs. *RNA.* 4: 1623-35.

van Dijk, E.L., Sussenbach, J.S., and Holthuizen, P.E. (2000). Distinct RNA structural domains cooperate to maintain a specific cleavage site in the 3'-UTR of IGF-II mRNAs. *J Mol Biol.* 300: 449-67.

van Dijk, E.L. and Holthuizen, P.E. (2001). Site-specific cleavage of the insulin-like growth factor II mRNAs. In: *mRNA binding proteins*. Eds. K. Sandberg and S. Mulrone. Kluwer Academic Publishers. MA, USA.

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CURRICULUM VITAE

Ik ben op 27 april 1972 geboren in Rotterdam. In 1990 behaalde ik het VWO diploma aan het Marnix Gymnasium te Rotterdam. In datzelfde jaar ben ik begonnen met de studie Biologie aan de Rijksuniversiteit Leiden. Tijdens de doctoraalfase volgde ik een bijvakstage bij de vakgroep 'Plantevirussen' in het Instituut voor Moleculaire Plantkunde onder begeleiding van Ir. E.A.G. van der Vossen en Prof. Dr. J.F. Bol. Vervolgens volgde ik een hoofdvakstage, eveneens in het Instituut voor Moleculaire Plantkunde in de vakgroep 'Rijstembryogenese' onder begeleiding van Dr. A.H. Meijer en Dr. J.H.C. Hoge. In augustus 1996 behaalde ik het doctoraal examen. Vanaf september 1996 was ik werkzaam als Onderzoeker in Opleiding bij de vakgroep Fysiologische Chemie waar ik, onder leiding van Dr. P.E. Holthuisen en Prof Dr. Ir. J.S. Sussenbach, het in dit proefschrift beschreven onderzoek verrichtte.

REFERENCES

- Adams,T.E., Epa,V.C., Garrett,T.P., and Ward,C.W. (2000). Structure and function of the type 1 insulin-like growth factor receptor. *Cell Mol. Life Sci.* 57, 1050-1093.
- Altman,S. (2000). The road to RNase P. *Nat. Struct. Biol.* 7, 827-828.
- Anisowicz,A., Bardwell,L., and Sager,R. (1987). Constitutive overexpression of a growth-regulated gene in transformed Chinese hamster and human cells. *Proc. Natl. Acad. Sci. U. S. A* 84, 7188-7192.
- Anisowicz,A., Zajchowski,D., Stenman,G., and Sager,R. (1988). Functional diversity of gro gene expression in human fibroblasts and mammary epithelial cells. *Proc. Natl. Acad. Sci. U. S. A* 85, 9645-9649.
- Arnold,T.E., Yu,J., and Belasco,J.G. (1998). mRNA stabilization by the *ompA* 5' untranslated region: Two protective elements hinder distinct pathways for mRNA degradation. *Exp. Cell Res.* 4, 319-330.
- Arraiano,C.M., Cruz,A.A., and Kushner,S.R. (1997). Analysis of the *in vivo* decay of the *Escherichia coli* dicistronic *pyrF-orfF* transcript: Evidence for multiple degradation pathways. *J. Mol. Biol.* 268, 261-272.
- Asfari,M., De,W., Noël,M., Holthuizen,P.E., and Czernichow,P. (1995). Insulin-like growth factor-II gene expression in a rat insulin- producing beta-cell line (INS-1) is regulated by glucose. *Diabetologia* 38, 927-935.
- Backlin,B.M., Gessbo,A., Forsberg,M., Shokrai,A., Rozell,B., and Engstrom,W. (1998). Expression of the insulin-like growth factor II gene in polychlorinated biphenyl exposed female mink (*Mustela vison*) and their fetuses. *Mol. Pathol.* 51, 43-47.
- Bae,S.K., Bae,M.H., Ahn,M.Y., Son,M.J., Lee,Y.M., Bae,M.K., Lee,O.H., Park,B.C., and Kim,K.W. (1999). Egr-1 mediates transcriptional activation of IGF-II gene in response to hypoxia. *Cancer Res.* 59, 5989-5994.
- Baker,J., Liu,J.P., Robertson,E.J., and Efstratiadis,A. (1993). Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* 75, 73-82.
- Barnes,C.A. (1998). Upf1 and Upf2 proteins mediate normal yeast mRNA degradation when translation initiation is limited. *Nucleic Acids Res.* 26, 2433-2441.
- Bartel,D.P., Zapp,M.L., Green,M.R., and Szostak,J.W. (1991). HIV-1 Rev regulation involves recognition of non-Watson-Crick base pairs in viral RNA. *Cell* 67, 529-536.
- Bartolomei,M.S., Zemel,S., and Tilghman,S.M. (1991). Parental imprinting of the mouse H19 gene. *Nature* 351, 153-155.
- Batey,R.T. and Doudna,J.A. (1998). The parallel universe of RNA folding. *Nature Struct. Biol.* 5, 337-340.

Battiste,J.L., Mao,H., Rao,N.S., Tan,R., Muhandiram,D.R., Kay,L.E., Frankel,A.D., and Williamson,J.R. (1996). Alpha helix-RNA major groove recognition in an HIV-1 rev peptide-RRE RNA complex. *Science* 273, 1547-1551.

Baxter,R.C. (2000). Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. *Am. J Physiol Endocrinol. Metab* 278, E967-E976.

Baxter,R.C. and Martin,J.L. (1989). Structure of the Mr 140,000 growth hormone-dependent insulin-like growth factor binding protein complex: determination by reconstitution and affinity-labeling. *Proc. Natl. Acad. Sci. U. S. A* 86, 6898-6902.

Beck,F., Samani,N.J., Senior,P., Byrne,S., Morgan,K., Gebhard,R., and Brammar,W.J. (1988). Control of IGF-II mRNA levels by glucocorticoids in the neonatal rat. *J Mol. Endocrinol.* 1, R5-R8.

Bedard,P.A., Alcorta,D., Simmons,D.L., Luk,K.C., and Erikson,R.L. (1987). Constitutive expression of a gene encoding a polypeptide homologous to biologically active human platelet protein in Rous sarcoma virus-transformed fibroblasts. *Proc. Natl. Acad. Sci. U. S. A* 84, 6715-6719.

Beelman,C.A. and Parker,R. (1995). Degradation of mRNA in eukaryotes. *Cell* 81, 179-183.

Bein,K., Ware,J.A., and Simons,M. (1998). Myb-dependent regulation of thrombospondin 2 expression - Role of mRNA stability. *J. Biol. Chem.* 273, 21423-21429.

Bellaoui,M., Pelletier,G., and Budar,F. (1997). The steady-state level of mRNA from the Ogura cytoplasmic male sterility locus in *Brassica* cybrids is determined post-transcriptionally by its 3' region. *EMBO J.* 16, 5057-5068.

Benard,L., Mathy,N., Grunberg-Manago,M., Ehresmann,B., Ehresmann,C., and Portier,C. (1998). Identification in a pseudoknot of a U.G motif essential for the regulation of the expression of ribosomal protein S15. *Proc. Natl. Acad. Sci. U. S. A* 95, 2564-2567.

Benard,L., Philippe,C., Dondon,L., Grunberg-Manago,M., Ehresmann,B., Ehresmann,C., and Portier,C. (1994). Mutational analysis of the pseudoknot structure of the S15 translational operator from *Escherichia coli*. *Mol. Microbiol.* 14, 31-40.

Bennett,A.L., Wilson,D.M., and Liu,F. (1983). Levels of insulin-like growth factors I and II in human cord blood. *J. Clin. Endocrinol. Metab.* 57(3), 609-612.

Berman,H.M., Bhat,T.N., Bourne,P.E., Feng,Z., Gilliland,G., Weissig,H., and Westbrook,J. (2000). The Protein Data Bank and the challenge of structural genomics [In Process Citation]. *Nat. Struct. Biol.* 7 Suppl:957-9, 957-959.

Bernstein,P.L., Herrick,D.J., Prokipcak,R.D., and Ross,J. (1992). Control of c-myc mRNA half-life in vitro by a protein capable of binding to a coding region stability determinant. *Genes Dev.* 6, 642-654.

Bichell,D.P., Kikuchi,K., and Rotwein,P. (1992). Growth hormone rapidly activates insulin-like growth factor I gene transcription *in vivo*. *Mol. Endocrinol.* 6, 1899-1908.

- Biddle,C., Li,C.H., Schofield,P.N., Tate,V.E., Hopkins,B., Engstrom,W., Huskisson,N.S., and Graham,C.F. (1988). Insulin-like growth factors and the multiplication of Tera-2, a human teratoma-derived cell line. *J. Cell Sci.* 90, 475-484.
- Binder,R., Horowitz,J.A., Basilion,J.P., Koeller,D.M., Klausner,R.D., and Harford,J.B. (1994). Evidence that the pathway of transferrin receptor mRNA degradation involves an endonucleolytic cleavage within the 3' UTR and does not involve poly(A) tail shortening. *EMBO J.* 13, 1969-1980.
- Binder,R., Hwang,S.P., Ratnasabapathy,R., and Williams,D.L. (1989). Degradation of apolipoprotein II mRNA occurs via endonucleolytic cleavage at 5'-AAU-3'/5'-UAA-3' elements in single-stranded loop domains of the 3'-noncoding region. *J. Biol. Chem.* 264, 16910-16918.
- Bisbal,C., Silhol,M., Laubenthal,H., Kaluza,T., Carnac,G., Milligan,L., Le Roy,F., and Salehzada,T. (2000). The 2'-5' oligoadenylate/RNase L/RNase L inhibitor pathway regulates both MyoD mRNA stability and muscle cell differentiation. *Mol. Cell Biol.* 20, 4959-4969.
- Biskobing,D.M., Fan,D., and Rubin,J. (1997). *c-fms* mRNA is regulated posttranscriptionally by 1,25(OH)₂D₃ in HL-60 cells. *Calcif. Tissue Int.* 61, 205-209.
- Bondy,C.A., Chin,E., and Zhou,J. (1993). Significant species differences in local IGF-I and -II gene expression. *Adv. Exp. Med. Biol.* 343:73-7, 73-77.
- Bonnieu,A., Roux,P., Marty,L., Jeanteur,P., and Piechaczyk,M. (1990). AUUUA motifs are dispensable for rapid degradation of the mouse *c-myc* RNA. *Oncogene* 5, 1585-1588.
- Borovjagin,A.V. and Gerbi,S.A. (1999). U3 small nucleolar RNA is essential for cleavage at sites 1, 2 and 3 in pre-rRNA and determines which rRNA processing pathway is taken in *Xenopus* oocytes. *J Mol. Biol.* 286, 1347-1363.
- Boshart,M., Weber,F., Jahn,G., Dorsch-Hasler,K., Fleckenstein,B., and Schaffner,W. (1985). A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* 41, 521-530.
- Brissenden,J.E., Ullrich,A., and Francke,U. (1984). Human chromosomal mapping of genes for insulin-like growth factors I and II and epidermal growth factor. *Nature* 310, 781-784.
- Brown,B.D. and Harland,R.M. (1990). Endonucleolytic cleavage of a maternal homeo box mRNA in *Xenopus* oocytes. *Genes Dev.* 4, 1925-1935.
- Brown,B.D., Zipkin,I.D., and Harland,R.M. (1993). Sequence-specific endonucleolytic cleavage and protection of mRNA in *Xenopus* and *Drosophila*. *Genes Dev.* 7, 1620-1631.
- Brown,E.J. and Schreiber,S.L. (1996). A signaling pathway to translational control. *Cell* 86, 517-520.

- Burd,C.G. and Dreyfuss,G. (1994). Conserved structures and diversity of functions of RNA-binding proteins. *Science* 265, 615-621.
- Butcher,S.E., Dieckmann,T., and Feigon,J. (1997). Solution structure of a GAAA tetraloop receptor RNA. *EMBO J.* 16, 7490-7499.
- Bycroft,M., Grunert,S., Murzin,A.G., Proctor,M., and St Johnston,D. (1995). NMR solution structure of a dsRNA binding domain from *Drosophila* staufen protein reveals homology to the N-terminal domain of ribosomal protein S5. *EMBO J.* 14, 3563-3571 [published erratum appeared in *EMBO J* 1995 Sep 1;14(17):4385].
- Calnan,B.J., Biancalana,S., Hudson,D., and Frankel,A.D. (1991). Analysis of arginine-rich peptides from the HIV Tat protein reveals unusual features of RNA-protein recognition. *Genes Dev.* 5, 201-210.
- Campbell,S., McLaren,E.H., and Danesh,B.J. (1996). Rapidly reversible increase in insulin requirement with interferon. *BMJ* 313, 92.
- Cannistraro,V.J. and Kennell,D. (1997). RNase YI* and RNA structure studies. *Nucleic Acids Res.* 25, 1405-1411.
- Cao,G.J., Kalapos,M.P., and Sarkar,N. (1997). Polyadenylated mRNA in *Escherichia coli*: Modulation of poly(A) RNA levels by polynucleotide phosphorylase and ribonuclease II. *Biochimie* 79, 211-220.
- Casey,J.L., Koeller,D.M., Ramin,V.C., Klausner,R.D., and Harford,J.B. (1989). Iron regulation of transferrin receptor mRNA levels requires iron-responsive elements and a rapid turnover determinant in the 3' untranslated region of the mRNA. *EMBO J* 8, 3693-3699.
- Chagnovich,D. and Cohn,S.L. (1997). Activity of a 40 kDa RNA-binding protein correlates with *MYCN* and *c-fos* mRNA stability in human neuroblastoma. *Eur. J. Cancer [A]* 33A, 2064-2067.
- Chagnovich,D., Fayos,B.E., and Cohn,S.L. (1996). Differential activity of ELAV-like RNA-binding proteins in human neuroblastoma. *J. Biol. Chem.* 271, 33587-33591.
- Chan,M.T. and Yu,S.M. (1998). The 3' untranslated region of a rice α -amylase gene functions as a sugar-dependent mRNA stability determinant. *Proc. Natl. Acad. Sci. USA* 95, 6543-6547.
- Chanfreau,G. and Jacquier,A. (1996). An RNA conformational change between the two chemical steps of group II self-splicing. *EMBO J.* 15, 3466-3476.
- Chen,J.L., Nolan,J.M., Harris,M.E., and Pace,N.R. (1998). Comparative photocross-linking analysis of the tertiary structures of *Escherichia coli* and *Bacillus subtilis* RNase P RNAs. *EMBO J.* 17, 1515-1525.
- Cheng,S.L., Zhang,S.F., Mohan,S., Lecanda,F., Fausto,A., Hunt,A.H., Canalis,E., and Avioli,L.V. (1998). Regulation of insulin-like growth factors I and II and their binding

- proteins in human bone marrow stromal cells by dexamethasone. *J Cell Biochem.* 71, 449-458.
- Chernokalskaya,E., Dompenciel,R., and Schoenberg,D.R. (1997). Cleavage properties of an estrogen-regulated polysomal ribonuclease involved in the destabilization of albumin mRNA. *Nucleic Acids Res.* 25, 735-742.
- Chernokalskaya,E., Dubell,A.N., Cunningham,K.S., Hanson,M.N., Dompenciel,R.E., and Schoenberg,D.R. (1998). A polysomal ribonuclease involved in the destabilization of albumin mRNA is a novel member of the peroxidase gene family. *RNA.* 4, 1537-1548.
- Christiansen,J., Kofod,M., and Nielsen,F.C. (1994). A guanosine quadruplex and two stable hairpins flank a major cleavage site in insulin-like growth factor II mRNA. *Nucleic Acids Res.* 22, 5709-5716.
- Ciesiolka,J., Michalowski,D., Wrzesinski,J., Krajewski,J., and Krzyzosiak,W.J. (1998). Patterns of cleavages induced by lead ions in defined RNA secondary structure motifs. *J. Mol. Biol.* 275, 211-220.
- Claverie-Martin,F., Wang,M., and Cohen,S.N. (1997). ARD-1 cDNA from human cells encodes a site-specific single-strand endoribonuclease that functionally resembles *Escherichia coli* RNase E. *J Biol. Chem.* 272, 13823-13828.
- Collins,M.K.L., Furlong,I.J., Malde,P., Ascaso,R., Oliver,J., and Rivas,A.L. (1996). An apoptotic endonuclease activated either by decreasing pH or by increasing calcium. *J. Cell Sci.* 109, 2393-2399.
- Cooke,H.J. and Elliott,D.J. (1997). RNA-binding proteins and human male infertility. *Trends Genet.* 13, 87-89.
- Corkins,M.R., Park,J.H., Davis,D.V., Slentz,D.H., and MacDonald,R.G. (1999). Regulation of the insulin-like growth factor axis by increasing cell number in intestinal epithelial (IEC-6) cells. *Growth Horm. IGF. Res.* 9, 414-424.
- Correll,C.C., Freeborn,B., Moore,P.B., and Steitz,T.A. (1997). Metals, motifs, and recognition in the crystal structure of a 5S rRNA domain. *Cell* 91, 705-712.
- Cusack,S. (1999). RNA-protein complexes. *Curr. Opin. Struct. Biol.* 9, 66-73.
- Czyzyk-Krzeska,M.F., Paulding,W.R., Lipski,J., Beresh,J.E., and Kroll,S.L. (1996). Regulation of tyrosine hydroxylase mRNA stability by oxygen in PC12 cells. *Adv. Exp. Med. Biol.* 410, 143-150.
- DaCosta,S.A., Schumaker,L.M., and Ellis,M.J. (2000). Mannose 6-phosphate/insulin-like growth factor 2 receptor, a bona fide tumor suppressor gene or just a promising candidate? *J Mammary. Gland. Biol. Neoplasia.* 5, 85-94.
- Danner,S., Frank,M., and Lohse,M.J. (1998). Agonist regulation of human β_2 -adrenergic receptor mRNA stability occurs via a specific AU-rich element. *J. Biol. Chem.* 273, 3223-3229.

- Daughaday, W.H., Emanuele, M.A., Brooks, M.H., Barbato, A.L., Kapadia, M., and Rotwein, P. (1988). Synthesis and secretion of insulin-like growth factor II by a leiomyosarcoma with associated hypoglycemia. *N. Engl. J. Med.* *319*, 1434-1440.
- Daughaday, W.H., Hall, K., Raben, M.S., Salmon, W.D., Jr., Brande, J.L., and Wyk, J.V. (1972). Somatomedin: proposed designation for sulphation factor. *Nature* *235*, 107.
- Daughaday, W.H. and Rotwein, P. (1989). Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. *Endocr. Rev.* *10*, 68-91.
- De la Cruz, J., Kressler, D., Tollervey, D., and Linder, P. (1998). Dob1p (Mtr4p) is a putative ATP-dependent RNA helicase required for the 3' end formation of 5.8S rRNA in *Saccharomyces cerevisiae*. *EMBO J.* *17*, 1128-1140.
- de Moor, C. H. Translation of Insulin-like Growth Factor 2 mRNAs. 1994. Thesis. Laboratory for Physiological Chemistry, Utrecht University, Utrecht, The Netherlands.
- de Moor, C.H., Jansen, M., Sussenbach, J.S., and Van den Brande, J.L. (1994). Differential polysomal localization of human insulin-like growth factor 2 mRNAs, both in cell lines and foetal liver. *Eur. J. Biochem.* *222*, 1017-1022.
- De Pagter-Holthuis, P., Jansen, M., van der Kammen, R.A., Van Schaik, F.M.A., and Sussenbach, J.S. (1988). Differential expression of the human insulin-like growth factor II gene. Characterization of the IGF-II mRNAs and an mRNA encoding a putative IGF-II-associated protein. *Biochim. Biophys. Acta* *950*, 282-295.
- de Wet, J.R., Wood, K.V., DeLuca, M., Helinski, D.R., and Subramani, S. (1987). Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell Biol.* *7*, 725-737.
- DeChiara, T.M., Efstratiadis, A., and Robertson, E.J. (1990). A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature* *345*, 78-80.
- DeChiara, T.M., Robertson, E.J., and Efstratiadis, A. (1991). Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* *64*, 849-859.
- Delihias, N. (1995). Regulation of gene expression by trans-encoded antisense RNAs. *Mol. Microbiol.* *15*, 411-414.
- Dell, G., Ward, A., and Engstrom, W. (1997). Regulation of a promoter from the mouse insulin like growth factor II gene by glucocorticoids. *FEBS Lett.* *419*, 161-165.
- Di Nicola, N.E., Fabbri, S., Bufardecchi, E., Baldi, M.I., Gandini, A.D., Mattoccia, E., and Tocchini-Valentini, G.P. (1997). The eucaryal tRNA splicing endonuclease recognizes a tripartite set of RNA elements. *Cell* *89*, 859-866.
- Dodson, R.E. and Shapiro, D.J. (1994). An estrogen-inducible protein binds specifically to a sequence in the 3' untranslated region of estrogen-stabilized vitellogenin mRNA. *Mol. Cell Biol.* *14*, 3130-3138.

- Dompenciel,R.E., Garnepudi,V.R., and Schoenberg,D.R. (1995). Purification and characterization of an estrogen-regulated *Xenopus* liver polysomal nuclease involved in the selective destabilization of albumin mRNA. *J. Biol. Chem.* 270, 6108-6118.
- Dong,B. and Silverman,R.H. (1995). 2-5A-dependent RNase molecules dimerize during activation by 2-5A. *J. Biol. Chem.* 270, 4133-4137.
- Douthwaite,S., Garrett,R.A., Wagner,R., and Feunteun,J. (1979). A ribonuclease-resistant region of 5S RNA and its relation to the RNA binding sites of proteins L18 and L25. *Nucleic Acids Res.* 6, 2453-2470.
- Doyle,G.A.R., Betz,N.A., Leeds,P.F., Fleisig,A.J., Prokipcak,R.D., and Ross,J. (1998). The *c-myc* coding region determinant-binding protein: a member of a family of KH domain RNA-binding proteins. *Nucleic Acids Res.* 26, 5036-5044.
- Drummond,I.A., Madden,S.L., Rohwer-Nutter,P., Bell,G.I., Sukhatme,V.P., and Rauscher,F.J., III (1992). Repression of the insulin-like growth factor II gene by the Wilms tumor suppressor WT1. *Science* 257, 674-678.
- Dufourny,B., Alblas,J., van Teeffelen,H.A., van Schaik,F.M., van der,B.B., Steenbergh,P.H., and Sussenbach,J.S. (1997). Mitogenic signaling of insulin-like growth factor I in MCF-7 human breast cancer cells requires phosphatidylinositol 3-kinase and is independent of mitogen-activated protein kinase. *J. Biol. Chem.* 272, 31163-31171.
- Ehretsmann,C.P., Carpousis,A.J., and Krisch,H.M. (1992). Specificity of *Escherichia coli* endoribonuclease RNase E: in vivo and in vitro analysis of mutants in a bacteriophage T4 mRNA processing site. *Genes Dev.* 6, 149-159.
- Enberg,G. and Hall,K. (1984). Immunoreactive IGF-II in serum of healthy subjects and patients with growth hormone disturbances and uraemia. *Acta Endocrinol. (Copenh)* 107, 164-170.
- Engstrom,W., Shokrai,A., Otte,K., Granerus,M., Gessbo,A., Bierke,P., Madej,A., Sjolund,M., and Ward,A. (1998). Transcriptional regulation and biological significance of the insulin like growth factor II gene. *Cell Prolif.* 31, 173-189.
- Fang,G. and Cech,T.R. (1993a). Characterization of a G-quartet formation reaction promoted by the beta-subunit of the *Oxytricha* telomere-binding protein. *Biochemistry* 32, 11646-11657.
- Fang,G. and Cech,T.R. (1993b). The beta subunit of *Oxytricha* telomere-binding protein promotes G-quartet formation by telomeric DNA. *Cell* 74, 875-885.
- Feng,J., Funk,W.D., Wang,S.S., Weinrich,S.L., Avilion,A.A., Chiu,C.P., Adams,R.R., Chang,E., Allsopp,R.C., and Yu,J. (1995). The RNA component of human telomerase. *Science* 269, 1236-1241.
- Florini,J.R., Magri,K.A., Ewton,D.Z., James,P.L., Grindstaff,K., and Rotwein,P.S. (1991). "Spontaneous" differentiation of skeletal myoblasts is dependent upon autocrine secretion of insulin-like growth factor-II. *J. Biol. Chem.* 266, 15917-15923.

- Floyd-Smith,G., Slattery,E., and Lengyel,P. (1981). Interferon action: RNA cleavage pattern of a (2'-5')oligoadenylate--dependent endonuclease. *Science* 212, 1030-1032.
- Frasca,F., Pandini,G., Scalia,P., Sciacca,L., Mineo,R., Costantino,A., Goldfine,I.D., Belfiore,A., and Vigneri,R. (1999). Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. *Mol. Cell Biol.* 19, 3278-3288.
- Gibson,T.J., Thompson,J.D., and Heringa,J. (1993). The KH domain occurs in a diverse set of RNA-binding proteins that include the antiterminator NusA and is probably involved in binding to nucleic acid. *FEBS Lett.* 324, 361-366.
- Gilley,D. and Blackburn,E.H. (1999). The telomerase RNA pseudoknot is critical for the stable assembly of a catalytically active ribonucleoprotein. *Proc. Natl. Acad. Sci. U. S. A* 96, 6621-6625.
- Giudice,L.C. (1992). Insulin-like growth factors and ovarian follicular development. *Endocr. Rev.* 13, 641-669.
- Gluckman,P.D. and Ambler,G.R. (1993). What is the function of circulating insulin-like growth factor-2 in postnatal life? *Mol. Cell Endocrinol.* 92, C1-C3.
- Gordon,D.A., Shelness,G.S., Nicosia,M., and Williams,D.L. (1988). Estrogen-induced destabilization of yolk precursor protein mRNAs in avian liver. *J. Biol. Chem.* 263, 2625-2631.
- Goya,L., de la,P.A., Ramos,S., Martin,M.A., Escriva,F., and Pascual-Leone,A.M. (1999). Regulation of insulin-like growth factor-I and -II by glucose in primary cultures of fetal rat hepatocytes. *J Biol. Chem.* 274, 24633-24640.
- Graham FL and van der Eb AJ (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52, 456-467.
- Granerus,G., Lonnqvist,B., Nystrand,J., and Roupe,G. (1998). Serum tryptase measured with B12 and G5 antibody-based immunoassays in mastocytosis patients and its relation to histamine turnover. *Br. J. Dermatol.* 139, 858-861.
- Granerus,M. and Engstrom,W. (1996). Growth factors and apoptosis. *Cell Prolif.* 29, 309-314.
- Granerus,M., Schofield,P., Bierke,P., and Engstrom,W. (1995). Growth factors and apoptosis in development. The role of insulin like growth factor I and TGFbeta1 in regulating cell growth and cell death in a human teratocarcinoma derived cell line. *Int. J. Dev. Biol.* 39, 759-764.
- Gray,S.G., Yakovleva,T., Hartmann,W., Tally,M., Bakalkin,G., and Ekström,T.J. (1999). IGF-II enhances Trichostatin A-induced *TGF 1* and p21^{Waf1, Cip1,Sdi1} expression in Hep3B cells. *Exp. Cell Res.* 253, 618-628.
- Greider,C.W. and Blackburn,E.H. (1989). A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. *Nature* 337, 331-337.

- Hall,K., Enberg,G., Ritzen,M., Svan,H., Fryklund,L., and Takano,K. (1980). Somatomedin A levels in serum from healthy children and from children with growth hormone deficiency or delayed puberty. *Acta Endocrinol. (Copenh)* *94*, 155-165.
- Hall,K. and Sara,V.R. (1984). Somatomedin levels in childhood, adolescence and adult life. *Clin. Endocrinol. Metab* *13*, 91-112.
- Herrick,D.J. and Ross,J. (1994). The half-life of c-myc mRNA in growing and serum-stimulated cells: influence of the coding and 3' untranslated regions and role of ribosome translocation. *Mol. Cell Biol.* *14*, 2119-2128.
- Hirai,K., Miyamasu,M., Yamaguchi,M., Nakajima,K., Ohtoshi,T., Koshino,T., Takaishi,T., Morita,Y., and Ito,K. (1993). Modulation of human basophil histamine release by insulin-like growth factors. *J Immunol.* *150*, 1503-1508.
- Holthuizen,P., van der Lee,F.M., Ikejiri,K., Yamamoto,M., and Sussenbach,J.S. (1990). Identification and initial characterization of a fourth leader exon and promoter of the human IGF-II gene. *Biochim. Biophys. Acta* *1087*, 341-343.
- Holthuizen,P.E., Cleutjens,C.B.J.M., Veenstra,G.J.C., van der Lee,F.M., Koonen-Reemst,A.M.C.B., and Sussenbach,J.S. (1993). Differential expression of the human, mouse and rat IGF- II genes. *Regul. Pept.* *48*, 77-89.
- Holthuizen,P.E., Steenbergh,P.H., and Sussenbach,J.S. (1999). Regulation of IGF gene expression. In *The IGF System. Molecular Biology, Physiology, and Clinical Applications.*, R.G.Rosenfeld and C.T.Roberts, eds. (Totowa, New Jersey, USA.: Humana Press.), pp. 37-61.
- Hua,J., Garner,R., and Paetkau,V. (1993). An RNasin-resistant ribonuclease selective for interleukin 2 mRNA. *Nucleic Acids Res.* *21*, 155-162.
- Hua,J. and Paetkau,V. (1996). A binding factor for interleukin 2 mRNA. *Nucleic Acids Res.* *24*, 970-976.
- Humbel,R.E. (1990). Insulin-like growth factors I and II. *Eur. J Biochem.* *190*, 445-462.
- Hunter,S.J., Daughaday,W.H., Callender,M.E., McKnight,J.A., McIlrath,E.M., Teale,J.D., and Atkinson,A.B. (1994). A case of hepatoma associated with hypoglycaemia and overproduction of IGF-II (E-21): beneficial effects of treatment with growth hormone and intrahepatic adriamycin. *Clin. Endocrinol. (Oxf)* *41*, 397-401.
- Hurley,L.H., Wheelhouse,R.T., Sun,D., Kerwin,S.M., Salazar,M., Fedoroff,O.Y., Han,F.X., Han,H., Izbicka,E., and Von Hoff,D.D. (2000). G-quadruplexes as targets for drug design. *Pharmacol. Ther.* *85*, 141-158.
- Ikejiri,K., Wasada,T., Haruki,K., Hizuka,N., Hirata,Y., and Yamamoto,M. (1991). Identification of a novel transcription unit in the human insulin-like growth factor-II gene. *Biochem. J.* *280*, 439-444.

Ioannidis,P., Havredaki,M., Courtis,N., and Trangas,T. (1996). In vivo generation of 3' and 5' truncated species in the process of c-myc mRNA decay. *Nucleic. Acids. Res* 24, 4969-4977.

Jacobs,S., Kull,F.C., Jr., Earp,H.S., Svoboda,M.E., Van Wyk,J.J., and Cuatrecasas,P. (1983). Somatomedin-C stimulates the phosphorylation of the beta-subunit of its own receptor. *J. Biol. Chem.* 258, 9581-9584.

Jacobson,A. and Peltz,S.W. (1996). Interrelationships of the pathways of mRNA decay and translation in eukaryotic cells. *Annu. Rev. Biochem.* 65:693-739, 693-739.

Jessen,T.H., Oubridge,C., Teo,C.H., Pritchard,C., and Nagai,K. (1991). Identification of molecular contacts between the U1 A small nuclear ribonucleoprotein and U1 RNA. *EMBO J* 10, 3447-3456.

Jhun,B.H., Meinkoth,J.L., Leitner,J.W., Draznin,B., and Olefsky,J.M. (1994). Insulin and insulin-like growth factor-I signal transduction requires p21ras. *J. Biol. Chem.* 269, 5699-5704.

Jones,J.I. and Clemmons,D.R. (1995). Insulin-like growth factors and their binding proteins: biological actions. *Endocr. Rev.* 16, 3-34.

Jones,T.R. and Cole,M.D. (1987). Rapid cytoplasmic turnover of c-myc mRNA: requirement of the 3' untranslated sequences. *Mol Cell Biol* 7, 4513-4521.

Kawahara,R.S. and Deuel,T.F. (1989). Platelet-derived growth factor-inducible gene JE is a member of a family of small inducible genes related to platelet factor 4. *J. Biol. Chem.* 264, 679-682.

Kelley,R.L. and Kuroda,M.I. (2000). Noncoding RNA genes in dosage compensation and imprinting. *Cell* 103, 9-12.

Kensch,O., Connolly,B.A., Steinhoff,H.J., McGregor,A., Goody,R.S., and Restle,T. (2000). HIV-1 reverse transcriptase-pseudoknot RNA aptamer interaction has a binding affinity in the low picomolar range coupled with high specificity. *J Biol. Chem.* 275, 18271-18278.

Kharrat,A., Macias,M.J., Gibson,T.J., Nilges,M., and Pastore,A. (1995). Structure of the dsRNA binding domain of E. coli RNase III. *EMBO J.* 14, 3572-3584.

Kiess,W., Yang,Y., Kessler,U., and Hoeflich,A. (1994). Insulin-like growth factor II (IGF-II) and the IGF-II/mannose-6-phosphate receptor: the myth continues. *Horm. Res. 41 Suppl 2:66-73*, 66-73.

Kim,K.W., Bae,S.K., Lee,O.H., Bae,M.H., Lee,M.J., and Park,B.C. (1998). Insulin-like growth factor II induced by hypoxia may contribute to angiogenesis of human hepatocellular carcinoma. *Cancer Res.* 58, 348-351.

Kim,N.W., Piatyszek,M.A., Prowse,K.R., Harley,C.B., West,M.D., Ho,P.L., Coviello,G.M., Wright,W.E., Weinrich,S.L., and Shay,J.W. (1994). Specific association of human telomerase activity with immortal cells and cancer. *Science* 266, 2011-2015.

- Kim,S., Kim,H., Park,I., and Lee,Y. (1996). Mutational analysis of RNA structures and sequences postulated to affect 3' processing of M1 RNA, the RNA component of Escherichia coli RNase P. *J Biol. Chem.* 271, 19330-19337.
- Koeller,D.M., Horowitz,J.A., Casey,J.L., Klausner,R.D., and Harford,J.B. (1991). Translation and the stability of mRNAs encoding the transferrin receptor and c-fos. *Proc. Natl. Acad. Sci. U. S. A* 88, 7778-7782.
- Kotani,K., Tsuji,M., Oki,A., Kashihara,T., Yamada,K., Kawakami,F., Tako,H., Okuno,G., Hizuka,N., and Aiba,M. (1993). IGF-II producing hepatic fibrosarcoma associated with hypoglycemia. *Intern. Med.* 32, 897-901.
- Kowalski,J. and Denhardt,D.T. (1989). Regulation of the mRNA for monocyte-derived neutrophil-activating peptide in differentiating HL60 promyelocytes. *Mol. Cell Biol.* 9, 1946-1957.
- Kurz,J.C. and Fierke,C.A. (2000). Ribonuclease P: a ribonucleoprotein enzyme. *Curr. Opin. Chem. Biol.* 4, 553-558.
- Kutoh,E., Schwander,J., and Margot,J.B. (1995). Cell-density-dependent modulation of the rat insulin-like-growth-factor-binding protein 2 and its gene. *Eur. J Biochem.* 234, 557-562.
- Lau,M.M., Stewart,C.E., Liu,Z., Bhatt,H., Rotwein,P., and Stewart,C.L. (1994). Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. *Genes Dev.* 8, 2953-2963.
- Lee,C.H., Leeds,P., and Ross,J. (1998). Purification and characterization of a polysome-associated endoribonuclease that degrades c-myc mRNA *in vitro*. *J. Biol. Chem.* 273, 25261-25271.
- Leeds,P., Kren,B.T., Boylan,J.M., Betz,N.A., Steer,C.J., Gruppuso,P.A., and Ross,J. (1997). Developmental regulation of CRD-BP, an RNA-binding protein that stabilizes c-myc mRNA *in vitro*. *Oncogene* 14, 1279-1286.
- Leighton,P.A., Ingram,R.S., Eggenschwiler,J., Efstratiadis,A., and Tilghman,S.M. (1995). Disruption of imprinting caused by deletion of the H19 gene region in mice [see comments]. *Nature* 375, 34-39.
- Leontis,N.B., Ghosh,P., and Moore,P.B. (1986). Effect of magnesium ion on the structure of the 5S RNA from Escherichia coli. An imino proton magnetic resonance study of the helix I, IV, and V regions of the molecule. *Biochemistry* 25, 7386-7392.
- Levinovitz,A. and Norstedt,G. (1989). Developmental and steroid hormonal regulation of insulin-like growth factor II expression. *Mol. Endocrinol.* 3, 797-804.
- Li,X.L., Blackford,J.A., Judge,C.S., Liu,M., Xiao,W., Kalvakolanu,D.V., and Hassel,B.A. (2000). RNase-L-dependent destabilization of interferon-induced mRNAs. A role for the 2-5A system in attenuation of the interferon response. *J. Biol. Chem.* 275, 8880-8888.

- Lindstein,T., June,C.H., Ledbetter,J.A., Stella,G., and Thompson,C.B. (1989). Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. *Science* 244, 339-343.
- Liu,J., Kahri,A.I., Heikkilä,P., Blum,W.F., and Voutilainen,R. (1994). Glucocorticoids increase insulin-like growth factor-II mRNA accumulation in cultured human pheochromocytoma cells. *J. Endocrinol.* 142, 29-35.
- Liu,J.P., Baker,J., Perkins,A.S., Robertson,E.J., and Efstratiadis,A. (1993). Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell* 75, 59-72.
- Louvi,A., Accili,D., and Efstratiadis,A. (1997). Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. *Dev. Biol.* 189, 33-48.
- Ludwig,T., Eggenschwiler,J., Fisher,P., D'Ercole,A.J., Davenport,M.L., and Efstratiadis,A. (1996). Mouse mutants lacking the type 2 IGF receptor (IGF2R) are rescued from perinatal lethality in *Igf2* and *Igf1r* null backgrounds. *Dev. Biol.* 177, 517-535.
- Madden,S.L. and Rauscher,F.J., III (1993). Positive and negative regulation of transcription and cell growth mediated by the EGR family of zinc-finger gene products. *Ann. N. Y. Acad. Sci.* 684:75-84, 75-84.
- Martin J.L and Baxter,R.C. (1999). IGF binding proteins as modulators of IGF action. In *The IGF System, Molecular Biology, Physiology and Clinical Applications*, Rosenfeld R. and Roberts C., eds. (Totowa, NJ: Humana), pp. 227-255.
- McKnight,S.L. and Kingsbury,R. (1982). Transcriptional control signals of a eukaryotic protein-coding gene. *Science* 217, 316-324.
- Meinsma,D., Holthuisen,P., Van den Brande,J.L., and Sussenbach,J.S. (1991). Specific endonucleolytic cleavage of IGF-II mRNAs. *Biochem. Biophys. Res. Commun.* 179, 1509-1516.
- Meinsma,D., Scheper,W., Holthuisen,P., Van den Brande,J.L., and Sussenbach,J.S. (1992). Site-specific cleavage of IGF-II mRNAs requires sequence elements from two distinct regions of the IGF-II gene. *Nucleic Acids Res.* 20, 5003-5009.
- Mineo,R., Fichera,E., Liang,S.J., and Fujita-Yamaguchi,Y. (2000). Promoter usage for insulin-like growth factor-II in cancerous and benign human breast, prostate, and bladder tissues, and confirmation of a 10th exon. *Biochem. Biophys. Res. Commun.* 268, 886-892.
- Minniti,C.P., Kohn,E.C., Grubb,J.H., Sly,W.S., Oh,Y., Muller,H.L., Rosenfeld,R.G., and Helman,L.J. (1992). The insulin-like growth factor II (IGF-II)/mannose 6-phosphate receptor mediates IGF-II-induced motility in human rhabdomyosarcoma cells. *J. Biol. Chem.* 267, 9000-9004.
- Mise,M., Arai,S., Higashitani,H., Furutani,M., Niwano,M., Harada,T., Ishigami,S., Toda,Y., Nakayama,H., Fukumoto,M., Fujita,J., and Imamura,M. (1996). Clinical significance of vascular endothelial growth factor and basic fibroblast growth factor gene expression in liver tumor. *Hepatology* 23, 455-464.

- Morrione,A., Valentinis,B., Xu,S.Q., Yumet,G., Louvi,A., Efstratiadis,A., and Baserga,R. (1997). Insulin-like growth factor II stimulates cell proliferation through the insulin receptor. *Proc. Natl. Acad. Sci. U. S. A* 94, 3777-3782.
- Moskaitis,J.E., Buzek,S.W., Pastori,R.L., and Schoenberg,D.R. (1991). The estrogen-regulated destabilization of *Xenopus* albumin mRNA is independent of translation. *Biochem. Biophys. Res. Commun.* 174, 825-830.
- Mouhieddine,O.B., Cazals,V., Kuto,E., Le Bouc,Y., and Clement,A. (1996). Glucocorticoid-induced growth arrest of lung alveolar epithelial cells is associated with increased production of insulin-like growth factor binding protein-2. *Endocrinology* 137, 287-295.
- Muller,M.M., Carrasco,A.E., and DeRobertis,E.M. (1984). A homeo-box-containing gene expressed during oogenesis in *Xenopus*. *Cell* 39, 157-162.
- Mullner,E.W. and Kuhn,L.C. (1988). A stem-loop in the 3' untranslated region mediates iron-dependent regulation of transferrin receptor mRNA stability in the cytoplasm. *Cell* 53, 815-825.
- Musco,G., Stier,G., Joseph,C., Castiglione Morelli,M.A., Nilges,M., Gibson,T.J., and Pastore,A. (1996). Three-dimensional structure and stability of the KH domain: molecular insights into the fragile X syndrome. *Cell* 19;85, 237-245.
- Nashimoto,M., Tamura,M., and Kaspar,R.L. (1999a). Selection of cleavage site by mammalian tRNA 3' processing endoribonuclease. *J. Mol. Biol.* 287, 727-740.
- Nashimoto,M., Wesemann,D.R., Geary,S., Tamura,M., and Kaspar,R.L. (1999b). Long 5' leaders inhibit removal of a 3' trailer from a precursor tRNA by mammalian tRNA 3' processing endoribonuclease. *Nucleic Acids Res.* 27, 2770-2776.
- Nielsen,D.A. and Shapiro,D.J. (1990). Estradiol and estrogen receptor-dependent stabilization of a minivitellogenin mRNA lacking 5,100 nucleotides of coding sequence. *Mol. Cell Biol.* 10, 371-376.
- Nielsen,F.C. and Christiansen,J. (1992). Endonucleolysis in the turnover of insulin-like growth factor II mRNA. *J. Biol. Chem.* 267, 19404-19411.
- Nielsen,F.C., Gammeltoft,S., and Christiansen,J. (1990). Translational discrimination of mRNAs coding for human insulin-like growth factor II. *J. Biol. Chem.* 265, 13431-13434.
- Nielsen,F.C., Ostergaard,L., Nielsen,J., and Christiansen,J. (1995). Growth-dependent translation of IGF-II mRNA by a rapamycin-sensitive pathway. *Nature* 377, 358-362.
- Nishimoto,I. (1993). The IGF-II receptor system: a G protein-linked mechanism. *Mol. Reprod. Dev.* 35, 398-406.
- Nissley,P. and Lopaczynski,W. (1991). Insulin-like growth factor receptors. *Growth Factors* 5, 29-43.

- Norstedt,G. and Moller,C. (1987). Growth hormone induction of insulin-like growth factor I messenger RNA in primary cultures of rat liver cells. *J. Endocrinol.* *115*, 135-139.
- Ogawa,O., Eccles,M.R., Szeto,J., McNoe,L.A., Yun,K., Maw,M.A., Smith,P.J., and Reeve,A.E. (1993). Relaxation of insulin-like growth factor II gene imprinting implicated in Wilms' tumour. *Nature* *362*, 749-751.
- Oka,Y., Rozek,L.M., and Czech,M.P. (1985). Direct demonstration of rapid insulin-like growth factor II Receptor internalization and recycling in rat adipocytes. Insulin stimulates ¹²⁵I-insulin-like growth factor II degradation by modulating the IGF-II receptor recycling process. *J. Biol. Chem.* *260*, 9435-9442.
- Oubridge,C., Ito,N., Evans,P.R., Teo,C.H., and Nagai,K. (1994). Crystal structure at 1.92 Å resolution of the RNA-binding domain of the U1A spliceosomal protein complexed with an RNA hairpin. *Nature* *372*, 432-438.
- Pastori,R.L., Moskaitis,J.E., Buzek,S.W., and Schoenberg,D.R. (1991a). Coordinate estrogen-regulated instability of serum protein-coding messenger RNAs in *Xenopus laevis*. *Mol. Endocrinol.* *5*, 461-468.
- Pastori,R.L., Moskaitis,J.E., and Schoenberg,D.R. (1991b). Estrogen-induced ribonuclease activity in *Xenopus* liver. *Biochemistry* *30*, 10490-10498.
- Pastori,R.L., Moskaitis,J.E., Smith,L.H., Jr., and Schoenberg,D.R. (1990). Estrogen regulation of *Xenopus laevis* gamma-fibrinogen gene expression. *Biochemistry* *29*, 2599-2605.
- Phizicky,E.M. and Greer,C.L. (1993). Pre-tRNA splicing: variation on a theme or exception to the rule? *Trends Biochem. Sci.* *18*, 31-34.
- Pleij,C.W. (1995). Structure and function of RNA pseudoknots. *Genet. Eng. (N. Y.)* *17*:67-80, 67-80.
- Pleij,C.W., Rietveld,K., and Bosch,L. (1985). A new principle of RNA folding based on pseudoknotting. *Nucleic Acids Res.* *13*, 1717-1731.
- Powell-Braxton,L., Hollingshead,P., Warburton,C., Dowd,M., Pitts-Meek,S., Dalton,D., Gillett,N., and Stewart,T.A. (1993). IGF-I is required for normal embryonic growth in mice. *Genes Dev.* *7*, 2609-2617.
- Price,W.A., Stiles,A.D., Moats-Staats,B.M., and D'Ercole,A.J. (1992). Gene expression of insulin-like growth factors (IGFs), the type 1 IGF receptor, and IGF-binding proteins in dexamethasone-induced fetal growth retardation. *Endocrinology* *130*, 1424-1432.
- Prokipcak,R.D., Herrick,D.J., and Ross,J. (1994). Purification and properties of a protein that binds to the C-terminal coding region of human c-myc mRNA. *J Biol Chem* *269*, 9261-9269.
- Puglisi,J.D., Chen,L., Blanchard,S., and Frankel,A.D. (1995). Solution structure of a bovine immunodeficiency virus Tat-TAR peptide-RNA complex. *Science* *270*, 1200-1203.

Raizis,A.M., Eccles,M.R., and Reeve,A.E. (1993). Structural analysis of the human insulin-like growth factor-II P3 promoter. *Biochem. J.* 289, 133-139.

Ramasharma,K. and Li,C.H. (1987). Human pituitary and placental hormones control human insulin-like growth factor II secretion in human granulosa cells. *Proc. Natl. Acad. Sci. U. S. A* 84, 2643-2647.

Ramos,A., Gubser,C.C., Varani,G. (1997). Recent solution structures of RNA and its complexes with drugs, peptides and proteins. *Curr.Opin.Struct.Biol.* 7, 317-323

Rauscher,F.J., III (1993). Tumor suppressor genes which encode transcriptional repressors: Studies on the EGR and Wilms' tumor (WT1) gene products. *Adv. Exp. Med. Biol.* 348, 23-29.

Rietveld,L.E., Holthuisen,P.E., and Sussenbach,J.S. (1997). Identification of a key regulatory element for the basal activity of the human insulin-like growth factor II gene promoter P3. *Biochem. J.* 327, 689-697.

Rietveld,L.E., Koonen-Reemst,A.M., Sussenbach,J.S., and Holthuisen,P.E. (1999). Dual role for transcription factor AP-2 in the regulation of the major fetal promoter P3 of the gene for human insulin-like growth factor II. *Biochem. J.* 338, 799-806.

Rinderknecht,E. and Humbel,R.E. (1978a). Primary structure of human insulin-like growth factor II. *FEBS Lett.* 89, 283-286.

Rinderknecht,E. and Humbel,R.E. (1978b). The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *J Biol. Chem.* 253, 2769-2776.

Rodenburg, R. J. Transcriptional regulation of the liver-specific promoter of the human insulin-like growth factor II gene. 1996. Thesis. Laboratory for Physiological Chemistry, Utrecht University, Utrecht, The Netherlands.

Rodenburg,R.J., Holthuisen,P.E., and Sussenbach,J.S. (1997). A functional Sp1 binding site is essential for the activity of the adult liver-specific human insulin-like growth factor II promoter. *Mol. Endocrinol.* 11, 237-250.

Romby,P., Westhof,E., Toukifimpa,R., Mache,R., Ebel,J.P., Ehresmann,C., and Ehresmann,B. (1988). Higher order structure of chloroplastic 5S ribosomal RNA from spinach. *Biochemistry* 27, 4721-4730.

Ross,J. (1995). mRNA stability in mammalian cells. *Microbiol. Rev.* 59, 423-450.

Rotwein,P. (1986). Two insulin-like growth factor I messenger RNAs are expressed in human liver. *Proc. Natl. Acad. Sci. USA* 83, 77-81.

Russell,A.G., Ebhardt,H., and Dennis,P.P. (1999). Substrate requirements for a novel archaeal endonuclease that cleaves within the 5' external transcribed spacer of *Sulfolobus acidocaldarius* precursor rRNA. *Genetics* 152, 1373-1385.

Saenger W (1984). Principles of Nucleic Acid Structure. (New York: Springer).

- Salmon, W.D. and Daughaday, W.H. (1957). A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. *J. Lab. Clin. Med.* *49*, 825.
- Sambrook J, Fritsch EF, and Maniatis T (1989). *Molecular cloning: A laboratory manual*. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).
- Scheper, W., Meinsma, D., Holthuizen, P.E., and Sussenbach, J.S. (1995). Long-range RNA interaction of two sequence elements required for endonucleolytic cleavage of human insulin-like growth factor II mRNAs. *Mol. Cell Biol.* *15*, 235-245.
- Schmidt, J.V., Levorse, J.M., and Tilghman, S.M. (1999). Enhancer competition between H19 and Igf2 does not mediate their imprinting. *Proc. Natl. Acad. Sci. U. S. A* *96*, 9733-9738.
- Schneid, H., Holthuizen, P.E., and Sussenbach, J.S. (1993). Differential promoter activation in two human insulin-like growth factor-II-producing tumor cell lines. *Endocrinology* *132*, 1145-1150.
- Schoenberg, D.R., Moskaitis, J.E., Smith, L.H., Jr., and Pastori, R.L. (1989). Extranuclear estrogen-regulated destabilization of *Xenopus laevis* serum albumin mRNA. *Mol. Endocrinol.* *3*, 805-814.
- Schuster, P., Stadler, P.F., and Renner, A. (1997). RNA structures and folding: From conventional to new issues in structure predictions. *Curr. Opin. Struct. Biol.* *7*, 229-235.
- Sen, G.C. and Lengyel, P. (1992). The interferon system. A bird's eye view of its biochemistry. *J. Biol. Chem.* *267*, 5017-5020.
- Senior, P.V., Tucci, J., Blythe, N.L., and Beck, F. (1996). Expression of IGF-II and H19 mRNA in the neonatal rat during normal maturation and after dexamethasone administration. *J. Mol. Endocrinol.* *17*, 217-223.
- Siomi, H. and Dreyfuss, G. (1997). RNA-binding proteins as regulators of gene expression. *Curr. Opin. Genet. Dev.* *7*, 345-353.
- Siomi, H., Matunis, M.J., Michael, W.M., and Dreyfuss, G. (1993). The pre-mRNA binding K protein contains a novel evolutionarily conserved motif. *Nucleic Acids Res.* *21*, 1193-1198.
- Steenbergh, P.H., Koonen-Reemst, A.M.C.B., Cleutjens, C.B.J.M., and Sussenbach, J.S. (1991). Complete nucleotide sequence of the high molecular weight human IGF-I mRNA. *Biochem. Biophys. Res. Commun.* *175*, 507-514.
- Stoeckle, M.Y. (1991). Post-transcriptional regulation of gro alpha, beta, gamma, and IL-8 mRNAs by IL-1 beta. *Nucleic Acids Res.* *19*, 917-920.
- Stoeckle, M.Y. (1992). Removal of a 3' non-coding sequence is an initial step in degradation of gro alpha mRNA and is regulated by interleukin-1. *Nucleic Acids Res.* *20*, 1123-1127.
- Stoeckle, M.Y. and Hanafusa, H. (1989). Processing of 9E3 mRNA and regulation of its stability in normal and Rous sarcoma virus-transformed cells. *Mol. Cell Biol.* *9*, 4738-4745.

- Sussenbach, J.S., Steenbergh, P.H., and Holthuisen, P. (1992). Structure and expression of the human insulin-like growth factor genes. *Growth Regul.* 2, 1-9.
- Suzuki, H., Veda, R., Takahashi, T., and Takahashi, T. (1994). Altered imprinting in lung cancer. *Nat. Genet.* 6, 332-333.
- Swartwout, S.G. and Kinniburgh, A.J. (1989). c-myc RNA degradation in growing and differentiating cells: possible alternate pathways. *Mol Cell Biol* 9, 288-295.
- Szewczak, A.A. and Moore, P.B. (1995). The sarcin/ricin loop, a modular RNA. *J Mol. Biol.* 247, 81-98.
- Tan, R., Chen, L., Buettner, J.A., Hudson, D., and Frankel, A.D. (1993). RNA recognition by an isolated alpha helix. *Cell* 73, 1031-1040.
- Teerink, H., Voorma, H.O., and Thomas, A.A.M. (1995). The human insulin-like growth factor II leader 1 contains an internal ribosomal entry site. *Biochim. Biophys. Acta Gene Struct. Expression* 1264, 403-408.
- Thorvaldsen, J.L., Duran, K.L., and Bartolomei, M.S. (1998). Deletion of the H19 differentially methylated domain results in loss of imprinted expression of H19 and Igf2. *Genes Dev.* 12, 3693-3702.
- Torres, A.M., Forbes, B.E., Aplin, S.E., Wallace, J.C., Francis, G.L., and Norton, R.S. (1995). Solution structure of human insulin-like growth factor II. Relationship to receptor and binding protein interactions. *J. Mol. Biol.* 248, 385-401.
- Ullrich, A., Gray, A., Tam, A.W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., and Chen, E. (1986). Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. *EMBO J.* 5, 2503-2512.
- van Dijk, E.L., Sussenbach, J.S., and Holthuisen, P.E. (1998). Identification of RNA sequences and structures involved in site-specific cleavage of IGF-II mRNAs. *RNA.* 4, 1623-1635.
- van Dijk, E.L., Sussenbach, J.S., and Holthuisen, P.E. (2000). Distinct RNA structural domains cooperate to maintain a specific cleavage site in the 3'-UTR of IGF-II mRNAs. *J Mol Biol* 300, 449-467.
- van Dijk, M.A., Rodenburg, R.J., Holthuisen, P., and Sussenbach, J.S. (1992). The liver-specific promoter of the human insulin-like growth factor II gene is activated by CCAAT/enhancer binding protein (C/EBP). *Nucleic Acids Res.* 20, 3099-3104.
- van Dijk, M.A., van Schaik, F.M., Bootsma, H.J., Holthuisen, P., and Sussenbach, J.S. (1991). Initial characterization of the four promoters of the human insulin-like growth factor II gene. *Mol. Cell Endocrinol.* 81, 81-94.

Voutilainen,R. and Miller,W.L. (1987). Coordinate tropic hormone regulation of mRNAs for insulin-like growth factor II and the cholesterol side-chain-cleavage enzyme, P450scc [corrected], in human steroidogenic tissues [published erratum appears in Proc Natl Acad Sci U S A 1987 Sep;84(17):6194]. *Proc. Natl. Acad. Sci. U. S. A* 84, 1590-1594.

Vreken,P. and Raue,H.A. (1992). The rate-limiting step in yeast PGK1 mRNA degradation is an endonucleolytic cleavage in the 3'-terminal part of the coding region. *Mol. Cell Biol.* 12, 2986-2996.

Vreken,P., van,d., V, de Regt,V.C., de Maat,A.L., Planta,R.J., and Raue,H.A. (1991). Turnover rate of yeast PGK mRNA can be changed by specific alterations in its trailer structure. *Biochimie* 73, 729-737.

Wagner,E.G. and Simons,R.W. (1994). Antisense RNA control in bacteria, phages, and plasmids. *Annu. Rev. Microbiol.* 48:713-42 , 713-742.

Wang,X., Kiledjian,M., Weiss,I.M., and Liebhaber,S.A. (1995). Detection and characterization of a 3' untranslated region ribonucleoprotein complex associated with human alpha-globin mRNA stability. *Mol. Cell Biol.* 15, 1769-1777 [published erratum appeared in *Mol Cell Biol* 1995 Apr;15(4):2331].

Wang,Z.Q., Fung,M.R., Barlow,D.P., and Wagner,E.F. (1994). Regulation of embryonic growth and lysosomal targeting by the imprinted *Igf2/Mpr* gene. *Nature* 372, 464-467.

Wang,Z.R., Day,N., Trifillis,P., and Kiledjian,M. (1999). An mRNA stability complex functions with poly(A)-binding protein to stabilize mRNA in vitro. *Mol. Cell. Biol.* 19, 4552-4560.

Wang,Z.R. and Kiledjian,M. (2000a). Identification of an erythroid-enriched endoribonuclease activity involved in specific mRNA cleavage. *EMBO J.* 19, 295-305.

Wang,Z.R. and Kiledjian,M. (2000b). The poly(A)-binding protein and an mRNA stability protein jointly regulate an endoribonuclease activity. *Mol. Cell Biol.* 20, 6334-6341.

Webber,A.L., Ingram,R.S., Levorse,J.M., and Tilghman,S.M. (1998). Location of enhancers is essential for the imprinting of *H19* and *Igf2* genes. *Nature* 391, 711-715.

Weiss,I.M. and Liebhaber,S.A. (1995). Erythroid cell-specific mRNA stability elements in the alpha 2-globin 3' nontranslated region. *Mol. Cell Biol.* 15, 2457-2465.

Weksberg,R., Shen,D.R., Fei,Y.L., Song,Q.L., and Squire,J. (1993). Disruption of insulin-like growth factor 2 imprinting in Beckwith-Wiedemann syndrome. *Nature Genet.* 5, 143-150.

Wennborg,A., Sohlberg,B., Angerer,D., Klein,G., and von Gabain,A. (1995). A human RNase E-like activity that cleaves RNA sequences involved in mRNA stability control. *Proc. Natl. Acad. Sci. U.S.A* 92, 7322-7326.

Westhof,E. and Fritsch,V. (2000). RNA folding: beyond Watson-Crick pairs. *Structure. Fold. Des* 8, R55-R65.

- Wickens,M., Anderson,P., and Jackson,R.J. (1997). Life and death in the cytoplasm: messages from the 3' end. *Curr. Opin. Genet. Dev.* 7, 220-232.
- Wimberly,B., Varani,G., and Tinoco,I., Jr. (1993). The conformation of loop E of eukaryotic 5S ribosomal RNA. *Biochemistry* 32, 1078-1087.
- Wisdom,R. and Lee,W. (1990). Translation of c-myc mRNA is required for its post-transcriptional regulation during myogenesis. *J Biol Chem* 265, 19015-19021.
- Wisdom,R. and Lee,W. (1991). The protein-coding region of c-myc mRNA contains a sequence that specifies rapid mRNA turnover and induction by protein synthesis inhibitors. *Genes Dev* 5, 232-243.
- Wright,C.V., Cho,K.W., Fritz,A., Burglin,T.R., and De Robertis,E.M. (1987). A *Xenopus laevis* gene encodes both homeobox-containing and homeobox-less transcripts. *EMBO J.* 20;6, 4083-4094.
- Yoshiko,Y., Hirao,K., and Maeda,N. (1998). Dexamethasone regulates the actions of endogenous insulin-like growth factor-II during myogenic differentiation. *Life Sci.* 63, 77-85.
- Zahler,A.M., Williamson,J.R., Cech,T.R., and Prescott,D.M. (1991). Inhibition of telomerase by G-quartet DNA structures. *Nature* 350, 718-720.
- Zapf,J. (1994). Role of insulin-like growth factor II and IGF binding proteins in extrapancreatic tumor hypoglycemia. *Horm. Res.* 42, 20-26.
- Zapf,J., Futo,E., Peter,M., and Froesch,E.R. (1992). Can "big" insulin-like growth factor II in serum of tumor patients account for the development of extrapancreatic tumor hypoglycemia? *J. Clin. Invest* 90, 2574-2584.
- Zapf,J., Walter,H., and Froesch,E.R. (1981). Radioimmunological determination of insulinlike growth factors I and II in normal subjects and in patients with growth disorders and extrapancreatic tumor hypoglycemia. *J. Clin. Invest* 68, 1321-1330.
- Zapp,M.L., Hope,T.J., Parslow,T.G., and Green,M.R. (1991). Oligomerization and RNA binding domains of the type 1 human immunodeficiency virus Rev protein: a dual function for an arginine-rich binding motif. *Proc. Natl. Acad. Sci. U.S.A* 88, 7734-7738.
- Zhan,S., Shapiro,D.N., and Helman,L.J. (1994). Activation of an imprinted allele of the insulin-like growth factor II gene implicated in rhabdomyosarcoma. *J. Clin. Invest* 94, 445-448.
- Ziehler,W.A., Day,J.J., Fierke,C.A., and Engelke,D.R. (2000). Effects of 5' leader and 3' trailer structures on pre-tRNA processing by nuclear RNase P. *Biochemistry* 39, 9909-9916.
- Zuker,M. (1989). On finding all suboptimal foldings of an RNA molecule. *Science* 244, 48-52.

Zuker,M. and Stiegler,P. (1981). Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. *Nucleic Acids Res.* 9, 133-148.

SAMENVATTING

Het humane insuline-achtige groeifactor II (IGF-II) speelt een belangrijke rol bij de embryonale ontwikkeling en is geassocieerd met tumorgroei. In gekweekte cellen kan IGF-II diverse effecten veroorzaken, afhankelijk van het celtype waarin het tot expressie komt, zoals groeistimulatie, of juist remming van groei en stimulatie van celdifferentiatie. Terwijl IGF-II een overlevingsfactor is in sommige systemen, induceert het in andere celtypes juist geprogrammeerde celdood (apoptose). IGF-II is dus betrokken bij diverse fysiologische processen, en het is derhalve waarschijnlijk dat IGF-II genexpressie nauwkeurig gereguleerd wordt. Inderdaad vindt er op verscheidene niveaus regulatie plaats; op het niveau van transcriptie (i.e. synthese van het 'boodschapper' mRNA) door middel van ontwikkelings- en weefsel-specifieke activiteit van vier verschillende promoters (i.e. de startplaats van transcriptie)(P1-P4) in het IGF-II gen. Transcriptie vanaf deze promoters leidt tot de vorming van mRNA moleculen die onderling verschillen in de lengte en samenstelling van hun 5' onvertaalde deel (5'-UTR). Dit leidt vervolgens tot verschillen in de translatie (eiwitproductie)-efficiëntie van deze mRNAs en genereert de mogelijkheid om IGF-II expressie te reguleren op het niveau van translatie. Verder is nog bekend dat IGF-II expressie gereguleerd wordt op het niveau van post-translationele bewerking van het IGF-II eiwit.

Tijdens de karakterisering van het humane IGF-II gen en de IGF-II mRNAs werd een RNA met een grootte van 1.8 kb gedetecteerd dat co-lineair is met het 3' uiteinde van het IGF-II gen. Dit RNA bleek gevormd te worden door plaats-specifieke endonucleolytische klieving in het 3' onvertaalde deel (3'-UTR) van de IGF-II mRNAs en niet door transcriptie vanaf een extra promotor. Het 1.8 kb RNA vertegenwoordigt dus het 3' klievingsproduct; het bestaat uit 3'-UTR sequentie en een poly(A) staart, en is zeer stabiel. Klieving van de IGF-II mRNAs genereert ook een 5' klievingsproduct, hetgeen de IGF-II coderende sequentie bevat. Dit 5' klievingsproduct is in tegenstelling tot het 1.8 kb 3' klievingsproduct onstabiel, en wordt niet meer getransleerd. Deze observaties hebben geleid tot de hypothese dat klieving van de IGF-II mRNAs kan fungeren als een additioneel mechanisme om IGF-II genexpressie te reguleren.

Begonnen werd met het in kaart brengen van de RNA gebieden die nodig zijn voor *in vivo* klieving. Hiertoe werden deleties aangebracht in een IGF-II minigen in een expressieplasmide voor transfectie-studies. Deze experimenten toonden aan dat er twee gebieden in de IGF-II 3'-UTR nodig zijn voor klieving: een sequentie van 323 nucleotiden rondom de klievingsplaats (element II), en een sequentie van 104 nucleotiden lang dat 2013 nucleotiden stroomopwaards gelegen is (element I). RNA-vouwings-studies lieten vervolgens zien dat element I, dat zeer rijk is aan C-nucleotiden, een stabiele duplex kan vormen met een G-rijk stroomafwaards gelegen gebied in element II. Direct stroomopwaards van de klievingsplaats in element II kunnen twee 'haarspeldlussen' gevormd worden, en de klievingsplaats zelf wordt voorspeld in een interne lus gelegen te zijn. De RNA gebieden die nodig zijn voor klieving kunnen dus onderverdeeld worden in drie verschillende vouwingsdomeinen: (1) het duplex domein, gevormd door de interactie tussen

nucleotiden -2108/-2029 (posities ten opzichte van de klievingsplaats) van element I en +18/+101 van element II, (2) het haarspeldlussen domein in het stroomopwaards gelegen deel (posities -133 tot -7) van element II, en (3) het klievingsplaatsdomein, gevormd door nucleotiden -161/-136 en -6/+16, met de klievingsplaats gelegen in een interne lus.

De studies beschreven in dit proefschrift begonnen met pogingen om eiwitten te identificeren die specifiek aan de hierboven beschreven RNA gebieden binden, en zo een rol zouden kunnen spelen bij klieving (Hoofdstuk 3). Hiertoe werden *in vitro* gesynthetiseerde RNA moleculen die deze gebieden bevatten geïncubeerd met extracten van humane Hep3B cellen, die endogeen IGF-II tot expressie brengen. Dit leverde een specifiek RNA-eiwit complex op dat alleen gevormd wordt in de aanwezigheid van het haarspeldlussen domein. Met behulp van puntmutaties werd de bindingsplaats van het eiwit nauwkeurig in kaart gebracht. Het complex bleek gevormd te worden halverwege de eerste van de twee haarspeldlussen. Wanneer puntmutaties die de eiwitbinding verstoren in het IGF-II minigen werden geïntroduceerd, leverde dit een gematigde reductie in klievingsefficiëntie op, hetgeen suggereert dat het RNA-eiwitcomplex niet essentieel is voor klieving, maar een modulerende rol in klieving zou kunnen hebben.

Hoofdstuk 3 beschrijft ook experimenten waarin het belang van de nucleotiden rondom de klievingsplaats voor klieving werd onderzocht. Verscheidene puntmutaties werden geïntroduceerd rondom de klievingsplaats in het IGF-II minigen, en het effect hiervan op de *in vivo* klievingsefficiëntie werd onderzocht. De resultaten tonen duidelijk aan dat de identiteit van specifieke nucleotiden rondom de klievingsplaats zeer belangrijk is, omdat vrijwel alle puntmutaties de klieving ernstig verstoren. De nucleotiden in het gebied direct naast de klievingsplaats van posities -6 tot +16 lijken belangrijker te zijn dan de nucleotiden in het hier tegenover liggende deel van posities -161 tot -136. Interessant genoeg is dit gebied ook duidelijk meer geconserveerd in de evolutie, hetgeen het belang van deze nucleotiden voor klieving onderstreept.

Hoofdstuk 4 beschrijft verdere experimenten om meer inzicht te krijgen in de rol van de verschillende RNA vouwingsdomeinen in klieving. Verscheidene deleties werden geïntroduceerd in het haarspeldlussen domein en de effecten op de voorspelde RNA vouwing en *in vivo* klievingsefficiëntie werden bestudeerd. Hieruit bleek dat alle deleties die de voorspelde RNA vouwing veranderen ook de klieving vrijwel volledig verstoren. Deleties die de wild type RNA vouwing intact laten hebben echter een veel minder drastisch effect op klieving, hetgeen aangeeft dat een specifieke vouwing rondom de klievingsplaats belangrijk is. Het haarspeldlussen domein is op zichzelf niet nodig voor klieving, omdat het geheel verwijderd kan worden zonder de klieving drastisch te verstoren, zolang de vouwing rondom de klievingsplaats maar intact blijft. Dit blijkt uit de resultaten met een mutant (Δ -135/-18) waarbij het gehele haarspeldlussen domein verwijderd is; deze mutant laat een redelijke klievingsefficiëntie zien van 47% van de wild type. Een opvallend resultaat echter werd verkregen met een mutant waarbij op een deel van de tweede haarspeldlus na het gehele haarspeldlussendomein verwijderd was (Δ -135/-72, Δ -60/-18). Hoewel de klievingsplaats zich bij deze mutant in de voorspelde wild type

configuratie bevindt, is de klievingsefficiëntie ernstig verstoord. Dit resultaat gaf aanleiding de computervoorspelling van de RNA-vouwing te verifiëren door middel van biochemische structuuranalyse van het RNA (Hoofdstuk 4). De resultaten van deze studies suggereren dat zowel in het wild type RNA als in mutant Δ -135/-18 de nucleotiden in de lus rondom de klievingsplaats niet ongepaard zijn, maar betrokken bij niet-Watson-Crick interacties, hetgeen een specifieke conformatie genereert. In mutant Δ -135/-72, Δ -60/-18 echter, gedragen deze nucleotiden zich als vrij in oplossing. Deze resultaten suggereren dat een specifieke conformatie, gevormd door niet-Watson-Crick interacties, belangrijk is voor klieving. In overeenstemming met deze hypothese werd gevonden dat puntmutaties rondom de klievingsplaats die de klieving verstoren ook de conformatie rondom de klievingsplaats veranderen in tegenstelling tot mutaties die geen effect hebben op klieving.

Hoofdstuk 5 laat experimenten zien die uitgevoerd zijn om meer inzicht te verkrijgen in de fysiologische functie van klieving van IGF-II mRNA. De kinetiek van klieving en de rol van klieving in de afbraak van IGF-II mRNA en eiwitproductie werd getest met behulp van IGF-II constructen onder controle van een tetracycline-induceerbare promotor. De niveaus van het IGF-II mRNA en het 3' klievingsproduct werden bepaald door Northern blot analyse. Na inductie van transcriptie werd een snelle toename gezien van de niveaus van het IGF-II mRNA; de niveaus van het 3' klievingsproduct begonnen pas geruime tijd later toe te nemen, hetgeen aangeeft dat klieving een traag proces is. In het omgekeerde experiment waarbij de transcriptie werd geremd, werd de afbraaksnelheid van wild type IGF-II mRNA vergeleken met de afbraaksnelheid van mutant IGF-II mRNA dat niet meer gekliefd wordt. Beide mRNAs werden met dezelfde snelheid afgebroken. Ook op het niveau van eiwitsynthese werd geen significant verschil gezien tussen het wild type IGF-II mRNA en de gemuteerde versie. Deze resultaten laten zien dat in het gebruikte testsysteem klieving een gering effect heeft op mRNA afbraak en ook op eiwitsynthese.

Regulatie van klieving onder invloed van externe factoren werd onderzocht in humane Hep3B cellen, die endogeen IGF-II tot expressie brengen en het mRNA klieven. Het effect van verscheiden externe factoren op de efficiëntie van klieving werd getest. De invloed van trichostatine A (TSA), een histon deacetylase remmer, dexamethason, een synthetisch glucocorticoïde, aan- of afwezigheid van serum (groeifactoren) in het medium, en celdichtheid werden onderzocht. De verkregen resultaten suggereren dat TSA, dexamethason, en serum geen invloed hebben op klieving. Celdichtheid echter, heeft wel effect op klieving; bij een toename van de celdichtheid werd een toename van de niveaus van het 3' klievingsproduct waargenomen, terwijl de niveaus van het volle lengte IGF-II mRNA gelijkbleven of afnamen, afhankelijk van de frequentie van medium verversing. We hebben dus waargenomen dat klieving in Hep3B cellen gereguleerd wordt afhankelijk van de dichtheid van de cellen. De betekenis van de toename van klieving bij hoge celdichtheid is echter niet duidelijk. De ongebruikelijke stabiliteit van het 3' klievingsproduct, die waarschijnlijk veroorzaakt wordt door de geconserveerde G-rijke sequentie aan het 5' uiteinde, maakt het aantrekkelijk te speculeren dat dit RNA

wellicht een intrinsieke functie vervult in de cel. Het zal interessant zijn deze mogelijkheid experimenteel te testen.

LIST OF PUBLICATIONS

Meijer, A.H., van Dijk, E.L., and Hoge, J.H. (1996).

Novel members of a family of AT hook-containing DNA-binding proteins from rice are identified through their in vitro interaction with consensus target sites of plant and animal homeodomain proteins. *Plant Mol Biol.* 31: 607-18.

Meijer, A.H., Scarpella, E., van Dijk, E.L., Qin, L., Taal, A.J., Rueb, S., Harrington, S.E., McCouch, S.R., Schilperoort, R.A., and Hoge, J.H. (1997).

Transcriptional repression by Oshox1, a novel homeodomain leucine zipper protein from rice. *Plant J.* 11: 263-76.

van Dijk, E.L., Sussenbach, J.S., and Holthuisen, P.E. (1998).

Identification of RNA sequences and structures involved in site-specific cleavage of IGF-II mRNAs. *RNA.* 4: 1623-35.

van Dijk, E.L., Sussenbach, J.S., and Holthuisen, P.E. (2000).

Distinct RNA structural domains cooperate to maintain a specific cleavage site in the 3'-UTR of IGF-II mRNAs. *J Mol Biol.* 300: 449-67.

van Dijk, E.L. and Holthuisen, P.E. (2001).

Site-specific cleavage of the insulin-like growth factor II mRNAs. In: *mRNA binding proteins*. Eds. K. Sandberg and S. Mulrone. Kluwer Academic Publishers. MA, USA.

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CURRICULUM VITAE

Ik ben op 27 april 1972 geboren in Rotterdam. In 1990 behaalde ik het VWO diploma aan het Marnix Gymnasium te Rotterdam. In datzelfde jaar ben ik begonnen met de studie Biologie aan de Rijksuniversiteit Leiden. Tijdens de doctoraalfase volgde ik een bijvakstage bij de vakgroep 'Plantevirussen' in het Instituut voor Moleculaire Plantkunde onder begeleiding van Ir. E.A.G. van der Vossen en Prof. Dr. J.F. Bol. Vervolgens volgde ik een hoofdvakstage, eveneens in het Instituut voor Moleculaire Plantkunde in de vakgroep 'Rijstembryogenese' onder begeleiding van Dr. A.H. Meijer en Dr. J.H.C. Hoge. In augustus 1996 behaalde ik het doctoraal examen. Vanaf september 1996 was ik werkzaam als Onderzoeker in Opleiding bij de vakgroep Fysiologische Chemie waar ik, onder leiding van Dr. P.E. Holthuisen en Prof Dr. Ir. J.S. Sussenbach, het in dit proefschrift beschreven onderzoek verrichtte.