H19 RNA Binds Four Molecules of Insulin-like Growth Factor II mRNA-binding Protein* 

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H19 RNA is a major oncofetal 2.5-kilobase untranslated RNA of unknown function. The maternally expressed H19 gene is expressed 90 kilobase pairs downstream from the paternally expressed insulin-like growth factor II (IGF-II) gene on human chromosome 11 and mouse chromosome 7; and due to their reciprocal imprinting and identical spatiotemporal expression, it is assumed that the two genes are functionally coupled. Here we show that human H19 RNA contains four attachment sites for the oncofetal IGF-II mRNA-binding protein (IMP) with apparent Kd values in the 0.4–1.3 nM range. The multiple attachment sites are clustered within a 700-nucleotide segment encoded by exons 4 and 5. This 3′-terminal segment targets H19 RNA to lamellipodia and perinuclear regions in dispersed fibroblasts where IMP is also localized. The results suggest that IMP participates in H19 RNA localization and provides a link between the IGF-II and H19 genes at post-transcriptional events during mammalian development.

The mammalian H19 gene was identified more than a decade ago. Despite intense efforts, the function of the gene product, H19 RNA, remains enigmatic (1). The H19 gene is transcribed by RNA polymerase II and encodes five exons and four unusually small introns (80–96 nucleotides in humans). The primary transcript is fully processed into a mature 2.5-kilobase polyadenylated RNA that is exported to the cytoplasm (2). The exons are considerably more conserved than the introns, and the mature H19 RNA does not exhibit any conserved open reading frames, arguing for a function of H19 as an untranslated RNA (3).

H19 RNA is expressed during fetal development, predominantly in tissues of mesodermal and endodermal origin, and down-regulated at birth (4). It is also expressed in several tumors in tissues that normally express H19 RNA only during fetal development, thus exhibiting the characteristics of an oncofetal factor (5). The spatiotemporal distribution of H19 RNA, remains enigmatic (1). The H19 gene is transcribed by RNA polymerase II and encodes five exons and four unusually small introns (80–96 nucleotides in humans). The primary transcript is fully processed into a mature 2.5-kilobase polyadenylated RNA that is exported to the cytoplasm (2). The exons are considerably more conserved than the introns, and the mature H19 RNA does not exhibit any conserved open reading frames, arguing for a function of H19 as an untranslated RNA (3).

The mammalian H19 gene was located 90 kilobase pairs downstream from the paternally expressed insulin-like growth factor II (IGF-II) gene on human chromosome 11 and mouse chromosome 7; and due to their reciprocal imprinting and identical spatiotemporal expression, it is assumed that the two genes are functionally coupled. Here we show that human H19 RNA contains four attachment sites for the oncofetal IGF-II mRNA-binding protein (IMP) with apparent Kd values in the 0.4–1.3 nM range. The multiple attachment sites are clustered within a 700-nucleotide segment encoded by exons 4 and 5. This 3′-terminal segment targets H19 RNA to lamellipodia and perinuclear regions in dispersed fibroblasts where IMP is also localized. The results suggest that IMP participates in H19 RNA localization and provides a link between the IGF-II and H19 genes at post-transcriptional events during mammalian development.

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§ The abbreviations used are: IGF-II, insulin-like growth factor II; IMP, IGF-II mRNA-binding protein; GFP, green fluorescent protein.

Vg1RB, Vg1 RNA-binding protein; IMP, IGF-II mRNA-binding protein; GST, glutathione S-transferase; PTB, polypyrimidine tract-binding protein; GFP, green fluorescent protein.
suggest that the H19 and IGF-II genes are linked mechanistically at the post-transcriptional level and that IMP participates in the subcytoplasmic localization of H19 RNA.

**EXPERIMENTAL PROCEDURES**

**H19 Constructs**—The human H19 cDNA (2313 base pairs) was generated by reverse transcription-polymerase chain reaction from human fetal liver total RNA (CLONTECH) using avian myeloblastosis virus reverse transcriptase (Amersham Pharmacia Biotech) and *Pfu* DNA polymerase (Stratagene). The amplified fragment was inserted into the *in vitro* transcription vector pGEM-11f (Promega) and into the *in vitro* intronless expression vector pCB6 (23). DNA fragments encoding H19 RNA segments C (positions 1–1613), E (positions 1612–2131), F (positions 1923–2008), and K (positions 2006–2313) were generated by polymerase chain reaction using full-length H19 cDNA as the template for *Pfu* DNA polymerase, and the amplified fragments were inserted into pGEM-11f and pCB6.

**In Vitro RNA Synthesis**—RNA segments were generated by *in vitro* transcription of linearized DNA templates with T7 RNA polymerase and purified by denaturing gel electrophoresis or by gel filtration in Microspin S-300 columns (Amersham Pharmacia Biotech). In UV cross-linking studies, a 10-μl random labeling reaction contained 100 μM unlabeled UTP and 3 μl of [α-32P]UTP (3000 Ci/mmol; Amersham Pharmacia Biotech), generating RNA with a specific activity of 30 Ci/mmol uridine monophosphate. In electrophoretic mobility shift analysis, the concentration of unlabeled UTP was lowered to 20 μM, thereby increasing the specific activity of uridine monophosphate in RNA to 150 Ci/mmol.

Antisense H19 RNA probes for *in situ* hybridization, incorporating either digoxigenin-11-UTP (Roche Molecular Biochemicals) or ChromaTide Red-5-UTP (Molecular Probes, Inc.), were generated by SP6 RNA polymerase (Roche Molecular Biochemicals) and subsequently exposed to partial base hydrolysis. The final concentrations of digoxigenin-11-UTP/Texas Red-5-UTP and UTP during *in vitro* transcription were 0.35 and 0.65 mM, respectively.

**UV Cross-linking**—In a final volume of 10 μl, 20–50 fmol of radiolabeled RNA (250 cpm) were incubated with 15 μg of total protein from a cytoplasmic extract of human HepG2 hepatocellular carcinoma cells for 25 min at room temperature in 20 mM Tris-HCl (pH 8.0), 140 mM KCl, 5 mM MgCl₂, 0.5 mM dithiothreitol, 2% glycerol, and 0.1 μg/μl *Escherichia coli* tRNA. Samples were chilled on ice, and 1 μl of 10% Ficoll-400 was added. Nondenaturing gel electrophoresis was carried out on a 5% polyacrylamide gel (21). RNA was digested with 1 μg of RNase A for 30 min at 37 °C. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

**Electrophoretic Mobility Shift Analysis**—In a final volume of 10 μl, 100–300 amol of radiolabeled RNA (2 cpm) were incubated with 1.0–2.0 nm recombinant IMP-1 or glutathione S-transferase-tagged poly(ADP-ribose) in glycerol and stored at 80 °C in aliquots containing

**Northern Analysis**—Total RNA was isolated from mouse embryos by the guanidinium thiocyanate method (25). RNA was denatured in glyoxal/dimethyl sulfoxide, separated on a 1% agarose gel, transferred to a Hybond-N membrane (Amersham Pharmacia Biotech), and hybridized with [α-32P]UTP-labeled cDNA probes. Autoradiography was carried out from 8 to 48 h, and the hybridization signals were visualized with a Fuji BAS 2000 Bioimager. A mouse H19 cDNA probe for Northern analysis was generated by polymerase chain reaction and includes positions 103–979, whereas the IGF-II and IMP cDNAs were coding region probes.

**Cell Culture and Cytoplasmic Extracts**—Human HepG2 hepatocellular carcinoma cells and mouse NIH 3T3 embryo fibroblast cells were obtained from the American Type Culture Collection and routinely maintained in RPMI 1640 medium supplemented with 10% fetal calf serum or in Dulbecco's modified Eagle's medium containing 10% calf serum. Stable transfectants of NIH 3T3 cells expressing GFP-IMP-1 were generated by retrovirus-mediated insertion of a construct encoding exon 1 (1328 nucleotides), 2 (135 nucleotides), 3 (113 nucleotides), 4 (123 nucleotides), and 5 (614 nucleotides) of H19 RNA and the three 5′-terminal RNA segments A (positions 1–252), B (positions 1–946), and C (positions 1–1613) (left panel) and from UV cross-linking of full-length H19 RNA and the four 3′-part RNA segments E (positions 1612–2131), F (positions 1612–2005), G (positions 1612–1880), and H (positions 1836–2008) (right panel). C, cross-linking of segment F in the presence (F) or absence (−F) of cytoplasmic extract or after treatment with proteinase K (+PK). Protein species and their sizes in kilodaltons are indicated to the left.

**In Situ Hybridization**—NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium with 10% calf serum. Cells were transiently transfected with LipofectAMINE (Life Technologies, Inc.) or Superfect

**FIG. 1.** UV cross-linking of [α-32P]labeled H19 RNA to a cytoplasmic extract of human HepG2 hepatocellular carcinoma cells. A, outline of full-length H19 RNA (2313 nucleotides) with contributing exons 1 (1328 nucleotides), 2 (135 nucleotides), 3 (113 nucleotides), 4 (123 nucleotides), and 5 (614 nucleotides) and of RNA segments used in the cross-linking experiments. B, autoradiographs from UV cross-linking of full-length H19 RNA and the three 5′-terminal RNA segments A (positions 1–252), B (positions 1–946), and C (positions 1–1613) (left panel) and from UV cross-linking of full-length H19 RNA and the four 3′-part RNA segments E (positions 1612–2131), F (positions 1612–2005), G (positions 1612–1880), and H (positions 1836–2008) (right panel). C, cross-linking of segment F in the presence (F) or absence (−F) of cytoplasmic extract or after treatment with proteinase K (+PK). Protein species and their sizes in kilodaltons are indicated to the left.
reagent (QIAGEN Inc.) according to the manufacturers' instructions. Briefly, 30,000 cells/cm² were seeded on poly-L-lysine-coated microscope slides 24 h prior to transfection. Cells were transfected with 2 μg/ml pCB6Δ1H19, pCB6Δ1H19ΔF, pCB6Δ1H19ΔH, pCB6Δ1E, or pCB6Δ1C and left for 48 h before the medium was changed to Dulbecco's minimal essential medium without phenol red for 3 h. The cells were then fixed in 4% (w/v) formaldehyde, 5% (w/v) acetic acid, and 0.9% (w/v) NaCl at room temperature for 30 min. After de- and rehydration, the slides were incubated with 5 ng/ml digoxigenin- or Texas Red-labeled and base-hydrolyzed antisense H19 RNA probe in 50% formamide, 900 mM NaCl, 5 mM EDTA, 50 mM sodium phosphate (pH 7.4), 5 × Denhardt's solution, 0.5% SDS, and 100 ng/ml salmon sperm DNA for 16 h at 42 °C and washed in 60% formamide, 300 mM NaCl, and 30 mM sodium citrate.

The slides incubated with digoxigenin-labeled RNA were subsequently incubated with alkaline phosphatase-coupled anti-digoxigenin (Roche Molecular Biochemicals) for 90 min at room temperature and developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche Molecular Biochemicals) for 5–10 min.

![Fig. 2](image)

**RESULTS**

**Identification of H19 RNA-binding Proteins by UV Cross-linking**—In an initial attempt to identify proteins that associate with H19 RNA, a UV cross-linking strategy was adopted. Full-length H19 RNA and seven RNA segments (Fig. 1) were randomly 32P-labeled and subjected to UV light at 254 nm in the presence of a cytoplasmic extract from human HepG2 hepatocellular carcinoma cells. Fig. 1B shows the autoradiographs from the resulting SDS-polyacrylamide gel electrophoresis of full-length H19 RNA and the three 5′-terminal RNA segments A–C (left panel) and the corresponding analysis of the four 3′-RNA segments E–H (right panel). Full-length H19 RNA exhibited three cross-links migrating at apparent masses of 50, 69, and 100 kDa. The 5′-terminal segments give rise to a cross-linking pattern similar to that of full-length H19 RNA, but with an additional signal at 60 kDa. The 50- and 100-kDa bands appear in cross-links with RNAs other than the H19 RNA segments, and the p50 species has tentatively been identified as the major core protein that associates unspecifically with RNA. If 4-thiouridine was incorporated during in vitro transcriptions and UV irradiation was carried out at 365 nm, a similar band pattern was observed.

Since a UV cross-linking profile can be sensitive to the magnesium ion concentration, cross-linking of segment F was examined at various concentrations of magnesium ions. Fig. 2A

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*S. Runge, F. C. Nielsen, J. Nielsen, J. Lykke-Andersen, U. M. Wewer, and J. Christiansen, unpublished data.*
To establish firmly that IMP and PTB are able to bind the H19 RNA and IGF-II leader 3 exhibit a virtually identical cross-linking pattern and suggest that the p69 and p60 cross-linking sites are to an RD rhabdomyosarcoma lysate are able to cross-link to IGF-II leader 3 (21). Moreover, we identified p60 as PTB and p69 as a member of the novel RNA-binding family termed IMP. Therefore, full-length H19 RNA and IGF-II leader 3 were cross-linked in parallel to a HepG2 cytoplasmic lysate and co-electrophoresed with H19 segment F cross-linked to the same unlabeled RNA and the RNA-protein complex are shown to the left. B, autoradiograph showing the mobility shift of 32P-labeled H19 segment H with recombinant GST-PTB in the concentration range of 0.25–1.5 nM, as indicated above each lane. The positions of unbound RNA and the RNA-protein complex are shown to the left.

**H19 RNA and IGF-II Leader 3 Are Cross-linked to the Same Proteins**—In a previous study of IGF-II mRNA-binding proteins, we showed that four proteins (p50, p60, p69, and p100) from an RD rhabdomyosarcoma lysate are able to cross-link to IGF-II leader 3 (21). Moreover, we identified p60 as PTB and p69 as a member of the novel RNA-binding family termed IMP. Therefore, full-length H19 RNA and IGF-II leader 3 were cross-linked in parallel to a HepG2 cytoplasmic lysate and co-electrophoresed with H19 segment F cross-linked to the same unlabeled RNA and the RNA-protein complex are shown to the left. B, autoradiograph showing the mobility shift of 32P-labeled H19 segment H with recombinant GST-PTB in the concentration range of 0.25–1.5 nM, as indicated above each lane. The positions of unbound RNA and the RNA-protein complex are shown to the left.

**H19 RNA Binds IGF-II mRNA-binding Protein**—Since the 5’-end of H19 RNA is unable to cross-link to p69 (Fig. 1) and since one molecule of IMP-1 binds to segment H in the 3’-part of H19 RNA, we examined whether additional IMP-binding sites are present in the 3’-end of H19 RNA. Mobility shift analyses of segments F–K were carried out at an IMP-1 concentration range of 0.25–1.75 nM, and Fig. 5 corroborates that segment H encompasses one IMP-binding site with an apparent $K_d$ of 0.4 nM. Furthermore, it reveals that segment G exhibits two binding sites with apparent $K_d$ values of 0.75 and 1.3 nM and that segment F, which includes both segments G and H, contains three binding sites with $K_d$ values of 0.4, 0.75, and 1.3 nM. Segment K at the 3’-terminus contains one binding site with an apparent $K_d$ of 1.0 nM. In an attempt to reduce further the size of the high affinity binding site in segment H, subsegments I and J were generated. Fig. 5 shows that none of the subsegments nor IGF-II leader 4 was able to bind IMP-1. We conclude that H19 RNA is able to bind four molecules of IMP-1.

**Putative RNA Secondary Structure of Segment H**—To gain insight into a putative RNA secondary structure of the high affinity attachment site, we subjected 5’-end-labeled segment H to probing with ribonucleases T1, T2, and V1 (24). Ribonuclease T1 monitors unpaired guanosines; ribonuclease T2 preferentially attacks unpaired adenosines; and ribonuclease V1 exhibits helical specificity. Fig. 6A is an autoradiograph showing the results of the enzymatic probing experiment, and Fig. 6B depicts the derived putative secondary structure. The striking feature of segment H is that besides two hairpins, it is unstructured in a classical Watson-Crick sense. The unstructured 60-kDa signal was predominant at concentrations at or below 1 nM, but was absent at 10 nM Mg$^{2+}$. The presence of 0.5 μg of 23 S rRNA was unable to change the cross-linking pattern substantially (Fig. 2B). We infer that H19 RNA cross-links to at least four protein species and that the 173-nucleotide segment H in the 3’-part exhibits a similar cross-linking profile.

**H19 RNA Binds Four Molecules of IMP-1**—Since the 5’-terminal 1.6 kilobases of H19 RNA are unable to cross-link to p69 (Fig. 1) and since one molecule of IMP-1 binds to segment H in the 3’-part of H19 RNA, we examined whether additional IMP-binding sites are present in the 3’-end of H19 RNA. Mobility shift analyses of segments F–K were carried out at an IMP-1 concentration range of 0.25–1.75 nM, and Fig. 5 corroborates that segment H encompasses one IMP-binding site with an apparent $K_d$ of 0.4 nM. Furthermore, it reveals that segment G exhibits two binding sites with apparent $K_d$ values of 0.75 and 1.3 nM and that segment F, which includes both segments G and H, contains three binding sites with $K_d$ values of 0.4, 0.75, and 1.3 nM. Segment K at the 3’-terminus contains one binding site with an apparent $K_d$ of 1.0 nM. In an attempt to reduce further the size of the high affinity binding site in segment H, subsegments I and J were generated. Fig. 5 shows that none of the subsegments nor IGF-II leader 4 was able to bind IMP-1. We conclude that H19 RNA is able to bind four molecules of IMP-1.
H19 RNA Binds IGF-II mRNA-binding Protein

**Expression of H19 RNA and IMP mRNA during Late Mouse Development**—Similar to IGF-II and H19 RNAs, IMPs are mainly expressed in the placenta, fetal skeletal muscle, liver, and epithelia (21, 4). To examine the temporal relation between the expression of H19 RNA and the family of IMP proteins, we subjected total RNA from mouse embryonic day 8.5 to birth to Northern analysis with H19, IMP-1, IMP-2, and IMP-3 cDNA probes (Fig. 7). Whereas H19 RNA expression was at its peak at embryonic day 17.5, global IMP-1, IMP-2, and IMP-3 expression attained a maximum at embryonic day 12.5, followed by a decline toward birth (21), indicating that IMPs exhibit maximal activity during the initial phase of H19 expression.

Subcytoplasmic Localization of H19 RNA—In the cytoplasm of transfected mouse NIH 3T3 embryo fibroblasts, IMP-1 is either evenly distributed or localized to the leading edge of the lamellipodia and perinuclear regions (21). Since the in vitro binding data in Fig. 5 demonstrate that IMP-1 bound H19 RNA with high affinity, a possible role of endogenous IMP in the subcytoplasmic distribution of transfected H19 RNA in NIH 3T3 cells was examined by in situ hybridization. The results show that the distribution of full-length H19 RNA is similar to that of IMP-1 (21) by being either evenly distributed in areas with confluent growth-arrested cells (88% of transfected cells) or, as illustrated in Fig. 8B, localized to the leading edge of lamellipodia and perinuclear regions in dispersed proliferating cells (42% of transfected cells). Untransfected cells in Fig. 8 show that no background reaction occurred with the employed in situ hybridization procedure. Excision of positions 1612–2005 from H19 RNA (H19ΔF RNA) removed three of the four IMP-1-binding sites, and the truncated RNA exhibited a diffuse cytoplasmic localization in both growth-arrested and proliferating NIH 3T3 cells (98% of transfected cells). Excision of the 173-nucleotide segment H (H19ΔH RNA), which encompasses the high affinity IMP-1-binding site (positions 1836–2008), reduced subcytoplasmic localization to 20% of the cells. Expression of H19 segment E (positions 1612–2313), which contains the entire complement of IMP-1- and PTB-binding sites in full-length H19 RNA, resulted in RNA accumulation at the leading edge of the lamellipodia and in perinuclear regions (69% of transfected cells) in a manner indistinguishable from full-length H19 RNA. Finally, H19 segment C (positions 1–1613), which lacks all IMP and PTB attachment sites, exhibited exclusive nuclear localization. Immunocytochemical staining of cells transfected with IMP-1 cDNA revealed that IMP-1 localized to similar subcytoplasmic domains as H19 RNA, and transfection of stably transfected cells expressing GFP-tagged IMP-1 with full-length H19 cDNA revealed overlapping localization of GFP-IMP-1 and H19 RNA within the same cell in Fig. 8C. We conclude that IMP-1 and H19 RNA localize to similar subcytoplasmic domains in NIH 3T3 cells and that segment E is sufficient to provide the complete localization response.

**DISCUSSION**

In this study, we show that four molecules of an embryonic IGF-II mRNA-binding protein (IMP-1), containing two RNA recognition motifs and four heterogeneous nuclear ribonucleoprotein K homology domains, associate specifically with the 3′-part of human H19 RNA. The subcytoplasmic localization of transfected H19 RNA reflects the localization of IMP-1 in lamellipodia and perinuclear regions of fibroblasts, and removal of three of the attachment sites leads to a de-localized distribution of the truncated RNA. The 3′-terminal 700 nucleotides of H19 RNA, which contain the full complement of the four attachment sites, are sufficient to mimic the localization pattern of full-length H19 RNA.

The syntenic nature of the IGF-II and H19 genes, their similar spatiotemporal expression, and their reciprocal imprinting are characteristics that have led to the assumption that the two gene products are functionally coupled. Although the biological significance of the IGF-II peptide is well established...
lished (28, 29), the lack of a clue to the biology of H19 RNA has hampered progress considerably. Here, we show that a family of embryonic RNA-binding proteins that were originally identified by the ability to bind to the IGF-II leader 3 mRNA also bind to H19 RNA, thus revealing a common trans-acting factor that may be involved in a mechanistic coupling of the IGF-II and H19 genes at post-transcriptional events such as nuclear export, cytoplasmic localization, and translation. Strictly speaking, we show that only three molecules of IMP-1 are primary RNA-binding proteins since it is conceivable that the fourth molecule associates with segment G via protein-protein interactions. This study identifies PTB as an additional common trans-acting factor that is able to associate with high affinity with the same RNA segments as the IMP family in both H19 RNA and IGF-II leader 3 (21). Interestingly, the theme of two trans-acting factors being able to associate with one RNA is also observed in the Xenopus Vg1 mRNA localization element where the two factors are Vg1RBP/Vera and VgRBP60 (30), the former being the ortholog of human IMP-3 and the latter being homologous to heterogeneous nuclear ribonucleoprotein I/PTB. The in situ hybridization data with the de-localized H19ΔF RNA, which lacks three IMP attachment sites and two PTB-binding sites, suggest that a similar molecular mechanism of localization may occur in the mammalian system. Since PTB is predominantly a nuclear protein (31) and since IMP is generally cytoplasmic, attachment of PTB to nascent RNA may provide a “nuclear experience” in the assembly of a translocation complex, so the proper conformation of the localization signal for subsequent IMP binding is achieved.

Although various sequence elements have been suggested as the binding sites of the homologous Vg1RBP/Vera (16, 17), zipcode-binding protein 1 (22), c-Myc coding region determinant-binding protein (32), and IMP (21) proteins, the recognition motifs are not known. Segment H in H19 RNA, which encompasses the high affinity binding site for recombinant IMP-1, was divided into two similarly sized subsegments I and J, neither of which was able to bind IMP-1 with high affinity. The most straightforward interpretation of this result is that the attachment site is severed. Alternatively, the two subsegments may contribute to a higher order RNA structure, or multiple RNA-protein interfaces may exist. To distinguish between the latter possibilities, segment H was subjected to the probing experiment with ribonucleases in Fig. 6, indicating the absence of a higher order interaction between subsegments I and J. Therefore, it is more likely that “unstructured” pyrimidine-rich elements in both subsegments may participate in the
recognition event. The possibility of at least two RNA-protein interfaces in the recognition event is in agreement with the multiple RNA-binding domains of the IMP family (21) and provides a rationale for the lack of a simple sequence element in the RNA targets identified so far. The RNA targets are found in the 3' untranslated region (Vg1 and β-actin mRNAs), in the translated region (c-Myc mRNA), and in the 5' untranslated region (IGF-II leader 3 mRNA) in varying numbers, which may influence the fate of a particular mRNA differently.

The binding data and the overlapping cytoplasmic localization pattern of H19 RNA and IMP-1 suggest that IMP-1 recognizes cis-elements in H19 RNA and participates in its subcytoplasmic localization. This suggestion is supported by the in situ hybridization analyses of H19ΔF RNA, H19ΔH RNA, and segment E, which show that the presence of an intact segment E with its four IMP attachment sites at the 3' terminus of H19 RNA is crucial for subcytoplasmic localization. The nuclear localization of segment C implies that the 300 nucleotides at the 3' terminus of H19 RNA, which are present in H19ΔF RNA, contain a signal for nuclear export, but that this segment is insufficient for subcytoplasmic localization. Multiple and even redundant cis-acting signals in RNA localization have been described in a few cases (33–35), but the lack of biophysical data regarding RNA-protein interactions and their stoichiometry makes general conclusions regarding the importance of multiple attachments of trans-acting proteins for efficient localization difficult.

H19 RNA belongs to a group of localized untranslated RNAs with members such as the Drosophila polar granule component RNA (36), the Xenopus and Drosophila Mtlr RNAs (37, 38), and the Xenopus Xlsirt RNAs (39), which play crucial roles during development. Among these, it is only Xlsirt RNAs that have been associated directly with post-transcriptional regulation of a messenger RNA. During Xenopus oogenesis, localization of the untranslated Xlsirt RNAs to the vegetal cortex of stage II/III oocytes is a prerequisite for the subsequent anchoring of the Vg1 mRNA at the vegetal cortex of stage III/IV oocytes (40). Since IMP-3 and Vg1RBP/Vera are orthologous proteins and since both IGF-II and Vg1 are secreted growth factors, a similar molecular mechanism of localization is appealing. However, the Xlsirt RNAs are localized by the early mitochondrial cloud-dependent pathway, whereas Vg1 mRNA is localized later in a microtubule-dependent manner (41), implying that Xlsirt RNAs do not bind Vg1RBP/Vera, which is in contrast to

**Fig. 8. In situ hybridization analysis of H19 RNA in transfected NIH 3T3 cells.** A, outline of the transfected cDNAs. B, results of hybridization with a digoxigenin-labeled antisense H19 RNA probe. H19ΔF lacks positions 1612–2005 in H19 RNA; H19ΔH lacks positions 1836–2008; segment E contains positions 1612–2313; and segment C contains positions 1–1613. No hybridization signal was detected in untransfected NIH 3T3 cells in any of the panels. C, immunocytochemical staining of IMP-1 cDNA-transfected NIH 3T3 cells with anti-IMP-1 antiserum (left panel) and in situ hybridization of cells expressing GFP-tagged IMP-1 with a Texas Red-labeled antisense H19 probe following transfection with full-length H19 cDNA (right panels). The arrow shows a lamellipodium with overlapping H19 RNA and GFP-IMP-1 expression.
the binding between H19 RNA and the IMP family reported in this study.

What is the function of H19 RNA? Since H19 RNA is expressed in a subset of instances of IGF-II production, whereas the opposite appears not to be the case, the most straightforward interpretation of the biological role of H19 RNA is one of a regulator of IGF-II expression. H19 knockout experiments (42, 43) suggest that H19 RNA is a negative regulator of IGF-II production since the resulting increase in neonatal weight is a hallmark of an increased IGF-II dosage (29). However, the increased levels of IGF-II mRNAs due to the relaxation of genomic imprinting in the H19 knockout mice might obscure a regulatory function of H19 RNA at the post-transcriptional level. Suggestions that H19 RNA could have a function in cis, in analogy with the mechanism of action of Xist RNA in X chromosome inactivation (44), have now been ruled out (45). The evidence regarding a possible function of H19 RNA in trans is limited, although analysis of single cells in a Wilms’ tumor suggests a functional interplay between H19 RNA and IGF-II mRNA (46). The combined evidence of the conservation of exons in comparison with introns (3) and the identity of the trans-acting factor presented in this study point to a riboregulator of importance in IGF-II production during mammalian development.

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