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The Equine IGF Genes

Structural and transcriptional features

Kerstin Otte

SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

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Abstract

Insulin-like growth factors 1 and 2 (IGF1 and IGF2) are small mitogenic peptides. IGF1 is mainly expressed in adult life, whereas IGF2 is one of the most ubiquitous growth factors in the mammalian embryo, where it plays an important role in the regulation of fetal growth. The encoding genes have been characterised in a number of species and show a complex structural organisation and regulation of expression. Furthermore, IGF2 it is subject to epigenetic modification and genomic imprinting.

The present study aimed at the basic structural and functional characterisation of the equine IGF1 and IGF2 genes.

cDNAs for both equine IGF1 and IGF2 were cloned and showed high sequence homology when compared to other species. Even more conserved is the deduced amino acid sequence of both peptides. The equine IGF2 gene was cloned and characterised. Sequence comparison revealed three non-coding leader exons and three coding exons. Downstream of the polyadenylation site a dinucleotide repeat sequence was identified. A novel structural element, an inverted repeat, is predicted. It is conserved between species and located in a region which is differentially methylated in the human and mouse genes and possibly involved in the imprinting mechanism. The inverted repeat acquires a stem-loop structure with a hybrid A/B-DNA conformation in the stem area and binds a specific, methylation sensitive protein which is developmentally regulated.

A number of transcripts were detected for both equine IGF1 and IGF2 genes. In the IGF2 gene, two promoters (P2 and P3) were shown to be active in fetal tissues and one promoter (P3) was found to be active in adult tissues. This represents a transcriptional pattern different from that in humans or rodents. Furthermore, IGF2 transcripts were located at tissue level using in situ hybridisation.

The equine IGF2 gene was physically mapped to ECA 12q13 and homoeology of this chromosome to HSA11 was revealed.

Key words: insulin-like growth factor, equine, cloning, transcription, physical localisation, stem-loop, imprinting

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Contents

Appendix

Papers I - IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Otte K and Engström W (1994). Insulin-like growth factor II in the horse: determination of a cDNA nucleotide sequence and expression in fetal and adult tissues. Gen Comp Endocrinol 96: 270-275

II. Otte K, Rozell B, Gessbo Å, Engström W (1996). Cloning and sequencing of an equine insulin-like growth factor I cDNA and its expression in fetal and adult tissues. Gen Comp Endocrinol 102: 11-15

III. Otte K, Choudhury D, Engström W, Rozell B. A conserved structural element in the equine IGF2 gene binds a methylation sensitive factor. Submitted to Nucleic Acids Res

IV. Raudsepp T, Otte K, Rozell B, Chowdhary BP (1997). FISH mapping of the IGF2 gene in horse and donkey - detection of homoeology with HSA11. Mamm Genome 8 (8): 569-572

Abbreviations

bp	base pair
BTA	Bos taurus
BWS	Beckwith-Wiedemann syndrome
CD	circular dichroism
CBP	cruciform binding protein
C/EBP	CAAT/enhancer binding protein
CNS	central nervous system
DAPI	4',6-diamidino-2-phenylindole
Dig	digoxigenin
DNA	deoxyribonucleic acid
ECA	Equus caballus
EAS	Equus asinus
FISH	flourescent insitu hybridisation
GH	growth hormone
GRE	glucocorticoid responsive element
HMG	high mobility group
HBB	haemoglobin β
HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homologue
HSA	Homo sapiens
IDDM2	insulin-dependent diabetes mellitus
IGF	insulin-like growth factor
IGF2/Mpr	IGF2/cation-independent mannose-6 phosphate receptor
IGFBP	IGF binding protein
INS	insulin
kb	kilo base
LAP	liver-enriched activating protein
LQT1	Long QT syndrome, Ward-Romano syndrome
MEU	Macropus eugenii
MMU	Mus musculus
mRNA	messenger RNA
mRNP	messenger ribonucleoprotein particle
NMR	nuclear magnetic resonance
OAR	Ovis aries
PFGE	pulse field gel electrophoresis
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
RT-PCR	reverse transcription PCR
SP	signal peptide
TH	tyrosine hydroxylase
UTR	untranslated region
WT-1	Wilms´ tumor suppressor gene 1

Introduction

The IGF system

The insulin-like growth factors IGF1 and IGF2 are members of a larger family of structurally related peptides which include insulin, IGF1, IGF2 and relaxin (Blundell and Humbel 1980, Dull et al 1984, Dafgård et al 1985) (Fig 1). The IGFs were discovered 40 years ago as molecules mediating GH action (Salmon and Daughaday 1957) and subsequent purification and amino acid sequence determination revealed two separate molecules (Rinderknecht and Humbel 1978a, Rinderknecht and Humbel 1978b).
Named after their high degree of homology with insulin, IGF1 and IGF2 are single chain polypeptides and consist of 70 and 67 amino acids, respectively (Daughaday and Rotwein 1989). Both mature peptides consist of four domains (A, B, C, and D). The A, B and C domains share strong homology with pro-insulin (Fig 1). Both IGFs are synthesised as precursors composed of a signal peptide, the mature peptide, and a trailer peptide. In IGF1, several signal and trailer peptides combine to make up different precursors. These are posttranslationally processed to yield identical mature peptides.
NMR solution structures have been determined for human IGF1 and IGF2 and predict structures similar to insulin, in accordance with earlier computer graphic modelling studies (Blundell et al 1978, Dafgård et al 1985, Cooke et al 1991, Sato et al 1992, 1993, Terasawa et al 1994, Torres et al 1995).

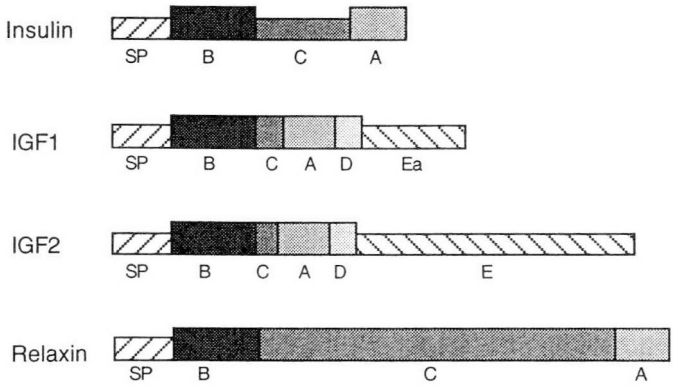

Fig 1. Structure of the members of the insulin peptide family. These proteins share structural similarities in the SP, A-, B-, C-, and D-domains as well as the E-peptides. Different domains in the proteins are indicated as boxes. Full size boxes indicate the mature peptide, while half size boxes indicate posttranslational removed domains. IGF1 is shown with the Ea peptide.

Physiology and functional role

Originally, the IGFs were considered to act in an endocrine manner as mediators of GH action. Later, they were shown to act also in a paracrine (local cell to cell response) or autocrine (cell responding to signalling molecules made by itself) fashion. The liver is the main source for plasma IGFs; IGF1 expression in the liver is highly regulated by GH and nutritional factors. In contrast to classical hormone action, the IGFs are synthesized by cells in nearly all tissues (Han et al 1987, Daughaday and Rotwein 1989) and often at developmental stages when these tissues are undergoing rapid growth. In addition, IGF receptors are expressed either by IGF-synthesising cells or by adjacent cells, thereby facilitating autocrine or paracrine actions.

In cultured cells, the IGFs stimulate a wide variety of growth and growth-related responses. They have been shown to induce proliferation and differentiation, to counteract apoptosis and enhance cell survival, and to exert insulin-like anabolic effects (rev Jones and Clemmons 1995).

The essential growth promoting functions of the IGFs during fetal development were demonstrated in knockout mice carrying null mutations for the genes encoding IGF1 and IGF2. Absence of IGF1 causes a decreased body weight at birth of around 60% of normal weight, while body proportions remain normal. These mice also show a reduced postnatal growth rate and high neonatal lethality (Liu et al 1993, Baker et al 1993). Mice lacking IGF2 show decreased prenatal growth rates and reach 60% of normal body weight at term; postnatal growth rates are normal (DeChiara et al 1990).

Creation of transgenic mice overexpressing IGFs have furthered the understanding of IGF function, as overexpression of a gene might amplify its action in vivo. These transgenics provide long-term exposure to IGFs and are capable of increasing IGF expression in specific tissues. Overexpression of human IGF1 in transgenic mice resulted in increased body weight and a tendency towards overgrowth was observed; variations in local expression caused disproportionate growth of specific tissues and spontaneous tumour formation (Mathews et al 1988, Coleman et al 1995, Reiss et al 1996, Bol et al 1997). Overexpressing IGF2 in transgenic mice affected body composition but not overall body growth; local transgene expression caused overgrowth of specific organs and increased development of tumours (Ward et al 1994, Wolf et al 1994, Rogler et al 1994, Bates et al 1995, van Buul-Offers et al 1995, Rossetti et al 1996).

Receptors

The IGFs are able to bind to and possibly exert their actions through three different membrane receptors, the insulin receptor, the type-1 IGF receptor and the type-2 IGF receptor, which is the equivalent of the IGF2/Mpr (Ullrich et al 1986, Morgan et al 1987). Each receptor-growth factor interaction shows different kinetic properties. The type-1 receptor has the highest affinity for IGF1, the type-2 receptor for IGF2, and the insulin receptor binds both molecules with low affinity (Steele-Perkins et al 1988, Nissley et al 1991, Werner et al 1992,).

The type-1 receptor is a heterodimeric trans-membrane tyrosine kinase consisting of two α/β subunits, with strong homology to the insulin receptor. After ligand binding and subsequent autophosphorylation of the receptor, a cascade of intracellular signalling takes place that mediates most of the biological effects of the IGFs (de Meyts et al 1994, rev Jones and Clemmons 1995). Recently, also the insulin receptor was shown to stimulate cell proliferation after binding of IGF2, but not IGF1 (Morrione et al 1997).

The type-2 IGF receptor is a monomer consisting of a large extracellular domain containing fifteen repeats of a cystein rich motif, a single transmembrane helix and a small cytoplasmic region. It binds mannose-6-phosphate and IGF2 at different sites of the molecule (Braulke et al 1988, Garmroudi and MacDonald 1994). IGF2 binding has not been shown to induce phosphorylation of this receptor, but has been suggested to cause receptor redistribution (Braulke and Mieskes 1992) and to promote exocytosis in insulin-secreting cells (Zhang et al 1997). Also, it might function as a scavenger which binds and degrades excess IGF2 in the embryo. This view is supported by the rescue of prenatal lethal IGF2/Mpr knockout mice through introduction of an IGF2 null allele (Barlow et al 1991, Filson et al 1993, Wang et al 1994).

Binding proteins

IGF1 and IGF2 peptides circulating in serum are mostly associated with high affinity binding proteins, IGFBPs (Baxter and Martin 1989). At least six different IGFBPs have been identified and cloned in man, along with six binding proteins from rat and several from pig, cow and sheep (rev Shimasaki et al 1991, rev Rechler 1993). Additional members have been proposed in rat and man (Chan and Nicoll 1994, Wilson et al 1997). The binding proteins differ in their biochemical and physiological characteristics as well as in their relative affinities for IGF1 and IGF2. The functions of the binding proteins have been proposed to prolong the half-life of the IGFs, to act as transport proteins for IGFs in serum and across the endothelial barrier of blood vessels, to inhibit or facilitate IGF action, and to store IGF peptides (Clemmons 1993, rev Jones and Clemmons 1995).

The molecular biology of the IGFs

IGF1 gene structure

The genomic organisation of the IGF1 gene has been determined in a number of species, including man (Rotwein et al 1986b), rat (Shimatsu and Rotwein 1987), sheep (Dickson et al 1991), chicken (Kajimoto and Rotwein 1989), and salmon (Kavsan et al 1993). cDNAs have been cloned from these and other species and sequence comparison reveals a generally conserved gene structure, although an increase in the size of introns occurred during evolution. Whereas the salmon IGF1 gene is less than 20 kb in length, the chicken IGF1 gene is ca 50 kb in length and the rat and human genes are from 80 to 100 kb in length. The IGF1 gene has been mapped to human chromosome 12 (Brissenden et al 1984, Tricoli et al 1984) and mouse chromosome 10 (Taylor et al 1991).

The IGF1 gene consists of up to 6 known exons (Fig 2). The chicken and mammalian genes contain two different 5′ leader exons (termed 1 and 2), whereas in xenopus and salmon IGF1 genes only exon 1 is present. In rat and man, the leader exons are arrayed in tandem with an intervening intron and each is driven by its own promoter (Adamo et al 1991, Kajiamoto and Rotwein 1991, Kim et al 1991, rev Sussenbach et al 1992) (Fig 2). Exons 3 and 4 are common to all IGF1 genes and encode parts of the signal peptide, the mature peptide and the common region of the E-peptide. The most 3′ exons encode different E-peptides. In chicken, the E-peptide is encoded by parts of the last two exons. In salmon and mammals, however, the two last exons are alternatively spliced which results in IGF1 mRNAs encoding multiple E-peptides (Rotwein et al 1986, Steenbergh et al 1991, Chew et al 1995).

IGF1 promoters

IGF1 promoter structures are highly conserved in all species analysed. The precise location and nature of the core promoters have not been determined, although minimal promoter regions have been suggested lately (Wang et al 1997). No TATA or CCAAT-box could be identified, nor are the promoter regions GC-rich, which might be the reason for multiple sites of transcription initiation in the IGF I promoters (Jansen et al 1991, Kim et al 1991, Hall et al 1992, Ohlsen et al 1993). However, specific binding of nuclear proteins has been observed (Thomas et al 1995, LeStunff et al 1995, An et al 1995, Pao et al 1995) and regions or elements that are responsive to transcription factors have been identified (Nolten et al 1994, 1995, Delany and Canalis 1995, Thomas et al 1996).

IGF1 gene expression

The transcription of IGF1 has been detected in a variety of tissues from several species (Rotwein 1986a, 1987, Lund et al 1986, Beck et al 1987, Tavakkol et al 1988, Wong et al 1989). IGF1 transcripts are most abundant in

adult tissues and the highest levels are found in adult liver; lower levels are present in a variety of non-hepatic tissues.
Transcripts containing leader exons 1 or 2 are distributed in a tissue-specific manner, with exon 1 containing transcripts being predominant in all rat and human tissues examined, and exon 2 derived transcripts occurring at relatively high levels in liver, and at low levels in some tissues as kidney, testes, lung and stomach. In addition, they appear at different times during development (Lowe et al 1987, Hoyt et al 1988, Adamo et al 1991, Jansen et al 1991).
Transcripts containing different E-peptide coding sequences are present at various levels: EaIGF1 mRNAs are predominant in all tissues examined, EbIGF1 transcripts show low abundance and EcIGF1 transcripts has only been detected in liver in low amounts (Lowe et al 1988, Nagaoka et al 1991, rev Ward and Ellis 1992, Chew et al 1995).
The IGF1 mRNA populations arising from the diversity of transcriptional mechanisms differ among species: salmon IGF1 mRNAs appear to be a single species, whereas xenopus, chicken, and mammalian IGF1 mRNAs occur as multiple species ranging in size from around 0.8 to around 7.5 kb. The differences in size depend mainly on the usage of the different polyadenylation sites (Lund et al 1989).

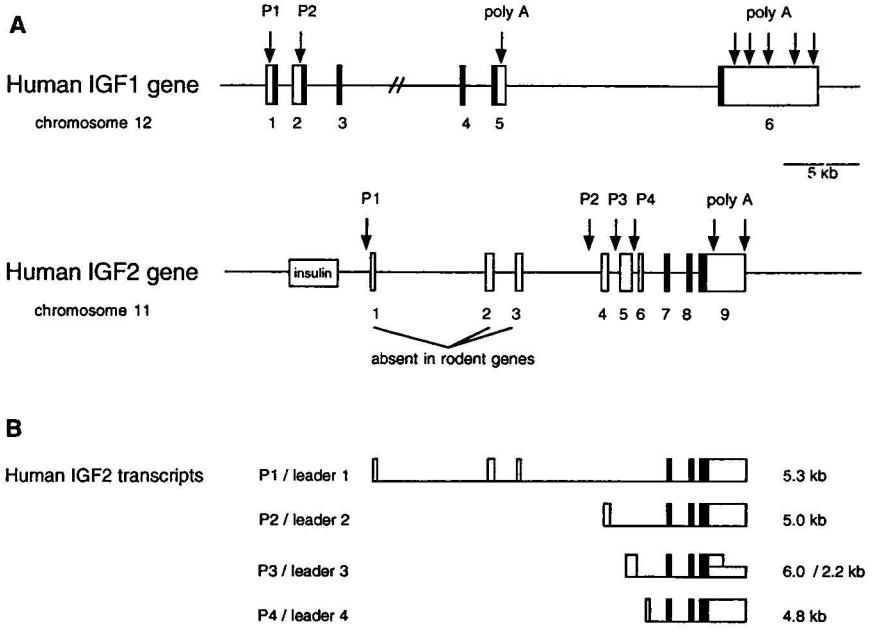

Fig 2: Schematic structure of human IGF1 and IGF2 genes. A) Exon/intron organisation, promoters and polyadenylation sites in the human IGF1 and IGF2 genes are indicated. Coding exons are shown as solid boxes, non coding sequences as open boxes and introns as lines. The chromosomal localisation is given on the left side. B) Transcripts and respective length derived from human IGF2 promoters P1-P4 are shown below. Half size boxes indicates differential usage of polyadenylation sites.

IGF2 gene structure

IGF2 cDNAs have been cloned from a variety of species and show strong sequence conservation between species including human (Bell et al 1984, Jansen et al 1985), rat (Dull et al 1984, Whitfield et al 1984), mouse (Bell et al 1986), sheep (O'Mahoney et al 1991), mink (Ekström et al 1993), cow (Boulle et al 1993), and pig (Catchpole and Engström 1990). Despite this, complete sequence information is only available for the rat and mouse IGF2 genes (Ikejiri et al 1990, Sasaki et al 1996) and large parts of the human and ovine genes (Dull et al 1984, de Pagter-Holthuizen et al 1986, 1987, Holtuizen et al 1990, Ohlson et al 1994).

The human IGF2 gene spans around 30 kb DNA on human chromosome 11p15.5 (Brissenden et al 1984, de Pagter-Holthuizen et al 1987, van Dijk et al 1991). The rodent IGF2 genes cover a 12 kb stretch and are located on mouse chromosome 7 and rat chromosome 1 (Frunzio et al 1986, Soares et al 1986, Rotwein and Hall 1990, Zemel et al 1992, Goldmuntz et al 1993) (Fig 3).

In all species hitherto examined, the IGF2 gene shows a complex genomic organisation. It consists of at least 9 exons in humans (Holthuizen et al 1991, Ikejiri et al 1991b), 9 exons in sheep (Ohlsen et al 1994) and 6 exons in rat and mouse (Frunzio et al 1986, Soares et al 1986, Ueno et al 1987, Rotwein and Hall 1990, Ikejiri et al 1990, 1991a) (Fig 2).

The 5´ exons are non-coding and driven by different promoters. In humans, four identified promoters (P1- P4) direct the tissue- and development-specific transcription of six leader exons (De Pagter-Holthuizen et al 1987, 1988, Holthuizen et al 1990), whereas in rodents only three promoters (P1-P3) have been identified that drive transcription of three leader exons (Soares et al 1986, Evans 1988, Matsagushi et al 1990). The additional, most 5´ located promoter P1 present in the human gene has also been identified in sheep and baboon (Jin et al 1993, Ohlsen et al 1994). The three 3´ exons are common to all IGF2 genes and encode a signal peptide, the mature peptide and a trailer peptide. The generated transcripts differ in their 5' untranslated regions, but have identical coding regions. In addition, heterogeneity in the 3´ region of the mRNAs is due to differential use of two polyadenylation sites (Fig 2).

IGF2 gene expression

The human IGF2 gene is expressed in a wide range of fetal tissues, including liver, adrenal and skeletal muscle with very high levels, kidney, skin and pancreas display intermediate levels. Low levels of IGF2 mRNA are detected in intestine, lung, heart, stomach, spleen and brain (Scott et al 1985, Gray et al 1987, Han et al 1987). After birth the IGF2 level declines significantly and tissues such as liver, kidney, skin, peripheral nerve, muscle, colon, uterus, stomach, hypothalamus, adrenal, granulosa cells and lung express IGF2 at low levels compared with fetal levels. Also in rodents IGF2 is abundantly expressed during fetal development and neonatal tissues, but levels decline

during late fetal development and persist in the adult only in the brain and spinal cord (Soares et al 1985, 1986, Brown et al 1986, Lund et al 1986, Beck et al 1987, Gray et al 1987, Murphy et al 1987, 1988, Lee et al 1990).

In humans, transcripts derived from P1 are only expressed in adult liver and choroid plexus/leptomeninges and contain an internal ribosomal entry site in their leader sequence (Ohlsson et al 1994, Li et al 1996, Teerink et al 1995). Transcripts derived from the P2 promoter are present at low levels in fetal liver but more abundantly in certain human tumour cell lines. These transcripts, as well as mRNA derived from P4, have been shown to be completely polysomal and actively engaged in protein synthesis (Ikejiri et al 1991b, Nielsen et al 1990, De Moor et al 1994). Transcripts derived from promoters P3 and P4 are abundant in many fetal tissues and non hepatic adult tissues, with the P3 promoter being predominantly used. Human P3 derived mRNAs are mainly present as part of untranslated mRNP and are selectively mobilised and translated in dispersed exponentially growing cells. In mouse, P3 transcipts were shown to disengage from polysomes during development (dePagter-Holthuizen et al 1987, 1988, Schofield and Tate 1987, Nielsen et al 1990, De Moor et al 1994, Newell et al 1994, Ohlsson et al 1994, Nielsen et al 1995, Li et al 1996). Expression from the P2, P3 and P4 promoters is reduced during adult life.
In rodents, IGF2 transcripts are derived from all three promoters during fetal life, with promoter P3 (homologous to human P4) being predominantly used. During adult life, transcription from all promoters is strongly repressed (Frunzio et al 1986, Soares et al 1986, Ueno et al 1988).

IGF2 transcripts are processed and thereby degraded by endonucleolytic cleavage downstream from the translation termination codons (3'UTR) in rat and man, respectively. The cleavage site is situated in a highly conserved and structured domain that exhibits two large hairpins and an intramolecular guanosine quadruplex (Christiansen et al 1994), providing binding sites for trans-acting factors (Scheper et al 1996a). The endonucleolytic cleavage of IGF2 transcripts seems to be growth-condition-dependent (Scheper et al 1996b).

IGF2 promoters
Characterisation of the IGF2 promoters identified a number of functional elements and transcription factor binding sites.
The human P1 promoter contains no TATA or obvious CCAAT box although there is a single functional SP1 site (van Dijk et al 1991, Rodenburg et al 1997). P1 is activated by C/EBP a and b and LAP (Van Dijk et al 1991, 1992, Sussenbach et al 1993, Rodenburg et al 1995) and may be down-regulated by two inverted repeat elements which are bound by a protein factor (Rodenburg et al 1996).

The human P2 promoter and the corresponding rodent P1 promoter are weak promoters. They contain no known consensus promoter elements and show great heterogeneity in their transcription start sites (Ueno et al 1987, Holthuizen et al 1990a, Van Dijk et al 1991).
The human P3 promoter is a typical RNA polymerase II driven promoter (dePagter-Holthuizen et al 1987, Van Dijk et al 1991) and contains TATA and CCAAT boxes along with two Sp1 sites and two Erg-1 binding sequences. Its structure and sequence is strongly conserved in all mammals examined (Soares et al 1986, Frunzio et al 1986, Evans et al 1988, O´Mahoney et al 1991, Boulle et al 1993). The human P3 promoter is repressed by binding of WT-1, (Drummond et al 1992) and might also be regulated by p53 (Zhang et al 1996). The rat P2 homologue was shown to be downregulated by glucocorticoids in the rat neonate (Levinovitz and Norstedt et al 1989, Kitraki et al 1992, Senior et al 1996) and several putative GREs have been suggested to mediate glucocorticoid action in human and rodent neonate (Beck et al 1988).
The human P4 promoter is strongly homologous to rodent P3 and contains a TATA box along with multiple Sp1 binding sites (Evans et al 1988, Van Dijk et al 1991). It is transactivated by the Ap1 complex (Caricasole et al 1993) and repressed by WT-1, like mouse P2 and P3 (Ward et al 1995, Duarte et al 1997).

Genomic Imprinting

Genomic imprinting is a form of developmental gene regulation which causes the expression of a gene according to its maternal or paternal origin (Solter 1988). A number of imprinted genes have been discovered to date, among those IGF2 (DeChiara et al 1991) and its receptor IGF2/Mpr (Barlow et al 1991).

Genomic imprinting of IGF2

The IGF2 gene is subject to genomic imprinting, such that only the paternal allele of IGF2 is transcribed while the maternal allele is silent. Uniparental allelic expression of IGF2 has been demonstrated in human (Giannoukakis et al 1993, Ohlsson et al 1993, Rainier al 1993), mouse (DeChiara 1991) and rat tissues (Pedone et al 1994), except human adult liver and human and murine CNS where both parental alleles are actively transcribed. In addition, promoter specific imprinting has been reported for human IGF2. During fetal life, promoters P2, P3 and P4 are expressed from the paternally derived allele in all IGF2 expressing tissues except the leptomeninges and choroid plexus where biallelic expression occurs. Promoter P1 is biallelically expressed both during fetal and adult life (Giannoukakis et al 1993, Ohlsson et al 1993, 1994, Vu and Hoffman 1994, Ekström et al 1995). Additionally, loss of IGF2 imprinting has been found in a number of human tumours (Ogawa et al 1993, Rainier et al 1993, Zhan et al 1994).

Mechanisms of genomic imprinting

The molecular basis of parental imprinting is still largely unknown, but the possibility of DNA methylation being involved in the imprinting mechanism is supported by knockout mice deficient for DNA methyltransferase that show aberrant expression of both IGF2 and the oppositely imprinted H19 gene (Li et al 1993).

Parent-specific methylation patterns have been detected in all imprinted genes examined, including IGF2, IGF2/Mpr and H19 (Stöger et al. 1993, Brandeis et al 1993, Feil et al 1994, Bartolomei et al 1993, Tremblay et al 1995). In the mouse IGF2 gene, two differentially methylated and DNase I hypersensitive regions have been found, one located 5′ of the first exon and one in the 3′ region of the gene. Both regions are more methylated on the expressed paternal allele (Sasaki et al 1992, Feil et al 1994, 1995). Interestingly, the methylation of the 3′ region is tissue-specific and appears to correlate with expression of the gene. Methylation of these regions, however, is not thought to constitute the primary imprinting signal but to play a part in maintaining the imprint (Razin and Cedar 1994).

Further support for DNA methylation governing genomic imprinting comes from studies using inhibitors of DNA methyltransferase, where an increased

expression (Eversole-Cire et al 1993, Hu et al 1996) or a switch from biallelic to monoallelic expression of IGF2 was observed upon demethylation (Barletta et al 1997).

Imprinting might also be regulated by more global mechanisms via a domain effect, addressing a cluster of imprinted genes. Mouse IGF2 is physically closely linked to the imprinted genes Mash2, Ins-2, H19 and $p57^{KIP2}$ (Guillemot et al 1995, Deltour et al 1995, Bartolomei et al 1991, Hatada and Mukai 1995). The H19 gene is imprinted in a manner opposite to that of IGF2, being expressed from the maternal allele and undergoing parental specific methylation (Bartolomei et al 1991, 1993, Ferguson-Smith et al 1993, Feil et al 1994, Tremblay et al 1995). Imprinting and expression of the H19 and IGF2 genes might be mechanistically linked. An enhancer competition model has been proposed, where promoters of both genes interact with a single enhancer element located downstream of H19 (Li et al 1993, Bartolomei et al 1993, Leighton et al 1995).

Furthermore, repeat sequences and alterations in chromatin structure have been suggested as additional features in the imprinting mechanism (Neumann et al 1995, Banerjee and Smallwood 1995).

Role of genomic imprinting

The question why imprinting exists is obviously important and a number of hypotheses have been proposed. Parental imprinting has been suggested to be an adaptation to prevent parthenogenetic development (Solter 1988), an expression of genetic conflicts between maternal and paternal genomes (Haig and Westoby 1989), an outcome of dominance modification (Sapienza 1989), a way of restraining placental growth (Hall 1990), a mechanism of growth factor regulation (Cattanach 1991), a consequence of host defence mechanisms (Barlow 1993) and a device to protect females against developing malignant germ-cell tumours (Varmuza and Mann 1994). The reciprocal imprinting of IGF2 and IGF2/Mpr providessome support for the genetic-conflict hypothesis (Haig and Graham 1991) which proposes that the maternally produced IGF2/Mpr functions as a sink to internalise and degrade paternally produced IGF2 before the growth factor can bind to its receptor.

Conserved chromosome regions

IGF2 linkage group

IGF2 is localised on human chromosome 11p15.5 (Brissenden et al 1984) closely linked to the INS and TH genes, in one of the most densely mapped chromosomal regions in the human genome. This region harbours several disease loci as BWS, IDDM2, and LQT1 (Junien and van Heyningen 1991, Higgins et al 1994). Several types of childhood tumours, including Wilms tumour, adrenocortical carcinoma, and rhabdomyosarcoma display a specific loss of maternal 11p15 alleles. This region also contains a cluster of imprinted genes, including $p57^{KIP2}$ situated 500 kb centromeric to IGF2 and H19 about 100 kb telomeric to IGF2 (Leibovitch et al 1991, Matsuoka et al 1996).

The mouse IGF2 gene maps to the distal region of chromosome 7, within a cluster of imprinted genes, including Mash2, $p57^{KIP2}$, Ins-2 and H19 (see section Genomic imprinting). This chromosomal region is highly conserved as compared to human 11p (fig. 3).

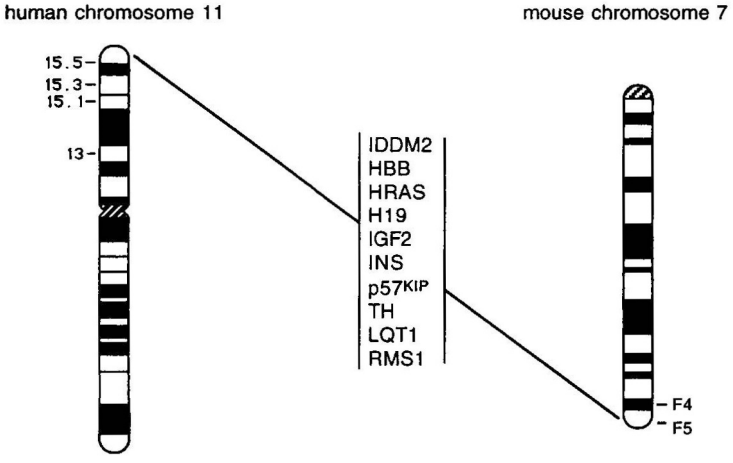

Fig. 3. Representation of the conserved autosomal region on human chromosome 11 and its homologous region on mouse chromosome 7.

Comparative Genomics

Conserved clusters of genes can be used for the transfer of genetic data from the well developed human or mouse gene maps to lower density maps and facilitate rapid assignment of functional genes (Hudson et al 1995, Dib et al 1996, Dietrich et al 1996). Gene maps are available for a number of animals including, cattle, sheep, pigs, and chickens. The equine gene map, however, is one of the most poorly developed with less than 200 established loci (Ellegren et al 1992, Bailey et al 1995, Breen et al 1997). A rapid expansion of the poorly developed equine gene map can be helped by comparative genome analysis and cross-species chromosome hybridisation utilising FISH has revealed conserved segments between most human and horse chromosomes (Raudsepp et al 1996).

Numerous equine genetic disorders are documented, but little is known about these conditions at DNA level (Bowling 1992). A developed physical and genetic linkage map of the horse genome will enable any future research on inherited disorders and their underlying genetics.

Previous research on equine IGFs

The issue of IGF1 and IGF2 levels in equine serum has only been adressed in a few studies. Both peptides are found in adult horse serum (Zangger et al 1987). By radioimmunoassay the IGF1-level was found to be 115 µg/ ml on average. The mean IGF2 concentration was determined by a radio-receptorassay and shown to be 149 µg/ ml. Thus, compared to humans and most other animals the horse is characterised by relatively low blood IGF concentrations. In a later study the mean plasma IGF1 concentration was demonstrated to be higher in male than in female horses (Ozawa et al 1995). No clear-cut difference in plasma concentrations was found between breeds with different weight characteristics (Ozawa et al 1995). In addition, five IGF binding proteins are present in equine blood as determined by a radio-ligand assay (Christensen et al 1997).

One study describes the expression of IGF2 in equine conceptuses at different stages of development (Lennard et al 1995). In northern blot analysis, an ovine IGF2 oligonucleotide probe hybridized to a total of seven transcripts (6.2-1.3 kb) in horse fetal liver. In situ hybridization using the same ovine probe demonstrated IGF2 gene expression in the fetus at all stages examined, predominantly in tissues of mesodermal origin.

Aims of the present study

The present thesis aimed at studying the basic properties of the IGF genes in the horse. In particular, the focus of the study was:

- to clone equine IGF1 and IGF2 cDNA, thus providing tools for further investigations.
- to elucidate the genomic sequence and structure of equine IGF2 and thereby make an extended interspecies sequence comparison possible.
- to study gene expression and promoter usage of equine IGF2.
- to physically localise IGF2 in the equine genome and reveal the homoeologous human chromosome.

Comments on methodology

In this chapter, only materials and methods that are not included in any paper will be discussed in detail. All other methods used in this thesis are thoroughly described in each publication, and will therefore only be briefly discussed.

Gene Cloning

Cloning of a specific cDNA can be performed by RT-PCR, taking advantage of gene regions with high homology between species for primer design. RT-PCR has the advantage of being comparatively fast, but will restrict the resulting cDNA to areas of high conservation between species. Moreover in most cases it will not provide full-length cDNAs. In addition, sequence errors may be introduced by the Taq-DNA polymerase. RT-PCR will therefore be the method of choice if the cDNA is to be used as a probe to access further features of the gene, e.g. expression, genomic cloning or evolutionary studies. An alternative method to provide full-length transcripts is the screening of a cDNA library. However, no suitable fetal equine cDNA library is currently available.

In order to clone the entire equine IGF2 gene including intron and exon regions, the equine IGF2 cDNA was used as a probe to screen a equine genomic phage library. To reach maximal sensitivity during the screen, a homologous equine cDNA and radioactive labelling was used instead of non-radioactive methods (e.g. Dig-labelling). An alternative method to clone large genomic DNA fragments would be the recently developed "long range PCR" (Barnes 1994), where a modified PCR protocol in combination with a proof-reading DNA polymerase enables the amplification of up to 15 kb DNA fragments. This procedure was tested prior to the library screening, but did not give satisfying results. There are several explanations for this, including the use of non-homologous PCR primers.

Studies on gene expression

Northern blot hybridisation was used in this study to examine expression patterns of the IGF1 and IGF2 genes in the horse. This method can determine the size and amount of any specific RNA in a certain tissue.

RNase protection analysis is more sensitive than Northern blot hybridisation to analyse gene expression and can also be used to quantify the amount of transcripts. In addition, RNase protection is less sensitive to degradation of the sample RNA. In the present study this technique was preferentially used to determine promoter usage of IGF2 in hepatic and non-hepatic equine tissues. Promoter-specific probes were subcloned from the isolated lambda phage containing the equine IGF2 gene. However, quantification of transcripts was not possible because adequate internal controls are at present not available for the horse.

Among hybridisation technologies, in situ hybridisation is unique in demonstrating of the presence of specific nucleic acids in their cellular environment. The method is based on hybridisation of a labelled probe directly to the tissue. To localise IGF2 transcripts at a cellular level, in situ hybridisation was performed on a variety of equine tissues.
In situ hybridisation was essentially performed according to Schaeren-Wiemers and Gerfin-Moser 1993. Briefly, tissues directly frozen in isopenthane cooled by liquid nitrogen, were sectioned in a cryostate and the sections attached to Vectabond treated glass slides. The sections were sequentially reacted with 4% paraformaldehyde in PBS for 10 min, washed in PBS for 3x 5 min and acetylated with acetic anhydride in triethanolamine. Prehybridisation was carried out at RT for 1 hour with a solution containing 50 % formamide, 5x SSC, 5x Denhardt's, 500 µg/ml of sheared and denatured salmon sperm DNA and 250 µg/ml of yeast tRNA. Digoxigenin labelled sense and antisense single-stranded RNA probes, corresponding to equine IGF2 exon 2 (fig 4) and the coding cDNA sequence, were produced using the Dig RNA Labeling Kit (Sp6/T7) (Boehringer). The respective probes were diluted 1/200 in prehybridisation buffer and hybridised with the sections over night at 57 °C. The slides were washed with 5x SSC at 57 °C for 5 min, treated with RNase A in NTE buffer at 37 °C for 30 min. Finally, they were washed in 0.2x SSC at 57 °C for 30 min followed by 0.2x SSC at RT for 5 min. The sections were developed with anti-Dig alkaline phosphatase and NBT/BCIP as recommended by Boehringer-Mannheim.

DNA-protein interaction

DNA binding of proteins can be studied by gel mobility shift assays. The method is based on the different mobility of a DNA-protein complex compared with the free DNA in a polyacrylamide or agarose gel. The advantages of the method are that the composition of the protein complex can be visualized, e g to distinguish monomer and dimer formation, and many samples can be analysed in parallel. However, the method is not completely quantitative and the exact binding site of the protein can not be determined.

Gene mapping

A physical gene map is a classification where loci are arranged with respect to their relative position on a chromosome. This can be performed by FISH with a labelled DNA probe on whole chromosomes or by hybridisation of a labelled DNA probe to large restriction fragments of genomic DNA separated by PFGE (Zemel et al 1992). In the present study, IGF2 was physically mapped to ECA12q13 by FISH using genomic subclones of the equine gene. A genetic linkage map arranges loci according to the frequency of recombination between polymorpic DNA markers. The repeat area in the equine IGF2 could be used to provide a linkage map of the gene. However, additional markers are required to accomplish this.

Results and Discussion

Cloning of equine IGF1 and IGF2 genes

cDNA cloning (papers I and II)
Prior to the present study no sequence information on equine IGF1 and IGF2 genes was available. In papers I and II, the cloning and subsequent sequencing of partial equine cDNAs for IGF1 and IGF2 using RT-PCR is described.

The reported equine IGF1 cDNA covers exons homologous to parts of human exon 1 (encoding parts of the signal peptide), exon 3 (encoding parts of the signal as well as mature peptide), and exon 4 (encoding parts of the mature and the E-peptide which is common to all human E-peptide variants).
The IGF1 nucleotide sequence and deduced amino acid sequence are both highly conserved among species. The coding region for the mature peptide shows 96% homology to human and pig sequences and 88% homology to rodent genes (Tab 1). The amino acid sequence is even more conserved. The mature equine IGF1 peptide consists of 70 amino acids and is 100% homologous to its human counterpart. The signal peptide is slightly less conserved with 97% homology to human and 91% to rodent peptides. Recently sequence information of the 3´ exons of equine IGF1 became available (Genbank Accnr: U85272, U85271, Feb 1997), coding for the entire common E-peptide as well as Ea and Eb peptides. Translation into amino acid sequence and comparison to human IGF1 reveals only one substitution in both the common E peptide (threonine to alanine), and in the Ea peptide (alanine to threonine). The Eb peptide is shorter than its human counterpart, comprising only 17 amino acids of which four are substituted

The amplified IGF2 cDNA covers parts of human exon 7 (encoding signal and parts of the mature peptide), exon 8 (encoding parts of the mature peptide), and the 5´ part of exon 9 (encoding parts of the mature and E peptide).
Sequence comparison of IGF2 shows high degrees of homology on both nucleotide and amino acid levels with other species. The coding region for the mature peptide displays a homology of 94% to pig and 92 and 90% to human and rat sequences (Tab 1). By combining the sequence data generated from equine IGF2 genomic clones (paper III), the nucleotide sequence coding for the entire IGF2 precursor pre-pro-peptide becomes available. The putative precursor protein includes a 24 amino acid leader peptide, a 67 amino acid mature paptide, and a 90 amino acid E peptide. The mature peptide is most conserved with a homolgy of 97% to its human counterpart (Tab 1). The only amino acid substitutions in the mature protein compared to its human counterpart are placed in the C-domain (isoleucine 35 was substituted with a valine and asparagine 36 with a serine), which shows together with the D-

domain in general the highest clustering of differences. The signal peptide shows a homology of 83% to human and 75 and 66% to rodent and pig peptides, respectively. The E-peptide is slightly less conserved with 81 and 71% homology to its human and rodent counterparts.

organism	*IGF1 nucleotide sequence*	*IGF1 amino acid sequence*	*IGF2 nucleotide sequence*	*IGF2 amino acid sequence*
pig	96	100	94	99
human	96	100	92	97
sheep	92	99	91	97
cattle	92	100	90	99
rat	88	96	90	96
mouse	88	95	89	93
xenopus	80	84	–	–

Tab. 1. Homology between horse and other species of nucleotide sequences coding for mature IGF peptides and deduced amino acid sequences. Values are given in percent.

There are several possible explanations for the remarkable degree of conservation of IGF DNA and protein sequences during evolution. It almost certainly reflects the structure/function relationships within these small peptides with multiple interactive surfaces. Biological responses of IGFs are dependent upon their interaction with cellular receptors, and distinct regions in domains A and B in the IGF peptides have been demonstrated to be important for type 1 and type 2 receptor binding (Baynes et al 1988, Bayne et al 1990, Clemmons et al 1990, Hodgson et al 1996, Shooter et al 1996, Jansson et al 1997), which might favour high degree of sequence conservation in these domains during evolution. The interaction of the IGFs with their binding proteins in the A and B domains, structures that only partly overlap with those determining receptor binding (Luthi et al 1992, Bach et al 1993, Francis et al 1993), places additional structural constraints on the growth factors. Taken together, there are several arguments for a low mutation rate of IGFs during evolution.

Genomic cloning of equine IGF2 (paper III)

The equine IGF2 cDNA (paper I) was used as a probe to screen a horse genomic phage library in order to clone the entire IGF2 gene (paper III). Two independent and overlapping clones were isolated, subcloned into bacterial vectors and sequenced. The equine IGF2 gene is the fourth gene (after human, mouse and rat) to be sequenced through intron and exon areas. Access to another IGF2 gene sequence provided an improved possibility of interspecies comparison to identify conserved elements.

The cloned region spans ca 20 kb of chromosomal DNA of which the equine IGF2 gene only represents around 10 kb in length. Sequence analysis and comparison with equine and human IGF2 transcripts revealed three leader exons in the 5´ region of the gene (exons 1, 2 and 3) in addition to three coding exons (exons 4, 5 and 6) (Fig 4).

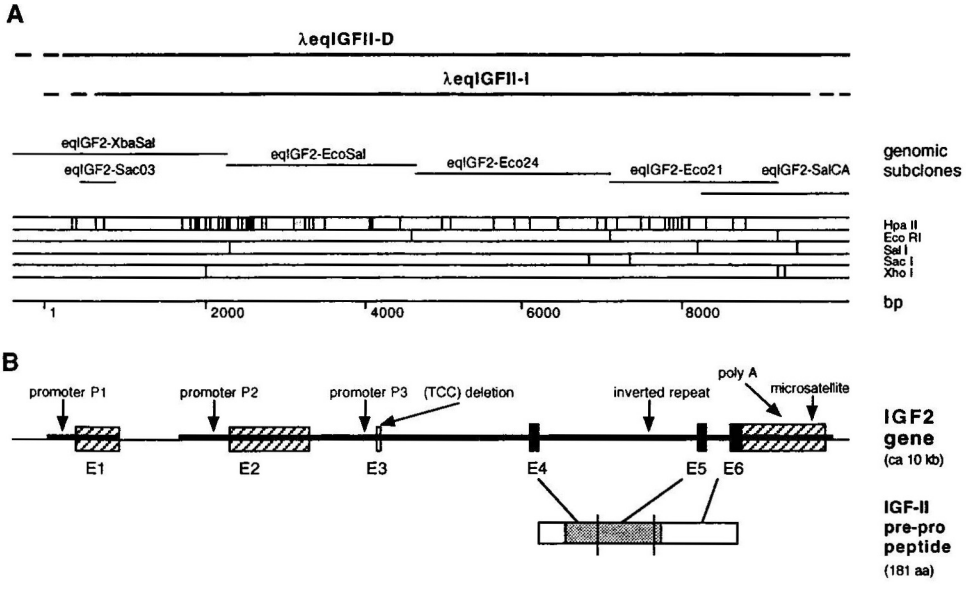

Fig 4. Physical map and gene organisation of equine IGF2. A) Two overlapping λ-clones and resulting subclones are indicated at the top, recognition sites for several enzymes below. B) Exon organisation, promoter location, polyadenylation site, (TCC) deletion, microsatellite and the inverted repeat are shown. Coding exons are represented by filled boxes and non-coding exons by open boxes. The sequenced regions is shown as a bold line, with an interruption in the 5´ region. The exon contribution to the deduced pre-pro-peptide is shown at the bottom, with the shaded area indicating the mature protein.

Equine exon 3 is the shortest of the exons and consists of only 81 bp. The $(TCC)_{10}$ repeat present in human and ovine corresponding exons is partly deleted, such that only a $(TCC)_2$ repeat remained. Whether this repeat region is functional remains to be determined. A putative polyadenylation site flanks the coding region in the last exon. Furthermore, a repeat area with a length of approximately 600 bp is located downstream of the polyadenylation site. Sequence data are not available beyond this point and it is possible that a more downstream polyadenylation site exists as seen in the human IGF2 gene. Therefore it cannot be excluded that the repeat area is present on mRNA level, which would facilitate studies on imprinting of the equine IGF2 gene.
No counterparts to human 5´ exons 1 -3 could be identified by Southern blot analysis of the isolated clones using corresponding human oligonucleotide probes. Also different experimental approaches did not identify additional exons: a) the library was rescreened with the 5´ genomic subclone eqIGFIIgSac03 (fig 4). However, Southern blot analysis of all additional clones revealed similarity to the previoulsly isolated phages; b) PCR was performed on equine genomic DNA with primers corresponding to human/ovine IGF2 exons 1, 2 or 3, and the closely linked insulin gene; c) RT-PCR was performed on RNA isolated from fetal and adult equine liver using primers corresponding to human/ovine IGF2 exons 1, 2 or 3 and equine exon 4.
Sequence comparison between the horse IGF2 gene and that of other species revealed a high degree of homology in the coding as well as non-coding exons. The intron regions generally bore no significant homology. There were some exceptions, however, including promoter regions for equine P2 and P3 and an inverted repeat upstream of exon 5.

Taken together, the overall intron/exon sturcture of the equine IGF2 gene is very conserved, although the intron regions seem to be generally shorter than in other species. The six identified exons are shared by all IGF2 genes analysed so far and there is no experimental evidence for the existance of additional 5´ exons.

Equine IGF gene expression

General analysis of equine IGF transcripts (papers I and II)
To examine expression of IGFs in a variety of equine tissues, the isolated IGF1 and IGF2 cDNAs were used in Northern blot experiments.
IGF1 transcripts were detected in adult equine tissues as liver, testis, myocardium, spleen, lung, kidney and liver as well as fetal liver. IGF1 mRNAs were present in two major forms of around 0.7 and 1.9 kb in length (Fig 5). IGF1 transcription in the horse appears to be similar to human and rodents where IGF1 mRNAs occur as multiple forms ranging from 0.8 to 7.5 kb in length (Lund et al 1986, Murphy et al 1987, Han et al 1988, Lund et al 1989). Here the differences in size mainly depend on the usage of multiple polyadenylation sites (Lund et al 1989). However, the larger IGF1 transcripts detected in these species were not detected in the horse. This might be due to an observed instability of the large IGF1 transcripts (Hepler et al 1990).

Examination of IGF2 expression identified two major transcripts in fetal liver along with some additional faint bands of a smaller size. In adult liver and testis one key transcript was found (Fig 5). These observations were generally confirmed by a later study of equine IGF2 transcription (Lennard et al 1995), where a total of 7 transcripts were identified in fetal liver and one major transcript along with a weaker signal in adult kidney. However, this study used a 45 bp oligonucleotide of ovine IGF2 sequence, bearing 4 bp mismatches.
The diversity of equine IGF2 transcripts might be due to a variety of regulatory processes. Human IGF2 transcripts are derived from multiple promoters during fetal life, are alternatively spliced and are modified by usage of a 3´ cleavage site; during adult life a more 5´ promoter is activated. Similar transcriptional mechanisms might be used in the horse to create transcripts of varying size and sequence. IGF2 plays an important role in embryonic and fetal development and a similar role might be suggested for the horse considering the abundance of transcripts present during equine development.

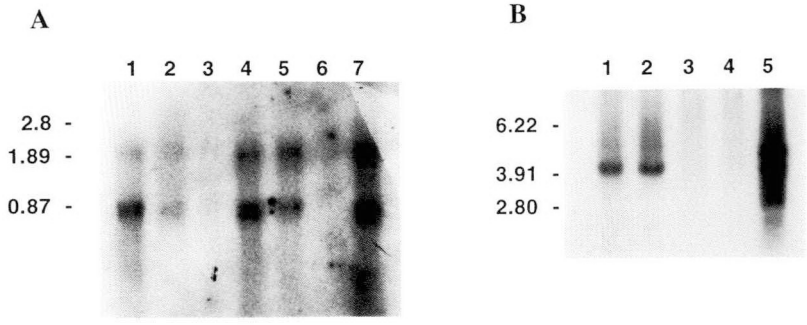

Fig 5. Equine IGF transcription assayed by Northern blot analysis. A) IGF1 transcripts in a) fetal horse liver, b) adult testis, c) adult myocardium, d) adult spleen, e) adult kidney, f) adult liver. B) IGF2 transcripts in a) adult liver, b) adult testis, c) fetal liver

Promoter usage of equine IGF2 (paper III)

Sequence comparison between species revealed two putative promoter regions preceeding equine exons 2 (P2) and 3 (P3) (Fig 4). These two promoters are highly conserved in their sequences as compared to corresponding regions in human, rodent and ovine genes. An additional promoter might be located upstream of equine exon 1 (P1), as determined in human and rodent genes, although as expected, no sequence conservation could be identified in this region.

To determine promoter usage during equine fetal and adult life, promoter-specific probes were constructed (Fig 6) and used in RNase protection analysis on fetal and adult hepatic and non-hepatic tissues (paper III). In fetal liver and kidney, P2 and P3 are transcriptionally active (Fig 6, lanes 3, 4, 9, 10), whereas transcripts derived from P1 could not be detected by RNase protection analysis (data not shown). Apparently the most abundant transcript in fetal liver and kidney is derived from P3. Promoter usage in adult liver and kidney changes markedly. Only transcripts from P3 are present in the adult tissues (Fig 6, lanes 5, 6, 11, 12). Therefore it seems that P3 is the main promoter throughout development and that P2 is downregulated in adult life. Promoter P1 seems to be inactive in all tissues and developmental stages hitherto examined. These results are in agreement with our previous Northern blot experiments that showed two main fetal and one adult hepatic transcript (paper I).

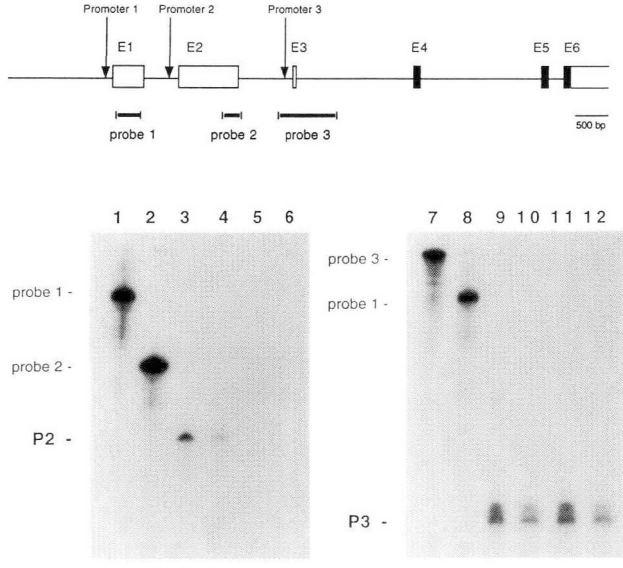

Fig 6. Promoter usage of IGF2 in fetal and adult equine tissues. A) The organisation of the equine IGF2 gene is indicated, showing the promoter-specific probes used. B) Usage of equine promoter P2 and P3 in fetal and adult tissues was assayed by RNase protection as shown below. Lane 1: probe 1, 2: probe 2, 3 -6: probe 2 with fetal liver, fetal kidney, adult liver, adult kidney, respectively. Lane 7: probe 3, 8: probe 1, 9-12: probe 3 with fetal liver, fetal kidney, adult liver, adult kidney, respectively.

The alternate promoter usage in horse IGF2 is clearly different from that in mouse and man. In mouse all three promoters are used during embryonic and fetal life. After parturition all promoters are downregulated and do not become reactivated in normal tissue at any stage of adult life. In man, there are three established promoters accounting for transcription during fetal life. These promoters continue to be active albeit at a much reduced level in adult life. However, there is a promoter (P1) located at the 5´end of the gene which is active in adult liver. The continued usage of a fetal promoter after birth in the horse suggests that an equine counterpart to human promoter P1 might not be present or inactive. This places the horse IGF2 gene in an intermediate functional position between human and mouse genes.

Tissue expression

The distribution of IGF2 transcripts was investigated in equine fetuses at 45 and 250 days of gestation (Otte et al, in preparation). Two probes were used, one corresponding to the protein-coding sequence (Fig 7A, C and E) and the other covering part of exon 2 (Fig 7B, D, and F). In the organs investigated both probes produced the same cellular distribution, although the coding sequence probe produced a more intense labelling reaction.

In liver at 45 days gestation only the hepatocytes display significant amounts of transcripts (Fig 7A and B). The transcripts are confined to the cords of hepatocytes, while the intervening blood-forming cells are negative. At this stage, the metanephric kidney is in its early stage of development. In situ hybridization reveals a similar distribution of IGF2 expressing cells using probes covering both exon 2 and the coding sequence (Fig 7C and D). Transcripts are mainly confined to the immature tubular sections, although the surrounding mesenchyme also show slight staining. In the kidney at 250 days of development IGF2 transcription is prominent in the immature glomeruli. At this stage the distribution suggest that IGF2 transcription is mainly confined to the glomerular endothelial cells (Fig 7E and F).

These results are in agreement with previous in situ hybridisation results on the distribution of IGF2 in equine fetuses (Lennard et al 1995). Also, the use of a non-radioactive technique offers better spatial resolution and allows single cell resolution allowing a more detailed analysis.

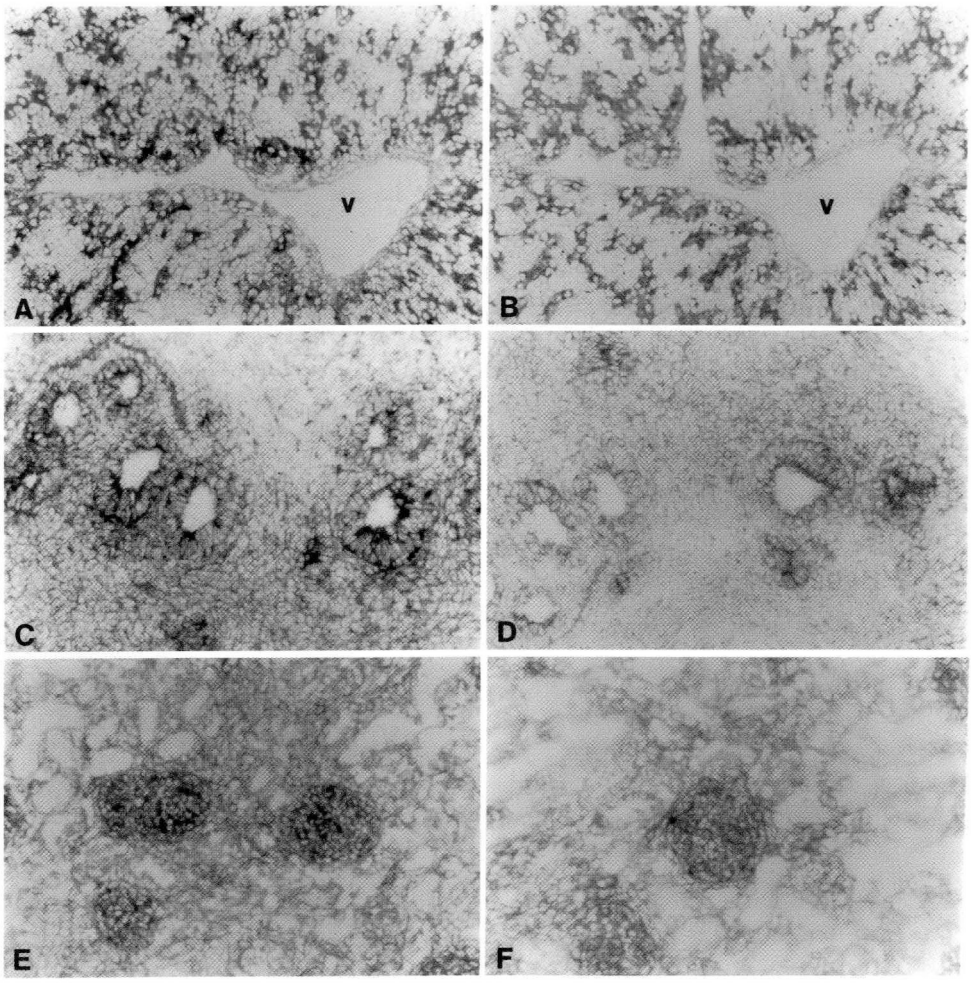

Fig 7. Distribution of IGF2 transcripts in fetal equine tissues as revealed by non-radioactive in situ hybridisation. A and B shows the distribution of the coding sequence and exon 2, in the liver at 45 days gestation, respectively. A liver vein is marked by v. In C and D the distribution in the metanephric kidney of the same fetus is shown. In E and F a kidney at 250 days gestation is shown. Both probes delinate the glomeruli. The distribution suggest an endothelial expression of IGF2. Original 62.5X.

Structural features of the IGF2 gene

Inverted repeat (paper III)

A novel structural element was identified in the equine IGF2 gene. It is located upstream of equine exon 4 and consists of two half-sites of 26 bp, which are separated by a 16 bp spacer, forming an inverted repeat (Fig 8). This inverted repeat displays a highly atypical distribution of nucleotides, one half-site consisting of a large excess of pyrimidines and the other dominated by purines. This inverted repeat is present in all species from which sequence information is available (human, rat and mouse IGF2 genes) and its location and sequence is conserved. Gross differences are only present in the spacer region. Interestingly, the surronding intron area was shown to be differentially methylated in human and mouse IGF2 genes and suggested to be involved in the imprinting mechanism (Feil et al 1994, Sasaki et al 1992, Brandeis et al 1993).

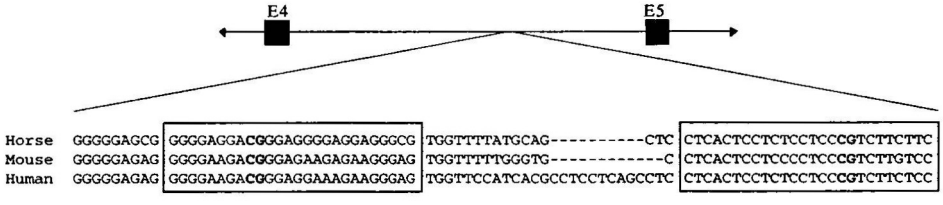

Fig 8. Location and sequence alignment of the inverted repeat element. It is located upstream of exon E5 and its sequence is conserved in corresponding introns of human, mouse and rat IGF2 genes. Conserved CpG dinucleotides are indicated in bold.

The identified inverted repeat has all properties to form a stem-loop structure (Fig 9). The sequence contains three CpG dinucleotides at positions 6, 26 and 68 (Fig 8 and Fig 9). Two of theses CpGs are in base-paired form in the predicted conformation and conserved between species. In order to determine stability of the postulated secondary structure, *in vitro* thermal melting studies were carried out on a corresponding oligonucleotide and its methylated counterpart. The observed high T_m (67.5 °C for native and 65.3°C for the methylated counterpart) and the monophasic character of the transition profiles suggest the formation of highly stable intramolecular duplexes at room temperature for both oligos. The unusual polypurine-polypyrimidine sequence of the inverted repeat suggests a triple helical conformation (Grabczyk et al 1995). However, subsequent CD analysis revealed a hybrid A/B conformation of DNA in the stem-area for both oligonucleotides.

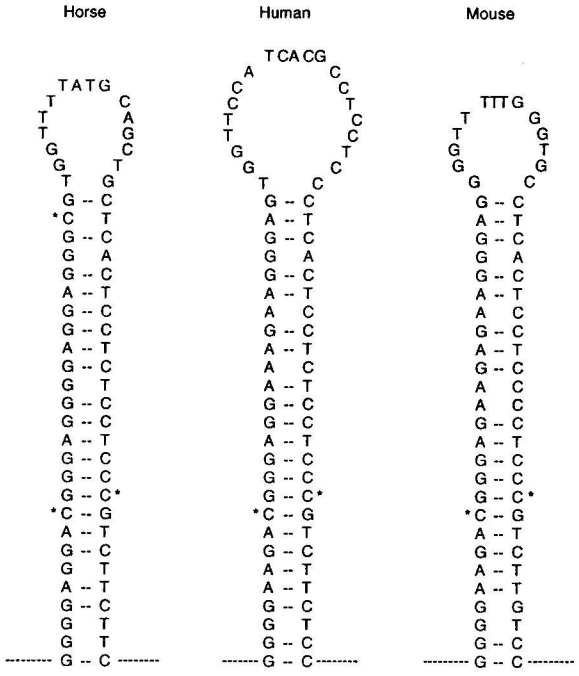

Fig 9. Postulated stem-loop structure for the inverted repeat in horse, human, and mouse IGF2 genes.
Also shown are possible cytosine-methylation sites (*).

To investigate if the postulated stem-loop structure is recognized and bound by specific protein components, protein-DNA interactions were studied by gel mobility shift assays. The annealed native oligonucleotide was bound by a specific protein component in extracts from equine fetal liver and fetal kidney (Fig 10, lanes 2, 9) and competition experiments verified the specificity of the interaction (lanes 3, 4, 10). In adult liver, however, a reduced binding activity was observed (lane 13), which indicates that the protein factor might be under developmental control. In order to determine whether methylation of CpG residues influences protein binding, gel mobility shift assays were carried out with a methylated oligonucleotide (see above). Protein binding was reduced in all tissues examined (lanes 5, 11, 15). These results indicate, that the stem-loop is recognised and bound by a specific, methylation sensitive protein component. Since the overall structure of both oligonucleotides appears to be similar, the protein might be sensitive to local conformational changes caused by methylation or by direct steric hindrance due to the presence of methyl groups.
Based on the the high similarity of the stem-loop in sequence and secondary structure compared to mouse and human, the methylation sensitive factor might well be conserved between species.

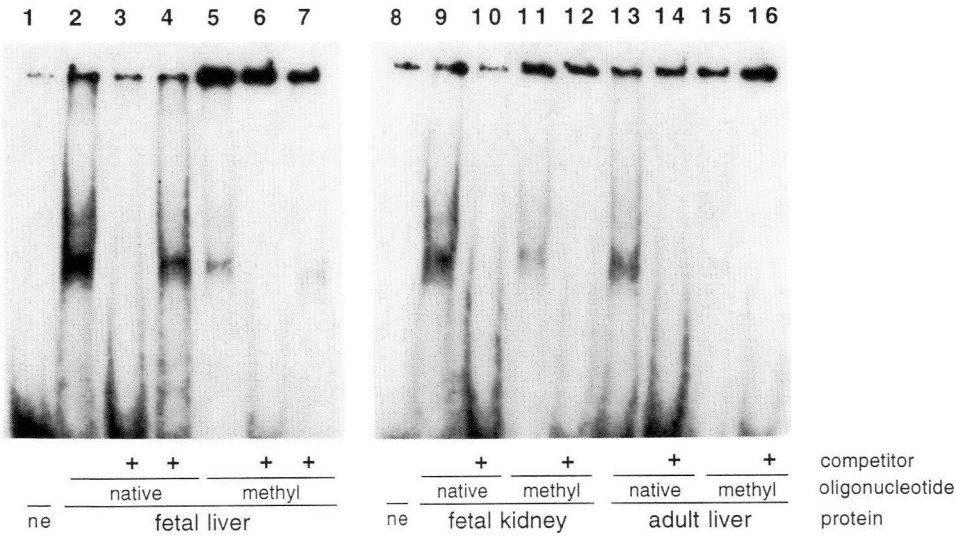

Fig 10. Protein binding to the stem-loop examined by gel mobility shift assays. Reactions contain protein extract from fetal liver (lanes 2-7), fetal kidney (lanes 9-12), and adult liver (lanes 13-16). Native oligonucleotide (native) was added in lanes 1-4, 9, 10, 13, and 14. The methylated counterpart (methyl) was added in lanes 5-7, 8, 11, 12, 15, and 16. Competition analysis for binding was performed by addition of 100x molar excess of unlabelled annealed inverted repeat (lanes 3, 6, 10, 12, 14, 16) or unrelated ds oligonucleotide (lanes 4, 7). Addition of competitor is indicated (+). Lanes 1 and 8 contain no protein extract (ne).

The formation of a stem-loop or cruciform structure of double stranded DNA, which is bound by a protein component has interesting implications. The inverted repeat is located in a GC rich area which has been established as a differentially methylated region in mouse and human IGF2 genes. The transcribed paternal allele is methylated while the silenced maternal allele is unmethylated on specific CpG residues (Brandeis et al 1993, Schneid et al 1993, Feil et al 1994). Since methylation of the paternal allele is tissue-specific and correlates directly with expression, the presence of a silencer element under epigenetic control has been suggested (Feil et al 1994). Inverted repeats and cruciform structures have previously been associated with regulation of gene expression (Horwitz et al 1988, McMurray et al 1991, Spiro et al 1993), as well as termination of transcription and attenuation (Rosenberg et al 1979). One possible role of the stem-loop structure presented in this study could therefore be to act as a structural silencer element which is recognised by specific protein factors depending on its methylation status. Proteins interacting with cruciforms have been reported, among those the HMG box proteins and CBP (Pearson et al 1996). All these proteins would be potential candidate peptides to recognise the presented stem-loop structure in human, rodent and horse IGF2 genes.

Only one methylation sensitive protein factor that binds to a differentially methylated region in an imprinted gene has previously been reported (Huntriss et al 1997). A methylation-dependent factor was shown to bind to a GC rich sequence in the promoter region of the imprinted mouse Xist gene. This region was also shown to be required for transcription.

The differential methylated region in IGF2 the gene which harbours the stem-loop is not thought to carry the primary imprinting signal (imprinting box), because allele specific methylation is established during development (Feil et al 1994). Any epigenetic tag imposed onto an imprinted gene must be established in the germline and propagated in the embryo. This has been shown for H19 and IGF2/Mpr genes, where a differentially methylated region is established in the germline which is resistant to preimplantation demethylation (Stöger et al 1993, Tremblay et al 1995). Lately new views regarding the nature of the imprinting signal have emerged. Sex-specific modifications of the chromatin conformation is proposed to be the primary event that establishes the imprint during gametogenesis. The allele-specific chromatin conformation might secondarily influence the accessibility of the imprinting box to the actitivity of the methylatransferase (Patterton and Wolffe 1996, rev Jaenisch 1997). In this context secondary structures as presented in this study might well be of importance.

Physical mapping of equine IGF2

(paper IV)
The IGF2 locus has to date been physically mapped in five species: man (Henry et al 1985, Morton et al 1986), sheep (Ansari et al 1994), cattle (Schmutz et al 1996), kangaroo (Toder et al 1996), and mouse (Beechey et al 1997). In the present study, three equine genomic clones have been used to physically map the IGF2 locus in horse and donkey (paper IV). This was achieved by FISH analysis through hybridisation of Dig-labelled IGF2 DNA to equine metaphase chromosomes. The IGF2 locus was mapped to chromosome 12q13 (ECA12), the long arm of this chromosome. In donkey, IGF2 mapped terminally on the long arm of a small submetacentric chromosome, which showed almost identical DAPI-banding patterns with equine chromosome 12.

In all seven species viz. cattle, sheep, human, kangaroo, mouse, horse and donkey, where IGF2 has been physically mapped, the gene is located on the terminal part of the chromosome arm. The terminal position of IGF2 in distantly related species might suggest that the localisation of this gene has been preserved during mammalian chromosome evolution. A similar situation is known of the gene encoding immunoglobulin gamma heavy chain, which tends to keep its relative position on the terminal part of chromosome arms in six distant mammalian species (Gu et al 1994) (Fig 11).

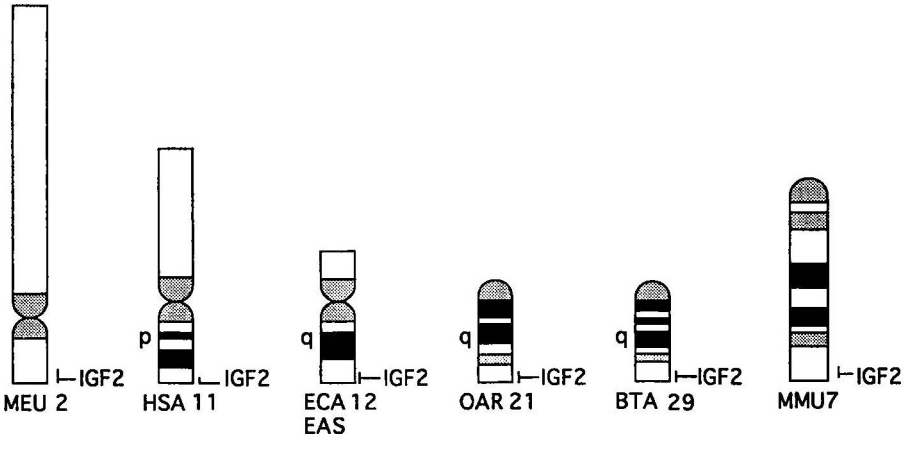

Fig 11. Physical localisation of the IGF2 gene. The gene occupies a terminal location on the chromosome in all species hitherto studied, including kangaroo, man, horse, donkey, sheep, cattle, and mouse.

In order to determine if even larger parts of the chromosomal region surrounding the IGF2 locus are preserved during mammalian evolution, microdissected probes of HSA11, containing the IGF2 gene, were used in FISH analysis on equine metaphase chromosomes. This cross species chromosome hybridisation revealed homoeology of HSA11 with both arms of ECA7 and both arms of ECA12. A previous FISH experiment demonstrated conserved segments for most of the human and horse chromosomes but failed in revealing homoeology of HSA11 (Raudsepp et al 1996). The present comparative chromosome painting results fill this gap and are in accordance with the mapping of IGF2 to ECA12. This shows that chromosomes of distantly related species sharing segmental homoeology also contain the same genes.

Mapping IGF2 in the horse and revealing segmental homoeology between HSA11 and ECA12 provides new possibilities for the human-horse comparative map which can be utilised for a rapid and organised assignment of functional gene to the horse gene map. Human IGF2 is situated on HSA11p15.5 in one of the most densely mapped regions in the human genome, harbouring several disease loci and a cluster of imprinted genes (see introduction). In this context the physical localisation of equine IGF2 and revealed homoeology between HSA11 and ECA12 could be of significance in studying the same phenomena in equids.

Future perspectives

The present study has focussed on the cloning and initial characterisation of equine IGFs. Special emphasis was given to the equine IGF2 gene and its structural organisation and transcriptional regulation. Much of its basic characteristics, including intron/exon organisation, promoter usage, tissue expression and localisation in the horse genome, have been described in this thesis. However, there are some features left to be investigated in order to proceed with the characterisation of this equine gene:

- IGF2 transcription was investigated by a variety of methods. Northern blot analysis revealed the number and length of transcripts, RNase protection showed the substantially different promoter usage compared to that in other species, and in situ hybridisation showed the tissue distribution of transcripts. However, the complete sequences of all transcripts and their different 5´ leader- and 3´ trailer regions are still not known. This is mainly due to the lack of suitable equine cDNA libraries. Construction and screening of adult as well as fetal equine cDNA libraries would provide useful tools for the characterisation of equine IGF2 transcripts.
- Human, ovine and baboon IGF2 genes harbour an additional promoter, which is located in the 5´ region of the IGF2 gene and is active in adult tissues. The present study was unable to identify a counterpart in the equine gene. Moreover, the continuous usage of a fetal promoter argues against the existance of an additional, adult specific promoter. However, this question will be conclusiveley resolved by screening an adult liver cDNA library and sequencing all leader regions in the present transcripts.
- One of the main question to be answered is whether or not the equine IGF2 gene is imprinted. The identified repeat region in the 3´ region of the gene could be used to investigate the imprinting status.
- The equine IGF2 gene was mapped to a chromosome that shows homoeology with HSA11. Since this human chromosome harbours IGF2, it would be of interest to investigate if the human IGF2 linkage group on HSA11p15.5 is conserved in the horse and if the same genes are present on ECA12.

This study revealed a novel, previously not described, structural feature which is present in the equine, mouse, human and rat IGF2 genes. This motif consists of an inverted repeat, which has been shown to form a highly stable secondary structure with a mixed A/B- DNA conformation in the stem area. Furthermore, this element has been shown to be bound by a specific, methylation sensitive protein in a developmental fashion. There are a number of interesting implications and questions emerging from these findings:

- Does the stem-loop structure have a biological relevance? Is it involved in the regulation of processes as transcription and/or imprinting? Is methylation in the inverted repeat or surrounding areas involved in a regulatory mechanism, and if so, in which way? These questions can be answered by genetic experiments, as e g mutagenesis in the repeat area and in vitro methylation studies of constructs containing the inverted repeat. Since the inverted repeat is conserved between species, it might be of advantage to perform these experiments in human or mouse systems. A wealth of information about regulation, imprinting and methylation status of the IGF2 gene is available for both the human and mouse genes. In addition, rodents can easily be used for transgenic experiments.
- Does the protein recognize a specific nucleotide sequence in the stem-loop? Is the loop region necessary for protein binding? Are the conserved CpG nucleotides important for binding? DNA footprinting or in vitro mutagenesis of the inverted repeat and subsequent gel mobility shift analysis might be used to identify nucleotides important for protein binding.
- Which protein component binds to the stem-loop? How does it recognize methylation of cytosine residues? These questions clearly implicate protein purification, sequencing and determination of its structure. Also for these experiments it might be advisable to use human or mouse systems, since a variety of cell lines are readily available to provide starting material for purification.

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