H19 sense and antisense transgenes modify insulin-like growth factor-II mRNA levels

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The oppositely-imprinted genes insulin-like growth factor-II (IGF2) and H19, a putative tumor suppressor, often show coordinate, reciprocal regulation and are believed to play a role in carcinogenesis. To explore the possible interactions between these genes, we stably transfected diHepG2 cells with a plasmid containing either the sense or the antisense H19 cDNA sequences and verified their expression by Northern analysis and by RNAse protection analysis. Levels of H19, IGF2 and β-actin mRNA were quantified by competitive RT-PCR analysis. Although H19 sense transgene overexpression (n = 24 clones) did not decrease the low, basal levels of IGF2 mRNA compared to control cells, levels of IGF2 mRNA were positively correlated with the levels of H19 antisense mRNA (P < 0.0001, n = 40 clones). Furthermore, the increase in IGF2 mRNA level was accompanied by an elevation of IGF-II peptide in conditioned media. To see if H19 mRNA had a specific effect on transcription, we also performed transient transfections with reporter gene constructs containing IGF2 promoter 3 in the presence of sense or antisense H19 cDNA sequences under control of a cytomegalovirus promoter. We show a lower reporter gene activity from reporter gene constructs in the presence of sense H19 cDNA than from those with antisense or neomycin. Our results suggest that H19 participates in the repression of IGF2, at least in part through effects on IGF2 transcription, an effect which may contribute to its action as a tumor suppressor.

Keywords: insulin-like growth factor-II; gene regulation; H19; tumor suppressor genes.

Human insulin-like growth factor (IGF-II) is an important mitogen which plays a role in normal fetal and postnatal growth, and also in tumorgenesis [1]. Regulation of the human IGF2 gene is very complex, both at the transcriptional and post-transcriptional level. Firstly, this gene is directed by four promoters which exhibit a tissue and development-specific expression pattern. Secondly, it includes sequence elements that are recognized by transcription factors (such as p53, WT-1 or C/EBPα), some of which are known to have tumor suppressor activity [2–4]. Thirdly, the minisatellite DNA polymorphism consisting of a variable number of tandem repeats (VNTR) at the human INS (insulin gene) 5′-flanking region has recently been shown to affect IGF2 transcription [5]. Finally, IGF-II mRNAs are subjected to differential polyadenylation, alternative splicing and site-specific endonucleolytic cleavage [4].

IGF2 is also epigenetically regulated [6], so that only the paternal allele is expressed in most tissues prenatally and in some tissues during postnatal life, a phenomenon referred to as genomic imprinting. Preferential loss of maternal alleles (loss of heterozygosity) and relaxation of parental imprinting (loss of imprinting) of IGF2 occur frequently in certain pediatric tumors, such as Wilms' tumors, adrenocortical carcinomas, hepatoblastomas and rhabdomyosarcomas, and in malignant childhood tumors (including uterine, lung and testicular tumors), suggesting that abnormal expression of IGF2 and/or of other 11p15.5 imprinted genes has a role in the pathogenesis of these diseases [7,8].

IGF2 lies only 110–200 kb centromeric to H19 [9], a gene whose transcript is not translated [10,11], but whose molecular evolution suggests a functional role for its mRNA [12]. Untranslated H19 is believed to play a role in tumor suppression (although this is controversial [13–15]) and, as for IGF2, it is an imprinted gene [16]. IGF2 and H19 are reciprocally imprinted, such that H19 is transcribed exclusively from the maternal allele in a tissue and developmentally regulated manner. H19 and IGF2 have been found to show coordinate, reciprocal regulation in a number of other situations: (a) although H19 and IGF2 show a similar tissuespecific pattern of expression, H19 is not expressed in the choroid plexus or leptomeninges, the only tissue where IGF2 is expressed bilaterally in mouse and human [17]; (b) methyltransferase knockouts, with no detectable methylation at the H19 domain, express both copies of H19 but neither of IGF2 [18]; (c) IGF2 expression has been shown to be biallelic in the mouse H19 knockout [19]; (d) Wilms' tumors with relaxation of IGF2 imprinting (i.e., with biallelic expression) show aberrant methylation of the maternal H19 copy and quantitatively repressed H19 [21]; (e) the level of H19 expression was found to be very low (and IGF2 expression very high) in adult adrenal carcinomas compared to normal adrenals [22]; and (f) in leukocytes, IGF2 in most individuals 'escapes' imprinting, but monoallelic expression is found in a minority. These imprinters are capable of expressing H19, whereas those that do not show imprint HGF2 expression do not express H19 [23].

Understanding the role of human H19 is complicated by the use of mouse H19 transgenes inserted into human cells, an
artificial situation in which cross-species trans-activating factors may be missing [24]. Furthermore, it is not yet clear whether the results from the more recent mouse H19 deletion mutants, suggesting that H19 expression is not necessary for imprinted IGF2 expression, can be extrapolated to humans, or that they rule out H19 trans effects on IGF2 regulation [25,26]. Indeed, Li et al. [27], have provided data which led them to propose a model whereby H19 may modify steady state IGF2 mRNA cytoplasmic levels as well as decreasing association of IGF2 mRNA with polysomes. They further suggest that, in mice, this regulatory role may have been lost.

In the present paper, we have explored the coordinate regulation of IGF2 and H19 further by studying the effect of H19 sense or antisense transgenes on the IGF2 mRNA level in stably and transiently transfected diHepG2 cells.

MATERIALS AND METHODS

Tissue cultures

DiHepG2 cells obtained from ATCC (HB-8065, Rockville, MD, USA) were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 2 mM L-glutamine and 1% sodium pyruvate. For the stable transfection experiments, a clonal cell line was selected (courtesy of M. Lambert, Genetics Service, Department of Pediatrics, Ste-Justine Hospital, Montreal, Quebec, Canada) which was disomic for chromosome 11 (diHepG2 cells) by fluorescent in situ hybridization (FISH) using digoxigenin-labeled chromosome-11-specific probes (Oncor, Gaithersburg, MD, USA).

Vector construction

For the stable transfections, the human H19 cDNA (bp +65 to +2248) kindly provided by S. Zemel (Department of Pediatrics, Yale University, New Haven, CT, USA) was subcloned into the HindIII site of pRC-CMV (Invitrogen, Carlsbad, CA, USA) in both orientations relative to the cytomegalovirus (CMV) promoter, and the sequence was verified from both ends.

To generate H19-IGF2 reporter gene constructs for transient transfections, we cloned the entire human H19 cDNA (bp -60 to +2248) into the EcoRI sites of pcdNA3 (Invitrogen) in both orientations relative to the CMV promoter, then CMV promoter and H19 sense or antisense were subcloned into SalI-XbaI sites of pcAT-Basic (CAT, chloramphenicol acetyltransferase; Promega, Madison, WI, USA). These constructs were termed pCMVH19+/CAT or pCMVH19−/CAT. As a control, we deleted the region between two EcoI sites (bp -922 and +2186) of pcdNA3 to have the neomycin cDNA directly downstream of the CMV promoter; this was then subcloned into SalI site of pcAT-Basic and termed pcMVNEO+CAT. IGF2 promoter P3 [5] was ligated into the XbaI site of these different plasmids directly upstream of the CAT reporter gene. These constructs were termed pCMVH19+/3CAT, pCMVH19−/3CAT or pCMVNEO/3CAT. A HindIII fragment containing the CMV promoter was also removed to generate control plasmids without this promoter and these constructs were termed pNEO/3CAT, pH19+/3CAT and pH19−/3CAT. A schematic diagram of all the constructs is included with the transient transfection results. The authenticity and directionality of all the constructs was verified by restriction enzyme analysis.

Transfections

For stable transfections, 20 μg of DNA vector was digested with PstI, ethanol precipitated and resuspended in 20 μL of Tris/EDTA. DiHepG2 cells were harvested by trypsinization from 75% confluent T-75 flasks, centrifuged at 200 g for 5 min and resuspended in calcium and magnesium free NaCl phosphate buffer, pH 7. DiHepG2 cells (1 × 10⁶/mL) were then transfected by electroporation using a 250-400 μF pulse from a Gene Pulser (Bio-Rad Labs, Hercules, CA, USA). Selection with G418 (400 μg/mL) (Geneticin, Life Technologies, Gaithersburg, MD, USA) started 48 h after the electroporation. After three weeks, G418-resistant (G418R) colonies were individually picked for further propagation.

For transient transfection assays, the procedure was as described in Paquette et al. [5]. Briefly, 3 × 10⁶ diHepG2 cells in 35-cm² multwell dishes in serum-free medium (Opti-MEM, Life Technologies) were transfected with 2 μg of each CAT construct and with 1 μg of pSVβ (a plasmid encoding β-galactosidase; Promega) using a cationic liposome formulation (Lipofectin, Life Technologies). The CAT and β-galactosidase assays were then performed 48 h later as described in [5].

Northern analysis

Total RNA (13 μg) from transfected diHepG2 cells was prepared [28], electrophoresed on 1% agarose-formaldehyde gel using standard procedures [29], blotted onto nylon membrane (MSI, Westborough, MA, USA) and hybridized with [α-32P]dATP probes labeled by random priming. For the H19 probe, a PCR-amplified fragment of the human H19 gene [30] was subcloned in pBluescript (Invitrogen) and a 0.5-kb Apal-HindIII fragment containing exons 4−5 cDNA was purified. For the IGF2 cDNA probe, a 1-kb PsI-XhoI fragment containing exons 1−9 was used, courtesy of Dr J. Sussenbach (Department of Biochemistry, Utrecht). Finally, a 2.1-kb BamHI fragment was used for β-actin cDNA probe.

cDNA synthesis and competitive RT-PCR analysis

Total RNA from clones of diHepG2 cells was isolated as described above and treated with RNase-free DNase (Promega). cDNA synthesis was performed as described in Paquette et al. [5]. The internal competitor standard was generated using PCR-based in vitro mutagenesis of IGF2, H19 or γ-actin as indicated [31,32]. For IGF2 PCR, the standard is 190 bp and consists of the identical sequence to that of the exon 9-derived IGF2 cDNA PCR product with an internal deletion of 46 bp. For H19 PCR, the standard is 488 bp and consists of the identical sequence to that of the exons 4−5-derived H19 cDNA PCR product with an internal deletion of 87 bp. For γ-actin PCR, the modified standard has lost a restriction site (BstEII) and gives a fragment of 612 bp instead of two smaller fragments of 360 bp and 252 bp (see below). Plasmids containing competitor were aliquoted in fractions of 0.01−1 × 10⁻¹ final for H19, 0.01−1 × 10⁻² final for IGF2 and 0.01−1 × 10⁻³ final for γ-actin. IGF2 and H19 PCR conditions have been described previously [5,30]. For the γ-actin PCR, the cDNA and competitor were mixed with sense (5'-GACACCAGGGCGGTGTCAGTGAG-3') and antisense (5'-GACGGTCGTGTCAGTGAGC-3') γ-actin primers (50 pmol each) in the buffer supplied by Life Technologies, supplemented with 2 mm MgCl₂, 0.2 mm of each dNTP including 2.5 μCi of [α-32P]dATP (Amersham Pharmacia Biotech) and 2 U of Taq DNA Polymerase (Life Technologies). The cycling parameters
consist of 30 cycles of 60 s at 94 °C and 60 s at 72 °C, followed by a final extension time of 10 min at 72 °C. PCR-amplified products were resolved electrophoretically, and quantified by phosphorimager analysis.

**RNase protection assay**

RNase protection assay was performed as described in the manufacturer's protocol (Promega). A PCR-amplified fragment of human H19 gene [30] was subcloned in both orientations in pRC/CMV (Invitrogen). The vectors were linearized at the unique HincII site, and served as templates in an in vitro transcription reaction using [α-32P]CTP and T7 RNA polymerase (Promega). A sense probe of 341 nucleotides (including 94 nucleotides of polylinker and bp +2754 to +3001 of the human H19 gene GenBank accession no. M32053) and an antisense probe of 501 nucleotides (including 94 nucleotides of polylinker and bp +3408 to +3001 of the human H19 gene) were then generated. The expected size of the major protected sense transcript fragment is 407 bp whereas that of the antisense transcript fragment is 125 bp (plus a smaller fragment of 42 bp not visualized) as the 247 bp probe includes an intron of 80 bp which will not be protected. Total RNA (15 μg) from stably transfected diHepG2 cells was hybridized to 5 × 10^6 c.p.m. radiolabeled probe overnight at 42 °C in a buffer containing 80% formamide, 1.0 mM EDTA, 40 mM Pipes (pH 6.4), and 0.2 M sodium acetate. Single-stranded RNA was then digested in a buffer containing 10 mM Tris/HCl (pH 7.5), 5 mM EDTA, 200 mM sodium acetate with 10 U of RNase ONE (Promega), and incubated for 1 h at 25 °C. The digestion was inactivated by adding stop solution containing 10% SDS and 1.0 mg/mL-1 tRNA. The products were precipitated with ethanol and analyzed on 6% polyacrylamide denaturing gels. Gels were exposed for up to 8 days to Fuji medical X-ray film with intensifying screens at -70 °C.

**IGF-II peptide immunoassay in conditioned medium**

The clones of diHepG2 cells were plated at 10^6 cells/well in six-well plates for 48 h, washed with serum-free MEM and incubated in serum-free MEM (1 mL per well) for an additional 48 h. Conditioned media were collected and centrifuged at 1000 g for 10 min. Following centrifugation by centrifrip-3 (Amicon, Beverly, MA, USA) or lyophilization, IGF-II content was measured after acid/ethanol extraction by ELISA assay (Diagnostic Systems, Webster, TX, USA). Cell counts were performed before and after the 48-h serum starvation.

**Data analysis**

Regression analysis and Student's t-test were performed using the STATVIEW software (Abacus Concepts, Berkeley, CA, USA).

**RESULTS**

We chose diHepG2 cells to explore the role of H19 in the regulation of IGF2 for several reasons: firstly, the IGF2 transcription pattern is similar to that of fetal liver; secondly, the nuclear factors are present for activation of all four promoters (P3 >> P4; P2 > P1), although P3 is by far the most important promoter [33]; thirdly, IGF2 mRNA is produced in quantities detectable by Northern analysis [33]; fourthly, the peptide IGF-II is also produced [34]; and finally, hepatoblastomas have been shown to retain imprinted expression of IGF2 [35].

Given that lines derived from tumors are often polyploid, and may thus possess abnormal gene dosages, we used a diHepG2-derived cloned cell line which we showed by fluorescent in situ hybridization to be diploid for chromosome 11 (diHepG2 cells) and to express both IGF2 and H19 by RT-PCR and by Northern analysis (data not shown).

Electroporation of these cells with the vector pRC/CMV alone (controls) or with the sense and antisense H19 cDNA sequences yielded multiple G418 clones, 69 of which were further characterized. The copy number of transgenes was analyzed by Southern hybridization for H19. Of ten randomly chosen clones (including those with the highest levels of transgene expression), each had a single or a double-copy insertion, as calculated by the relative signal intensities on phosphorimager analysis normalized to the endogenous signal (data not shown).

Production of IGF2 transcripts and of H19 sense and antisense transcripts was confirmed by Northern analysis and by RNase protection assay analysis, respectively. The two major 6.0-kb and 2.3-kb IGF2 transcripts (expressed in these cells from P3 promoter) were detected in all clones by Northern analysis, with the highest levels observed in the antisense clones. To evaluate the RNA loading, Northern blots were exposed to Fuji medical X-ray film with intensifying screens at -70 °C.

**Fig. 1. Northern blot analysis of H19 and IGF2 total RNA in stably transfected diHepG2 cell lines with controls. H19 sense or antisense constructs.** (A) Hybridization with an H19 exon 4.5-specific cDNA probe. Lanes 1 and 2 correspond to RNA from two different control clones, lanes 3 and 4 to RNA from two different sense clones, and lanes 5 and 6 to RNA from two different antisense clones. (B) The same blot stripped and rehybridized with an IGF2 cDNA probe as described in the text. (C) The same blot stripped and rehybridized with a 15-aminoterminal cDNA probe as described in the text.
Fig. 2. RNase protection assay analysis of H19 transcripts in diHepG2 cell lines stably transfected with H19 sense or H19 antisense constructs. (A) Lane designations are MW, molecular mass; Probe +, undigested sense probe; Probe −, undigested antisense probe; Probe actin, undigested actin probe. The last two lanes (Probe +/RNA; Probe −/RNA) represent the undigested probes mixed with tRNA to identify nonspecific bands and to verify if probes are contaminated with RNase. Lanes containing RNase-treated probes confirmed completeness of digestion (data not shown). (B) Sense transfectants (+, top row) hybridized with antisense −, second row) or sense (+, second row) probe. (C) Sense and antisense transfectants (+ or −, respectively, top row) hybridized with antisense probe (−, second row). The expected size of the major protected sense transcript fragment is 407 bp. (D) Sense and antisense transfectants hybridized with sense probe. The expected size of the major protected antisense transcript fragment is 125 bp. NS, nonspecific bands seen in undigested probe control lane.

(in duplicate) were stripped and rehybridized with a β-actin cDNA probe as an unchanging control transcript. The ratio between the major 6-kb IGF2 mRNA and the β-actin mRNA determined by ImageQuant phosphorimager software was: 0.10 ± 0.02 for control clones (cells transfected with the pRCE/CMV vector alone); 0.12 ± 0.06 for sense clones and 0.23 ± 0.04 for antisense clones (Fig. 1B,C). At exposure times which permitted detection of IGF2 transcripts, H19 RNA was detected in sense-transfected cells but not in antisense-transfected or in control cells (Fig. 1A).

RNase protection assay analysis, however, confirmed transcription of the H19 transgenes, no doubt due to the increased sensitivity of this technique compared to Northern blot analysis. The expected protected fragment of 407 bp from the sense transfectants was clearly detected after hybridization with the antisense probe (Fig. 2B,C) but not with the sense probe (Fig. 2B). Inversely, in antisense transfectant clones, this fragment (corresponding to the endogenous transcript) was weakly detected (Fig. 2C). Furthermore, the predicted protected fragment of 125 bp is only seen in antisense transfectants after hybridization with the sense probe; as expected, overexposure of the X-ray film never revealed this fragment in sense transfectants (Fig. 2D).

Given the levels of H19 sense and antisense transcript expression, a highly sensitive competitive RT-PCR analysis was next used to more precisely quantify H19, IGF2 and γ-actin mRNA from three control, 24 sense and 40 antisense clones. Figure 3A shows a representative RT-PCR assay for each of the clones; linearity of the three competitive RT-PCR assays over the range of competitors used was confirmed by regression analysis. Figure 3B summarizes the results obtained in triplicate for each gene; each point corresponds to one clone. For each clone, the amount of IGF2, H19 and γ-actin mRNA was expressed as the ratio (in arbitrary units) of the intensity of the specific PCR product to the intensity of the internal competitor. In order to permit comparisons between clones, the IGF2 and H19 data were normalized to γ-actin to control for quality of RNA and efficiency of the reverse transcription reaction. In addition, parallel PCR amplification of the reverse transcription reaction following incubation either with or without reverse transcriptase was routinely performed to ensure that no genomic DNA was present in the PCR reactions.

Both the sense and antisense transfectant clones showed variability in the amount of transcript expression as expected, given the random integration of the constructs. We observed that H19 sense transgene overexpression, although very high in some clones (up to 70-fold higher), did not influence the level of IGF2 mRNA compared to control cells. However, although observed H19 antisense transcript expression was lower in comparison with sense clones, IGF2 mRNA levels increased up to a maximum of approximately eightfold (Fig. 3B) in clones expressing the highest levels of H19 antisense transcripts. More importantly, a regression analysis comparing H19 antisense and IGF2 transcription levels showed a highly significant positive and linear correlation between the two (p < 0.0001), as seen in Fig. 3C.

The increase of IGF2 mRNA level in the H19 antisense transfectants was accompanied by a significant increase in IGF-II peptide production, as determined by an IGF-II ELISA on conditioned media obtained from clones in which IGF2 mRNA levels were shown to be the highest (Fig. 4; P < 0.05). In contrast, the production of IGF-II in H19 sense transfectants was the same as in the control cells.

We next examined the effects of H19 mRNA on reporter gene activity driven by the third IGF2 promoter (IGF2 P3), to see if H19 mRNA has specific transcriptional effects, and to verify if a local production of H19 RNA (in cis, i.e. on the same DNA strand) is required to see an effect on IGF2 P3. We hypothesized that with this approach, we would also exclude a direct effect of H19 antisense on this promoter. We performed transient transfections of diHepG2 cells with constructs containing both IGF2 P3 placed upstream of the CAT reporter gene and sequences of sense or antisense H19 cDNA under control of a cytomegalovirus promoter. Transfection efficiency was monitored by cotransfection with a plasmid encoding the β-galactosidase gene (pSVB). Multiple experiments were
performed at different transfection efficiencies. Results are shown normalized to CAT activity of a control construct containing neomycin instead of H19 sense or antisense (pCMVvNGEO/3CAT) following correction for β-galactosidase activity (Fig. 5A). When H19 was placed in an antisense orientation (pCMV/H19-3CAT), levels of CAT activity were not significantly different from the control pCMVvNGEO/3CAT construct ($0.375 < P < 0.4$). In contrast, in the presence of H19 sense cDNA (pCMV/H19+/3CAT), we observed a significantly decreased level of reporter gene activity relative to pCMVvNGEO/3CAT ($P < 0.0005$). Constructs in which IGF2 P3 promoter elements were omitted, thus placing the CAT gene without any promoter control, failed to produce significant CAT activity, thereby excluding cytomégalovirus promoter-directed CAT transcription.

Another set of plasmid constructs was designed in which the cytomégalovirus promoter segments were omitted. Results are shown normalized to CAT activity of a control plasmid with neomycin instead of H19 sense or antisense (pNEO/3CAT) following correction for β-galactosidase activity. In these experiments, the pH19+3CAT construct was not significantly different from pH19-3CAT ($P > 0.9$), excluding an effect of the H19 DNA on the activity of the IGF2 P3 promoter (Fig. 5B). It should be noted that the activity of P3 in the absence of cytomégalovirus promoter is comparable to what we have shown previously [5].

Fig. 3. Competitive RT-PCR analysis of IGF2. H19 and γ-actin mRNA levels of different clones after stable transfection of dHepG2 cells with control, H19 sense or H19 antisense constructs. (A) Examples of the three competitive RT-PCR assays using a range of internal-standard amounts to assess the linearity of the assay. The relative concentration of endogenous transcript in each clone is that concentration of internal competitor added at which the intensity of the specific PCR product is near that of the internal standard (competitor amount in femtomoles; for IGF2 lane 1 = 1 × 10^{-5}, lane 2 = 1 × 10^{-4}, lane 3 = 1 × 10^{-5}, lane 4 = 1 × 10^{-4}, lane 5 = 1 × 10^{-5}; for H19 lane 1 = 1 × 10^{-4}, lane 2 = 1 × 10^{-5}, lane 3 = 1 × 10^{-4}, lane 4 = 1 × 10^{-5}, lane 5 = 1 × 10^{-6}; for γ-actin lane 1 = 1 × 10^{-5}, lane 2 = 1 × 10^{-6}, lane 3 = 1 × 10^{-5}, lane 4 = 1 × 10^{-6}, lane 5 = 1 × 10^{-5}). (B) Each point corresponds to a clone to = 24 sense clones, n = 40 antisense clones) and represents the value in arbitrary units determined in triplicate for each competitive PCR reaction of IGF2 mRNA level as a function of H19 sense or antisense mRNA level, determined as described in Materials and methods. In addition, all clones were normalized for the γ-actin mRNA content in order to permit comparisons between clones. Three control clones are included for comparison, and lie close to the origin. (C) Regression analysis was performed on antisense clones and shown to be highly significant ($R^2 = 0.6575, P < 0.0001$).
**DISCUSSION**

A functional significance for the reciprocal regulation of H19 and IGF2 was initially suggested by indirect evidence that H19 may be acting as a suppressor of IGF2 and/or of other genes involved in growth. For example, maternal loss of 11p15.5 was correlated with the appearance of tumors in the Beckwith-Wiedemann syndrome of fetal macromesia [36]. Also, in the mouse model of Beckwith-Wiedemann syndrome, involving transactivation of IGF2, it was shown that the Igf2 transgenes were repressed during development and the endogenous IGF2 locus became hyperactivated, concomitant with a reduction in H19 mRNA [37]. Evidence supporting a tumor suppressor role for H19 was obtained in vitro because, when cell lines derived from embryonal tumors were transfected either with H19 genomic DNA constructs [38] or with an H19 cDNA construct [13], tumorigenesis was decreased. Unfortunately, IGF2 expression was not evaluated in these studies.

Reactivation of the silent Igf2 maternal allele and the resultant overgrowth seen in the initial H19 knockout models raised expectations that the tumor suppressor role of H19 was via its effects, in cis, on Igf2 silencing [19,20]. More recent studies deleting the H19 transcriptional unit found no loss of imprinting of IGF2 in neonatal liver [25,26] and only a small relaxation of IGF2 imprinting in skeletal muscle, which resulted in a 17% increase of IGF2 mRNA [26]. These and other experiments [39-41] suggest that H19 mRNA is not required to maintain the imprinted expression of IGF2, and that absence H19 transcription does not always imply biallelic IGF2 expression, at least in these mouse models.

Recruitment of the second (maternal) allele may not be the only mechanism leading to increased IGF2 mRNA, however. Li et al. [27] have shown, by in situ hybridization, an inverse correlation between H19 expression and cytoplasmic levels of IGF2 mRNA in a Wilms' tumor; cells negative for H19 showed a two to threefold increase in IGF2 mRNA. Furthermore, H19 mRNA was shown to be associated with polysomes and thus may also participate in modulating translation of IGF2 mRNA.

We used diHepG2 cells disomic for chromosome 11 to study the effects of a decrease in the level of H19 transcripts on the expression level of IGF2 using an H19 antisense approach. IGF2 mRNA levels were increased up to eightfold; the magnitude of this increase was directly proportional to the level of H19 antisense transcription even at levels of expression which were severalfold lower than the levels of H19 sense transcription achieved. Our inability to detect high levels of antisense H19 transcripts may be explained by the degradation of the RNA-RNA hybrid molecules by enzymes that selectively cleave RNA sense/antisense duplexes [42]. The mechanism of action of the H19 antisense is assumed to be via its ability to decrease endogenous H19 levels. An alternative possibility is that the antisense transcripts may have more direct effects on IGF2 transcription. One of the rationales for proceeding to the transient transfection studies was to address this possibility. If the antisense transcripts could directly act on the major HepG2 IGF2 promoter, then its expression in tandem to this promoter should have resulted in an increase in reporter gene activity compared to the control constructs. This was not the case.

The modest increase in IGF-II peptide production compared to the marked increase in mRNA may be explained by the differential polysomal localization of human IGF2 mRNA seen in diHepG2 cells and fetal liver. Previous studies have shown that the majority of the 6.0-kb transcripts have sedimented as a 100S particle, indicating that this mRNA is sequestered as ribonucleoprotein, and is therefore completely untranslated [43]. The fact that cells were not grown under the same culture conditions for the two assays, may also make extrapolations between mRNA levels and peptide levels difficult. For IGF-II peptide measurements, the cells were incubated for 48 h in serum-free MEM in order to ensure that serum-derived IGF-II would not interfere in the IGF-II ELISA. Indeed, in growth arrested cells, an increase (up to 87%) of 6.0-kb mRNA is found within ribonucleoprotein particles; therefore these transcripts are highly post-transcriptionally regulated [44].

A puzzling finding was the observation that H19 sense transgene overexpression, although very high (up to 70-fold higher), did not affect the level of IGF2 mRNA compared to control cells. This suggested that there was either a threshold effect of H19 on IGF2 or that the transeptional product of the randomly integrated H19 transgene was not accessible to IGF2, i.e., cannot act when the two are in trans.

To see if H19 mRNA has a specific effect on transcription, we performed transient transfections with constructs containing both a reporter gene driven by IGF2 P3 and sense or antisense H19 cDNA sequences under control of a cytomegalovirus promoter. We show a significantly decreased level of reporter gene activity (P ≤ 0.0005) from reporter gene constructs containing sense H19 cDNA (pCMVH19+3CAT), relative to those with antisense H19 cDNA (pCMVH19-3CAT) or neomycin (pCMVNEO+3CAT).

Transient transfection results, in contrast to stable transfection results, show that sense H19 mRNA repressed transcriptional activity of IGF2 P3, perhaps because a local production of H19 mRNA (on the same DNA strand) is required. It should also be noted that in transient transfections, P3 is present in large numbers and not saturated by endogenous transcriptional regulators (including perhaps endogenous H19).

Conversely, seemingly in contrast with the stable transfection results, transient transfection results show that antisense H19 RNA does not effect the transcriptional activity of IGF2 P3. This could be explained by a level of the H19 endogenous transcript (the target of the antisense H19) insufficient to interact with P3-CAT, which is in excess.

Our results do not allow us to determine if the effects of the H19 transcripts are allele-specific, but they do indirectly...
support a role for the H19 gene in IGF2 transcription. What is also clear, however, is that the increase in IGF2 mRNA level of up to eightfold, which was observed when antisense H19 transcripts were stably overexpressed, cannot be due simply to a doubling of gene dosage, as would be seen if the cells had undergone relaxation of imprinting. We hypothesize therefore that H19 may participate in IGF2 gene regulation independent of allelic usage. Interestingly, the converse (i.e. regulation of H19 by IGF2 gene overexpression) did not occur in our model of human embryonal kidney fibroblast cells stably transfect with full-length IGF2 cDNA, as we have previously shown [45]. Precedents do exist both for RNA as a transcriptional regulator [46–48], and for the presence of untranslated, possibly regulatory, RNAs within other imprinted regions [49–54].

In conclusion, we have shown, in stable transfections, that an antisense H19 transgene increases IGF2 mRNA and IGF-2 peptide levels. Furthermore, in transient transfections, we show a significantly lower level of IGF2 P3 activity from reporter gene constructs containing sense H19 cDNA, relative to those with antisense H19 or neomycin cDNA. These data further emphasize the need to support a tumor suppressor role for H19, via its ability to regulate IGF2, and we hypothesize that H19 mRNA is affecting IGF2 transcription. It will be important to determine if the regulation is allelic-specific or independent of imprinting.

ACKNOWLEDGMENTS

These studies were supported by the Cancer Research Society, Inc., the AUPPEL-UREF (Fellowship to F.W.), the Canadian Medical Research Council (grant # 23103-MP-29654-CFCA-15953 to C.D.), the Fonds de Recherche en Santé du Québec (Chevreuil-Bourgeois no. 6050 to C.D.) and the Research Center of the Hôpital Sainte-Justine. The authors would also like to thank Dr Nicole Lemieux for the FISH analysis and Dr Constantin Polychronakos and Dr Guy Van Vliet for their thoughtful review of the manuscript.

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