The mouse H19 locus mediates a transition between imprinted and non-imprinted DNA replication patterns

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Genes subject to genomic imprinting generally occur in clusters of hundreds of kilobases. These domains exhibit several gamete of origin-dependent manifestations, including a pattern of asynchronous replication when studied by fluorescence in situ hybridization (FISH). We find a transition from asynchronous replication at the imprinted mouse H19 gene to synchronous replication at the downstream Rpl23 gene, the human homologue of which appears to be non-imprinted. Two-colour FISH demonstrates that this transition is due solely to a difference in replication timing between the upstream and downstream chromatin on the later-replicating (maternal) chromosome. This difference is lost in mice deleted for the H19 gene body and 9.9 kb of upstream DNA when this deletion is maternally inherited, with synchronous replication patterns extending over 110 kb upstream from the deleted area. No effect is seen when the deletion is paternally inherited. The presence of a boundary element in this region has been suggested by observations of position-independent expression of H19-containing transgenes and the blocking of accessibility of downstream enhancers to the upstream Igf2 and Ins2 genes on the maternal chromosome. The FISH studies presented here demonstrate the insulation of replication patterns within the imprinted domain from downstream, non-imprinted chromatin, mediated by an element at the H19 locus which is subject to genomic imprinting.

INTRODUCTION

Genomic imprinting refers to the establishment of a gamete-determined group of epigenetic modifications that remain through growth and differentiation. This process characteristically results in changes in gene expression, cytosine methylation, chromatin structure and replication timing within the imprinted domain (reviewed in refs 1–3), with similar physical organization of sex-specific recombination patterns (4,5). As these manifestations occur in different patterns on the paternal and maternal chromosomes, obvious effects include expression of certain genes from the paternal chromosome only, and of other genes exclusively from the maternal chromosome. The regions affected by genomic imprinting are generally large, up to hundreds of kilobases in size. It is not known how these imprinted domains are established or circumscribed.

We studied the physical circumscription of the mouse chromosome 7F imprinted domain, which includes the imprinted Ins2, Igf2 and H19 genes (6–8). The extent of its syntenic human region has been analysed. The centromeric end of the human chromosome 11p15.5 imprinted domain has been proposed to lie between the biallelically expressed hNAP2 and maternally expressed p57kip2 genes (9). Telomeric to the domain are three biallelically expressed genes, RPL23 [ribosomal protein, L23, the symbol assigned to the gene provisionally reported as L23MRP (10)], 2G7 and TNNT3, suggesting a boundary between the maternally expressed H19 and RPL23 (10,11). In each case, the authors emphasize that since imprinting of gene expression can be extremely tissue and developmental stage specific [as found for insulin 2 (6)], the finding of biallelic expression in the tissues studied may not exclude imprinting at these loci. Moreover, within imprinted domains, there are genes that escape imprinting, such as the biallelically expressed Th (12). To address this concern, Tsang et al. (10) studied a second component of the imprinted epigenotype, cytosine methylation, at the RPL23 locus. They did not find gamete of origin-dependence of methylation patterns at this locus, supporting their proposal that RPL23 lies outside the imprinted domain.

We mapped the mouse Rpl23 gene using a human cDNA probe, confirming that synteny with human is maintained in mouse as it is in rat (10,11). By studying replication patterns using fluorescence in situ hybridization (FISH), we were able to test for a boundary to the imprinted domain between H19 and Rpl23. Replication asynchrony appears to be less dependent on cell type compared with the other components of the imprinted epigenotype (12). These FISH studies therefore complement the suggestive human expression and methylation data (10,11) with an assay of increased specificity.

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RESULTS AND DISCUSSION

Primary mouse splenocyte cultures stimulated with concanavalin A were analysed. The pattern of hybridization foci was analysed for each probe on 100 consecutive bromodeoxyuridine (BrdU)-positive cells, as in other studies (12). Figure 1 shows the results of FISH studies of replication timing in C57Bl/6 mouse splenocytes. The proportions of these cells showing a single/single, single/double or double/double pattern of hybridization are shown as percentages. Probes mapping upstream from H19 show a substantially proportion of nuclei which exhibit asynchronous replication patterns. The downstream cosmid containing the Rpl23 gene shows a markedly decreased proportion of single/double hybridization foci similar to that of non-imprinted chromatin. This supports the conclusions from expression and methylation studies of the syntenic human region that the imprinted domain extends to a point between H19 and RPL23 (10).

Our next question was whether the replication within the imprinted domain occurs completely independently from that in the downstream, non-imprinted region. To address this, we used two-colour FISH, the H19-containing cAH probe labelled with digoxigenin and the Rpl23-containing c11.18 probe labelled with biotin. The replication pattern of both probes was documented for each of 50 cells (non-BrdU selected). The first analysis applied was the determination of replication order in cis, as previously performed in studies of the human β-globin domain (13). We found the proportion of chromosomes in which c11.18 replicates prior to cAH is substantially higher (23%) than the proportion with earlier cAH replication (6%). This indicates that, overall, the downstream, non-imprinted region replicates earlier in the cell cycle than the upstream, imprinted region. This analysis was broken down further in order to compare the relative replication timing on each chromosome (Fig. 2). cAH and c11.18 concordantly showed pairs of single foci in 20% of nuclei and pairs of double foci in 22% of nuclei. In each case when both cAH and c11.18 showed single/double patterns (10%), the single foci were always present on the same chromosome, with the double foci occurring on the homologue. Of note was the coincidence of a single/double pattern for cAH and a double/double pattern for c11.18 in a high proportion (28%) of nuclei. This shows that on the chromosome with earlier cAH replication [previously found to be the paternally derived chromosome (12)], replication of cAH occurs at the same time as the downstream, non-imprinted chromatin, while on the other chromosome the cAH region is not only replicating later than its homologue but also later than the downstream c11.18 region. The transitions from asynchronous to synchronous and later to earlier replication patterns are therefore due to a difference in replication timing on the maternal chromosome only, with no discernible difference in replication patterns between upstream and downstream chromatin on the paternal chromosome.

In mouse, it has been shown that the regulation of imprinted expression of the genes in this region is dependent on the accessibility of promoters to enhancers located downstream from H19 (14,15). It has been proposed that the patterns of expression observed are the result of promoter competition for these enhancers (15,16). Deletion of the actively transcribed H19 gene and 9.9 kb of upstream DNA on the maternal chromosome allows access of the maternal Ins2 and Igf2 genes to the enhancers, supporting this model (14). These results have also been suggested to support the presence of a chromatin insulator normally active on the maternal chromosome (14), as enhancer blocking is a characteristic property of boundary elements (17). Given the results of the two-colour FISH experiments indicating the insulation of replication patterns on the maternal chromosome, we wanted to test whether this H19 deletion also affected the insulation of replication patterns.

Splenocytes from mice heterozygous for this H19 deletion (14) were prepared for FISH analysis. A striking change to synchronous replication patterns was found in mice deleted for the H19 gene body and 9.9 kb of upstream sequence when the deletion was inherited maternally (Fig. 3A). This was consistent for each of the three cosmids upstream from H19 which had detected asynchronous replication in splenocytes from non-deleted mice. No effect was seen in mice inheriting the deletion paternally (Fig. 3B). The H19 gene body/upstream DNA knockout therefore affects not only the expression patterns of upstream genes (14) but...
also the replication patterns of >120 kb upstream from the gene body, without affecting downstream replication patterns. This effect is dependent on the parental origin of the deletion, with no effect observed for the paternally inherited deletion.

Replication asynchrony in imprinted regions is a characteristic finding of FISH studies. The timing of replication does not simply reflect gene expression differences, as the imprinted H19 and Igf2r genes are paradoxically expressed from the later-replicating chromosome (12). The data presented here are comparable with previous studies of this region (12), although our proportion of cells showing double signals is higher. This may be due to our use of primary rather than transformed cell lines, allied with differences in cell preparation and imaging techniques. As this study compares patterns in cis, involving the use of a number of probes on the same cell preparations, such differences have no bearing on the conclusions reached. It is possible that the hybridization patterns observed using FISH actually reflect delayed sister chromatid separation on one chromosome, given the observations of synchronous replication in imprinted regions using non-FISH-based assays (18,19). The interpretation of the data presented here depends only on the FISH observation being a feature of imprinted domains, reflecting a difference in chromatin organization which determines either true asynchronous replication or synchronous replication with asynchronous sister chromatid separation.

We find evidence using FISH of a limit to the mouse chromosome 7F imprinted domain in the vicinity of the H19 gene, a finding consistent with studies of the homologous region in human. We also find that deletion of an area at this transition changes the replication patterns within the normally imprinted domain when the deletion is maternally inherited. The deleted area therefore contains an element required for the establishment or maintenance of the replication patterns of the upstream region. Given the location of the deletion at the transition to non-imprinted chromatin, we favour the model of an imprinted boundary element within the deleted area, active on the maternal chromosome only. Alternative possibilities are that the maternally inherited knockout affects the chromatin organization of the paternal translocation in trans (20) or that the presence of prokaryotic DNA (the neo\textsuperscript{\textregistered} gene) affects the normal organization of this domain (21). The model of an imprinted boundary element is consistent with the enhancer-blocking property of the H19 deletion previously described (14), as well as the observation that H19-containing transgenes are expressed in a position-independent manner (22,23), a characteristic of boundary element-containing constructs. A transgenic assay in Drosophila has shown that a silencer is located within the 3 kb upstream from the H19 promoter (24). While boundary elements are distinguished from silencers by having no intrinsic effects on promoters or enhancers (25), the suppressor of hairy wing boundary element can act as a silencer in a genetic background lacking the mod(mdg4) protein (26). The silencing activity in Drosophila of the element upstream from the H19 promoter does not, therefore, exclude its possible contribution to chromatin insulation in mammalian cells. Also potentially informative is the recent knockout of the 3 kb H19 gene body without removing upstream DNA, which also allowed expression of the upstream Igf2 gene on the maternal chromosome but only to 25% of levels of expression seen in the deletion including 9.9 kb of upstream DNA (27). One interpretation of these results is that the knockout removed part of a boundary element at H19, allowing some access of the downstream enhancers across a weakened residual element.

This study shows that FISH can be used as a tool to analyse the organization of imprinted chromatin. The FISH data presented strongly support the model of an imprinted chromatin insulator at the H19 locus. Canonical boundary elements are not defined using FISH, but rather by the demonstration of blocking of the effects of enhancers on promoters (17). Therefore, we define the element identified by this study as an insulator of replication patterns rather than as a boundary element. More focused studies
Figure 3. (A) Splenocytes were analysed from mice inheriting maternally a deletion of the 3 kb H19 gene body and 9.9 kb of upstream DNA, replaced with a neomycin resistance gene (Neo) (13). Synchronous replication was observed not only for the downstream c17.2 region but also throughout the 120 kb upstream from H19. (B) Analysis of mice inheriting paternally the deletion of the H19 gene body and 9.9 kb of upstream DNA. No alteration in replication patterns compared with non-deleted mice (Fig. 1) was observed.

are required to determine the presence, extent and nature of a boundary element within the deleted region. While several boundary elements have been described to date (17,25,28–30), none has been found to be subject to genomic imprinting. Defining a boundary element at the H19 locus will provide a useful model for studying the epigenetic control of this class of chromatin regulators.

MATERIALS AND METHODS

Cosmid subcloning
Apart from the cAH cosmid (31), cosmids were subcloned from non-chimeric yeast artificial chromosomes (YACs) spanning this region into the SuperCos-1 vector (Stratagene) using supplied protocols. Rpl23 was mapped using the human cDNA for RPL23 (10) probed against the cosmid library. Sequence homologous with the second human exon was identified in the downstream 4.6 kb EcoRI restriction fragment shown in Figure 1 (GenBank accession No. U71209). Cosmid DNA was prepared using a commercial alkaline lysis/ion exchange kit (Qiagen).

Fluorescence in situ hybridization
The wild-type cells analysed were cultured from the spleen of a female C57Bl/6 mouse, while the H19 knockout mouse splenocytes were derived from the offspring of male or female mice deleted for the H19 gene body and 9.9 kb of upstream DNA, mated with C57Bl/6 mice. Standard techniques were used for mouse splenocyte culture, harvesting and slide preparation (31). Cell cultures were pulsed with 100 µM BrdU prior to harvesting to allow the subsequent identification of cells in S phase (32). Probe labelling and FISH techniques have been described previously (33), while BrdU detection was performed using a biotin-conjugated antibody to BrdU detected with avidin-conjugated fluorescein isothiocyanate (FITC). For each probe, the efficiency of hybridization and percentage of BrdU-positive cells were determined, following which the hybridization patterns for 100 BrdU-positive cells were documented. Only hybridizations with efficiencies of at least 85% were used for subsequent studies. Digital images were analysed using a Zeiss epifluorescence microscope with a cooled CCD camera (Photometrics PM512) controlled by software described previously (34).
Greyscale images were captured separately with filter sets for DAPI, fluorescein and rhodamine.

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