COMMENTARY

Allele-specific gene expression in mammals: The curious case of the imprinted RNAs

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Mammals exhibit two epigenetic phenomena whose consequence is the silencing of one of two wild-type copies of a gene — X chromosome inactivation in females and parental imprinting of selected autosomal genes. As the mechanisms underlying these forms of gene dosage control are being elucidated, some striking similarities between them are being revealed. Of these similarities, none is more curious than the involvement of two noncoding RNAs in these processes, *Xist*, in the case of X chromosome inactivation, and *H19*, an imprinted RNA on chromosome 7. This article will explore the potential evolutionary relationship between X chromosome inactivation and imprinting by focusing on these unusual RNAs.

The association between imprinting and *H19* RNA

The first imprinted gene identified in mammals was found, by chance, in the course of studies intended to elucidate the function of the fetal-specific growth factor, insulin-like growth factor II. DeChiara et al. (1990) had generated a mutation in the gene using homologous recombination in embryonic stem cells. When that mutation was inherited from fathers, the heterozygous offspring were 60% of normal size, but when the gene was maternally inherited, the offspring were normal-sized. The authors explained this genetic mystery by showing that only the paternal copy of the *Igf2* gene is active in most tissues of the fetus (DeChiara et al. 1991).

Igf2 had been mapped to the distal third of mouse chromosome 7 in a region that had been implicated in imprinting by genetic analysis (Searle and Beechey 1990). Embryos that inherit the chromosomal region containing *Igf2* exclusively from mothers die late in gestation. If the lack of *Igf2* was the only defect in these embryos, they should have survived, suggesting that the distal end of chromosome 7 contained at least one other imprinted gene. *H19* was identified shortly thereafter as the second imprinted gene in the region (Bartolomei et al. 1991). Unexpectedly, its expression was the opposite to that of *Igf2*, that is, it was maternally expressed.

The discovery of *H19*'s allele-specific expression only served to fuel the mystery surrounding this gene. It was originally identified in a differential screen for genes that were coregulated with an endoderm- and fetal-specific gene, α-fetoprotein (AFP) (Pachnis et al. 1984, 1988). Since then, it has been identified in a variety of such screens, including ones to identify genes induced by differentiation of fibroblasts into muscle (Davis et al. 1987) and embryonic stem cells into primitive endoderm (Poirier et al. 1991). What made it unusual was the absence of a conserved open reading frame in the "mRNA," despite the fact that its gene structure and biosynthesis pointed to its being a conventional mRNA (Brannan et al. 1990). The patterns of sequence conservation among mammalian versions of the RNA suggested that aspects of both primary and secondary structure, not protein coding capacity, were being conserved (Tilghman et al. 1992). Although Leibovitch et al. (1991) used an antibody to detect a 29-kD protein purported to be encoded by the human *H19* gene, the specificity of the antibody has never been demonstrated, and the putative protein product is not conserved in any other mammalian species.

The association between X chromosome inactivation and *XIST* RNA

Several years ago a second noncoding RNA that was expressed in an allele-specific manner was discovered in mammals. *XIST* RNA was first identified in humans as a transcript that mapped to the X chromosome inactivation center (XIC), a site required in *cis* for inactivation of the X chromosome (Brown et al. 1991). Surprisingly, its expression was derived exclusively from the inactive X chromosome, a property that is unique to *XIST*. Like *H19*, it encodes no open reading frame conserved in the two mammalian homologs—human and mouse—identified to date (Brockdorff et al. 1991, 1992; Pizzuti et al. 1991; Brown et al. 1992). In fact, Hendrich et al. (1993) have recently shown that the degree of sequence conservation among *H19* and *XIST* homologs is very similar (see Fig. 3, below).

The *XIST* RNA in humans and its counterpart, *Xist* RNA in mice, were localized to the nucleus, and possibly

even to the inactive X chromosome, although at the moment the in situ hybridization studies on which this conclusion is based cannot distinguish between the inactive X as the site of *XIST* transcription versus its localization (Brockdorff et al. 1992; Brown et al. 1992). Biochemical fractionation studies, on the other hand, place the majority of the human and mouse *H19* RNAs in 28S–30S cytoplasmic particles, although they also indicate that a portion of the RNA may be nuclear (Brannan et al. 1990). This difference in localization represents a potentially significant difference in the properties of the two RNAs. However, in light of the fact that the two experiments were performed using very different methods, this difference could be more apparent than real. Until it is established in what cellular compartment each RNA functions, the significance of these studies remains uncertain.

The role of DNA methylation in the allele-specific expression of *Xist* and *H19*

The parallels between *H19* and *XIST* extend to their mode of expression in mammals, in the sense that each is expressed within a chromosomal context in which one or more neighboring gene(s) is maintained in a silent configuration. In the case of *H19*, it is the 5′ *Igf2* gene that is silent on the maternal chromosome. For *XIST*, the silent neighbors encompass most of the inactive X chromosome. Recent work suggests that DNA methylation may underlie at least some part of the mechanisms controlling this allele-specific transcription.

The problem that allele specificity poses is very similar for imprinting and X chromosome inactivation: The transcriptional machinery must distinguish between the expressed and silent copies, and that distinction must be stable and heritable through many cell divisions. In the case of imprinting, this distinction is based solely on chromosomal origin—maternal versus paternal—and not on any instrinsic information in the DNA sequences of the alleles. One could envisage at least two ways in which DNA methylation could participate in allele-specific expression of genes. First, it could act as the primary mark that is established during gametogenesis to distinguish the alleles. Alternatively, DNA methylation could be a response to a primary mark, functioning to maintain differential gene expression. Distinguishing between these possibilities is crucial for understanding the mechanism of imprinting.

The argument in favor of a central role for DNA methylation in at least the early stages of *Xist* expression has recently come from work from Rastan's laboratory (Norris et al. 1994). The choice of which X chromosome to inactivate in eutherian mammals is a random one in all somatic cells. The only exceptions are the trophectoderm and endodermal cells in the extra-embryonic membranes, among the first cells to differentiate in early embryos. There the choice is paternal inactivation, and, in that sense, X chromosome inactivation can be considered to be imprinted (Takagi and Sasaki 1975; West et al. 1977). Norris et al. (1994) have recently shown that the paternal *Xist* allele becomes demethylated during meiosis and remains so until fertilization. They argue that the hypomethylation permits paternal-specific *Xist* expression by the four-cell stage, and thereby directs paternal-specific X chromosome inactivation in the earliest tissues to differentiate. Between the morula and blastocyst stages, a genome-wide demethylation (Monk et al. 1987; Kafri et al. 1992) presumably erases the paternal methylation in all other uncommitted embryonic cells, and the counting mechanism that inactivates all but one X chromosome with random choice is initiated. Even in these cells, the pattern of DNA methylation around the *Xist* gene is again consistent with a negative role for methylation, as demonstrated by methylation over the silent allele on the active X chromosome (Norris et al. 1994).

DNA methylation was first implicated in autosomal parental imprinting when it was noticed that some transgenes were methylated in a parental-specific manner, usually when inherited from mothers, and the methylation served to silence expression from the transgene in at least one instance (Hadchouel et al. 1987; Reik et al. 1987; Sapienza et al. 1987; Swain et al. 1987; Chaillet et al. 1991). After the first endogenous imprinted genes were identified, parental-specific methylation patterns were found in their environs, but the patterns were not always what was expected. For example, the insulin-like growth factor II receptor (*Igf2r*) gene, which is maternally expressed in mice (Barlow et al. 1991), is selectively methylated on the expressed maternal allele within an intron and on the silent paternal allele on the promoter (Stoger et al. 1993). However, only the maternal methylation is set during gametogenesis and maintained throughout development, a requirement for the mark. Likewise, a region upstream of the active paternal allele of *Igf2* is differentially methylated (Sasaki et al. 1991). The differential methylation of the active alleles of these two imprinted genes ran counter to the expectation that methylation would perform a negative regulatory function. *H19*, on the other hand, seemed to fulfill this expectation beautifully in that an 8- to 10-kb domain that included ~5 kb of its promoter and the gene itself were heavily methylated only on the silent paternal copy (Bartolomei et al. 1993; Brandeis et al. 1993; Ferguson-Smith et al. 1993).

The importance of DNA methylation in maintaining the imprint on these genes has been demonstrated recently by Li et al. (1992, 1993), who analyzed the impact of genome-wide demethylation in embryos lacking the maintenance methylase, DNA methyltransferase. They showed that in homozygous mutant embryos, the loss of DNA methylation results in the activation of the silent paternal *H19* gene, but the silencing of both *Igf2* and *Igf2r*. Thus, for *H19*, DNA methylation acts negatively, whereas it must be a positive regulator for the other two genes.

Are *XIST* and *H19* involved in Allele-specific expression?

The presence of genes for noncoding RNAs within at

least two imprinted domains raises the possibility that they have some functional role to play, either in their own imprinting, or the imprinting of neighboring genes. The attractiveness of considering RNA as a regulatory molecule for imprinting is its ability to act in *cis*, unlike proteins, which must be translated in the cytoplasm. On the basis of its map position and its exclusive transcription from the inactive X chromosome, *XIST* could be the long sought after X chromosome inactivation center as has been suggested by several investigators. A word of caution is needed, however, because the defined genetic boundaries of *Xce* extend well beyond the borders of the *XIST* gene (P. Cooper et al. 1993; Heard et al. 1993; Lafreniere et al. 1993; Leppig et al. 1993). Notwithstanding this proviso, circumstantial evidence in favor of a role for *XIST* in X chromosome inactivation has been steadily mounting. *XIST* is not transcribed in XY males, which do not undergo X chromosome inactivation (Brockdorff et al. 1991; Brown et al. 1991). Allelic differences at the X chromosome inactivation center in mice can affect the likelihood that an X chromosome will be chosen for inactivation in XX females cells (Cattanach et al. 1969; Cattanach and Papworth 1981; Johnson and Cattanach 1981). X chromosomes with "strong" *Xce* alleles are more likely to remain active than those with "weak" alleles. Those alleleic differences inversely correlate with levels of *Xist* RNA expression, in that the X chromosomes with strong *Xce* alleles express less *Xist* than those with weak alleles (Brockdorff et al. 1991). Finally, the activation of *Xist* precedes X chromosome inactivation in mouse embryos, and therefore its expression is not a consequence of the process (Kay et al. 1993). Definitive evidence, however, awaits the analysis of mutations of the gene in mice. However, for the purposes of this article, we will accept the hypothesis that the decision to transcribe the *Xist* gene is mechanistically crucial to the silencing of the other genes on that X chromosome. Whether it is the act of *Xist* transcription per se, or the RNA product, acting in *cis* to maintain its own transcription or silence that of the neighboring genes remains a central issue for exploration.

For *H19*, there are no data that argue that its RNA product is involved in either its own imprinting or the imprinting of *Igf2*. However, several observations suggest the possibility that the transcriptional imprinting of the *H19* and *Igf2* genes are mechanistically linked, suggesting at least an indirect role for *H19* in *Igf2* imprinting. First, the two genes are very tightly linked and lie within 75 kb of one another on the distal end of chromosome 7 (Zemel et al. 1992). Second, they are expressed in an identical pattern during embryogenesis in cells of endoderm and mesoderm origin, and then repressed in most tissues after birth (Lee et al. 1990; Poirier et al. 1991). Several years ago, we proposed that the reciprocal imprinting of these two genes is mediated by a competition between them for a common set of regulatory elements (Fig. 1; Bartolomei and Tilghman 1992; Bartolomei et al. 1993). The competititon is driven by paternal-specific methylation of the *H19* gene, which silences that allele, thereby permitting *Igf2* transcription. On the maternal chromosome, the *H19* gene is fully active due to the absence of DNA methylation, and so by default, the maternal *Igf2* gene is not transcribed.

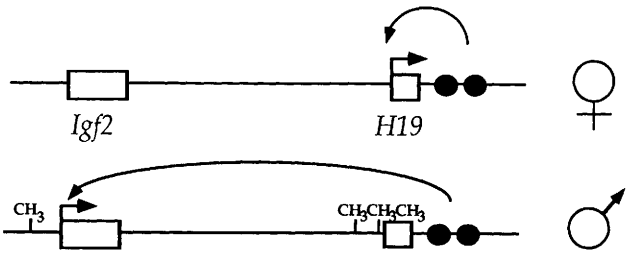

Figure 1. The enhancer competition model to explain the opposite imprinting of *H19* and *Igf2*. (□) The *H19* and *Igf2* genes, with the horizontal arrows indicating the transcribed alleles. (●) The two *H19* enhancers. The positions of allele-specific methylation of the paternal chromosome are indicated by the CH_3 symbols. The single-lined arrows leading from the enhancers indicate the engagement of the enhancers with the *H19* gene on the maternal allele and the *Igf2* gene on the paternal allele. The data for the methylation at *Igf2* are taken from Ferguson-Smith et al. (1993), and the data for *H19* from Bartolomei et al. (1993).

Three observations are consistent with the model: The paternal methylation of *H19* acts as a negative signal, as judged by the reactivation of the paternally inherited gene in embryos lacking DNA methyltransferase (Li et al. 1993); one of the strong enhancers downstream of the *H19* gene is hypersensitive on both alleles, indicating that it is actively engaged in transcription on both parental chromosomes (Sasaki et al. 1992; Bartolomei et al. 1993); and the *H19* gene, transposed to other chromosomal locations as a transgene, is still imprinted, unlike the majority of *Igf2* transgenes (Bartolomei et al. 1993; Lee et al. 1993). The model implies that the proximity of *Igf2* to *H19* facilitiates its imprinting, that is, that the control of allele specificity of both genes resides at the *H19* locus.

An evolutionary link between the imprinting and X chromosome inactivation?

The parallels between imprinting and X chromosome inactivation in early embryos raises the possibility that the two phenomena are related in evolution. X chromosome inactivation represents one solution to the ancient problem of dosage compensation in eukaryotes. Unlike dosage compensation in *Caenorhabditis elegans*, where *both* X chromosomes in XX hermaphrodites are downregulated to equalize expression of X-linked genes in XO and XX animals, dosage compensation in mammals is characterized by the down regulation of only *one* of the two chromosomes. This form of dosage compensation has been documented in both eutherian and marsupial mammals. In marsupials, however, X chromosome inactivation differs in significant ways from X chromosome inactivation in eutherians, because it is both incomplete and can be reactivated in tissue culture without de-

methylating agents such as 5-azacytidine (for review, see D.W. Cooper et al. 1993). The most striking difference, however, is the preferential inactivation of the paternal X chromosome, similar to what is observed in the extraembryonic tissues of the mouse embryo (Cooper et al. 1971; Sharman 1971). The more distantly related monotremes such as the platypus have incomplete differentiation of the X and Y chromosomes, and for this reason the study of X chromosome inactivation in this species has been difficult. However, cytological studies have suggested that one of the two X chromosomes is late replicating, a hallmark of X chromosome inactivation in other mammals, suggesting the possibility of inactivation in this species as well (Murtagh 1977).

Two hypotheses have been proposed to explain the evolution of parental imprinting. The simpler of the two proposes that imprinting arose in placental mammals as a mechanism to regulate tightly the rapid growth phase associated with the second half of gestation once organogenesis is complete (Solter 1988). Oversized fetuses not only endanger their own survival but the survival of their littermates and mother as well. Haig and his colleagues (Moore and Haig 1991) have provided a second model, whereby imprinting represents a tug-of-war between the desire of the male to sire many large fetuses with many females to ensure that his genes will be propagated in the population, versus the advantage to the female to conserve her resources with any single litter to ensure many future offspring. That is, the male's genome is using imprinting to deliver signals for growth while the female's genome is providing a compensatory brake.

Both models readily accommodate the imprinting of *Igf2* and its binding protein, *Igf2r*, both of which are involved in growth regulation. In addition, the direction of their imprints, that is, the paternal expression of the positive growth regulator, *Igf2*, (DeChiara et al. 1991) and the maternal expression of the negative growth regulator, *Igf2r* (Barlow et al. 1991), are predictions of the Haig hypothesis. Likewise, the phenotype of uniparental disomies of chromosome 11, in which maternal inheritance of the proximal third of the chromosome leads to reduction in size of mice, while paternal inheritance leads to the reverse (Cattanach and Kirk 1985), would also lend support to either model.

Both models predict that oviparous animals, such as birds and amphibians, will not display imprinting, which is consistent with reports that parthenogenesis can be achieved in these species. However, they make different predictions about the time in vertebrate evolution when imprinting arose. The first model requires that the growth phase of embryonic development should occur in utero, and therefore one might expect that imprinting arose at the time that marsupials, whose progeny grow outside the uterus in late gestation, and eutherian mammals diverged. The Haig model, on the other hand, requires only that the mother contribute resources to the offspring, either before or after birth, and therefore all lactating mammals, including monotremes that lay eggs (such as the platypus), should exhibit imprinting. However, one other condition would also have to hold: The species must be nonmonogamous for the two parental genomes to be engaged in their tug-of-war.

The evolution of the different reproductive strategies represented by eutherian mammals, marsupials, and monotremes is thought to have occurred in a very short period of time between 145 and 130 million years ago (Novacek 1992). It has not been established to date whether either marsupials or monotremes display imprinting, but it obvious that X chromosome inactivation in marsupials is essentially equivalent to autosomal imprinting, in that there is a parental bias (Chandra and Brown 1975). If marsupials have an *Xist* gene, its expression would therefore be imprinted as well, as it is in the early mouse embryo.

Are *H19* and *Xist* homologs?

Thus, the stage is set to ask whether *XIST* and *H19* arose as the result of convergent evolution, or whether they share a common ancestor. To establish common ancestry, we normally rely on two hallmarks of relatedness, the intron–exon structure of the genes and direct sequence comparison. Using both of these criteria, the case for the relatedness of *H19* and *Igf2* is equivocal at best.

At first glance, it is apparent that the sizes of the genes and their transcripts are quite different (Fig. 2). The *H19* gene is composed of five exons, separated by four very small intervening sequences and codes for a 2.5-kb transcript in both humans and mice (Pachnis et al. 1988; Brannan et al. 1990). *Xist/XIST*, on the other hand, is a large gene, with a transcript ~17 kb in length (Brockdorff et al. 1992; Brown et al. 1992). The number of exons of the *Xist* gene is not conserved in mammals, because the human gene has two extra exons at its 3′ end that are differentially spliced. What is similar between the overall structures of *H19* and *Xist* is the placement of large outside exons that are separated by very small exons. The differential splicing of the human transcript already suggests a mechanism whereby the absolute number and size of the exons could change, especially in a gene

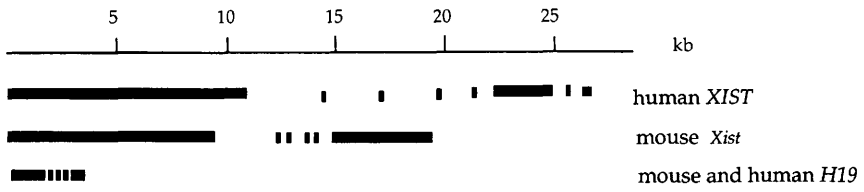

Figure 2. The structures of the *H19* and *XIST* genes. The exons for the human *XIST*, mouse *Xist*, and *H19* genes are indicated by the solid boxes, with the spaces between representing the introns. (Adapted from Pachnid et al. 1988; Brannan et al. 1990; Brockdorff et al. 1992; Brown et al. 1992).

where the normal constraints imposed by maintaining the triplet genetic code are not working.

At the level of sequence similarity, the case for an evolutionary relationship between *H19* and *Xist* is harder to make. The problem lies in the fact that there is only modest primary sequence conservation, even within the mammalian *Xist* or *H19* gene families. As Figure 3 illustrates, both genes exhibit regions of considerable sequence identity, interrupted by regions of relative nonidentity as well as by insertions/deletions in one sequence relative to the other. This confounds attempts to find similarities between the gene families. The overall degree of sequence identity between mouse and human *H19* is only 77% (Brannan et al. 1990). For *XIST*, the overall degree of conservation is even less, although, as is the case for *H19*, regions such as exon 4 are relatively well conserved (Brockdorff et al. 1992; Brown et al. 1992; Hendrich et al. 1993). When the sequences of the small internal exons of *H19* and *XIST* are examined for similarities, and when the most conserved regions of both genes were compared, no significant homologies (>60% over 24 bases without the introduction of gaps) were evident.

One striking aspect of the sequence conservation within the *H19* gene family is the apparent preservation of secondary structure (Tilghman et al. 1992). This is evident in the presence of long energetically favorable stem–loop structures, which are conserved between homologs by virtue of compensatory base substiututions in the stems. A particularly noteworthy example within *H19* is shown in Figure 4. When the *XIST*/*Xist* RNAs were examined for similar structures, several were detected (Fig. 4). As was the case for *H19*, the longest of these coincided with the most conserved region of the RNA. However, when the primary sequences of these two regions were compared between the gene families, no similarities were apparent. Although the conservation of secondary structure shared by both RNAs is striking, it by no means argues in favor of a common ancestor for these genes, only that the secondary structure of both RNAs is important to their functions.

Three aspects of these structures suggest that they may be important in the overall activity of the two RNAs. First they occur in regions whose primary sequence is also being conserved; second, when base changes occur, they often occur at compensatory positions in the stem–loop; third, they each represent the most energetically favorable stem–loop predicted on the basis of the STEMLOOP program, primarily because of the long length of the stem, 35 and 36 bp. When comparable studies were performed on human and rodent AFP and albumin mRNAs, two RNAs whose sequences are diverging rapidly enough to provide some basis for comparison with *H19* and *Xist* (~75% identity between either mouse and human AFP or between rat and human albumin), only two stem–loop structures were conserved

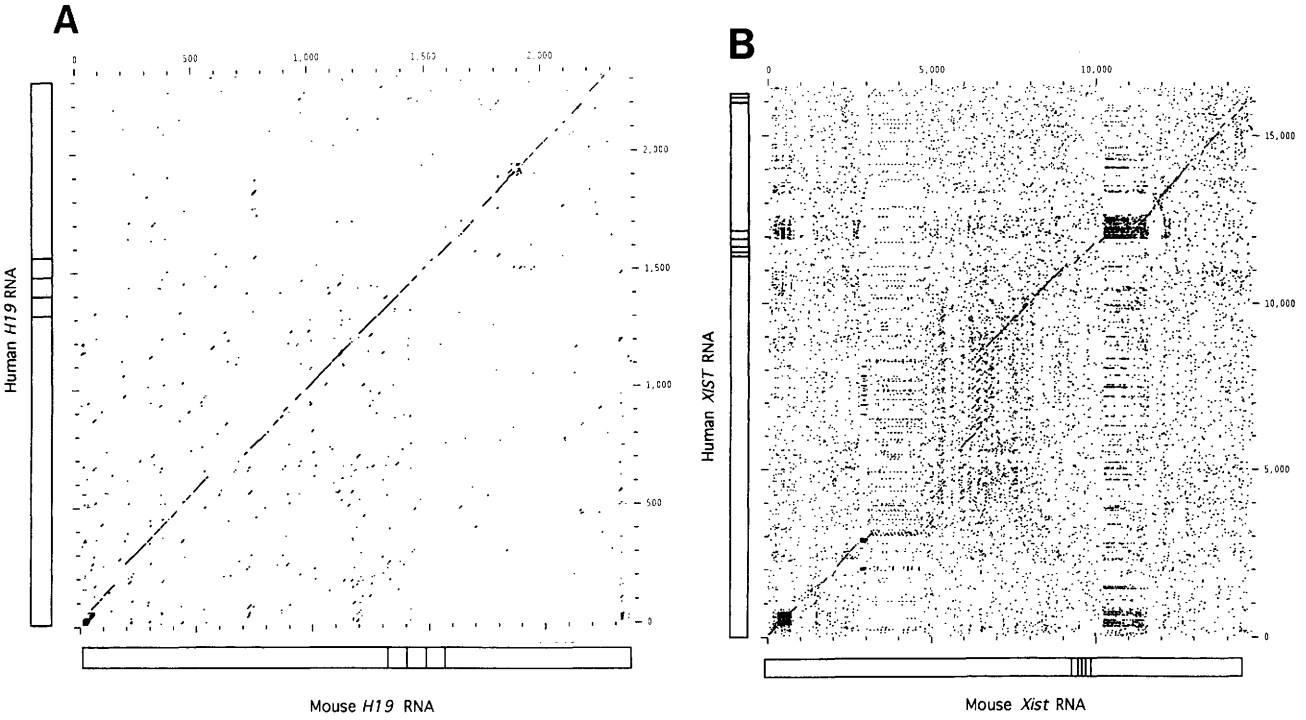

Figure 3. Sequence conservation within the human and mouse *H19* genes (*A*) and between the human and mouse *XIST* genes (*B*). The sequences of the mature mouse and human *XIST* RNAs and the mature mouse and human *H19* RNAs were compared using the COMPARE program of the Genetics Computer Group (GCG) using a window of 21 bases and a stringency of 66%, and plotted using the program DOTPLOT. The correspondence of the bases with the exons of each gene are indicated on the sides of the plots. (Sequences taken from Pachnis et al. 1988; Brannan et al. 1990; Brockdorff et al. 1992; Brown et al. 1992).

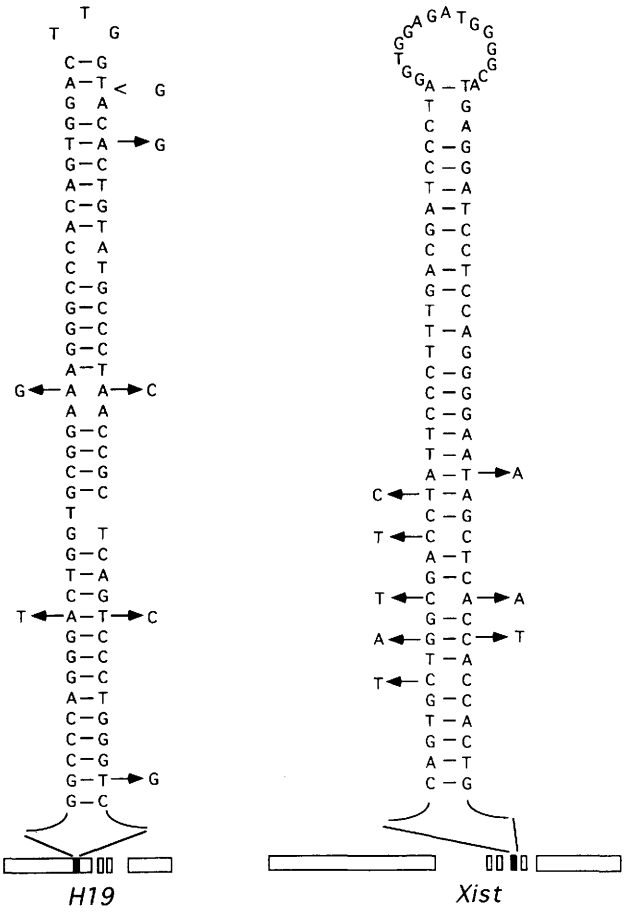

Figure 4. Conserved stem–loop structures in the *H19* and *Xist* genes. The most conserved region of the mouse *H19* gene, within exon 1, is drawn in an extended stem–loop. The bases differing between the mouse and human genes are indicated by the arrows. The most conserved region of the mouse *Xist* gene, within exon 4, is drawn similarly, with the base differences in the human gene indicated by the arrows.

between human and rat albumin mRNA, with stems ranging between 10 and 13 bases in length. No energetically favorable conserved stem–loop was detected in comparisons between human and mouse AFP.

Both human and mouse *XIST/Xist* contain a number of short tandem repeats throughout their lengths (Brockdorff et al. 1992; Brown et al. 1992). Although the repeat lengths vary in number between *XIST* and *Xist*, their positions and sequence motifs are conserved. There is no comparable extensive pattern of simple sequence repeats in *H19*; however, a short region at the 5′ end of both the human and mouse *H19* RNAs contains the sequence TGGGGG repeated 8–10 times. In neither gene is the significance of these repeats clear.

Future prospects

In the absence of primary sequence conservation between *XIST/Xist* and *H19* to aid in establishing evolutionary relatedness, one must await the analysis of mutations in the two genes to ascertain whether they are performing similar functions in X chromosome inactivation and imprinting. Demonstrating a similar function would certainly strengthen the likelihood that the two RNAs shared a common ancestor, although by no means would this prove it. It will also be essential for any model that ascribes a function to these RNAs to establish when in mammalian speciation they arose and whether the times coincided with the evolution of X chromosome inactivation and imprinting, respectively. This particular piece of the puzzle may also help to determine which of the two forms of dosage compensation came first. Finally, one strong prediction of a model that proposes a function for RNAs in genomic imprinting is the presence of RNA-coding genes at other imprinted loci. Molecular analysis of three other imprinted loci in the mouse and human are under way in several laboratories. The most extensive of these, at the locus encoding the maternally expressed *Igf2r* gene in the mouse, has failed to reveal any additional imprinted genes to date (Barlow et al. 1991).

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K Pfeifer and S M Tilghman

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