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An Antisense RNA Regulates the Bidirectional Silencing Property of the *Kcnq1* Imprinting Control Region

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The *Kcnq1* imprinting control region (ICR) located in intron 10 of the *Kcnq1* gene is unmethylated on the paternal chromosome and methylated on the maternal chromosome and has been implicated in the manifestation of parent-of-origin-specific expression of six neighboring genes. The unmethylated *Kcnq1* ICR harbors bidirectional silencer activity and drives expression of an antisense RNA, *Kcnq1ot1*, which overlaps the *Kcnq1* coding region. To elucidate whether the *Kcnq1ot1* RNA plays a role in the bidirectional silencing activity of the *Kcnq1* ICR, we have characterized factor binding sites by genomic footprinting and tested the functional consequence of various deletions of these binding sites in an episome-based system. Deletion of the elements necessary for *Kcnq1ot1* promoter function resulted in the loss of silencing activity. Furthermore, interruption of *Kcnq1ot1* RNA production by the insertion of a polyadenylation sequence downstream of the promoter also caused a loss of both silencing activity and methylation spreading. Thus, the antisense RNA plays a key role in the silencing function of the ICR. Double-stranded RNA (dsRNA)-mediated RNA interference is unlikely to be involved, as the ICR is active irrespective of the simultaneous production of dsRNA from the genes it silences.

Most of the 70 mammalian imprinted genes identified so far are organized into clusters. It has been suggested that the clustering of genes enables sharing of *cis*-acting elements located distances several kilobase pairs away. This notion is corroborated by the fact that the differentially methylated regions associated with the imprinted clusters play a crucial role in the maintenance of parent-of-origin-specific gene expression patterns which are hence called imprinting control regions (ICRs). The imprinted cluster at the distal end of mouse chromosome 7 and the orthologous human chromosome 11 has been extensively studied. It harbors six maternally expressed genes, *Impt1*, *Ipl*, *Cdkn1c*, *Kcnq1*, *Ascl2*, and *H19*, and the paternally expressed *Kcnq1ot1*, *Ins2*, and *Igf2* genes (18, 20). Several lines of evidence obtained from mouse targeted deletion experiments suggest that this cluster is organized into two expression subdomains by two ICRs, those of *H19* and *Kcnq1* (3, 25). The mode of action of the *H19* ICR has been well characterized, in contrast to that of the *Kcnq1* ICR. The *H19* ICR, located 2 kb upstream of the *H19* promoter, controls the monoallelic expression of the *H19* and *Igf2* genes by insulating communication between the downstream *H19* enhancers and the *Igf2* promoter (10). The chromatin insulator property of the *H19*/*Igf2* ICR is regulated by a chromatin insulator protein, CTCF (6, 11, 23).

The *Kcnq1* ICR located in intron 10 of the *Kcnq1* gene is methylated on the maternal chromosome, but on the paternal chromosome, it is unmethylated and associated with an antisense RNA, *Kcnq1ot1* (13, 22). The significance of the *Kcnq1* ICR in the imprinting of neighboring genes came from studies demonstrating that the *KCNQ1* ICR is hypomethylated in 40% of sporadic Beckwith-Wiedemann syndrome (BWS) patients (13, 22). Recently, it has been documented that BWS patients with hypomethylation at the *KCNQ1* ICR revealed a marked decrease in the expression of the nearby *CDKN1C* gene (1). Targeted deletion experiments of the 3.6-kb *Kcnq1* ICR on a paternally inherited mouse chromosome 7 and a similar deletion on the human chromosome 11 propagated in human chicken hybrid cells revealed derepression of the normally silent paternal alleles of the *Ipl*, *Slc22a1l*, *Cdkn1c*, *Kcnq1*, *Tssc4*, and *Ascl2* genes (3, 8). Taken together, these results strongly suggest that the *Kcnq1* ICR plays a critical role in controlling the imprinting of neighboring genes

We and others have documented that the *Kcnq1* ICR behaves as a bidirectional silencer (2, 15, 24). The bidirectional silencing function of the *Kcnq1* ICR could be due to the presence of *cis*-acting elements, which inactivate neighboring reporter and endogenous genes by associating with transcriptional repressor molecules. Alternatively, the silencing could result from antisense RNA transcription. Although the *Kcnq1* ICR associates with an antisense RNA on the paternal chromosome in a manner reminiscent of *Igf2r*, it is unclear whether the *Kcnq1* ICR employs similar or distinct mechanisms in bidirectional silencing of neighboring genes (21, 29). Earlier studies have mapped the *Kcnq1ot1* promoter to the three DNaseI-hypersensitive sites located 40 bp upstream of the *Kcnq1ot1* start site (15). Here, we used genomic footprinting to fine map the transcription factor binding sites occupied when the *Kcnq1* ICR is active. Using selective deletions of these

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binding sites as well as polyadenylation site insertion, we could show that the production of the *Kcnq1ot1* RNA is essential for silencing activity in a manner that does not appear to involve double-stranded RNA (dsRNA)-mediated silencing of the flanking reporter genes. DNA methylation spreading over the flanking sequences, which occurs as a consequence of silencing (24), is lost in these mutants, suggesting that the production of *Kcnq1ot1* RNA plays a critical role in the bidirectional spreading of inactive chromatin structures.

MATERIALS AND METHODS

In vivo footprinting. DNaseI and dimethyl sulfate (DMS) footprinting were performed as described previously (4). In brief, approximately five million Hep-3B cells, containing episomal plasmids with the *Kcnq1* ICR, were permeabilized with 0.4% NP-40 and treated with 10 to 20 μg of DNase I at 4°C for 3 min. In a similar way, DNase I treatment was carried out on mouse fetal liver cells which had been made into single-cell suspensions by treatment of fetal liver tissue with trypsin at a 1-mg/ml final concentration for 30 min at 37°C. DNase I-treated DNA was extracted with phenol-chloroform. For DMS footprinting, about five million Hep-3B cells were treated with 0.1% DMS, and the DNA from the treated cells was prepared by using a genomic DNA preparation kit (Promega). Chemical sequencing reactions and piperidine cleavage were performed as described previously (4).

Ligation-mediated PCRs were carried out by using a procedure described previously (4). The primers used for footprinting were as follows: for the footprint 1 lower strand, GCAGGCTAGGTCGTACAA (primer 1), TGTTTCCTC TTGGCCAATAAAAATA (primer 2), and TGGCCAATAAAAATAGTCAG CACAGAGC (primer 3) were used; for the footprint 1 upper strand, TAGAC CGTAACCACG (primer 1), AATCCAAAATGAGGCCGACCACACC (primer 2), and AAAATGAGGCCGACCACACCGGGCCT (primer 3) were used. The ligation-mediated PCRs were separated on a urea-5% acrylamide gel, dried, and autoradiographed.

Plasmid cloning strategies. ΔFp and ΔCTCF deletions in the *Kcnq1* ICR were made by creating AgeI restriction enzyme sites flanking the respective deletion by Stratagene quick-change site-directed mutagenesis followed by restriction digestion and religation. For the deletion of the whole DNase I footprint (ΔFp), AgeI sites were created 199 bp upstream of the *Kcnq1ot1* start site (by changing TCCTGT to ACCGGT) by using forward primer 5′-GCT AGG TCG TAC AAA CCG GTA CAA GCT CAC CC-3′ and reverse primer 5′-GGG TGA GCT TGT ACC GGT TTG TAC GAC CTA GC-3′ and 150 bp downstream of the *Kcnq1ot1* start site (by changing TACGGT to ACCGGT) by using forward primer 5′-TCT CCT CGG CGT GGT ACC GGT CTA GTA CACCT-3′ and reverse primer 5′-AGG TGT ACT AGA CCG GTA CCA CGC CGA GGA GA-3′. For the ΔCTCF deletion, AgeI sites were created 10 bp upstream of the *Kcnq1ot1* start site (by changing ACCCGT to ACCGGT) by using primers GGTAGGTGGCC TACCGGTTGTCGCTAGGAGG and CCTCCTAGCGACAACCGGTAGGC CACCTACC and 150 bp downstream of the *Kcnq1ot1* start site (this site was created by using primers as described above). The *Kcnq1* ICR fragment carrying these deletions was cloned into the PH19 vector at a unique NotI site. The 510-bp simian virus 40 (SV40) polyadenylation sequence was amplified from the pREP4 vector (Invitrogen) by using primer sequences flanked with AgeI restriction sites 5′-TATAACCGGTCCGTAGTCAGGTTTAGTTCG-3′ and 5′-TAT AACCGGTCGCCATTTGACCATTCACCA-3′. The SV40 polyadenylation fragment was cloned into the *Kcnq1* ICR fragment in the PS4 episomal plasmid at AgeI sites 150 bp downstream of the *Kcnq1ot1* start site, created by site-directed mutagenesis with primers described above, and 750 bp downstream of the *Kcnq1ot1* start site with primers 5′-TCTGAGTCAGCACGGCACCGGTC GCAAGTCCGCGT-3′ and 5′-ACGCGGACTTGCGACCGGTGCCGTGCTG ACTCAGA-3′. The episomal plasmid Δ*H19*-PS4 was generated by digesting the PS4 episomal plasmid with BstZ17I, which removes the promoter and most of the coding region of the *H19* gene, followed by religation.

Strand-specific RT-PCR. Reverse transcription-PCR (RT-PCR) analysis of gene expression was performed on strand-specific cDNA templates. Two micrograms of RNA was used as a template for cDNA synthesis with a mixture of strand-specific primers for the *H19* gene (sense primer, 5′-GAATGTTGAAGG ACTGAGGG-3′; antisense primer, 5′-CACGTCCTGTAACCAAAAGT-3′) and for the hygromycin resistance gene (sense primer, 5′-TCCAGAAGAAGA TGTTGGCG-3′; antisense primer, 5′-CGGTCAATACACTACATGGCG-3′) as described previously (17). For GAPDH (glyceraldehyde-3-phosphate dehydrogenase) amplification, forward primer CCTTCATTGACCTCAAC and reverse primer AGTTGTCATGGATGACC were used. cDNAs were PCR amplified for 25 cycles (94°C for 45 s, 57°C for 30 s, and 72°C for 1 min). The PCR products were quantified with a Fuji FLA-3000 Phosphorimager.

The episome silencer-insulator assay. The pREP4-based episomal vectors were transfected into JEG-3 cells as described previously (10), and total RNA was extracted 4 days after transfection. The RNase protection assay (RPA) was performed as previously described with a 365-bp *H19* antisense probe, a 150-bp GAPDH antisense probe as the control (10), and a 270-bp *Kcnq1ot1* probe. Ten micrograms of RNA was hybridized with [α-^{35}S]CTP-labeled antisense probes (600,000 cpm/reaction for *H19* and *Kcnq1ot1* and 40,000 cpm/reaction for GAPDH) overnight at 45°C. All procedures were performed according to the manufacturer's protocol for the RPAIII kit (Ambion). Quantification of individual protected fragments was done by using a Fuji FLA-3000 Phosphorimager. The *H19* and *Kcnq1ot1* expression was corrected with respect to both internal control (GAPDH) and episome copy number as determined by Southern blot analysis of BglII-restricted DNA hybridized with *H19* and *PDGFB* probes (10).

To assess the effect of *Kcnq1* ICR-mediated bidirectional silencing on a hygromycin resistance gene, equimolar concentrations of episome-based plasmids were transfected into JEG-3 cells. Following transfection, the cells were selected with 150 μg of hygromycin until all the cells died on the control plate, which contained the cells incubated with transfection reagent without any episomal plasmid DNA. Following selection, the drug-resistant colonies were stained with hematoxylin and counted.

RESULTS

Genomic footprinting analysis of the *Kcnq1ot1* promoter. Previously, three DNase I-hypersensitive sites around the *Kcnq1ot1* promoter were fine mapped (9). An independent study revealed that the *Kcnq1ot1* transcription start site maps to 40 bp downstream of the third DNase I-hypersensitive site (15). We were therefore interested in identifying the crucial *cis*-acting elements responsible for the *Kcnq1ot1* promoter activity and relating this to the bidirectional silencing property of the *Kcnq1* ICR. To this end, we used a genomic footprinting approach on episomal plasmid stably propagated in cultured cells containing the *Kcnq1* ICR in the PS4 orientation (in which the *Kcnq1ot1* transcript faces towards the *H19* reporter gene) (see Fig. 2). As can be seen in Fig. 1A, one DNase I footprint spanning 300 to 350 bp was uncovered within the ICR. This footprint region appeared covered, almost continuously, with transcription factors. Bioinformatic analysis of this region suggested potential binding sites for the following factors: OCT-1, CCAAT, YY1, GATA, CTCF, and CREB (highlighted in boldface type in Fig. 1B). We could confirm CTCF and YY1 interaction within this region by chromatin immunoprecipitation analysis (data not shown). Changes of reactivity toward DMS were also observed within the DNase I footprinted regions (Fig. 1A). The most notable changes of reactivity were obtained at the putative YY1, GATA-1, CREB, and CTCF binding sites. We verified that the DNase I footprint obtained with our episome-based system was comparable to those found in vivo by carrying out genomic DNase I footprinting analysis of mouse fetal liver cells obtained from day 14.5 embryos. The footprints obtained from the episomal system and fetal liver cells are highly similar (data not shown), indicating that factor occupancy can be recapitulated in the episome-based system in a manner reminiscent of the in vivo situation.

Targeted deletion of the DNase I footprint in the *Kcnq1ot1* promoter results in bidirectional activation of reporter genes. To measure the bidirectional silencing activity of the wild-type and modified *Kcnq1* ICRs, we used an episome-based vector, PH19 (formerly referred to as pREPH19A) (10). An important

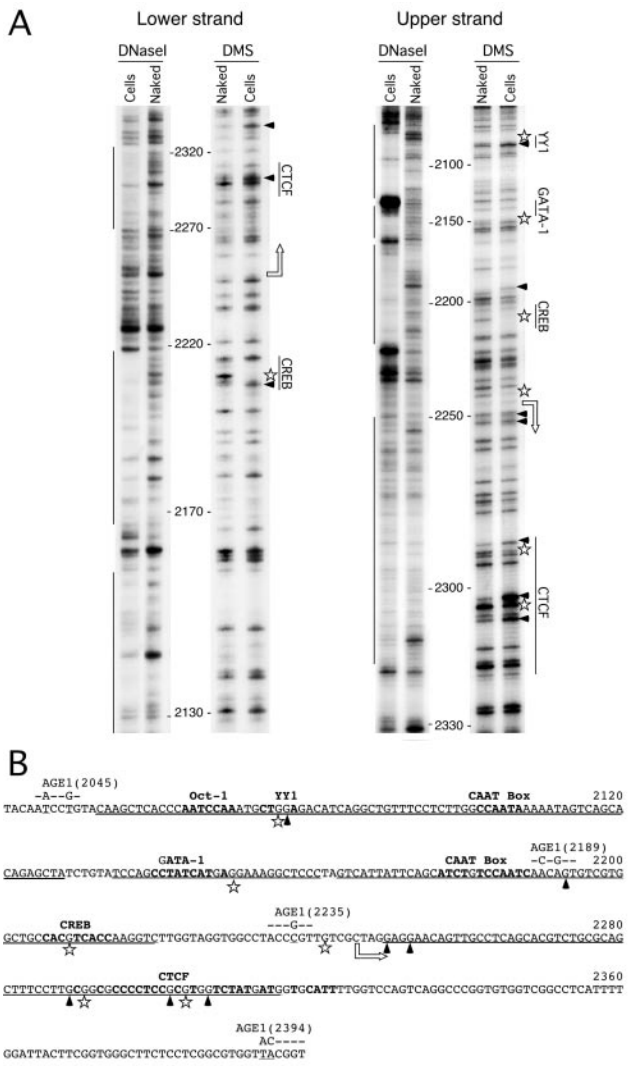

FIG. 1. Genomic footprinting analysis of the *Kcnq1ot1* promoter. (A) DNase I and DMS genomic footprinting analyses of the lower and upper strands of the *Kcnq1* ICR. The analyses were performed by using cultured cells transfected with the PS4 episomal plasmid containing the *Kcnq1* ICR (Fig. 2). The genomic footprints were deduced by comparison with the pattern obtained following in vitro treatment with either DNase I or DMS of naked DNA (Naked). The DNase I footprint visible using cells transfected with the ICR is shown on the left-hand side. Guanine residues that were protected from DMS in cells are marked with stars, and those that showed hyperreactivity are marked with arrowheads. The numbering within the ICR is indicated in the middle. The transcription start site of *Kcnq1ot1* gene is marked by a curved arrow. (B) Sequence of the upper strand of the *Kcnq1ot1* promoter showing predicted *cis*-acting elements (boldface type), DNase I footprinted region on the upper strand (underlined type), and changes of reactivity toward DMS on the upper strand (represented by stars and arrowheads). The mutations that allowed creation of the AgeI sites used to target deletions of key binding sites are represented above the sequence.

advantage associated with the episome-based vector system is that it avoids position-dependent effects of neighboring chromatin on the transcriptional activity of a reporter gene. More importantly, it can recapitulate the in vivo chromatin conformation (10), making it more suitable for studying the transcriptional activity of a given gene in a native chromatin conformation. The PH19 plasmid harbors the mouse *H19* and hygromycin resistance genes as reporter genes (Fig. 2). We inserted the *Kcnq1* ICR into PH19 at various strategic positions relative to the reporter *H19* and hygromycin resistance genes (PS4, NS11, and PC3 in Fig. 2). These episomal constructs were transiently transfected in cultured cells, and the activity of both the *H19* and *Kcnq1ot1* genes was analyzed by RPA 4 days after transfection. Activity of the hygromycin resistance gene was analyzed by counting the number of resistant colonies obtained after selection with hygromycin. The reporter gene activities for each construct are represented relative to the control PH19 (*H19* and hygromycin resistance genes) or PS4 (*Kcnq1ot1*) vectors. As can be seen in Fig. 2, the *Kcnq1* ICR, placed at various strategic positions in the PS4, NS11, and PC3 plasmids, repressed *H19* and hygromycin resistance gene activity severalfold. When the ICR was inserted between the *H19* and hygromycin resistance genes, the repression affected both markers to a similar extent, irrespective of the orientation of the *Kcnq1* ICR (compare PS4 and NS11 with PH19). Furthermore, even when the ICR was placed upstream of the hygromycin resistance gene in the same orientation, both the *H19* and the hygromycin resistance genes were silenced (PC3 in Fig. 2). Therefore, the bidirectional silencing activity of the *Kcnq1* ICR does not appear to result from opposing transcription between the ICR and the genes it silences.

Next, we set out to address the functional role of the transcription factor binding sites detected by genomic footprinting in the bidirectional silencing property of the *Kcnq1* ICR. To this end, using site-directed mutagenesis, we introduced AgeI restriction sites into the *Kcnq1ot1* promoter region within the *Kcnq1* ICR in order to allow selective deletion of the footprinted region (Fig. 2). These mutations did not affect the activity of the ICR (compare AgeI-PS4 with PS4 in Fig. 2). Deletion of the whole DNase I footprint (ΔFp) resulted in a loss of *Kcnq1ot1* transcription and a parallel loss of silencing of both the *H19* and hygromycin resistance reporter genes (compare ΔFp-PS4 with PS4 and PH19 in Fig. 2). These results indicate that the corresponding fragment is essential for both *Kcnq1ot1* transcription and silencing activity.

To explore the potential role of the CTCF target site in the *Kcnq1* ICR function, we selectively deleted the DNase I footprint spanning the CTCF target site. The deletion encompassing the CTCF target site affected neither the *Kcnq1ot1* transcription nor the bidirectional silencing activity of the *Kcnq1* ICR (compare ΔCTCF-PS4 with PH19 in Fig. 2). Even though the footprinted region is essential for *Kcnq1* ICR activity, it is not sufficient, as the insertion of this fragment alone into PH19 provided neither *Kcnq1ot1* promoter activity nor hygromycin resistance gene silencing activity (data not shown), suggesting that there are additional *cis*-acting elements playing a critical role in *Kcnq1ot1* transcription. Taken together, mutagenic analysis of the *Kcnq1ot1* promoter allowed better characterization of the elements essential for antisense transcription and revealed that there was an absolute correlation between *Kcnq1ot1* transcription and bidirectional silencing activity for all constructs, thus pointing towards a key role for *Kcnq1ot1* transcription in silencing.

Truncation of the *Kcnq1ot1* RNA results in the loss of bidirectional silencing. Deletion of promoter elements could in-

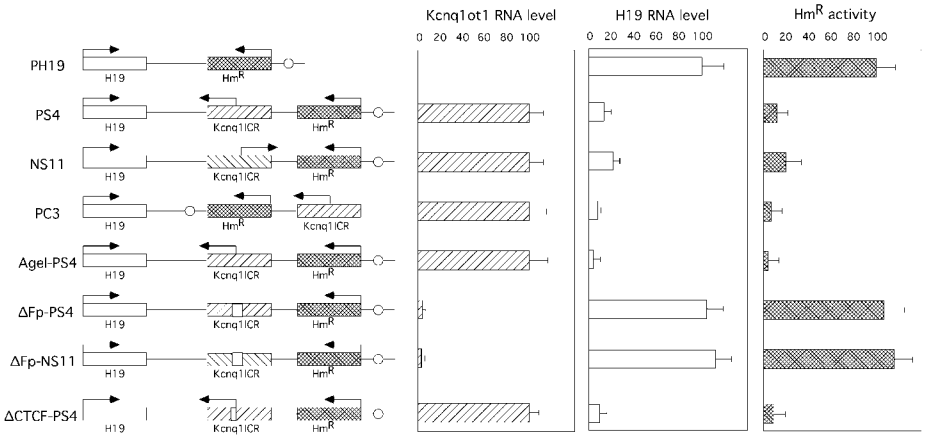

FIG. 2. Targeted deletion of the promoter of *Kcnq1ot1* results in the loss of bidirectional silencing. Left panel, arrangement of the wild-type and mutant *Kcnq1* ICRs and the reporter *H19* and hygromycin resistance genes within the episomal plasmids. The circles indicate the location of the SV40 enhancer present in all tested constructs. PH19 is the parent plasmid without ICR. In PS4, NS11, and PC3, the intact 3.6-kb ICR has been inserted into PH19 in various orientations and positions as shown. In AgeI-PS4, the *Kcnq1* ICR mutated to introduce the AgeI sites flanking the footprinted region has been inserted in the PS4 orientation. Right panel, activity of the three genes analyzed for each construct shown as bar graphs. The activity of the reporter genes is represented as percent expression levels. For convenience, the activity of the control vectors (PH19 for *H19* and hygromycin resistance genes and PS4 for the *Kcnq1ot1* gene) was assigned a value of 100%, and the values obtained with mutant episomal plasmids were related to this value. The expression levels of the *H19* (open bars) and *Kcnq1ot1* (hatched bars) genes were quantified by RPA 4 days after transient transfection of the various episomes into JEG-3 cells. The percentages of expression levels were calculated after normalization against total input RNA by using GAPDH mRNA as an internal standard after correction for episomal copy numbers (quantified by Southern hybridization of DNA from transfected cells probed with both an episome-specific probe and, as an internal control probe, the *PDGF* gene). The hygromycin resistance gene activity (filled bars) was analyzed by counting the number of hygromycin-resistant clones. The mean of at least three independent experiments is indicated for each construct, with error bars showing deviation.

dependently affect antisense transcription and bidirectional silencing. To check whether the antisense RNA has a direct role in the silencing property of the *Kcnq1* ICR, we interfered with *Kcnq1ot1* RNA production by inserting the SV40 polyadenylation sequence 0.15, 0.75, or 1.7 kb downstream from the transcription start site (Fig. 3A) and tested the effect of these insertions as described above. *Kcnq1ot1* RNA was analyzed by RPA using a probe spanning the position 0.43 to 0.7 kb downstream from the start site. The insertion of the polyadenylation sequence indeed truncated the *Kcnq1ot1* RNA, as no RNA was detected with the probe hybridizing downstream of the inserted sequence in the PS4-polyA0.15 construct (Fig. 3B). When the polyadenylation sequence was inserted 0.75 and 1.5 kb downstream, the detection of high levels of *Kcnq1ot1* RNA revealed that the inserted sequence did not affect promoter activity (Fig. 3B). However, even though the antisense promoter is fully functional, the bidirectional silencing property of the *Kcnq1* ICR is lost upon the polyadenylation sequence insertions, as revealed by the analysis of *H19* and hygromycin resistance reporter gene expression (Fig. 3B). Thus, it is not

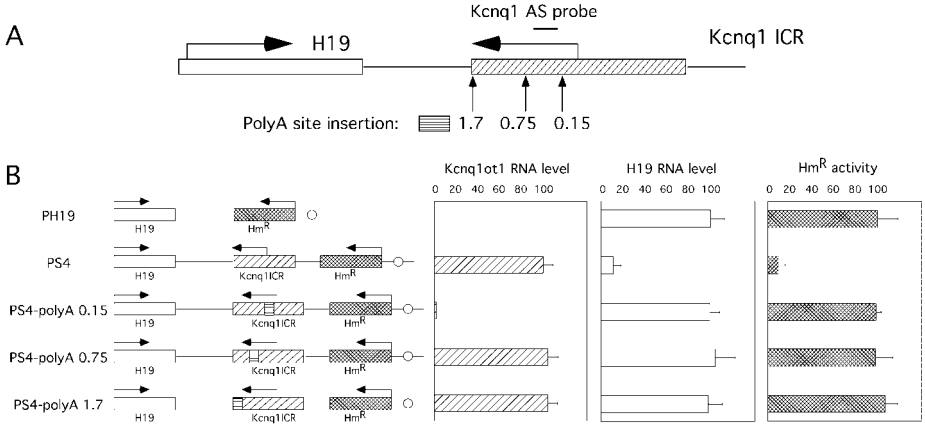

FIG. 3. Truncation of *Kcnq1ot1* RNA by the SV40 polyadenylation sequence results in the loss of bidirectional silencing. (A) Diagram depicting the insertions of the SV40 polyadenylation sequence [poly(A) site] at 0.15, 0.75, and 1.7 kb downstream from the *Kcnq1ot1* transcription start site within the PS4 construct. (B) Left panel, arrangement of the ICR and reporter genes in the constructs tested with the various polyadenylation sequence insertions shown in Fig. 3A; right panel, bar graph showing the corresponding relative expression levels of the *H19*, *Kcnq1ot1*, and hygromycin resistance genes normalized as described in the legend of Fig. 2.

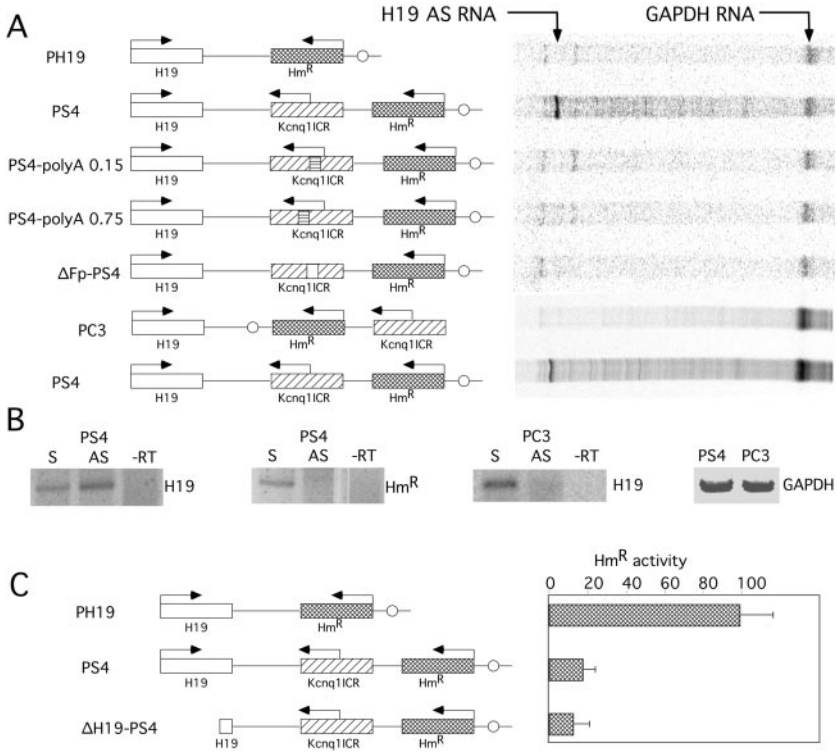

FIG. 4. Overlapping transcription of *Kcnq1ot1* and *H19* occurs sometimes but is not essential for silencing. (A) Left panel, maps of the episomal plasmids analyzed; right panel, autoradiogram of an RPA showing that antisense transcription in the *H19* coding region occurs only with the PS4 construct and is thus not correlated to *H19* activity. (B) RT-PCR analyses of the sense (S) and antisense (AS) transcription occurring within the *H19* and hygromycin resistance (Hm^R) genes within the PS4 and PC3 constructs. −RT is a no-reverse-transcriptase control. GAPDH is an internal control corresponding to RT-PCR detection of the GAPDH mRNA. (C) Left panel, maps of the PS4 episomal plasmid with the *H19* gene deletion and of the control plasmid analyzed; right panel, bar graph showing the relative levels of corresponding hygromycin resistance gene activities.

the promoter activity per se but the production of the *Kcnq1ot1* RNA that is important for silencing.

dsRNA is not involved in the bidirectional silencing of the *Kcnq1* ICR. Even though the *Kcnq1* ICR silences the two reporter genes simultaneously irrespective of its orientation and site of insertion, its activity could involve the production of dsRNA that could elicit a short-interfering RNA response. For example, in the PS4 episomal plasmid, the *Kcnq1ot1* and *H19* reporter gene promoters face each other. This finding prompted us to analyze whether *Kcnq1ot1* transcription proceeds through the *H19* coding region. RPA revealed that transcription in the *H19* gene region was indeed observed on the opposite strand with an active *Kcnq1ot1* promoter in the PS4 orientation (Fig. 4A). This antisense transcription within the *H19* gene was lost upon deletions or polyadenylation sequence insertions that inactivated the *Kcnq1* ICR silencing activity (Fig. 4A). However, the *H19* gene could be silenced by the ICR even in the absence of antisense transcription. This is visible for the PC3 construct in which the ICR faces the *H19* gene but is separated from it by the hygromycin resistance gene (Fig. 2). For this construct, no *H19* antisense transcription was detected by RPA (Fig. 4A). These results were confirmed by RT-PCR analyses that detected *H19* antisense transcription from the PS4 but not from the PC3 construct (Fig. 4B). Furthermore, RT-PCR revealed the absence of detectable antisense transcription within the hygromycin resistance gene from the PS4 episome, showing that the 3.6-kb *Kcnq1* ICR does not harbor bidirectional transcriptional activity (Fig. 4B).

To further test whether transcription of both strands of the *H19* gene plays a role in the bidirectional silencing, we deleted the promoter and most of the coding region of the *H19* gene (ΔH19-PS4 in Fig. 4C). The removal of the *H19* gene did not affect the silencing property of the *Kcnq1* ICR, as the hygromycin resistance gene is still repressed. Taken together, these results show that bidirectional silencing is not correlated with the production of dsRNA, which might be formed between the sense and antisense strands of the *H19* and hygromycin resistance reporter transcripts. Given the absence of any sequence similarity between the silencing *Kcnq1ot1* transcript and either of the reporter genes, we conclude that an RNA interference (RNAi) mechanism is not involved in the silencing of the reporter genes.

Absence of methylation spreading in the truncated *Kcnq1ot1* RNA and *Kcnq1ot1* promoter mutants. It was previously documented that the *Kcnq1* ICR has a capacity to spread DNA methylation over the flanking *H19* reporter gene and that this feature occurred as a consequence of silencing (24). In this investigation, we have shown that the production of antisense RNA is crucial for bidirectional silencing. Therefore, we were interested in determining whether the production of the *Kcnq1ot1* RNA could be linked to the spreading of DNA methylation over the flanking *H19* reporter gene. To this end,

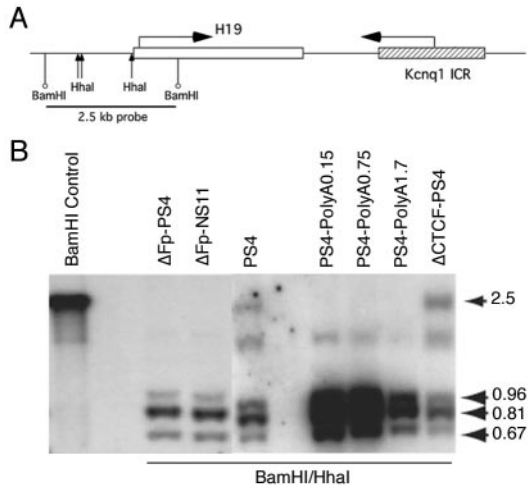

FIG. 5. CpG methylation spreading promoted by the *Kcnq1* ICR is lost upon *Kcnq1ot1* promoter deletions or polyadenylation site insertions. (A) Diagram showing the region of the PS4 plasmid that was analyzed for methylation. Below the *H19* coding and promoter regions, the HhaI sites used to assess the methylation status are represented alongside the BamHI sites used. The indicated 2.5-kb BamHI fragment was used as a probe. (B) Southern blot showing that the methylation of the *H19* promoter, which is seen with the wild-type *Kcnq1* ICR, is lost upon *Kcnq1ot1* promoter deletions and polyadenylation site insertions.

we carried out Southern blot hybridization analysis using the methylation-sensitive restriction enzyme HhaI (Fig. 5A) on the episomes carrying the *Kcnq1* ICR with *Kcnq1ot1* promoter deletions and SV40 polyadenylation sequence insertions at various positions. In this analysis, in the absence of DNA methylation, BamHI/HhaI cleavages generate three bands at 0.67, 0.8, and 0.95 kb. Methylation of the three HhaI sites results in the appearance of a 2.5-kb band. The DNA methylation analysis was performed 9 days after transfection and at that time revealed partial methylation of the *H19* gene. It was previously documented that DNA methylation occurs as a consequence of silencing, as the silencing of the *H19* gene precedes DNA methylation. In addition, it has been shown that methylation intensity over the *H19* gene progressively increases with time in culture (24). This level of CpG methylation was lost when the length of the *Kcnq1ot1* RNA was reduced by the insertion of the polyadenylation sequence and by the deletions of the *Kcnq1ot1* promoter that resulted in the loss of *Kcnq1ot1* transcription, namely, ΔFp but not ΔCTCF (Fig. 5B). Despite the overloading of the PS4-poly(A) episomal DNA, no methylation of either HhaI site was detected. Taken together, these observations suggest that the *Kcnq1ot1* RNA is directly or indirectly involved in the spreading of DNA methylation over the flanking *H19* reporter gene.

DISCUSSION

We have presented here a strategy aimed at identifying key *cis* regulatory elements within the *Kcnq1* ICR that trigger and maintain long-range bidirectional silencing. First, genomic footprinting approaches fine mapped a DNaseI footprint that appeared identical between mouse fetal liver and in vitro-propagated episomes harboring the central ICR domain. Second, by the selective deletion of the DNaseI footprinted region, we were able to identify pivotal *cis* elements controlling *Kcnq1ot1* transcription, bidirectional silencing and methylation spreading. Third, truncation of the *Kcnq1ot1* RNA by insertion of a polyadenylation sequence at several positions resulted in the loss of silencing as well as DNA methylation spreading, indicating that the production of the *Kcnq1ot1* RNA is crucial for silencing. Fourth, neither the insertion of the ICR in various orientations and positions nor the removal of the *H19* gene sequences transcribed opposite to *Kcnq1ot1* in several constructs inhibits the silencing property of *Kcnq1ot1*, indicating that RNAi is unlikely to be the mechanism involved in silencing.

Previous work has documented that the *Kncq1* ICR is responsible for long-range silencing (3, 24). Our observations documented here suggest that this feature involves the transcript of the *Kcnq1ot1* gene. The evidence for this conclusion is based primarily on the effects of the introduction of transcriptional termination signals 0.15, 0.75, and 1.7 kb away from the transcriptional start site. In each instance, including that of the mutant that left the entire coding potential of the *Kcnq1* ICR fragment [poly(A), 1.7 kb] intact, the bidirectional silencing property was significantly reduced. While it was a formal possibility that the antisense RNA silencing depended on a dsRNA effect by annealing to the sense transcripts in the PS4 orientation (towards the *H19* gene), the removal of the *H19* promoter and a major part of the *H19* gene neutralized *H19* expression without reducing the silencing effect. In addition, silencing was not affected by the modification of the position and orientation of the *Kcnq1* ICR fragment relative to the *H19* gene, as exemplified in the NS11 and PC3 episomal constructs, and gene silencing occurred irrespective of dsRNA production within the repressed genes, as exemplified for the *H19* gene in the PC3 construct or the hygromycin resistance gene in the PS4 construct. The transcripts generated from the *Kcnq1ot1* promoter in the PS4, PC3, and PS4-polyA1.7 constructs all have the 1.7-kb-long *Kcnq1ot1* 5′ end in common, but they end at different positions. When silencing is not induced (PS4-polyA1.7), the transcript ends shortly after the ICR fragment. When silencing is induced, the transcripts are much longer. With the PS4 construct, it is at least 5 kb long and its 3′ part consists of the antisense *H19* sequence. With the PC3 construct, it is presumably 6.0 kb long and its 3′ part consists of the sense hygromycin resistance gene sequence, as it is likely to be terminated at the polyadenylation sequence of the hygromycin resistance gene since no antisense *H19* RNA was detected. This finding suggests that the silencing by the *Kcnq1* ICR requires the *Kcnq1ot1* transcription beyond the *Kcnq1* ICR fragment irrespective of the identity of the region transcribed. We therefore propose that the silencing property of the *Kcnq1ot1* transcript is intimately related to the duration of its association with the site of transcription. The prolongation of the transcriptional process might simply increase its chances of recruiting silencing factors. These considerations could also apply to the *Air* transcript of the *Igf2r* locus which, like the *Kcnq1ot1* transcript, is a bidirectional silencer and extends more than 100 kb from its transcriptional start site (14). While this fact has prompted suggestions that the *Air* transcript inactivates *Igf2r* by promoter occlusion, this can hardly apply to

the *Kcnq1ot1* transcript which does not extend to the *Kcnq1* sense promoter (16).

Based on earlier literature, it is clear that RNA can trigger silencing in at least two distinct ways. In one way, dsRNA formed as a result of overlapping transcripts triggers RNAi, resulting in targeted recruitment of heterochromatic complexes to specific chromosomal loci (19, 26). Alternatively, the newly transcribed RNA may directly recruit repressor complexes, thereby silencing neighboring genes, as exemplified in the case of *Xist* (28). We reason that the antisense RNA-mediated silencing of genes located on both sides of the *Kcnq1* ICR might be similar to the latter alternative, as silencing is restricted not only to overlapping genes but also to nonoverlapping genes. An earlier study has compared the process of X inactivation and genomic imprinting with RNAi-mediated heterochromatinization in *Schizosaccharomyces pombe* (27). We believe that such a process may not be applicable in the *Kcnq1* ICR-mediated silencing. However, it cannot be ruled out that a dsRNA specifically involving the *Kcnq1ot1* RNA triggers repeat-associated small-interfering RNA-mediated heterochromatinization that subsequently spreads to the neighboring region (5).

It was previously documented that there is a temporal delay between silencing and DNA methylation spreading, suggesting that there is an intermediate step in the silencing process (24). This situation is reminiscent of what is seen in the *Xist*-mediated X inactivation. The loss of methylation spreading in the truncated versions of *Kcnq1ot1* RNA due to a poly(A) site insertion at various positions in the *Kcnq1* ICR points to the possibility that the *Kcnq1ot1* RNA may be involved in the heterochromatinization of flanking sequences. We presume that *Kcnq1ot1* transcription results in the recruitment of silencing factors associated with histone deacetylases and histone methyltransferases, thus modifying the chromatin into an inactive state, which later forms a target for DNA methylation.

Our observation that the transcription product of the *Kcnq1ot1* gene has silencing properties brings up a paradox which it has in common with other antisense transcripts such as *Tsix* and *Air* (12, 21). How does the antisense gene escape the silencing effects of its own transcript? The DNaseI footprints provide a potential key to this enigma since they cover a range of potential transcription factor binding sites such as CTCF, OCT-1, and GATA-1, all of which have been implicated in the maintenance of transcriptionally active chromatin. It is therefore possible that these sites have dual properties by being responsible not only for basal transcription but also for maintaining transcriptionally active chromatin that protects or prevents bidirectional silencing on the paternal allele from spreading into this region. Consistent with this view, it has been demonstrated in a recent report that the promoter of the *Kcnq1ot1* on the paternal chromosome is acetylated on the tails of histones H3 and H4 and methylated at lysine 4 of H3, which is a signature for active chromatin, while on the maternal allele, lysine 9 methylation, which is a signature for inactive chromatin, is observed (7).

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