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Biallelic transcription of *Igf2* and *H19* in individual cells suggests a post-transcriptional contribution to genomic imprinting

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The *H19* and insulin-like growth factor 2 (*Igf2*) genes in the mouse are models for genomic imprinting during development. The genes are located only 90 kb apart in the same transcriptional orientation [1], but are reciprocally imprinted: *Igf2* is paternally expressed while *H19* is maternally expressed. It has been suggested that expression of *H19* and repression of *Igf2* (or the converse) on a given chromosome are mechanistically linked and that the parental imprint operates at the level of transcription [2]. Although expression of *Igf2* and *H19* is thought to be monoallelic, the data have so far been obtained exclusively by looking at steady-state RNA levels using techniques that reflect the average activity of the genes in a cell population [3,4]. Here, we have adapted a fluorescent *in situ* hybridisation (FISH) method to detect nascent RNA molecules of *Igf2* and *H19* at the initial transcription sites in the nuclei of wild-type mouse embryonic liver cells. Nine different transcription patterns were observed, reflecting a high heterogeneity of transcription at the single-cell level. Our observations suggest that regulation of *Igf2* and *H19* by parental imprinting is much more complex than previously proposed and acts at both transcriptional and post-transcriptional levels.

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Received: 2 July 1999
Revised: 3 August 1999
Accepted: 6 September 1999

Published: 11 October 1999

Current Biology 1999, 9:1199–1202

0960-9822/99/$ – see front matter
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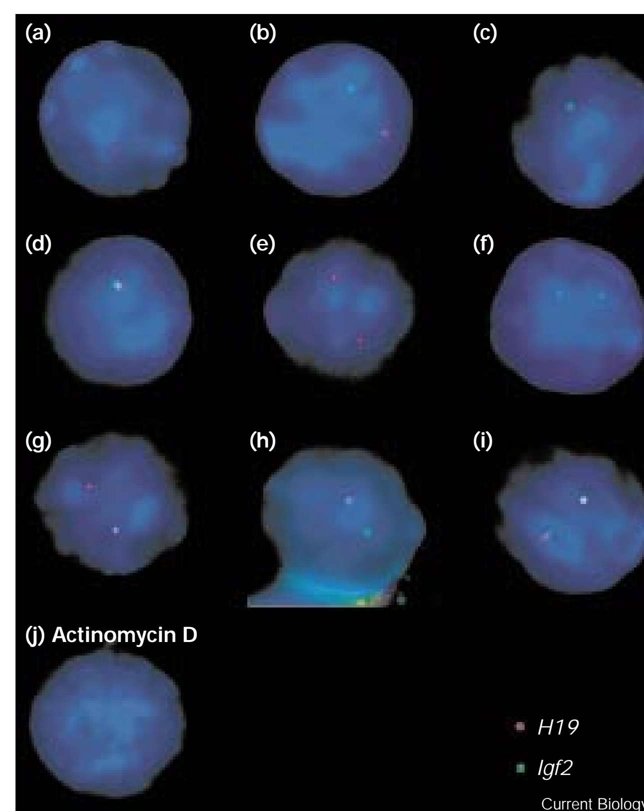
Results and discussion

The RNA FISH technique has been used previously to detect primary β-globin transcripts [5]. Applying the technique to cells derived from the foetal liver at 13.5 days of gestation (E13.5), which at this stage of development comprise a mixture of hepatocytes and hematopoietic precursors, we detected strong reproducible nuclear signals using control β-globin probes. In this technique, DNA is not denatured and thus remains inaccessible to the probe. In addition, the signals were sensitive to RNAse treatment, confirming the detection of RNA. Despite proteinase treatment, cytoplasmic remnants can persist occasionally around nuclei. In these cases, cytoplasmic signals can also be detected. As reported by Wijgerde *et al.* [5], we found that over 80% of globin-transcribing nuclei (hematopoietic cells) displayed two signals, that is, two transcriptional foci, indicating that the biallelic transcription of the β-globin gene was being efficiently detected (data not shown).

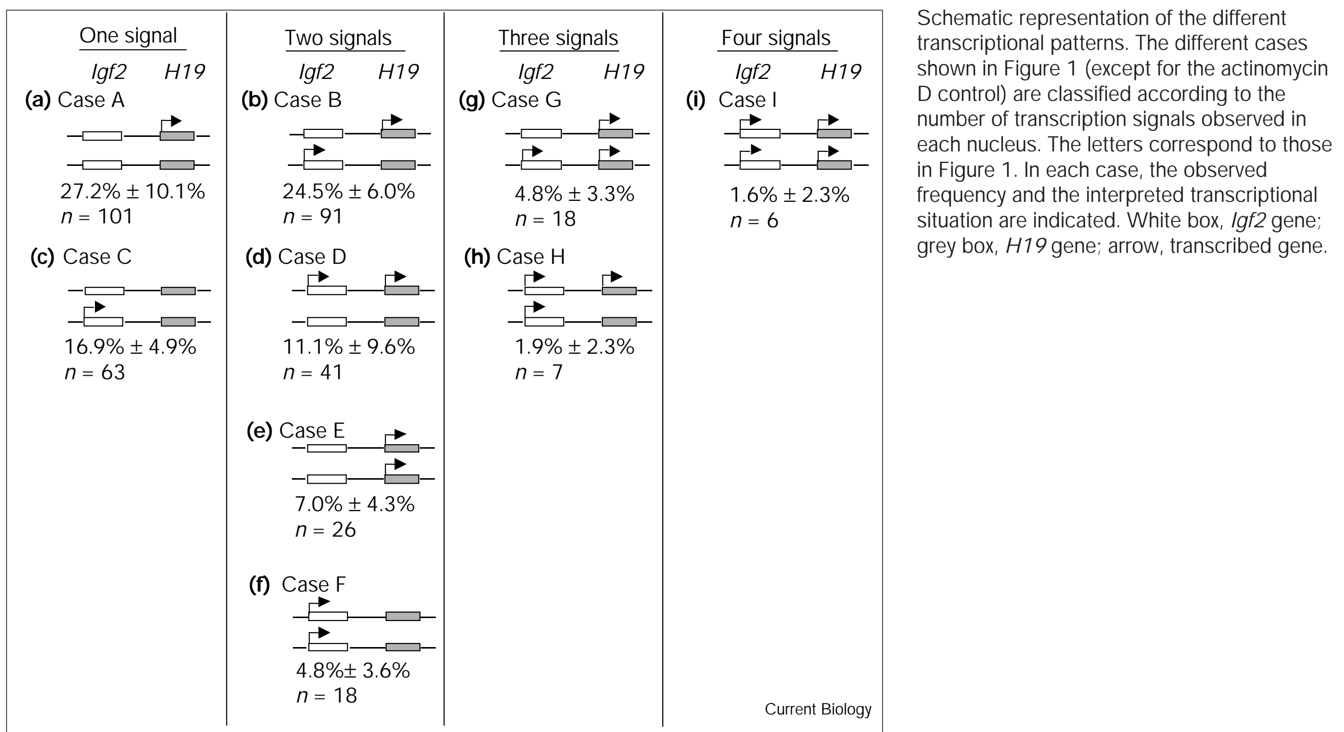
Using identical conditions except for a slight modification of the cell permeabilisation method (see Materials and methods), we detected *H19* and/or *Igf2* transcriptional foci in about half of the cells, consistent with the fact that these

Figure 1



Analysis of *H19* and *Igf2* primary transcripts by double-colour RNA FISH in nuclei of E13.5 foetal liver cells. The *Igf2* probe is labelled with fluorescein isothiocyanate (FITC, green), the *H19* probe with Texas Red (red) and the nuclear DNA is stained with 4′,6-diamidino-2-phenylindole (DAPI, blue). **(a–i)** Nine different patterns were observed with respect to the number and location of specific signals. None of these signals persisted after RNAse treatment of the slides. **(j)** Cells treated with the transcriptional inhibitor actinomycin D showed a drastic reduction in the intensity and number of signals.

Figure 2



Schematic representation of the different transcriptional patterns. The different cases shown in Figure 1 (except for the actinomycin D control) are classified according to the number of transcription signals observed in each nucleus. The letters correspond to those in Figure 1. In each case, the observed frequency and the interpreted transcriptional situation are indicated. White box, *Igf2* gene; grey box, *H19* gene; arrow, transcribed gene.

genes are expressed in hepatocytes (Figure 1). In these experiments, the *Igf2* probe was detected with fluorescein green and the *H19* probe with Texas red.

A total of nine different transcription patterns were observed. In addition to the expected situation with one *H19* and one *Igf2* signal per nucleus (Figure 1b), we observed nuclei exhibiting only a single transcriptional focus for *H19* (Figure 1a) or nuclei with a single transcriptional focus for *Igf2* (Figure 1c). This indicates that the two genes are not always transcribed simultaneously in a cell. The parental origin of the transcripts cannot be determined in these experiments. We can only assume that the majority of the single red and green signals come, respectively, from the maternal *H19* and paternal *Igf2* allele. In Figure 1d, simultaneous transcription of one *H19* and one *Igf2* allele is taking place, as in Figure 1b, except that in this case, the green and red signals are in close or overlapping positions. As *H19* and *Igf2* are located only 90 kb apart on chromosome 7, this pattern is most likely the result of transcription of the two genes from the same chromosome, either paternal or maternal. These two possibilities cannot be discriminated on the basis of the present data.

Cells were also found with two *H19* signals (Figure 1e) or two *Igf2* signals (Figure 1f), indicating that biallelic transcription of either gene can occur in normal embryonic liver cells. Moreover, we also observed transcription of both genes on one chromosome associated with transcription of either *H19* (Figure 1g) or *Igf2* (Figure 1h) on the other chromosome. Even simultaneous biallelic transcription of both genes was found (Figure 1i). A formal possibility is that these patterns reflect heterogeneity in the cell population. It seems more likely, however, that each individual cell goes through various transcriptional states during the cell cycle.

Six independent experiments using double-stranded DNA probes were performed, and over 700 nuclei were scored. The frequency of each pattern of transcription was calculated as the fraction of all cells having at least one signal for *H19* or *Igf2* (which represent about half of the nuclei) and the results are summarised in Figure 2 together with the most likely interpretations that fit the observed patterns.

Although the majority of cells did display monoallelic transcription of either *H19* (Figure 2, case A) or *Igf2* (case C) or both (cases B and D), 20% of the cells exhibited biallelelic transcription of one or both genes (cases E–I). Thus, in one out of six *H19*-expressing cells, both alleles were transcribed and, in one out of eight *Igf2*-expressing cells, both alleles were also transcribed. In addition, only 24.5% of the cells showed the pattern expected if expression of *H19* negatively affects expression of *Igf2* (case B). Statistical analysis indicated, however, that there was some degree of correlation between the transcription of *H19* and *Igf2*, as biallelic transcription of *Igf2* is less likely ($p < 0.01$, as determined by

χ^2 test) in a nucleus transcribing one or both *H19* alleles than in a nucleus in which *H19* is silent, which is consistent with previous reports [6].

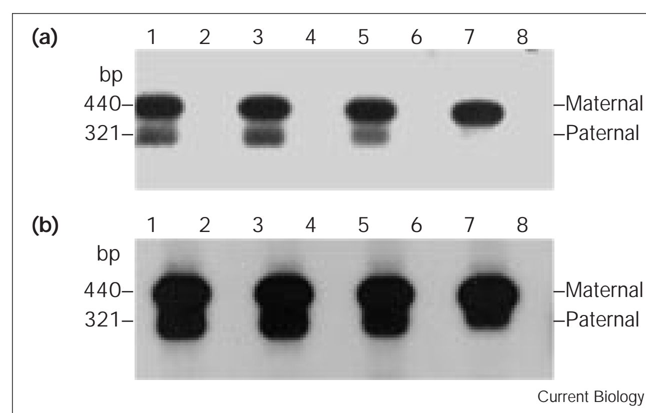
No antisense transcripts have ever been described for *H19*. Nevertheless, we repeated the experiment using single-stranded oligonucleotide probes that can detect only sense transcripts for each gene. The same nine transcription patterns were observed with similar frequencies as with the double-stranded probes (A = 42; B = 14; C = 15; D = 20; E = 13; F = 6; G = 10; H = 5; I = 3; for the double-stranded probes, see Figure 2), indicating that the observed patterns are not due to the presence of antisense transcripts.

Antisense transcripts overlapping with the *Igf2* mRNA have been reported. These transcripts are, however, also synthesised from the paternal chromosome [7]. Therefore, their detection cannot account for the two transcriptional foci frequently observed with the *Igf2* probe.

Low-level expression (about 5%) of the paternal *H19* allele has been reported in mutant mice in which the maternal allele has been deleted and replaced by the *neo* gene cassette [6]. Similar observations were also reported for the maternal *Igf2* allele in heterozygous null mutant mice [8]. Our results provide evidence that transcription of the paternal *H19* allele and maternal *Igf2* allele occurs not only in mutant tissues but also in normal wild-type mouse cells. In addition, the extent of biallelic transcription is higher than expected from previous data [6,8].

To confirm the presence of paternal *H19* transcripts in the nuclei, we performed an RT–PCR assay on total and nuclear RNA extracted from embryonic liver cells (Figure 3). The embryos were obtained by mating of a B6/D2 F1 female and a BalbC male. These strains display an *Msp*I polymorphism in the first exon of *H19*, which allows the two parental alleles of *H19* to be distinguished [9]. As expected, the maternal *H19* transcripts were found in the three independent nuclear RNA samples (lanes 1,3,5). In addition, a second weaker band, corresponding to the paternal *H19* transcript was also easily detected in nuclear RNA samples. The amount of paternal *H19* RNA was estimated to be around 15% of the total *H19* nuclear transcripts. In contrast, when total RNA extracts were examined by RT–PCR under the same conditions, paternal H19 transcripts were hardly detectable (lane 7). A faint band appeared on the autoradiograph only after prolonged exposure (lane 7). Therefore, paternal transcripts are highly enriched in the nuclear fraction. Somehow, paternal *H19* transcripts do not reach the cytoplasm with the same efficiency as maternally derived *H19* transcripts. One possibility is that paternal transcripts are less stable, and may even be degraded before reaching the cytoplasm.

Figure 3



RT–PCR assay of *H19* transcripts. Allele-specific expression of *H19* transcripts was monitored in three independent nuclear RNA preparations (lanes 1–6) and in total liver RNA (lanes 7,8). The *Msp*I digest of the amplification products results in a 440 bp and a 321 bp fragment for the maternal and paternal transcript, respectively. Controls without reverse transcription (lanes 2,4,6,8) show that the preparations were free of genomic DNA. **(a)** Short exposure. **(b)** The same blot after several hours' exposure.

That maternal and paternal H19 transcripts do not have identical post-transcriptional fates may result from the different chromatin structures around the promoter and around the entire gene between the two parental alleles of H19 (and of other imprinted genes). Such parental-allele-specific epigenetic imprints at the promoter level might be responsible for the assembly of different RNA-binding protein complexes on the nascent maternal and paternal transcripts. Recent evidence indicates that an increasing number of RNA-binding proteins seem to play a role not only in transcription but also in post-transcriptional processes such as splicing and transport [10]. Both synthesis and maturation of a transcript appear to be physically linked in the same functional protein complex. It is thus possible that differences in the proteins recruited onto the nascent paternal and the maternal H19 transcripts account for their different fates. Whatever the mechanism, our data show that the inhibition of transcription by parental-origin-dependent epigenetic imprint is not absolute and that post-transcriptional factors are also likely to be involved in the process that results in the preferential accumulation of maternal H19 transcripts in the cytoplasm of embryonic liver cells.

Materials and methods

Cell preparations

Embryos were obtained by B6/D2 F1 intercross. E13.5 livers were dissected out and mechanically disrupted by pipetting in PBS. The cell suspension was then spread onto Superfrost$^+$ slides (O. Kindler Gmbh & Co) and fixed in a 4% formaldehyde/5% acetic acid solution. After successive PBS washes, the slides were stored in 70% ethanol at –20°C. For transcription inhibition tests, cells were preincubated for 5–20 min in a 5 μg/ml actinomycin D solution (Sigma) at 37°C before spreading on slides.

FISH probes

The β-globin probe was prepared using a mix of four DNP-labelled oligonucleotides specific for the β-major globin introns (kindly provided by Peter Fraser). The labelling of *H19* and *Igf2* probes was done on purified inserts. For *H19*, a 2 kb plasmid insert coding for the full-length mouse cDNA [4] was labelled with biotin (Boehringer Mannheim) by nick translation (Life Technologies). For *Igf2*, the mouse genomic fragment (plasmid kindly provided by Wolf Reik) was labelled with digoxygenin (Boehringer Mannheim) by nick translation (Life Technologies). The detection of sense H19 transcripts was done using a mixture of the following three DNP-labelled 50-mers (from Eurogentec): H19-E6, TACTCGCTCTACCTGCACCCAACCTCC-CTCCCTAGAAACTCATTCATTCT; H19-E7, TTCAAGAGTGGC-TCTGGCAAAGTCCCAAGTTTGCCAGAGCCTCAATAACT; H19-E8, ATGACTGCCCTTCTGTCCTCTCCATCACACCGGACCATGTCAG TCTTTC. The sense *Igf2* transcripts were also detected with a mixture of three biotin-labelled oligonucleotide probes (from Eurogentec): IGF-E7, GGAAGCAGCACTCTTCCACGATGCCACGGCTGCGACGGTTG GCACGGCTT; IGF-E8, GGCCTGAGAGGTAGACACGTCCCTC-TCGGACTTGGCGGGGGTGGCACAGT; IGF-E9, GCCTGCGCA-GAGGCCTGCCTGCCCTCCTGCGTGCCCGCCGG-GGTCGCATG.

Primary transcript in situ *hybridisation*

Following storage, cells were rehydrated in 0.1 M Tris/0.15 M NaCl, then permeabilised with either 0.01% pepsin (Sigma) for *H19* and *Igf2* detection, or 0.001% pepsin for β-globin detection, in 0.01 M HCl at 37°C for 5 min. To inactivate pepsin and to preserve the nuclear structure, cells were post-fixed in a 3.7% formaldehyde/PBS solution for 5 min. After two PBS washes, cells were dehydrated in 70%, 90% and 100% ethanol solutions. Controls for RNAse sensitivity were performed by incubating cells at room temperature for 10 min in a PBS solution containing 10 μg/ml RNAse A (Sigma) prior to post-fixation. *H19* and *Igf2* probes were diluted at 1–5 ng/μl in a hybridisation mixture containing 50% formamide, 2 × SSC, 200 ng/μl salmon sperm DNA, 50 ng/μl Cot1 DNA (Life Technologies), 5 × Denhardts, 1 mM EDTA and 50 mM sodium phosphate, pH 7. They were then denatured at 80°C for 5 min, and set at 37°C for 30 min during the preannealing period. In the case of oligonucleotide probes, the formamide concentration was decreased to 25%, and the denaturation and preannealing steps omitted. Probes were applied to the slides, and hybridisation was carried out under coverslips at 37°C overnight in a humid chamber. After hybridisation, the slides were washed three times for 10 min in 2 × SSC at 42°C and finally rinsed in TNT solution (0.05% Tween-20, 0.15 M NaCl, 0.1 M Tris-HCl).

Immunocytochemical detection

Each labelled probe was detected by a three-step procedure. Biotin was detected using successively Texas Red-conjugated avidin DCS (Vector labs), biotinylated anti-avidin D antibody (Vector labs) and another Texas Red-conjugated avidin DCS step. Digoxygenin was detected using sheep fluorescein-conjugated anti-digoxygenin serum (Boehringer Mannheim), rabbit fluorescein-conjugated anti-sheep antibodies (Vector labs) and goat fluorescein-conjugated anti-rabbit antibodies (Vector labs). DNP was detected using a rat anti-DNP monoclonal antibody (Monosan), donkey Texas Red-conjugated anti-rat antibodies (Jackson ImmunoResearch Laboratories) and goat Texas Red-conjugated anti-horse antibodies (Jackson ImmunoResearch Laboratories). All dilutions were prepared in 0.5% blocking reagent (Boehringer Mannheim) diluted in 0.15 M NaCl, 0.1 M Tris-HCl. Each incubation step was done at room temperature for 30 min, and slides were subsequently washed twice for 5 min in TNT solution. Finally, the coverslips were mounted in Vectashield containing 0.5 μg/ml 4',6-diamidino-2-phenylindole (DAPI). Fluorescence was detected by epifluorescence/CCD microscopy.

Nuclear RNA preparation and RT–PCR

B6/D2 F1 female mice were crossed with Balb/C males. E13.5 livers were dissected and Dounce-homogenised. The cell suspension was then layered onto a 1.2 M sucrose solution and centrifuged at 1,500 rpm for 20 min. The nuclear pellet was recovered and the RNA extracted using the RNAxel kit (Eurobio). The RNA was then treated by DNAse RQ1 (Promega). We performed RT–PCR experiments (Access RT–PCR kit, Promega) with 30 amplification cycles, using primers described previously [9], then the products were digested by *Msp*I, southern blotted and revealed with a radioactively end-labelled *H19*-specific oligonucleotide (5′-GGTTACAGGACGTGGCGGCTGGCT-3′). Expression levels were quantitated using a Phosphorimager.

Acknowledgements

We are indebted to Peter Fraser for his help and support throughout these studies. We thank Roger Karess for critical reading of the manuscript and Rolf Ohlsson and Jean-Jacques Panthier for helpful suggestions. This work was supported by a grant from the Association pour la Recherche sur le Cancer (#6935 and #9845).

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