Development 120, 361-368 (1994)
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Overlapping patterns of *IGF2* and *H19* expression during human development: biallelic *IGF2* expression correlates with a lack of *H19* expression

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SUMMARY

The spatial patterns of *IGF2* and *H19* gene expression are strikingly similar during parts of human embryonic/fetal and early postnatal development. Notable exceptions were found with the ciliary anlage of the embryonic retina and the choroid plexus/leptomeninges, where transcripts from the *IGF2* but not the *H19* locus could be detected. Moreover, in contrast to the other tissue samples examined, the choroid plexus/leptomeninges expressed both parental *IGF2* alleles. Whilst RNase protection analysis revealed a weak activity of the P1 promoter in the choroid plexus/leptomeninges, the P2, P3 and P4 promoters were all active wherever *IGF2* was expressed. We discuss these observations with respect to a hypothesized coordinated control of the reciprocally imprinted and closely linked *IGF2* and *H19* loci.

Key words: in situ hybridization, promoter usage, tissue-specific parental imprinting, *IGF2*, H19, human development

INTRODUCTION

A subset of mammalian genes are expressed from only one allele in a manner dependent on the parental origin (Surani, 1991). To date, four genes have been shown to be parentally imprinted in the mouse: *Igf2r* (Barlow et al., 1991) and *H19* (Bartolomei et al., 1991) are preferentially expressed from the maternal allele while *Snrpn* (Leff et al., 1992) and *Igf2* (DeChiara et al., 1991) are primarily expressed from the paternal allele. In humans, *IGF2* (Ohlsson et al., 1993; Giannoukakis et al., 1993; Rainier et al., 1993; Ogawa et al., 1993) and *H19* (Rainier et al., 1993) have been shown to be parentally imprinted in the same direction as in the mouse. The *IGF2* and *H19* loci are of particular interest since, in addition to their reciprocal imprinting patterns, they are closely linked on chromosome 11 in humans. In addition, the spatiotemporal patterns of expression of these genes appear very similar during mouse embryogenesis (Lee et al., 1990; Poirier et al., 1991). These reciprocal patterns of imprinting appear to have been lost during the generation of a subtype of Wilms' tumours since both parental alleles of *IGF2* (Rainier et al., 1993; Ogawa et al., 1993) and *H19* (Rainier et al., 1993) were expressed. Biallelic expression of *Igf2* has, however, been observed in the choroid plexus and leptomeninges of the mouse during normal development (DeChiara et al., 1991).

IGF-II gene expression patterns appear to be evolutionarily conserved during mammalian development although notable exceptions have been reported. In man (Ohlsson et al., 1989a; Brice et al., 1989), mouse (Lee et al., 1990) and pig (unpublished observation), IGF-II expression appears to be first activated in trophectodermal derivatives after postimplantation. In the mouse, *Igf2* expression first appears in the embryo proper at the late primitive streak/neural plate stage suggesting a role for IGF-II in the formation of the heart, derivatives of the foregut and cranial aspects of neural crest derivatives (Lee et al., 1990). During later stages of intrauterine development, a more extensive expression pattern is found, with organ-specific similarities among rodents (Stylianopoulou et al., 1988a; Bondy et al., 1990; Lee et al., 1990; Ayer-LeLievre et al., 1991; DeChiara et al., 1991) and man (Han et al., 1988; Brice et al., 1989). Some species differences may exist, however, since no expression could be detected in rat adrenal cortex and pancreas and comparatively low levels of *Igf2* transcripts were found in rat kidney while corresponding human embryonic tissues express *IGF2* at high levels (Stylianopoulou et al., 1988a; Han et al., 1988; Brice et al., 1989). Considerable interspecies differences can be found during postnatal regulation of *IGF2* gene transcription: In the rat, its expression declines after birth in all tissues except for choroid plexus and leptomeninges (Stylianopoulou et al., 1988b), while postnatal expression has been demonstrated in several other human tissues (reviewed by Rechler and Nissley, 1990). The liver is the likely source of serum IGF-II in adult humans (Jansen et al., 1990) produced from *IGF2* transcripts derived from the

human-specific P1 promoter (de Pagter-Holthuizen et al., 1988; Sussenbach, 1989). Three other promoter regions with counterparts in mouse and rat have been identified in the human *IGF2* gene (Jansen et al., 1990). These have been referred to as 'fetal promoters', although adult tissues such as the pregnant and nonpregnant endometrium express P2-, P3- and P4-derived *IGF2* transcripts (Glaser et al., 1992). Although considerably less information is available on the expression patterns of the *H19* gene in comparison with *IGF2*, it has been noted that these two genes show strong similarities in their temporal and spatial patterns of expression (Lee et al., 1990; Poirier et al., 1991). This is of particular interest since these two loci appear to express opposite functions (DeChiara et al., 1991; Hao et al., 1993).

The purpose of this study was to examine the extent of overlap in the *IGF2* and *H19* expression patterns during some aspects of human development. Although these loci are coexpressed in a large variety of cell types within several tissues, the choroid plexus/leptomeninges and the ciliary anlage of the retina were notable exceptions, since they expressed the *IGF2* but not *H19* locus at detectable levels. Moreover, we show that *IGF2* is monoallelically expressed in all tissues examined except for the choroid plexus/leptomeninges during early postnatal development. These results are discussed with respect to possible shared transcriptional control mechanisms between *IGF2* and *H19.*

MATERIALS AND METHODS

Tissue specimens

Human embryos from first trimester pregnancies were obtained from therapeutic terminations carried out at the Huddinge university hospital and the Karolinska hospital, with the permission of the local ethical committees. All specimens were, so far as could be assessed, morphologically and physiologically normal. Gestational age was assessed by ultrasound analysis, crown-rump lengths and patient information. In addition, the postmortem tissues of a newborn human patient who died 16 weeks following birth at 26 weeks of gestation were analyzed. The likely cause of death was a fulminant bacterial infection. Since the choroid plexus epithelium cannot be separated from the leptomeningal component, the sample of this specimen is referred to as choroid plexus/leptomeninges. The morphologically normal liver samples were obtained from 9 and 18 months old children undergoing operations for hepatoblastoma. The tissue specimens were either snap-frozen for DNA/RNA extraction or subjected to standard histological procedures.

Nucleic acid preparation

Genomic DNA was prepared by repeated phenol/chloroform extractions of tissue cells lysed with 0.5% SDS and proteinase K (200 μg/ml final concentration). Total cellular RNA was prepared as has been described previously (Chomczynski and Sacchi, 1987).

PCR amplifications

The polymorphic $(C\text{-}A)_n$ repeats of *IGF2* were PCR-amplified as has been described (Ohlsson et al., 1993). The P1 promoter probe of *IGF2* was generated by PCR-amplifying the clone pALP7 (kindly provided by Dr P. Schofield, Cambridge) using the following primers: CAATCTGCACCTTTCCTGAG and CTCACATACCTCAGCTCCAG. These primers gave a product including exon 1 and about 285 bases of 5′ flanking sequence which was subcloned into the pCRII vector (Stratagene) to generate the pP1TE clone. Exon 4 and parts of exons 3 and 5 of *H19* were PCR-amplified according to Zhang and Tycko (1992). The resulting fragment was subcloned into pCRII (Stratagene) to generate the pH19VT clone. The identity of each clone was verified by sequencing both ends using the Sequenase kit (USB).

RNA probes

^{35}S-labeled antisense *IGF2* and *H19* riboprobes for in situ hybridization analysis were generated from a 572 bp *Hin*fI-*Pst*I human cDNA *IGF2* insert, cloned into pGem-3 (pHIGF2; Ohlsson et al., 1989b) and a 863 bp human *H19* fragment (see above) inserted into the pCRII vector (pH19VT). Riboprobes from these templates were transcribed from supercoiled plasmids, yielding probes with similar specific activity (approximately 250 Ci/mmol) and size (600-700 bases). The exon-specific riboprobes (derived from the genomic clone hINS-3 (kind gift of Dr G. Bell) (see Fig. 4A) were generated from linearized Bluescript templates: P2, a *Pst*I/*Sac*I fragment of 340 bp, linearized with *Eco*RI; P3, an *Eco*RI/*Sal*I fragment of 425 bp, linearized with

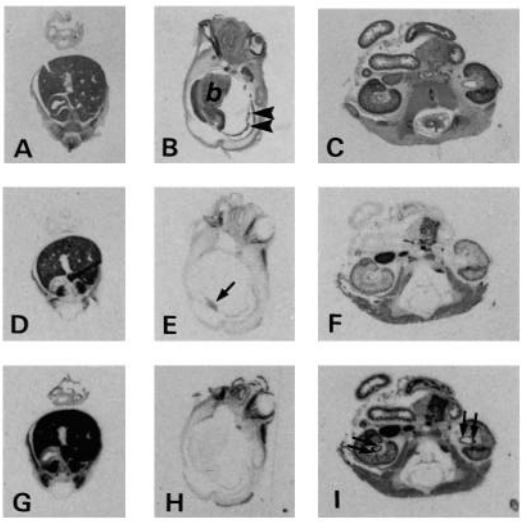
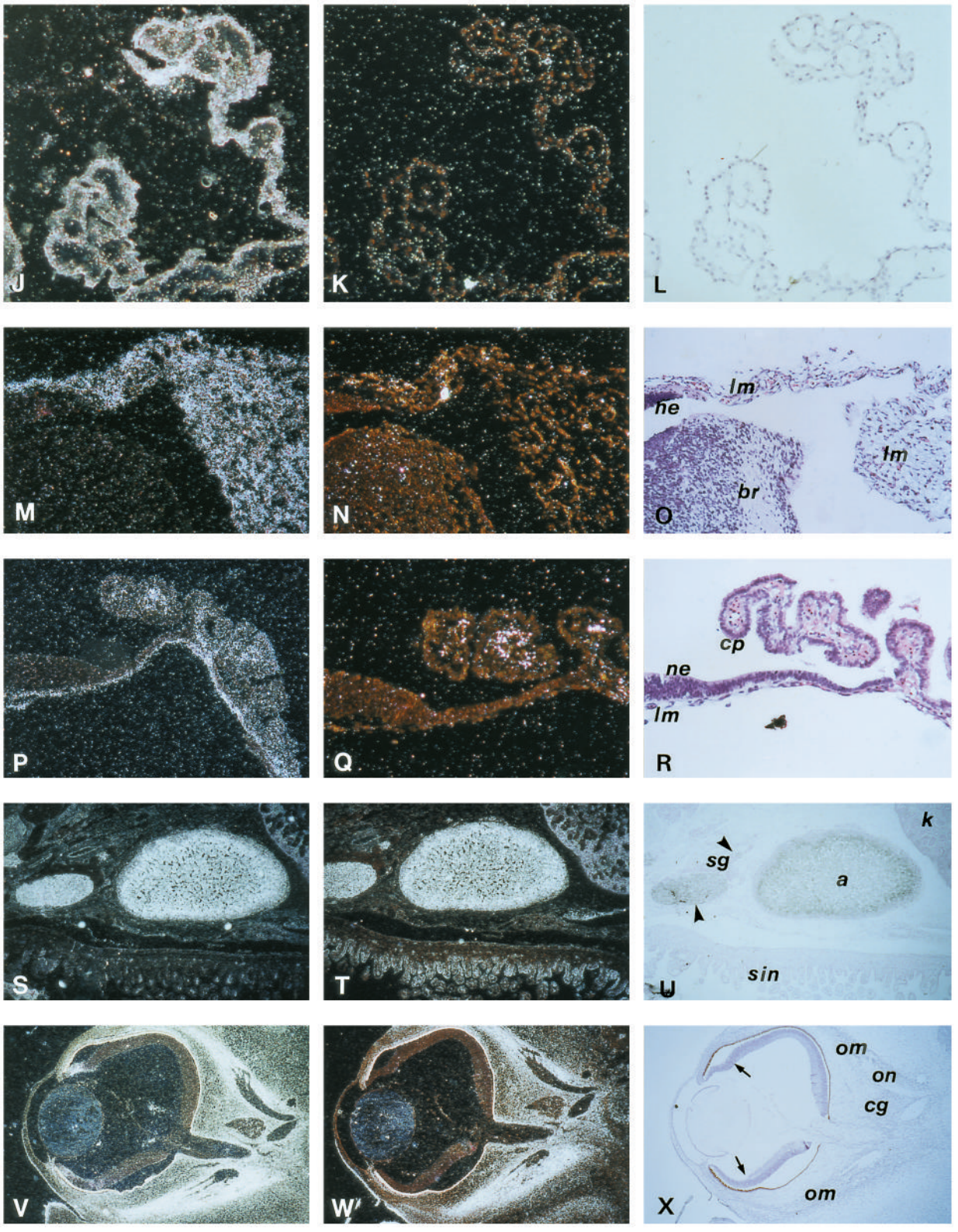


Fig. 1. Expression of *IGF2* and *H19* genes in consecutive sections of human embryonic/fetal and early postnatal specimens. (A-C) Hematoxylin/eosin stained sections of embryos/fetus at 7.0, 7.5 weeks and 10.5 weeks postconception, respectively. Autoradiographs on X-ray film of adjacent sections show *IGF2* (D-F) and *H19* (G-I) expression patterns. (J,K) Analysis of *IGF2* and *H19* expression, respectively, in the choroid plexus of a human patient who was prematurely born at 26 weeks of gestation and died 16 weeks postnatally. (L) Bright-field view of K. (O,R) Hematoxylin/eosin-stained leptomeninges and choroid plexus/leptomeninges, respectively, in sections of embryos at 7.0 and 7.5 weeks postconception. Consecutive sections were hybridized to ^{35}S-labelled, antisense *IGF2* (dark-field views in M and P) and *H19* (dark-field views in N and Q) riboprobes. (U) Hematoxylin/eosin-stained thin section of small intestine and some retroperitoneal organs in a 10.5 weeks old fetus. (S,T) Dark-field views of the expression of *IGF2* and *H19*, respectively, in consecutive sections. (X) Bright-field view from a horizontal section over an embryonic eye (at 7.5 weeks postconception); (V,W) dark-field views of *IGF2* and *H19* expression patterns, respectively, in adjacent sections. Double arrowheads, choroid plexus/leptomeninges; thin arrow, leptomeninges (also shown in panels M-O); double thin arrows, transitional epithelium in calyx of the kidney; single arrowheads, paraganglia; fat arrows, junction between retina proper and ciliary portion of the retina; a, adrenal gland; br, brain; cg, ciliary ganglion; cp, choroid plexus; k, kidney; lm, leptomeninges; ne, neuroepithelium; om, ocular muscle; on, oculomotor nerve; sg, sympathetic gangion; sin, small intestine. Magnifications are: (A-I) ×1.9; (J-L) ×210; (M-O) ×105; (P) ×80; (Q,R) ×105; (S-X) ×18.

*Eco*RI; P4, a *Sma*I/*Pst*I fragment of 330 bp, linearized with *Sal*I. Antisense RNA probe of the P1 promoter clone was generated from a *Hin*dIII-linearized template.

For RNase protection analysis, antisense ^{32}P-labeled P1-P4 promoter-specific riboprobes were generated from linearized subclones of exons 4, 5 and 6, as described above (specific activity approximately 900 Ci/mmol). An antisense ^{32}P-labeled riboprobe (specific activity of approximately 90 Ci/mmol) diagnostic for the polymorphic region of the exon nine of *IGF2* was generated from the *Hin*dIII-linearized pPA1 plasmid (T7 polymerase) (Ohlsson et al., 1993). The simultaneous detection of *IGF2/H19* transcripts by RNase protection used antisense riboprobes from *Xho*I-linearized pHIGF2



(covering bases 131 to 260 in human *IGF2* sequence; accession number XO 7868 in GenBank) and *Sac*I-linearized pH19VT (covering bases 3036 to 3376 in human *H19* sequence; accession number M32053 in GenBank) vectors, respectively. The specific activities of these probes were approximately 250 Ci/mmol.

RNase protection analysis

For the identification of heterozygous alleles of *IGF2*, ^{32}P-labeled antisense RNA probes (100,000 cts/minute) generated from pPA1 template were annealed to 5 μl of PCR incubation mixture (corresponding to approximately 50 ng of *IGF2* exon nine DNA) at 30°C for 15 hours using the hybridization solution of the RNase protection kit (Ambion). For a corresponding RNase protection analysis of cellular RNA, the same amount of radiolabeled probe was annealed to 10 μg of total cellular RNA at 45°C for 15 hours. The comparison of *IGF2* and *H19* transcript levels was performed as above by annealing 200,000 cts/minute of *IGF2* and *H19* RNA probes, generated from pHIGF2 and pH19VT templates, respectively, to 1.5 μg of total cellular RNA. The promoter usage was similarly analyzed by annealing 500,000 cts/minute of exon-specific, antisense ^{32}P-labeled riboprobes with 5 μg of total cellular RNA. Following removal of excess probe by RNase degradation according to the manufacturers protocol, the denatured samples were analyzed on 6% acrylamide-urea sequencing gels.

In situ hybridization analysis

Serial 5 μm sections from formaldehyde-fixed and paraffin-embedded human embryos and fetuses were used for in situ hybridizations. No anomalies were seen and since hybridization patterns were concordant in all specimens they were accepted for evaluation of normal development. In situ hybridization analyses were performed as has been described previously (Ohlsson et al., 1989b). Sections were hybridized to ^{35}S-labeled riboprobes at 56°C overnight and washed stringently prior to RNase treatment as described previously (Ohlsson et al., 1989b). Following application of Ilford K5 photographic emulsion (diluted 1:1 in 2% glycerol) and exposure for 3-16 days at 4°C, sections were counterstained with Mayer's hematoxylin and mounted.

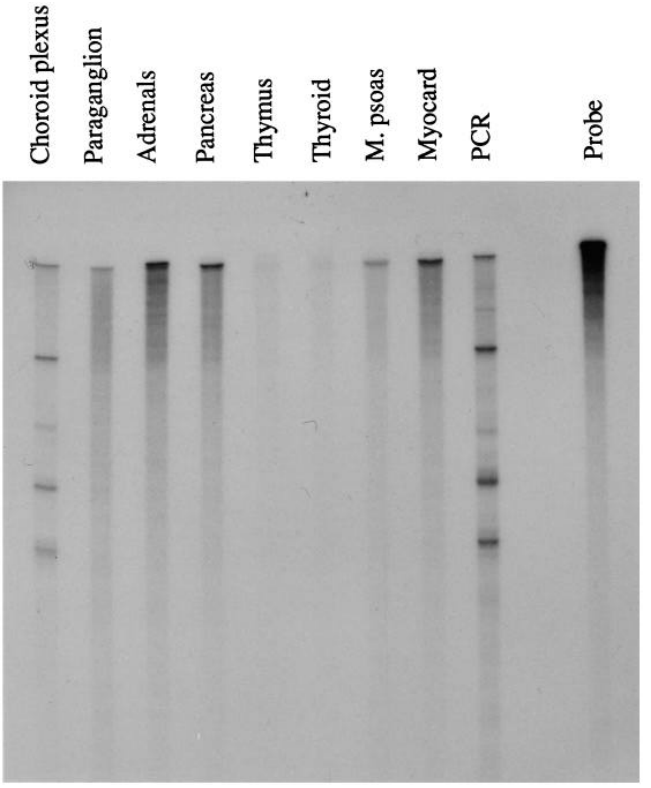


Fig. 2. Tissue-specific parental imprinting of *IGF2* during early human postnatal development. Total cellular RNAs extracted from different tissues of a early human postnatal patient (see legend of Fig. 1) were analyzed for allelic usage by RNase protection using a ^{32}P-labelled antisense riboprobe, specific for the polymorphic $(C\text{-}A)_n$ repeat region of exon nine of *IGF2*. The PCR lane depicts PCR-amplified *IGF2* exon nine of DNA extracted of the pancreas.

RESULTS

Spatiotemporal coexpression of *IGF2* and *H19*

It has previously been noted that there is a strong similarity between the *IGF2* and *H19* gene expression patterns during murine embryogenesis (Lee et al., 1990; Poirier et al., 1991). To examine this issue in some detail during parts of human embryonic and fetal development, we analyzed the expression of *H19* and *IGF2* in adjacent sections of embryos/fetuses. Fig. 1 shows that there is a strikingly similar pattern of *IGF2* and *H19* expression in several human embryonic and early fetal specimens, with the particular exceptions of the choroid plexus, leptomeninges and the ciliary portion of the embryonic retina, which all express *IGF2* but not *H19*. The lack of *H19* expression in the choroid plexus appears to persist into at least early postnatal development (Fig. 1K). In contrast, *H19* is comparatively more active than *IGF2* (although showing the same spatial pattern of expression) in the epithelium of the small intestine (Fig. 1S,T), myocardium, epithelium of urinary collecting system and respiratory epithelium (data not shown). We conclude that the spatiotemporal patterns of *IGF2* and *H19* expression are very similar in the examined specimens, with the ciliary body, choroid plexus and leptomeninges as the most prominent exceptions.

Biallelic expression of IGF2

Since *IGF2* and *H19* expression patterns did not overlap in the choroid plexus/leptomeninges which expresses both parental alleles of *Igf2* in the mouse (DeChiara et al., 1991), we next addressed if *IGF2* is biallelically expressed in the corresponding human tissues. RNase protection analysis of PCR-amplified polymorphic $(C\text{-}A)_n$ repeats of exon nine of *IGF2* (Ohlsson et al., 1993) showed that an early postnatal patient carried heterozygous *IGF2* alleles (Fig. 2). When the same RNase protection approach was employed for the expressed *IGF2* transcripts and compared with the polymorphisms of the PCR-amplified DNA, it was evident that *IGF2* was monoallelically expressed (albeit at varying levels) in tissues such as the paraganglion, the pancreas, the thyroid, the thymus, the adrenals, the myocardium and the muscle of psoas (Fig. 2). We also conclude that the same parental allele, in all likelihood the paternally derived, was expressed in all of these tissues. In contrast, the choroid plexus/leptomeninges tissue expressed both parental alleles (Fig. 2). We conclude that human *IGF2* is biallelically expressed in a tissue-specific manner in a similar or identical way to that of the mouse (DeChiara et al., 1991).

To determine whether or not the choroid plexus/leptomeninges of the newborn patient also displayed the lack of *H19* expression demonstrated for other specimens of earlier

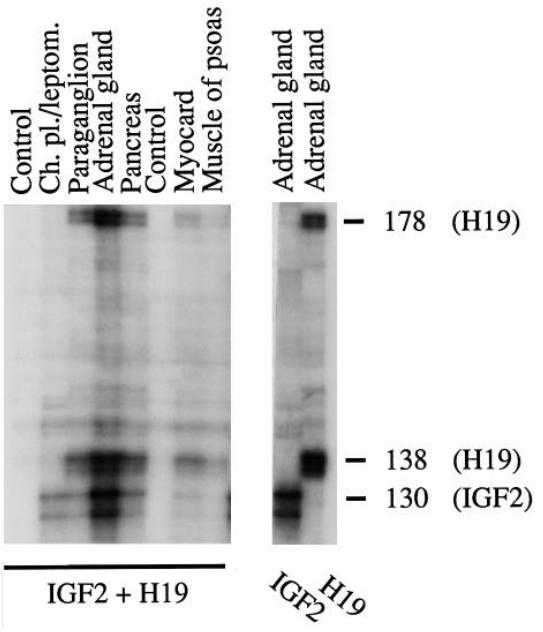


Fig. 3. Choroid plexus/leptomeninges express *IGF2* but not *H19*. The relative levels of *IGF2* and *H19* transcripts in various tissues of an early postnatal human patient (see legend of Fig. 1) were analysed by RNase protection. Antisense *IGF2* and *H19* riboprobes were annealed to total cellular RNA samples as indicated in Fig. 2. The dominant *IGF2* transcript-derived bands around 130 bases correspond to bases 131 to 260 of exon 9. The two *H19* transcript-derived fragments (138/178 bases) result from a polymorphic difference between the RNA probe and RNA substrate in the exon 5 region. The sizes of the protected probe fragments were estimated from a sequence ladder. Control, yeast RNA control; Ch.pl/leptom., choroid plexus/leptomeninges. The bottom left of the figure indicate samples that were simultaneously analyzed for *IGF2* and *H19* expression (marked IGF2 + H19). The bottom right shows the separate analysis of *IGF2* and *H19* expression in the adrenal gland sample, allowing the identification of the protected *IGF2* and *H19* RNA fragments.

human development (Fig. 1), we examined the relative levels of *IGF2* and *H19* transcripts. Fig. 3 shows a simultaneous RNase protection analysis using probes encompassing part of exon 5 of *H19* (see Zhang and Tycko, 1992) and the coding sequence of *IGF2*, respectively, of the same samples analysed above for the allelic usage of *IGF2*. The results again show that there is a correlation between the levels of *H19* and *IGF2* expression in all tissue samples analyzed except for the choroid plexus/leptomeninges which did not express *H19* at a detectable level. Moreover, in situ hybridization analysis of an adjacent portion of the choroid plexus/leptomeninges sample used in the RNase protection analysis above shows that, while *IGF2* is abundantly expressed in this tissue (Fig. 1J), we failed to detect a reliable *H19* signal (Fig. 1K) except in some of the invading blood vessels (data not shown). Biallelic expression of *IGF2* in the human choroid plexus/leptomeninges correlates, therefore, with a lack of *H19* expression.

IGF2 promoter usage

IGF2 transcripts originate from at least four different promoters (Sussenbach, 1989; Holthuizen et al., 1990). To address whether or not the tissue-specific biallelic expression of *IGF2* reflected coordinated changes in the pattern of promoter usage, we analyzed P1-P4 promoter-driven transcription. This was performed by annealing ^{32}P-labeled exon-specific antisense RNA probes (Fig. 4A) to the same RNA samples that were analyzed for allele-specific expression (see above), followed by RNase protection analysis. Fig. 4B shows that the major band(s) for each protected promoter probe were similar or identical to the ones expected from previous studies (Sussenbach, 1989; Holthuizen et al., 1990). Hence, for the P2, P3 and P4 promoter probes, protected transcripts of about 340, 132 and 93-103 bases, respectively, could detected (Fig. 4B). We have on numerous occasions with a large variety of RNA samples observed additional less abundant transcripts such as those shown in Fig. 4B. The possibility that these may represent hitherto unreported *IGF2* transcript species is currently analysed. The results show that the three 'fetal' promoters (P2, P3 and P4) were active in the choroid plexus, paraganglia and adrenal glands with minor variations in the promoter usage within each tissue sample (Fig. 4B). Conversely, low levels of P1-derived transcripts with the protected size of 116-118 bases, which is the expected (Sussenbach, 1989), could be detected only in choroid plexus/leptomeninges (the lanes depicted liver show promoter usage in a sample from an 9 month infant serving as a marker). Interestingly, in one informative infant liver specimen (18 months postnatally) with an active P1 promoter (data not shown), both *IGF2* alleles are expressed (Fig. 4C).

To assess if a cell-type-specific promoter usage exists within the various organs during development, the expression of *IGF2* in thin sections of human embryos was analysed by using exon-specific ^{35}S-labeled RNA probes. Although the probes revealed that the *IGF2* promoter usage/splicing patterns appear to be more complex than previously realized (Fig. 4B), the different probes nonetheless uncovered an almost identical pattern of *IGF2* expression. Typically, here represented by the retroperitoneal organs, the results show that P3 promoter-derived transcripts were the most abundant *IGF2* mRNA species in all tissues analyzed and were indistinguishable from the described cDNA probe pattern (Fig. 5B,C). The probes specific for the P2 and P4 promoters yielded less distinct but similar, if not identical hybridization patterns as the P3 promoter-specific probe (Fig. 5D,E). P1 promoter-derived transcripts evaded sensitivity of detection since these could not be detected by in situ hybridization analysis, even in infant and adult liver samples (data not shown).

DISCUSSION

The *IGF2* and *H19* loci are closely linked and reciprocally imprinted in both mouse and man. In addition, these two loci show striking similarities in their expression patterns during mouse embryogenesis. We show here that the *IGF2* and *H19* genes are extensively coexpressed during human embryonic/fetal development. The striking similarities in their expression patterns apply, therefore, also to humans. These results are consistent with the proposals that the imprinting processes and the overlapping expression patterns of *IGF2* and *H19* are coordinatedly controlled (Surani, 1991; Zemel et al., 1992; Brandeis et al., 1993; Bartolomei et al., 1993). Since it has pre-

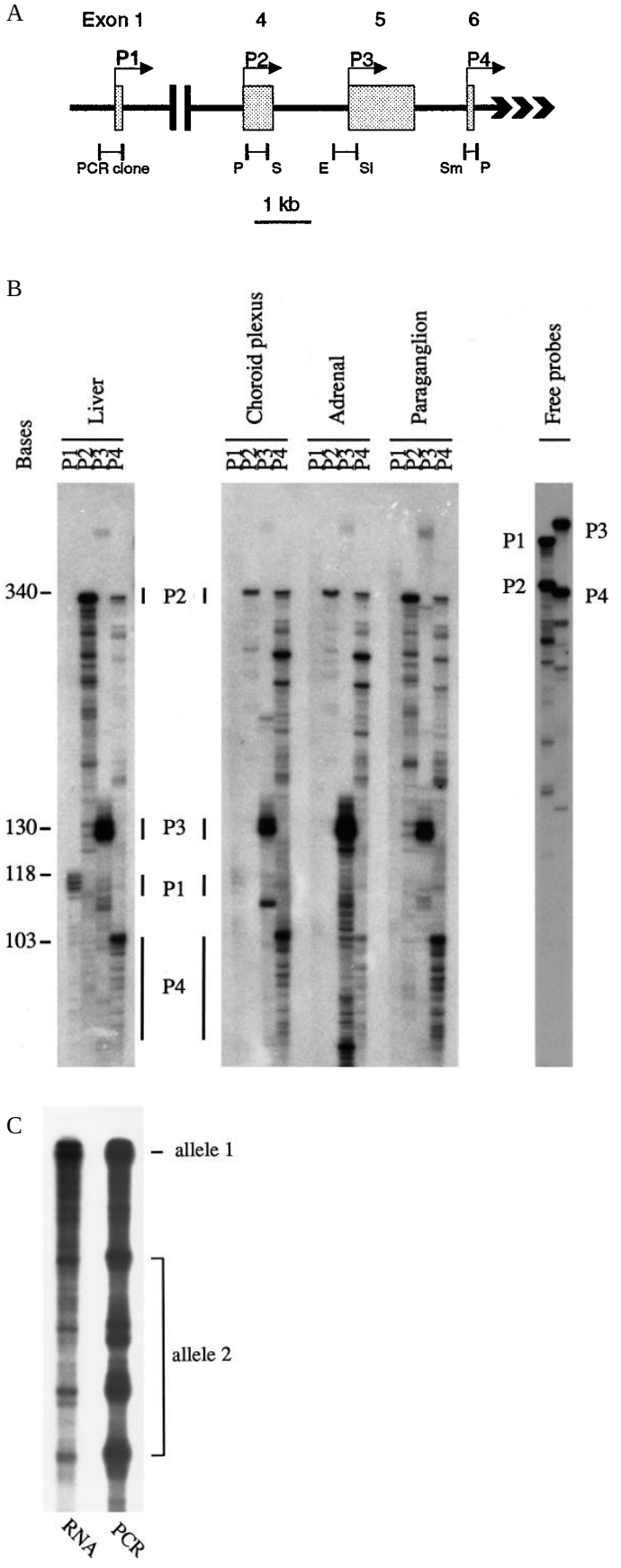


Fig. 4. Promoter usage in human tissues expressing *IGF2* mono- or biallelically. (A) Map of the exon-specific riboprobes. (B) RNase protection analysis of *IGF2* promoter usage in choroid plexus/leptomeninges, adrenals and paraganglia (the same samples as analyzed in Figs 2 and 3). A morphologically normal part of a liver tissue of a 9 month child suffering from hepatoblastoma served as a reference for P1-P4-derived transcripts. Each promoter probe was individually annealed to each cellular RNA sample. The expected transcript species derived from each of the four promoters (and their sizes estimated from a sequence ladder) are indicated in the figure. While the P1, P2 and P3 promoter-derived transcripts appear as doublets or triplets (due to nibbling of the RNases), the wider range (93-103 bases) of P4-derived transcripts may reflect alternate cap site usage (Sussenbach, 1989). (C) A morphologically normal liver of an 18 month infant expresses *IGF2* biallelically. The analysis of RNA and PCR-amplified DNA covering the polymorphic $(C\text{-}A)_n$ repeat region of exon nine was performed as described in the legend of Fig. 2 (see also Materials and Methods).

viously been shown that *Igf2* is not imprinted in the choroid plexus and leptomeninges in the mouse, we considered it interesting to investigate if these tissues express both parental *IGF2* as well as *H19* alleles during human prenatal development. Precedence for the biallelic expression of both *IGF2* and *H19* loci can be found in a subtype of Wilms' tumour forms, although this lack of imprinting was considered to reflect a pathological condition (Rainer et al., 1993; Ogawa et al., 1993). We report here that, in one informative sample, *IGF2* was biallelically expressed in the choroid plexus/leptomeninges but monoallelically expressed in all other internal organs examined. Surprisingly, the *H19* locus was not expressed at detectable levels in the choroid plexus/leptomeninges (and the ciliary anlage of the embryonic retina) in embryonic/fetal as well as postnatal tissue samples. This is in contrast to the observations of Zhang and Tycko who showed that human fetal leptomeninges express *H19* monoallelically (Zhang and Tycko, 1992). It is probable, however, that the *H19* expression in the leptomeninges detected by the more sensitive RT-PCR method, originates from invading blood vessels. Since we present only one informative example of biallelic *IGF2* expression in the choroid plexus/leptomeninges, the generality of the allelic *IGF2* usage in this tissue could be challenged. However, it is the expected result since a similar observation was previously reported in the mouse (DeChiara et al., 1991).

The transcriptional unit of *IGF2* encompasses some 32 kb (Sussenbach, 1989). Since the P1 and P4 promoters are approximately 24 kb apart (Sussenbach, 1989), it might be informative to discover whether or not these promoters are coordinately expressed. We show here that whenever *IGF2* is expressed during human embryonic/fetal development, the P2, P3 and P4 promoters (separated by at least 5 kb) appears to be active although with some variation in relative strength. In contrast, we could detect expression of P1 promoter-derived transcripts in the choroid plexus/leptomeninges alone during early human postnatal development. These observations hint that the usage of the P2, P3 and P4 promoters but not of the P1 promoter can be coordinated irrespective of whether *IGF2* is mono- or biallelically expressed. Although a similar correlation could be observed in a human infant liver specimen, such a link remain inconclusive, however, until the allelic usage for each individual promoter has been examined directly.

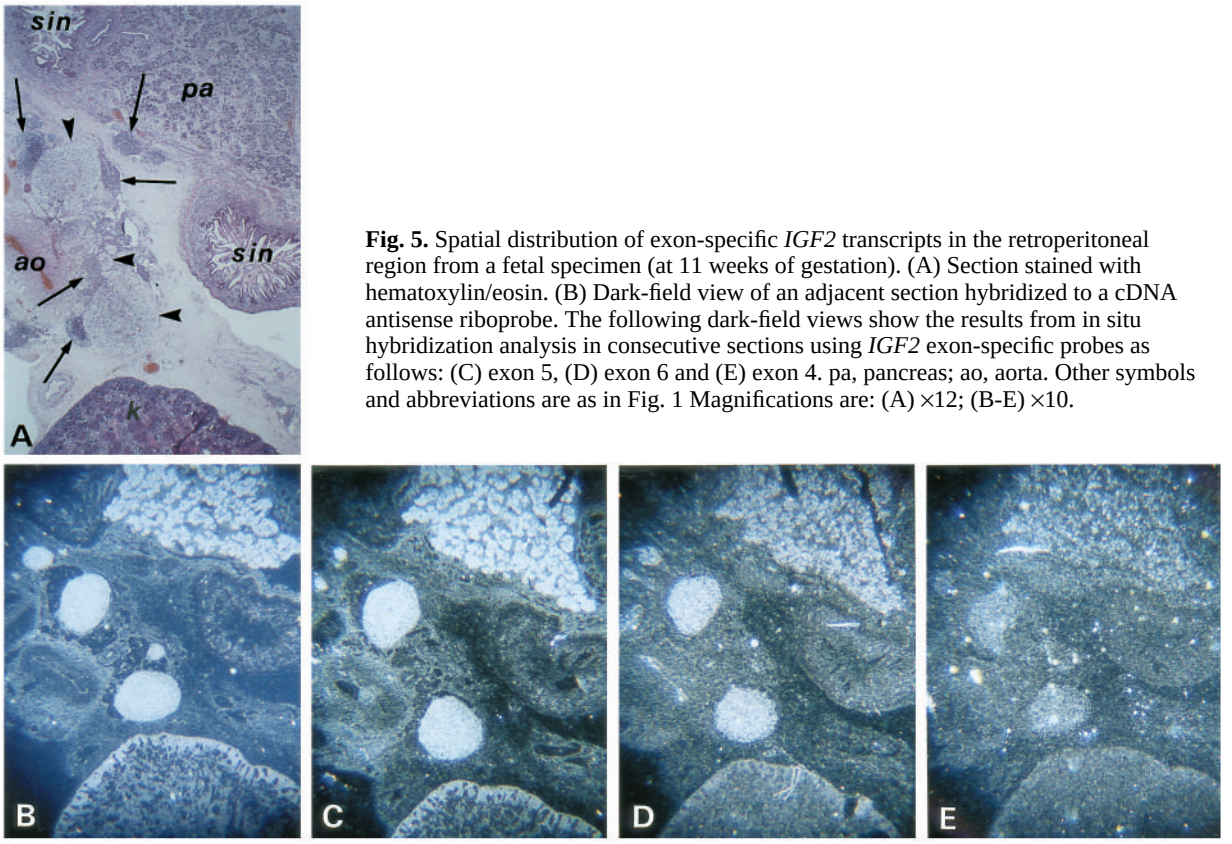


Fig. 5. Spatial distribution of exon-specific *IGF2* transcripts in the retroperitoneal region from a fetal specimen (at 11 weeks of gestation). (A) Section stained with hematoxylin/eosin. (B) Dark-field view of an adjacent section hybridized to a cDNA antisense riboprobe. The following dark-field views show the results from in situ hybridization analysis in consecutive sections using *IGF2* exon-specific probes as follows: (C) exon 5, (D) exon 6 and (E) exon 4. pa, pancreas; ao, aorta. Other symbols and abbreviations are as in Fig. 1 Magnifications are: (A) ×12; (B-E) ×10.

The mechanism of parental imprinting is poorly understood at present. The nature of the epigenetic mark on one or both parental alleles of imprinted loci has not yet been identified although CpG methylation has been implicated in the process of parental imprinting for both *H19* (Ferguson-Smith et al., 1993) and *Igf2r* (Stöger et al., 1993). This link suffers, however, from the uncertainty of whether allele-specific CpG methylation patterns are the cause or consequence of the allele-specific expression patterns. The inactivation of X-linked genes, for example, precedes CpG methylation (Riggs and Pfeifer, 1992). It is interesting, however, that, while the chromatin structure of both parental *IGF2* alleles appears accessible for nucleases (Sasaki et al., 1992), differences both in CpG methylation patterns as well as accessibility of nucleases in the chromatin between the parental *H19* alleles in the mouse have been reported (Ferguson-Smith et al., 1993). The establishment of these differences may reflect independent or interdependent events. Based upon circumstantial evidence, it has previously been proposed that the expression and reciprocal imprinting patterns of *IGF2* and *H19* are coordinated (Surani, 1991; Zemel et al., 1992; Brandeis et al., 1993; Bartolomei et al., 1993). An overriding transcriptional control element for *IGF2* is consistent with the apparently coordinated *IGF2* promoter usage as discussed above. However, a common transcriptional control of *IGF2/H19* would not fit with the lack of *H19* expression in the choroid plexus/leptomeninges, which expresses *IGF2* biallelically, unless some form of hierarchy is invoked so that *IGF2* and *H19* are not normally allowed to be expressed from the same chromosome. In such a scenario, biallelic *IGF2* expression would not be consistent with simultaneous expression of *H19*. The locus control region of the β-globin locus is a precedent for a distal element (separated from the transcription units by up to 60 kb) showing developmentally controlled preferences for different transcription units within this locus (Epner et al., 1992). In this analogy, the expression patterns of *IGF2* and *H19* in the choroid plexus/leptomeninges could be established by a single event such as the repression of the maternal *H19* allele (it should not be essential here whether or not the paternal and maternal *H19* alleles are repressed by the same molecular mechanism except in the extreme possibility that the allele-specific expression of *IGF2* is indirectly governed by the epigenetic imprint of *H19*). Other possibilities, such as the tissue-specific loss of or inability to recognize epigenetic imprints as well as cell-type-specific enhancer elements which are refractory to epigenetic imprints, cannot be ruled out at this stage. These alternatives may, however, require at least two independent events (activation and repression of both parental alleles of *IGF2* and *H19*, respectively) to establish the *IGF2/H19* expression patterns observed in the human choroid plexus/leptomeninges. Whatever the nature of the involved regulatory mechanisms, they should differ (at least in part) between mouse and man, since mouse embryonic choroid plexus/leptomeninges appear to express a parentally imprinted *H19* (Svensson et al., unpublished data).

We are very grateful for valuable advice from Dr Gary Franklin and skilful technical assistance from Virpi Töhönen. This work was supported by the Swedish Cancer Research Foundation (RmC; grants 1955 and 3271), Swedish Natural Science Council (NFR; grant nrs B-Bu-4494-301-303) and research funds of the Karolinska Insitute.

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(Accepted 18 November 1993)