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 γ-*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 tumorogenesis [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 adulthood 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*, 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 tissue-specific pattern of expression, *H19* is not expressed in the choroid plexus or leptomeninges, the only tissue where *IGF2* is expressed biallelically 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 [19,20]; (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 imprinted *IGF2* 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, Montréal, Québec, Canada) which was disomic for chromosome 11 (diHepG2 cells) by fluorescent *in situ* hybridization (FISH) using digoxigenin-labeled chromosome-11-specific probes (Coatasome 11) from 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 *Hin*dIII 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 *Eco*RI sites of pcDNA3 (Invitrogen) in both orientations relative to the CMV promoter, then CMV promoter and *H19* sense or antisense were subcloned into *Sal*I-*Xba*I 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 *Eag*I sites (bp +922 and +2186) of pcDNA3 to have the neomycin cDNA directly downstream of the CMV promoter; this was then subcloned into *Sal*I site of pCAT-Basic and termed pCMVNEO/CAT. *IGF2* promoter P3 [5] was ligated into the *Xba*I site of these different plasmids directly upstream of the CAT reporter gene. These constructs were termed pCMVH19+/3CAT, pCMVH19−/3CAT or pCMVNEO/3CAT. A *Hin*dIII 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 *Pvu*I, 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/P_i, pH 7. DiHepG2 cells ($1 \times 10^7 \cdot mL^{-1}$) were then transfected by electroporation using a 250-V, 500 μF pulse from a Gene Pulser (Bio-Rad Labs, Hercules, CA, USA). Selection with G418 (400 $\mu g \cdot mL^{-1}$) (Geneticin, Life Technologies, Gaithersburg, MD, USA) was started 48 h after the electroporation. After three weeks, G418-resistant ($G418^r$) colonies were individually picked for further propagation.

For transient transfection assays, the procedure was as described in Paquette *et al.* [5]. Briefly, 3×10^5 diHepG2 cells in 35-cm^2 multiwell dishes in serum-free medium (Opti-MEM, Life Technologies) were cotransfected 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 detailed [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 magnacharge nylon membrane (MSI, Westborough, MA, USA) and hybridized with [α-^{32}P]dATP probes labeled by random priming. For the *H19* probe, a PCR-amplified fragment of the human *H19* gene [30] was subcloned in pRc/CMV (Invitrogen) and a 0.5-Kb *Apa*I-*Hin*dIII fragment containing exons 4–5 cDNA was purified. For the *IGF2* cDNA probe, a 1-kb *Pst*I-*Xho*I fragment containing exons 1–9 was used, courtesy of Dr J. Sussenbach (Department of Biochemistry, Utrecht). Finally, a 2.1-kb *Bam*HI 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), and 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 (*Bst*EII) 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} fmol for *H19*, 0.01–1×10^{-2} fmol for *IGF2* and 0.01–1×10^{2} fmol 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′-GACAC-CAGGGCGTCATGGTG-3′) and antisense (5′-GCAGCTCG-TAGCTCTTCTCC-3′) γ-*actin* primers (50 pmol each) in the buffer supplied by Life Technologies, supplemented by 2 mM $MgCl_2$, 0.2 mM of each dNTP including 2.5 μCi of [α-^{32}P]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 *Hin*cII site, and served as templates in an *in vitro* transcription reaction using [α-^{32}P]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 5×10^5 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 another 48 h. Conditioned media were collected and centrifuged at 1000 *g* for 10 min. Following concentration by centriprep-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^r 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 transcription), 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-kb and 2.3-kb *IGF2* transcripts (expressed in these cells from P3 promoter) were routinely detected in all clones by Northern analysis, with the highest levels observed in the antisense clones. To evaluate the RNA loading, Northern blots

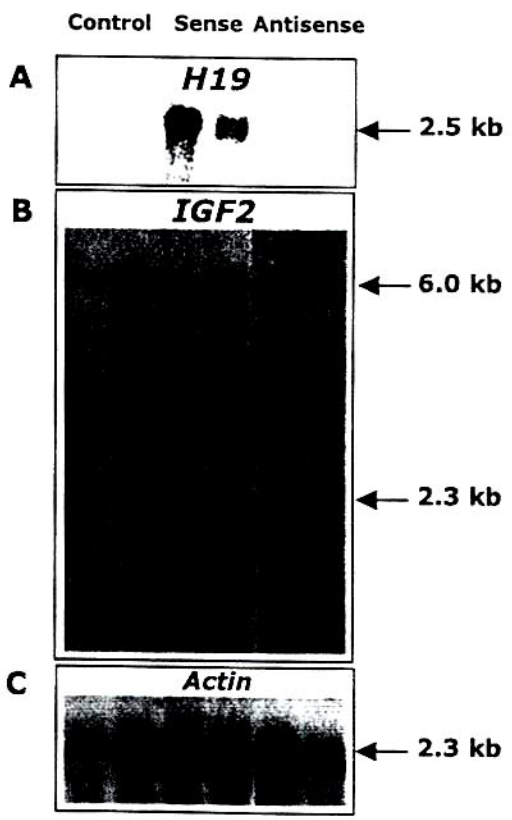

Fig. 1. Northern blot analysis of *H19* and *IGF2* total RNA in stably transfected diHepG2 cell lines with controls, *H19* sense or antisense constructs. (A) Hybridization with an *H19* exon 4,5-specific cDNA probe. The two first lanes correspond to RNA from two different control clones, lanes 3 and 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 *β-actin* cDNA probe as described in the text

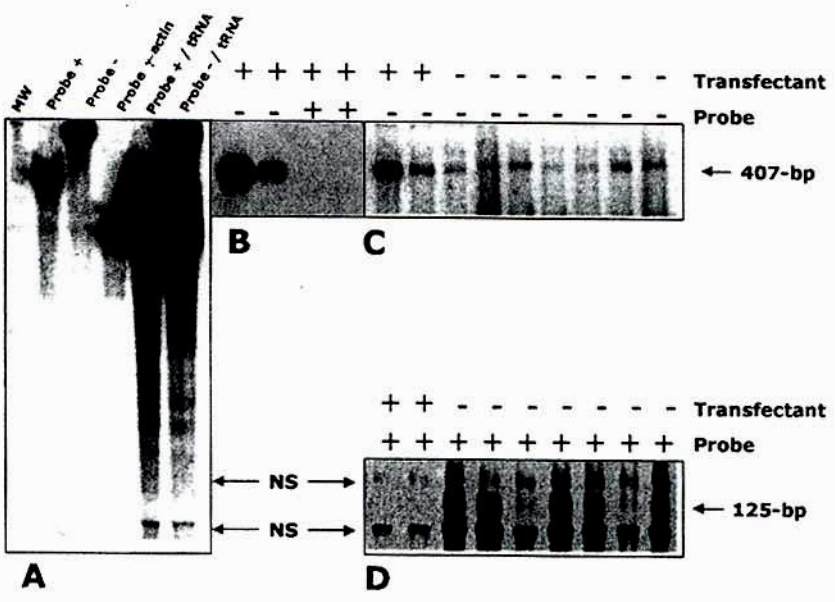

Fig. 2. RNase protection assay analysis of *H19* transcripts in diHepG2 cell lines stably transfected with *H19* sense or *H19* antisense constructs. (A) Lane designations are MW, molecular mass; Probe +, undigested sense probe; Probe −, undigested antisense probe; Probe actin, undigested actin probe. The last two lanes (Probe +/tRNA; Probe −/tRNA) 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 pRc/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 controls 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 genes; 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 transgene 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 (pSVβ). 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 (pCMVNEO/3CAT) following correction for β-galactosidase activity (Fig. 5A). When *H19* was placed in an antisense orientation (pCMVH19–/3CAT), levels of CAT activity were not significantly different from the control pCMVNEO/3CAT construct ($.375 < P < 0.4$). In contrast, in the presence of *H19* sense cDNA (pCMVH19+/3CAT), we observed a significantly decreased level of reporter gene activity relative to pCMVNEO/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 cytomegalovirus promoter-directed CAT transcription.

Another set of plasmid constructs was designed in which the cytomegalovirus 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 cytomegalovirus promoter is comparable to what we have shown previously [5].

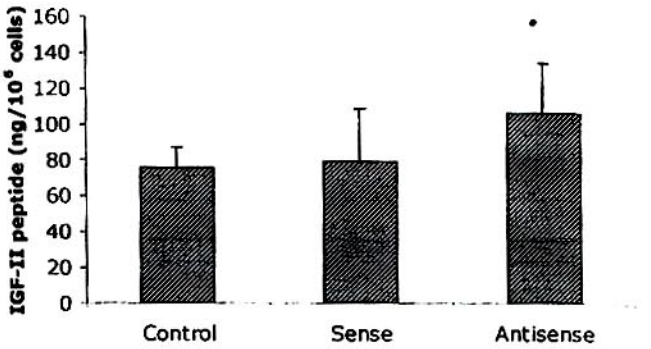

Fig. 4. IGF-II peptide quantitation in conditioned media of different clones after stable transfection of diHepG2 cells with control, *H19* sense or *H19* antisense constructs. Each column corresponds to the mean of three experiments performed on a different clone (± SD, $n = 5$ control clones, $n = 2$ sense clones, $n = 6$ antisense clones). IGF-II levels in the conditioned media were determined as described in Materials and methods. Antisense clones contained higher mean IGF-II than control or sense clones. Significance was achieved ($P < 0.05$) using the one-tailed Student's *t*-test.

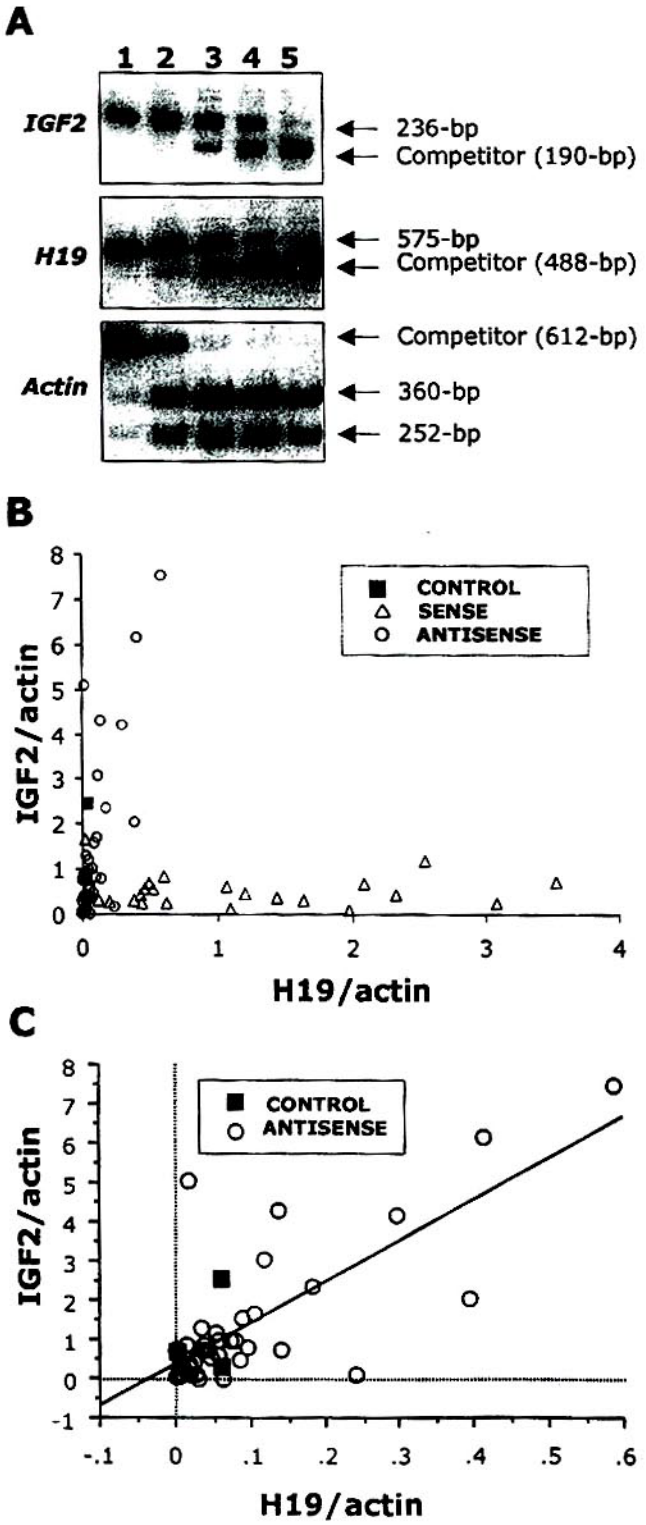

Fig. 3. Competitive RT-PCR analysis of *IGF2*, *H19* and γ-*actin* mRNA levels of different clones after stable transfection of diHepG2 cells with control, *H19* sense or *H19* antisense constructs. (A) Examples of the three competitive RT-PCR assays using a range of internal-standard amounts to assess 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 $1 = 1 \times 10^{-5}$, lane $2 = 1 \times 10^{-4}$, lane $3 = 1 \times 10^{-3}$, lane $4 = 1 \times 10^{-2}$, lane $5 = 1 \times 10^{-1}$; for *H19* lane $1 = 1 \times 10^{-4}$, lane $2 = 1 \times 10^{-3}$, lane $3 = 1 \times 10^{-2}$, lane $4 = 1 \times 10^{-1}$, lane 5 = 1; for γ-*actin* lane $1 = 1 \times 10^{2}$, lane $2 = 1 \times 10^{1}$, lane 3 = 1, lane $4 = 1 \times 10^{-1}$, lane $5 = 1 \times 10^{-2}$). (B) Each point corresponds to a clone ($n = 24$ 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 ($R^2 = 0.575$; $P < 0.0001$).

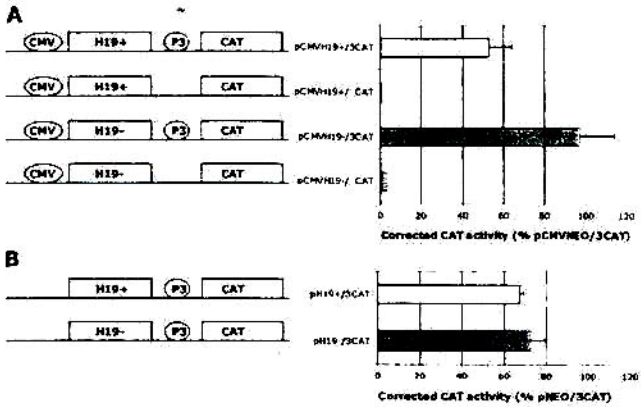

Fig. 5. Effects of the *H19* mRNA on IGF2-P3 driven CAT expression in transiently transfected diHepG2 cells with *H19* sense or *H19* antisense constructs. Generation of the reporter gene constructs is described in Materials and methods. To control the efficiency of transfection, each construct was cotransfected with a plasmid encoding the β-galactosidase gene (pSVβ). All transfections were performed in triplicate. The results of CAT activity, corrected by β-galactosidase activity, are presented normalized to pCMVNEO/3CAT in (A) ($n = 6$ experiments) and to pNEO/3CAT in (B) ($n = 2$ experiments) (mean ± SE).

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 macrosomia [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 fragments [38] or with an *H19* cDNA construct [13], tumorogenicity 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* knock-out 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 absent *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 transcriptional 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 ($P \leq 0.0005$) 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 transfected with full-length IGF-II cDNA, as we have previously shown [45]. Precedents do exist both for RNA as a transcriptional regulator [46–48], and for the presence of nontranslated, 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-II 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 offer further evidence 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 allele-specific or independent of imprinting.

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