Conserved characteristics of heterochromatin-forming DNA at the 15q11-q13 imprinting center

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Nuclear matrix binding assays (NMBAs) define certain DNA sequences as matrix attachment regions (MARs), which often have cis-acting epigenetic regulatory functions. We used NMBAs to analyze the functionally important 15q11-q13 imprinting center (IC). We find that the IC is composed of an unusually high density of MARs, located in close proximity to the germ line elements that are proposed to direct imprint switching in this region. Moreover, we find that the organization of MARs is the same at the homologous mouse locus, despite extensive divergence of DNA sequence. MARs of this size are not usually associated with genes but rather with heterochromatin-forming areas of the genome. In contrast, the 15q11-q13 region contains multiple transcribed genes and is unusual for being subject to genomic imprinting, causing the maternal chromosome to be more transcriptionally silent, methylated, and late replicating than the paternal chromosome. We suggest that the extensive MAR sequences at the IC are organized as heterochromatin during oogenesis, an organization disrupted during spermatogenesis. Consistent with this model, multicolor fluorescence *in situ* hybridization to halo nuclei demonstrates a strong matrix association of the maternal IC, whereas the paternal IC is more decondensed, extending into the nuclear halo. This model also provides a mechanism for spreading of the imprinting signal, because heterochromatin at the IC on the maternal chromosome may exert a suppressive position effect in cis. We propose that the germ line elements at the 15q11-q13 IC mediate their effects through the candidate heterochromatin-forming DNA identified in this study.

epigenetic | fluorescence *in situ* hybridization | gametogenesis | gene regulation | matrix-attachment regions

Human chromosome 15q11-q13 is subject to genomic imprinting. This 2-Mb region contains multiple imprinted genes, most of which are paternally expressed (1) except for *UBE3A,* which is maternally expressed in parts of the brain (ref. 2; Fig. 1). The domain also exhibits other manifestations of genomic imprinting, such as differences in methylation (1), DNA replication timing (3, 4) and chromatin sensitivity to nucleases (5, 6). Mutations involving this domain result in Prader–Willi syndrome (PWS), if there is a genetic or epigenetic failure of the paternal 15q11-q13 contribution, whereas the phenotypically distinct Angelman syndrome (AS) results from the loss of the maternal 15q11-q13 contribution (1). Mechanisms for a genetic failure of a parental contribution include deletion of the imprinted region or uniparental disomy (UPD) of the chromosome. Thus, PWS arises from deletions of paternal 15q11-q13 or maternal UPD for chromosome 15, whereas the converse mechanisms occur in AS (1).

An epigenetic or functional failure of a parental contribution occurs in 2–5% of PWS and AS patients. For example, a father may transmit a chromosome 15 with a maternal imprinted pattern on its q11-q13 region. The offspring inherits two chromosomes 15, one from each parent, but both with a maternal imprint of the q11-q13 region, causing the PWS phenotype (1, 5, 7–10). The converse situation, the transmission of a paternal imprinting pattern on a maternally inherited chromosome 15, is described in patients with AS (1, 9, 10). It has been proposed that these imprinting mutations (IMs) block the imprint switch process during paternal or maternal gametogenesis for PWS and AS, respectively (1, 5, 9–11). PWS IM patients have small deletions clustering at the small nuclear ribonucleoprotein N (*SNRPN*) upstream reading frame (*SNURF*)-*SNRPN* promoter, whereas AS IM deletions cluster approximately 35 kb upstream. Nevertheless, these small deletions result in abnormal DNA methylation and gene expression throughout the imprinted 2-Mb domain in 15q11-q13 (1). These observations indicate that germ line imprinting is not directed independently at each individual gene within the domain, but is established in a hierarchical manner, initially directed at the region defined by the deletion clusters, which subsequently directs the imprinting of the 2 Mb as a whole. The region defined by the PWS and AS IM deletion clusters has therefore been referred to as the imprinting center (IC) of 15q11-q13 (1). A 42-kb deletion involving the mouse *Snurf-Snrpn* promoter has a similar effect to disrupt distant gene expression in cis, indicating evolutionary conservation of the IC (12).

The IC is therefore the region of primary mechanistic importance in the process of genomic imprinting of 15q11-q13. How the IC performs this function is unknown. The putative maternal-to-paternal switch element, active in the male germ line and defined by deletions in PWS IM cases, colocalizes with the *SNURF-SNRPN* promoter (1, 13). This can act as a silencer in *Drosophila* (14), although it is unknown whether this reflects the presence of a transcriptional silencer also active in mammalian cells. The putative paternal-to-maternal switch element active in oogenesis is located approximately 35 kb upstream from the *SNURF-SNRPN* promoter and includes one of the alternatively spliced exons of the *SNRPN U* (*SNRPN* upstream) transcript (8, 10, 15). A splice site mutation in an upstream exon has been found in a nondeleted AS patient with an IM (11), raising the possibility that the mRNA itself acts in cis to regulate imprint switching, although this is presently unproven. It has been proposed that trans-acting factors specific to the female germ line interact with the *SNRPN U* transcript to cause heterochromatin formation (11, 16). Resetting this maternal imprint in spermatogenesis is suggested to involve displacement of silencer proteins from the *SNURF-SNRPN* promoter (1, 5, 14). These proposals constitute a working model for the mechanism of IC function while further regulatory properties of the region are sought.

Abbreviations: AS, Angelman syndrome; FISH, fluorescence *in situ* hybridization; IC, imprinting center; IM, imprinting mutation; MAR, matrix attachment region; NMBA, nuclear matrix binding assay; ORE, oogenesis-responsive element; PWS, Prader–Willi syndrome; *SNRPN,* small nuclear ribonucleoprotein N; *SNURF, SNRPN* upstream reading frame; *ZNF127/Zfp127,* zinc finger protein 127.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF130843 (5′*Snrpn,* 6.8 kb), AF130844 (5′ *ZNF127,* 6.2 kb), and AF130348 (5′ *Zfp127,* 7.2 kb).].

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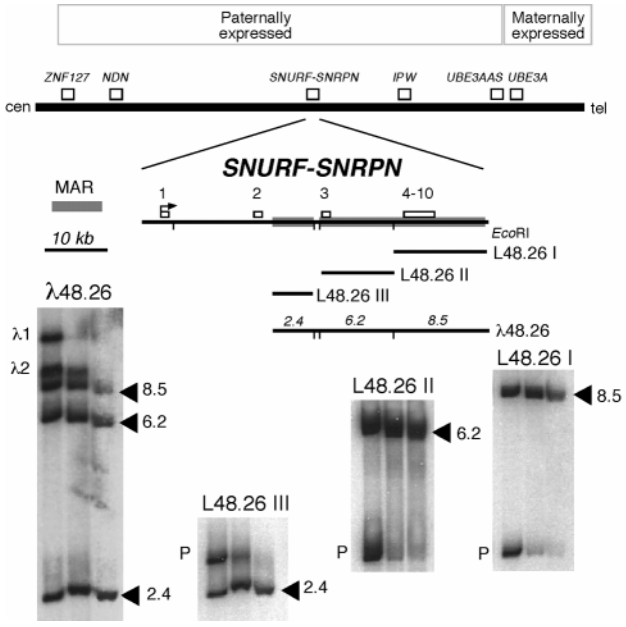

Fig. 1. NMBAs of *Eco*RI-digested phage λ48.26 and its plasmid subclones. In each panel, the left lane is unassayed probe and the second and third lanes are the results of NMBAs in the presence of 100 and 300 μg/ml of cold competitor DNA, respectively. The three *Eco*RI bands marked with arrowheads are the insert fragments binding more strongly than both of the phage vector arms (λ1 and λ2) or the plasmid vector (P). Densitometry was used to confirm that the adjusted relative ratio of insert to vector DNA was high, as calculated previously (17). The relative ratios for the 300 μg/ml lanes are 40.9 (L48.26III), 15.1 (L48.26II), and 39.0 (L48.26I). The region analyzed at *SNURF-SNRPN* therefore contains a large MAR (gray bar).

We have previously proposed that nuclear matrix attachment regions (MARs) are involved in the mechanism of genomic imprinting (17). MARs are periodical DNA sequences identified by their capacity to bind to isolated nuclear matrices *in vitro* that can colocalize with epigenetic regulatory sequences such as enhancers or repressors of transcription (18–20), origins of DNA replication (21), as well as cis-acting regulators of chromatin structure (20, 22) and cytosine methylation (22, 23). They have been found to regulate gene transcription in reporter constructs introduced as transgenes, whereas the same constructs introduced in somatic cells showed no regulatory role (22). These results were interpreted as indicating a functional dependence for MARs on passage through the germ line. These heterogeneous properties suggest a model in which a subset of MARs is functionally dependent on exposure to one, but not the other, mammalian germ line. The resulting regulatory effects in cis would be recognized as genomic imprinting.

To assess the possible role of MARs in the imprinting of 15q11-q13, we used an *in vitro* assay to test the IC region and the cis-regulated zinc finger protein 127 (*ZNF127*) gene for the presence of MARs, as well as the mouse loci homologous with both regions. We find MARs to be present and conserved at each locus. Moreover, we demonstrate a parent of origin-dependent difference in matrix association *in vivo* using a fluorescence *in situ* hybridization (FISH) approach. The striking density of the MARs at the IC suggests their active role in gametogenesis-determined heterochromatin formation that results in genomic imprinting.

Materials and Methods

Nuclear Matrix Binding Assays (NMBAs). Nuclei were prepared from mouse hepatocytes (17) and human HeLa cell or lymphoblast nuclei as described (24). Nuclei were digested with DNase I at 100 ng/ml in the presence of 1 mM CaCl2 at ambient temperature for 1 hr. The nonmatrix proteins were then extracted with 2.0 M NaCl for 10 min on ice, after which the resultant nuclear matrices were washed and stored. These nuclear matrix preparations were stable at −20°C in the presence of protease inhibitors for at least 1 yr.

The DNA clones used for this analysis from the 15q11-q13 IC (7, 25), the mouse *Snurf-Snrpn* (26), the human *ZNF127* (27), and mouse *Zfp127* (28) loci have all been previously published. Restriction digestions of these clones were performed as indicated in the figure legends. The DNA was carefully quantified and end labeled radioactively (29) for use in the NMBA.

Binding assays were performed by incubation of nuclear matrices with 2 ng of radiolabeled probe and 100 or 300 μg/ml of unlabeled competitor *Escherichia coli* genomic DNA. Probe DNA bound to the nuclear matrix was separated by centrifugation followed by proteinase K digestion to destroy the nuclear matrices. Samples were run on agarose gels with unassayed radiolabeled probe for comparison. Specific nuclear matrix binding was defined for cloned DNA fragments that continued to show strong autoradiographic signals despite the presence of increasing quantities of competitor DNA. The quality of new nuclear matrix preparations was tested by performing assays by using the well-characterized *Igk* intronic MAR (18) as a positive control (not shown). The relative strength of binding was quantified by densitometry by using the National Institutes of Health IMAGE program (rsb.info.nih.gov/nih-image). Adjusted relative binding ratios were calculated and positive results defined as previously described (17), by using both phage and plasmid DNA as internal negative controls in all assays.

Sequence Analysis. Sequence data were available for some of the regions studied (GenBank accession nos. U41304, AF063659, U19106, and U19107), with the remainder determined by using automated fluorescent DNA sequencing (Applied Biosystems) (available through GenBank). Published motifs suggested to be characteristic of MARs (30) include a number we found to occur solely because of high (A+T) content (17), so these were excluded from our analyses. A MACVECTOR (Oxford Molecular Group, Oxford, UK) subsequence file was constructed consisting of specific motifs only (available from john.greally@yale.edu). After filtering the DNA sequence to remove repetitive sequences (ftp.genome.washington.edu/cgi-bin/RepeatMasker) as described previously (31), the occurrence of specific motifs on each strand was identified. The base composition function of the same program was used to quantify (A+T) content. Interspecies comparison was performed by using the Pustell DNA matrix function of MACVECTOR. Windows of 35 bp with identity of 65% or greater on either strand were identified by using a hash value set at 1 for maximum sensitivity.

FISH of Halo Nuclei. Human fibroblasts from a normal female and a female PWS patient with a 15q11-q13 deletion were grown on poly-D-lysine-coated culture slides (Falcon). When 60% confluent, the cells were made permeable with 0.5% Nonidet P-40 for 5 min, followed by extraction with 2.0 M NaCl in the presence of 200 mM sodium citrate and 2.5 μM spermidine, pH 7.0. Ethanol was added to 1.6% after 5 min, after which the solution was immediately replaced by 300 mM NaCl/30 mM sodium citrate/100 ng/ml 4′,6-diamidino-2-phenylindole (DAPI) dilactate, pH 7.0. This was replaced with 150 mM NaCl/15 mM sodium citrate/100 ng/ml DAPI dilactate, pH 7.0, after 5 min and incubated for a further 5 min. The slides were then dehydrated in a series of ethanol washes. These nuclear halo preparations were stored in 100% ethanol at −20°C.

Bacterial artificial chromosome clones with the human IC and *HPRT* loci were labeled by nick translation with digoxigenin and biotin, respectively. A chromosome 15-specific α satellite probe, pTRA-20 (32), was likewise labeled with CY3. Slides were denatured in 70% formamide for 2 min at 73°C. Four hundred nanograms of each probe, 10 μg of human Cot-1 DNA (GIBCO), and carrier salmon sperm DNA were denatured in 50% formamide at 70°C for 10 min and preannealed at 37°C for 10 min. Detection of the hapten labels was with FITC and avidin-CY3.5. Fluorescence microscopy by using a charge-coupled device camera allowed capture of grayscale images that were pseudocolored and merged by using PHOTOSHOP 5.0 (Adobe Systems, Mountain View, CA).

Results

Establishment of NMBA Conditions. Fig. 1 shows an example of a positive result in the NMBA, by using the λ48.26 clone spanning part of the human *SNURF-SNRPN* locus. The first lane in each gel is radiolabeled unassayed *Eco*RI-digested probe, to show where the bands migrate electrophoretically. NMBAs with 100 and 300 μg/ml of competitor DNA are shown in lanes 2 and 3, respectively. In these lanes, the phage vector bands are almost completely deficient, showing that the amounts of competitor DNA used are sufficient to prevent nonspecific matrix binding. Strong binding is seen of all three insert bands relative to the λ arms (lanes 2 and 3, Fig. 1). To confirm that the binding observed is specific, we analyzed subclones in a plasmid vector as described (17). The three plasmid subclones (L48.26 III, II, and I) all show markedly strong binding of insert compared with the vector control, confirmed quantitatively by densitometry (Fig. 1). The λ48.26 clone therefore contains an extensive MAR defined by the three *Eco*RI restriction fragments examined.

Analysis of the Human Chromosome 15q11-q13 IC and the Homologous Mouse Region. A summary of the results of extended NMBA analyses of the 15q11-q13 IC and *SNURF-SNRPN* gene is shown is Fig. 2. All the primary NMBA data are available as supplemental material on the PNAS web site (see www.pnas.org) or from the corresponding author. Previous studies of MAR frequencies would predict one MAR of 1 kb or less in the 68.9 kb tested (17, 33). However, the density of MARs at the IC/*SNURF-SNRPN* region is unusually high: of the 68.9 kb tested, a total of 49.1 kb are positive for MARs (gray boxes on map, Fig. 2*a*).

We then studied the mouse region homologous with the IC. In mouse, both imprinting of the syntenically organized genes on mouse chromosome 7C (1, 2, 13, 28) and the presence of an IC (12) have been demonstrated. Assuming that this conservation of imprinting and IC function should be reflected by conservation of the responsible epigenetic regulatory elements, we analyzed the mouse *Snurf-Snrpn* locus with NMBAs (Fig. 2*b*; see www.pnas.org). Of the 29.4 kb assayed at mouse *Snurf-Snrpn*, 18.0 kb were positive. The interspecies conservation of MARs is therefore striking not only in terms of their physical locations but also in terms of their density.

Sequence analysis shows the frequency of MAR motifs to be considerable in the human *SNURF-SNRPN* and mouse *Snurf-Snrpn* regions, except at the exon 1 region found not to bind in the NMBA (see www.pnas.org). The occurrence of these motifs suggests that the MARs identified biochemically occur throughout the restriction fragments analyzed. The density of MAR motifs is even greater than described for the MAR sequences in the mouse *Ins2/H19* region (17). Our next question was whether the conservation of MAR organization occurred because of conservation of DNA sequence. The vertical gray lines in Fig. 2 show the extent of the sequences compared. Apart from short regions of sequence identity at the *SNURF-SNRPN* promoter, exon 1 and within intron 1 (5, 26), the DNA sequence is mostly

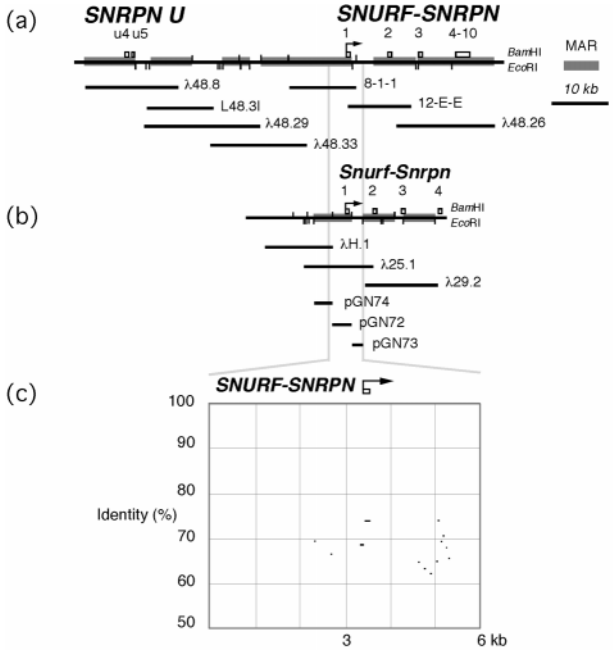

Fig. 2. A summary of the results of NMBAs for (*a*) the human 15q11-q13 IC/*SNURF-SNRPN* region and (*b*) the mouse *Snurf-Snrpn* locus. Exons 1–10 of the *SNURF-SNRPN* gene (13) and two exons of the *SNRPN U* transcript (11, 15) are shown. NMBAs were performed on each of the genomic clones shown (primary data in www.pnas.org). The gray bars on the human and mouse maps show the extent of MARs identified. (*a*) In total, 49.1 kb of the 68.9-kb region at the human IC/*SNURF-SNRPN* gene region are biochemically defined as MARs by using the NMBA. (*b*) The organization of MARs at the homologous mouse region is similar in terms of the density and distribution of MARs. (*c*) Sequence comparison [percent identity plots (31)] reveals extensive divergence of sequence at these loci, except at the immediate promoter/exon 1 region of *SNURF-SNRPN*/*Snurf-Snrpn* and within intron 1. Conservation of MARs is therefore not accounted for by identity of sequence between these two species.

divergent between the two species (Fig. 2*c*). The conservation of MAR organization is therefore occurring in a DNA sequence-independent manner.

Analysis of the *ZNF127* and *Zfp127* Loci. We then tested a locus regulated in cis by the IC in humans and mice for the presence of MARs. Here the organization of MARs is again conserved (Fig. 3 *a* and *b*; see www.pnas.org) with very divergent DNA sequences of the MAR-containing regions (Fig. 3*c*). Sequence analysis again showed a concordance between MAR motif frequency and the results of the NMBA, with a sharp diminution of motif frequency in the areas negative in the NMBA (see www.pnas.org). The presence of conserved MARs at the *ZNF127/Zfp127* loci supports the candidacy of MARs as mediators of the distant propagation of the IC signal in cis.

FISH of Halo Nuclei. We performed FISH on nuclear haloes to test whether association with the nuclear matrix differs between homologous chromosomes in an imprinted region. A problem with nuclear halo preparations is a nonuniformity of extraction within a preparation. We therefore used multicolor FISH and a unique set of controls to find individual suitable cells, circumventing this problem. By using cell lines derived from females, a probe from the *HPRT* locus showed not only the matrix association at that locus (34) but also the difference between homologues because of facultative heterochromatin organization of the inactive X chromosome. As a further control, we used an α satellite probe from chromosome 15 (32) to detect a region of

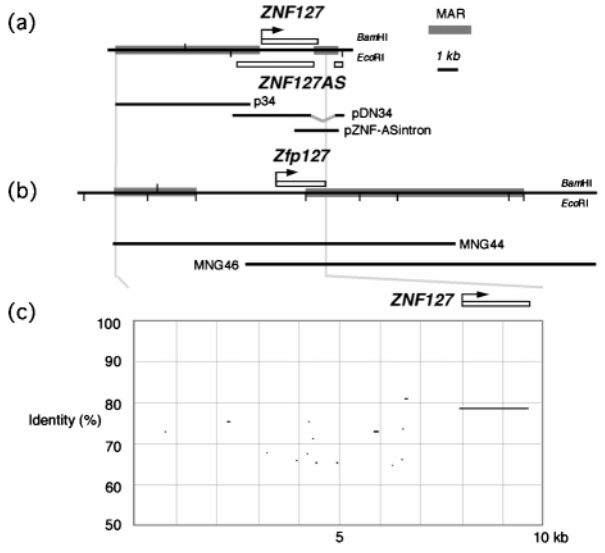

Fig. 3. MARs are present and conserved at the orthologous (*a*) human *ZNF127* and (*b*) mouse *Zfp127* loci. There are upstream and downstream MARs at each locus (*a* and *b*) and DNA sequence divergence (*c*) similar to that found at the IC despite the conservation of MARs. Note that the *ZNF127/Zfp127* loci are intronless, with a conserved antisense gene, *ZNF127AS/Zfp127as*, at this locus (27, 28).

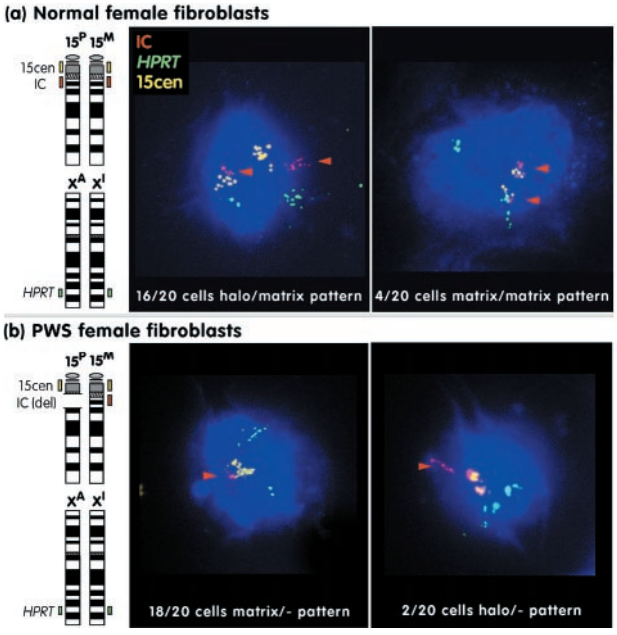

Fig. 4. The relationship of the 15q11-q13 IC to the nuclear matrix depends on its parental origin. The halo nuclei analyzed were those in which both copies of the chromosome 15 α satellite repeat (15cen, yellow) are present within the nuclear matrix (visible on 4′,6-diamidino-2-phenylindole counterstaining, blue), and one of the two copies of the *HPRT* probe (*HPRT*, green) is confined to the nuclear matrix with the homologue associated, at least in part, with the surrounding DNA halo. The IC probe is shown as the red signal in these images. (*a*) In a normal female, the majority (16/20) of analyzed halo nuclei show one IC signal solely within the nuclear matrix, the other partially within the DNA halo. The remaining DNA haloes (4/20) showed both signals to be present within the matrix. (*b*) In a patient with PWS caused by a deletion of the paternal 15q11-q13 region, the sole signal is from the maternal chromosome, found only in the matrix in the majority (18/20) of halo nuclei.

DNA undergoing constitutive heterochromatin organization. We selected halo nuclei that preserved relationships with the nuclear matrix while being extracted sufficiently to show differences between euchromatic and heterochromatic DNA organization (i.e., those with one *HPRT* signal associated completely with the nuclear matrix and the other associated, at least in part, with the nuclear halo, and both α satellite signals completely within the nuclear matrix; Fig. 4). In normal female cells, the predominant pattern of signals (16/20) was that of discordance for matrix association between the IC homologues, with the IC on one chromosome completely contained within the nuclear matrix and the other emanating into the nuclear halo (Fig. 4*a*). The remaining cells (4/20) showed both IC probes within the nuclear matrix. To ascertain whether these patterns depended on parental origin of the chromosomes, we used fibroblasts from a female PWS patient with a large deletion of the paternal 15q11-q13 region (D. J. Driscoll, personal communication). Hence, the only IC present is that on the maternal chromosome. In 18/20 halo nuclei, the single signal was present within the nuclear matrix (Fig. 4*b*), suggesting that the pattern observed in normal cells is because of the containment of the maternal chromosome within the nuclear matrix and the partial extraction of the paternal chromosome into the nuclear halo. We therefore conclude that parent-of-origin-dependent differences in epigenetic organization at the IC are reflected by differences in association with the nuclear matrix.

Discussion

The starting hypothesis was that MARs are candidates for mediating the epigenetic differences between homologous chromosomes that define genomic imprinting. This was based on the prior demonstration that MARs influence gene expression, DNA replication, chromatin structure, and cytosine methylation in cis, and can be influenced by passage through the germ line (18–22). This hypothesis is supported by our experimental data showing interspecies conservation of MARs at the mechanistically important IC of human 15q11-q13/mouse 7C and the distant regulated *ZNF127/Zfp127* loci, and further supported by the demonstration of parent of origin-dependent differences in matrix association at the IC by using a controlled FISH approach.

This appears to be the first time that MAR organization has been shown to be conserved at orthologous loci in animal genomes. In plants, the two species of grass were compared at the *Sh2/A1* loci and found to have a strikingly similar organization of MARs (35). Both our study and the plant comparison (35) found minimal sequence similarity at the conserved MARs. Nuclear matrix proteins bind within the minor groove of DNA (36), in which nucleotide discrimination is difficult (37). This may account not only for the degeneracy of the sequence motifs characterizing MARs (17) but also the sequence divergence allowed between species conserving MAR organization. The degree of evolutionary sequence divergence therefore appears to be occurring within constraints that maintain binding to the nuclear matrix.

The high density of MARs at the IC has not been previously described for gene-containing regions of the genome. The initial mapping studies of MARs were performed on bulk genomic DNA or focused on regions known to contain genes. The results were concordant, with a frequency of one MAR of less than 1 kb every 50–100 kb (17, 33). The lowest frequency was observed at the human α-globin locus [none in 140 kb studied (ref. 38 and J.M.G. and D. R. Higgs, unpublished work)] with a higher frequency at the *Drosophila H1/H3* histone gene region [$1 \leq 0.7$ kb MAR every 5 kb (39)]. In contrast, the genomic regions with a high density of MARs are more notable for their capacity to form heterochromatin than for their association with genes. Examples of these regions include centromeric and other satellite repeats from *Drosophila* (40), mouse (41–43), and human

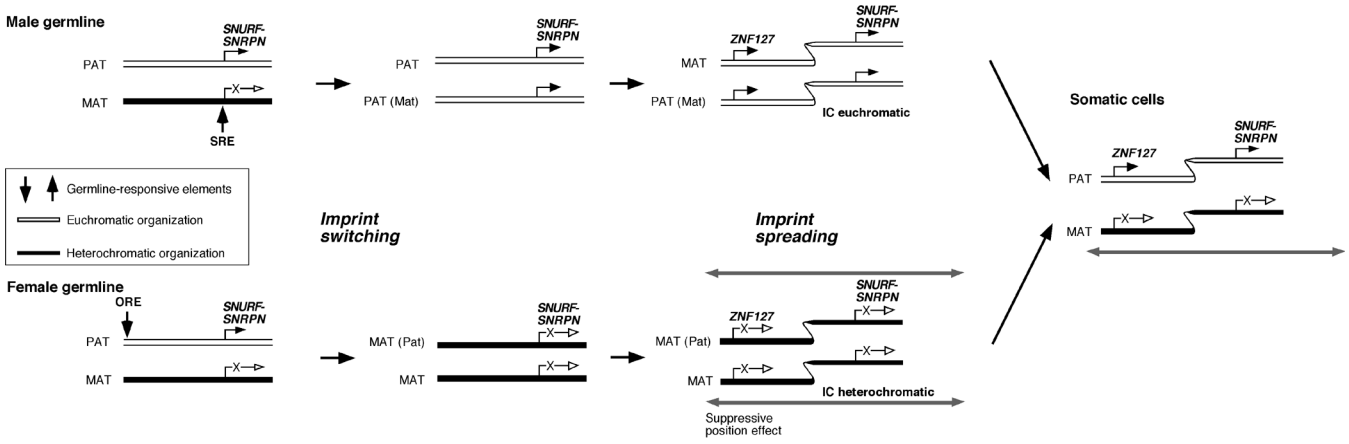

Fig. 5. A model for the function of the 15q11-q13 IC. The locations and target chromosomes for the putative germ line imprint switch elements are represented by vertical arrows marked oogenesis-responsive element (ORE) (responsible for switching the paternal to a maternal imprint in the female germ line) and spermatogenesis-responsive element (SRE) (responsible for maternal to paternal switching in the male germ line). We propose that the maternal chromosome is inherited with a heterochromatic organization of the MAR sequences identified in this study. The function of the SRE (*SNURF-SNRPN* promoter) as a switch element in this model is to displace the heterochromatin constituents, whereas the ORE is directing the formation of heterochromatin at the adjacent MARs on the paternal chromosome. This model does not require imprinted expression of the *SNRPN U* transcript or *SNURF-SNRPN* during gametogenesis but does require that *SNURF-SNRPN* be expressed on the maternal chromosome in the male germ line, for which there is evidence in mouse (50). The presence of the heterochromatin on the maternal chromosome is likely to be associated with suppressive effects in cis, allowing the proposal of a position effect model for imprint spreading to regulate the 2-Mb domain.

DNA (44), the individual repeats of which were found to act biochemically as MARs. These satellite repeats constitutively form heterochromatin in these organisms. As the sequences are repeated manyfold in tandem, the physical size of the aggregate of MAR DNA is comparable with or in excess of the size of the MARs at the IC.

Some heterochromatin-forming DNA sequences have been found to contain a higher than expected frequency of MAR motifs (45). A total human genomic MAR fraction used as a FISH paint on metaphase chromosomes decorates sites of constitutive heterochromatin formation (46). The proteins binding to MAR sequences such as topoisomerase II or RAP-1 are respectively recognized to colocalize with (47) or be constituents of (48) heterochromatin. However, the most compelling evidence that MAR DNA can form heterochromatin comes from functional studies. A defining functional characteristic of heterochromatin is its capacity to exert a suppressive (position) effect. Expression of a MAR-binding protein, MATH20, in *Drosophila* relieved position effect exerted by pericentromeric repetitive DNA (49), previously shown to bind to the nuclear matrix (40). In summary, MARs can be defined in a context-dependent manner that appears to correlate with their functional properties. Isolated DNA sequences of 1 kb or less that bind to the nuclear matrix within a gene-rich euchromatic region are defined as MARs, with epigenetic regulatory properties as discussed above. However, DNA sequences with the same capacity to bind to the nuclear matrix present in large continuous blocks are instead defined by their property of forming heterochromatin. The density of sequences at the IC binding to the nuclear matrix resembles the latter pattern of heterochromatin-forming DNA.

On the basis of our NMBA and FISH observations, we suggest that the IC appears to use potential heterochromatin-forming DNA differently on the paternal and maternal chromosomes. The increased methylation, decreased transcription, and later replication of the maternal 15q11-q13 chromosomal region in somatic cells indicate a relatively heterochromatic organization compared with that of the paternal chromosome, consistent with the differences in condensation of the two alleles seen by FISH. We extend the prior models of IC function (see Introduction) by proposing that the germ line-responsive elements establish local epigenetic changes by determining whether heterochromatin is formed at the sequences we identified as MARs (Fig. 5). In this model, the female germ line acts on the upstream ORE [or its transcribed product (11)] to initiate heterochromatin formation at local MAR DNA. The male germ line acts on the downstream spermatogenesis-responsive element to disrupt this heterochromatin formation. The activity of the *SNURF-SNRPN* promoter in the male germ line [described for mouse *Snurf-Snrpn* (50)] may be the specific disruptive agent of heterochromatin organization, as previously proposed (10). Imprint switching would therefore involve both the active formation of heterochromatin in the female germ line and its active disruption in the male germ line, initiated by the germ line-responsive elements and spread locally by the sequences we identify as MARs.

Local Epigenetic Changes at the IC Propagate Their Effects in cis. If heterochromatin is formed at the IC as a response to the female germ line environment, then a likely consequence is a suppressive position effect. Such position effects described in *Drosophila* are caused by the influence of heterochromatic regions on juxtaposed eukaryotic genes and have been found to occur over at least 2 Mb (51). A simple model for propagation of imprinting in cis from the IC is therefore the exertion of a suppressive position effect by the heterochromatin at the IC on the maternal chromosome and the absence of such an effect on the paternal chromosome. Because MAR DNA has the properties associated with heterochromatin formation, MARs are periodic throughout the genome, and they have the ability to physically interact to form chromatin loops (48), the question arises whether MARs could be propagating position effects. The finding of MAR sequences at the *ZNF127/Zfp127* loci supports this possibility.

In summary, we have found conserved MARs in the 15q11-q13 imprinted domain. At the IC, these MARs are unusually large, suggesting a mechanism for IC gamete responsiveness mediated by the propensity of these MARs to form heterochromatin. Formation of heterochromatin at the IC provides a mechanism for spreading of the imprinting signal in cis by position effect, utilizing MARs located adjacent to imprinted genes distant from the IC. Moreover, the emphasis on gamete-

dependent heterochromatin formation highlights the parallels with genomic imprinting occurring in invertebrates. In both *Sciara coprophila* (52) and *Planococcus sp.* (mealybugs) (53), heterochromatin formation of entire chromosomes is both gamete of origin-dependent and sex-determining. In mealybugs, the DNA sequences forming heterochromatin have been found to bind to nuclear matrices (54). The recent demonstration of genomic imprinting in *Drosophila* (55) is of particular interest, because the mechanism appears to involve gamete-determined position effect mediated by adjacent heterochromatic DNA. If there were found to be mechanistic similarities between imprinting in mammals and these egg-laying organisms, such similarities could have interesting implications in terms of the evolutionary pathway of genomic imprinting.

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