NF-Y Regulates the Antisense Promoter, Bidirectional Silencing, and Differential Epigenetic Marks of the Kcnq1 Imprinting Control Region*

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Antisense transcription has been shown to be one of the hierarchies that control gene expression in eukaryotes. Recently, we have documented that the mouse Kcnq1 imprinting control region (ICR) harbors bidirectional silencing property, and this feature is linked to an antisense RNA, Kcnq1ot1. In this investigation, using genomic footprinting, we have identified three NF-Y transcription factor binding sites appearing in a methylation-sensitive manner in the Kcnq1ot1 promoter. By employing a dominant negative mutant to the NF-Y transcription factor, we have shown that the NF-Y transcription factor positively regulates antisense transcription. Selective mutation of the conserved nucleotides in the NF-Y binding sites resulted in the loss of antisense transcription. The loss of antisense transcription from the Kcnq1ot1 promoter coincides with an enrichment in the levels of deacetylation and methylation at the lysine 9 residue of histone H3 and DNA methylation at the CpG residues, implying a crucial role for the NF-Y transcription factor in organizing the parent of origin-specific chromatin conformation in the Kcnq1 ICR. Parallel to the loss of antisense transcription, the loss of silencing of the flanking reporter genes was observed, suggesting that NF-Y-mediated Kcnq1ot1 transcription is critical in the bidirectional silencing process of the Kcnq1 ICR. These data highlight the NF-Y transcription factor as a crucial regulator of antisense promoter-mediated bidirectional silencing and the parent of origin-specific epigenetic marks at the Kcnq1 ICR. More importantly, for the first time, we document that NF-Y is involved in maintaining the antisense promoter activity against strong silencing conditions.

Several lines of evidence have revealed that antisense transcription is one of the key regulators of eukaryotic gene expression (1). Transcriptome analysis in humans and in mice has revealed the presence of more than 2500 antisense transcripts, indicating a crucial role for antisense RNAs in the regulation of mammalian gene expression (2). Although the role of antisense transcription in gene regulation has been well established in the recent past, the mechanisms underlying the antisense RNA-mediated repression are yet to be investigated. It has recently been demonstrated that antisense RNAs produced from the imprinting control regions (ICRs)1 play a key role in the manifestation of the parent of origin-specific gene expression patterns in the imprinted clusters (3, 4). One of these imprinted clusters, located at the distal end of the mouse chromosome 7, has been relatively well investigated. This cluster divided into two sub-expression domains by the H19 ICR and the Kcnq1 ICR. The Kcnq1 ICR, which is located in intron 10 of the Kcnq1 gene, has been shown to be involved in regulation of the imprinting of the flanking genes Kcnq1, Cdkn1c, Ascl2, Tssc4, Tssc3, and Slc22a11 (5). It is methylated on the maternal allele and unmethylated on the paternal allele. The unmethylated paternal Kcnq1 ICR allele harbors a promoter for a long transcript, Kcnq1ot1. This transcript is oriented in an antisense orientation to the Kcnq1 transcript (6, 7).

We have earlier documented that the Kcnq1 ICR behaves as a bidirectional silencer (8). More recently, by selectively deleting the region spanning the antisense promoter and attenuating the antisense transcription by poly(A) sequence, we have shown that the production of an antisense RNA, Kcnq1ot1, is critical in the bidirectional silencing property of the Kcnq1 ICR (3). In this instance, the antisense transcription not only silences the overlapping genes but also the non-overlapping genes, thus distinguishing this phenomenon from the other cases, where antisense transcription leads to the silencing of the overlapping gene (9, 10). The antisense promoter activity is regulated by the parent of origin-specific epigenetic marks at the Kcnq1 ICR. Little is known how these epigenetic marks are laid down during gametogenesis (during early embryonic development) and maintained thereafter. Moreover, it has not been investigated whether the antisense promoter activity plays an important role in the maintenance of differential epigenetic marks at the Kcnq1 ICR. More importantly, loss of differential epigenetic marks at the human KCNQ1 ICR has been observed in >40% of sporadic Beckwith-Wiedemann syndrome patients. This suggests that understanding the molecular mechanisms underlying the establishment and the maintenance of these epigenetic marks will provide more insights into

1 The abbreviations used are: ICR, imprinting control region; RPA, RNase protection assay; ChIP, chromatin immunoprecipitation; DMS, dimethyl sulfate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; cpm, counts/minute.
NF-Y Regulates Silencing and Epigenetic Marks of Kcnq1 ICR

the complex bidirectional silencing process mediated by the Kcnq1 ICR. Interestingly, although the silencing of flanking genes, which are spread over several hundreds of kilobases, occurs due to antisense transcription, it is unclear how the transcriptional competence is maintained at the antisense promoter under strong silencing conditions.

Taken together, these observations indicate that antisense promoter activity plays an equally important role in the parent of origin-specific expression patterns at the Kcnq1 locus. We therefore were interested in understanding the role of antisense promoter activity in silencing and in the maintenance of differential epigenetic marks at the Kcnq1 ICR. Using genomic footprinting, we have earlier uncovered CCAAT boxes in the antisense promoter region (3). CCAAT boxes are present in all sorts of promoters and have been shown to play an important regulatory role. They are usually recognized by NF-Y, a trim-eric protein complex that consists of three subunits: NF-YA and histone-like NF-YB and NF-YC, all of which are required for DNA binding (11). In this study, we have identified NF-Y as the CCAAT activator and have set out to address the specific function of NF-Y binding sites in antisense transcription, bidirec- tional silencing, and the maintenance of differential epigenetic marks. By using the genomic footprinting approach, we have shown that occupancy of NF-Y binding sites is methylation-sensitive. By selective deletion of the promoter region containing the NF-Y binding sites, we showed that this region is important for antisense promoter as well as silencing activity. By employing dominant negative mutants of NF-YA and by selectively mutating the crucial nucleotides in all three NF-Y binding sites, we demonstrated that NF-Y occupancy plays an important role in the antisense promoter activity and that antisense promoter activity is critical for the silencing property of the Kcnq1 ICR. Interestingly, increased levels of deacetyl- ation and methylation of the lysine 9 residue of histone H3 and methylation of CpG residues ablated the antisense promoter function, suggesting that the trimer plays an important role in maintaining the differential epigenetic marks at the Kcnq1 ICR on the parental alleles. Taken together, these observations suggest that NF-Y-mediated antisense promoter activity is crucial for the maintenance of imprinting at the Kcnq1 locus.

EXPERIMENTAL PROCEDURES

In Vivo Footprinting—DNasel footprinting was performed as described earlier (12). Briefly, 5 million Hep-3B cells, containing episomal plasmids inserted with the Kcnq1 ICR in methylated and unmethylated versions (see Ref. 13 for methylation conditions), were permeabilized with 0.4% Nonidet P-40 and treated with 10–20 μg of DNasel at 4 °C for 3 min. For DMS footprinting, ~5 million Hep-3B cells were treated with 0.1% DMS, and the DNA from the treated cells was prepared using a genomic DNA preparation kit (Promega). Chem- ical sequencing reactions and piperidine cleavage were performed as described earlier (12).

Ligation-mediated PCR reactions were carried out using a proce- dure described earlier (12). The primers used for footprinting were as follows: upper strand, primer 1, 5'-TAGACCGTAACCCAGC-3'; primer 2, 5'-AATCCCAAAATGAGGGGACACACC-3'; primer 3, 5'-AAAAAG- GAGCCGACCCACCGGCTT-3'. The ligation-mediated-PCR re- actions were separated on a 5% urea-acrylamide gel, dried, and autoradiographed.

Plasmid Cloning Strategies—ΔCAT deletion in the Kcnq1 ICR was made by creating AgeI restriction enzyme sites flanking the NF-Y binding sites. One AgeI site was created at 198 bp upstream of the Kcnq1ot1 start site (by changing TCCTG to ACCCGT) using a forward primer 5'-GCT AGG TCA GGA TGA GAT AC-3' and a reverse primer 5'-GGG TGA TGT GCT ACC CTG TAG TAT-3'. A second AgeI site was created 10 bp upstream of the Kcnq1ot1 start site (by changing ACCCGG to ACCCGT) using a forward primer 5'-GTTGAGGCGCTTACCAGGTGGTTGAGG-3' and a reverse primer 5'-CTTCTAGGGCAACCGGATGGCCACCCCTAG-3') using Stratagene QuickChange site-directed mutagenesis followed by restriction digestion and re-ligation. The Kcnq1 ICR fragment carrying this deletion was cloned into the PH19 vector at a unique NotI site. The PS4CAT1 (carrying mutation at the CCAAT Box 1), PS4CAT1&2 (carrying mutations at the CCAAT Box 1 and 2), and PS4CAT1–3 (carrying mutations at the CCAAT Box 1, 2, and 3) constructs were made in the following manner. Initially, we generated mutations at all three NF-Y binding sites by changing the conserved nucleotides. In the case of CCAAT Box 1, we changed CCAAT to CCGGT and for CCAAT Box 2, we changed CCAAT to CCGGA. In the CCAAT Box 3, we changed CCAAT to CCATC. All of these mutations were created using the Stratagene QuikChange site-directed mutagenesis kit, using primers shown in Table I. The Kcnq1 ICR fragments carrying mutations at CCAAT Box 1, CCAAT Box 1 and 2, and CCAAT Box 1–3 were cloned at the unique NotI site of the PH19 episomal plasmid.

The Episome Silencer/Insulator Assay—The pREP4-based episomal vectors were transfected into JEG-3 cells, according to the method described previously (14), and total RNA was extracted 4 days after transfection. The RNase protection assay was performed as described previously using a 365-bp H19 antisense probe, a 150-bp GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antisense probe as control (14). The RNase protection reaction was carried out in the presence of 150 μg ofyps and hybridized with α-32P-labeled antisense probe (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 RPAII kit (Ambion). Quantification of individual protected frag- ments was done using a Fuji FLA 3000 phosphorimaging device. The H19 and Kcnq1ot1 expressions were corrected with respect to both internal control (GAPDH) and the drug-resistant colony number as determined by Southern blot analysis of BglII-restricted DNA hybridized with H19 and PDGFB probes (14).

To assess the effect of Kcnq1 ICR-mediated bidirectional silencing on a hygroycin resistance gene, equimolar concentrations of episomal-based plasmids were transfected into JEG-3 cells. Following transfec- tion, 15,000 cpm/10 fmol of radiolabeled probe was hybridized with cytosine DNA from the cells died in 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.

Electrophoretic Mobility Shift Assay—Nuclear extracts were pre- pared according to Dignam et al. (15). Oligonucleotides containing NF-Y binding sites (see Table I) were end-labeled with γ-32P-ATP. For the electrophoretic mobility shift assay, the binding reaction was performed for 30 min at room temperature in 30 μl of binding buffer containing 10,000 cpm/10 fmol of labeled probe, one μg of poly(dI·dC), and nuclear extract containing 5.0 μg of protein. For the supershift assay, the nuclear extracts and antibodies were pre-incubated for 20 min at room temperature before the radiolabeled probe was added, followed by an- other 30-min incubation. Separation of unbound labeled DNA from the DNA-protein complexes was carried out on a 4% non-denaturing acry- amide gel at 200 V at 4 °C. The gels were dried, and autoradiography was performed overnight.

Chromatin Immunoprecipitation (ChIP)—Single cell suspension of embryonic day 14.5 mouse fetal liver (Mus musculus domesticus) was prepared using the protocol described earlier (16). Briefly, liver tissue was minced in phosphate-buffered saline supplemented with 5 mM MgCl2, 2 mM CaCl2, and 1% glucose) and then incubated with trypsin (1 mg/ml) and crude DNasel (5 μg/ml) to release the cells from clumps. The action of trypsin was blocked by 10% fetal calf serum. The cells were washed twice with phosphate-buffered saline and resuspended in minimal essential medium (Invitrogen). The mouse fetal liver cells and Hep-3B cells transfected with episomal plasmids PS4CAT1–3 and PS4,

| Name          | Sequence (5‘→3’)                  | Orientation |
|---------------|-----------------------------------|-------------|
| CCAAT1 (wt)   | TTTACCATCTTTCATCCATCAACAGCTGTCCTG | F           |
| CCAAT1 (wt)   | CCAAGCATGTGGTTCAGGATGGGTTGAA      | R           |
| CCAAT1 (mut)  | ATTTACCATCTTTCATCCATCAACAGCTGTCCTG| F           |
| CCAAT1 (mut)  | CCAACACCTTTTTCCAGGATGGGTTGAA      | R           |
| CCAAT2 (wt)   | GTTCCATTTTTTTTTTCAGGCGAAAGGAC    | F           |
| CCAAT2 (mut)  | GTTCCATTTTTTTTTTTTCAGGCGAAAGGAC  | F           |
| CCAAT3 (wt)   | TGTCTGCTGACTATTTTTTCCGGCCAAGGAAAC| F           |
| CCAAT3 (wt)   | TGTACAGCACTTTTTCCGGCCAAGGAAAC    | F           |
| CCAAT3 (wt)   | TCTCCAGGCTGTCCACCATCCATCGTCCACAG| F           |
| CCAAT3 (wt)   | TCTCCAGGCTGTCCACCATCCATCGTCCACAG| F           |
| CCAAT3 (wt)   | TCTCCAGGCTGTCCACCATCCATCGTCCACAG| F           |
| CCAAT3 (wt)   | TCTCCAGGCTGTCCACCATCCATCGTCCACAG| F           |

TABLE I

Oligonucleotides used in the electrophoretic mobility shift assay and site-directed mutagenesis

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containing Kcnq1 ICR in methylated and mock-methylated versions, were cross-linked, as described earlier (17). Following isolation of nuclei and sonication to shear the DNA, the NF-Y-containing DNA-protein complexes were immunoprecipitated using an NF-Y antibody and protein A 4 Fast Flow-Sepharose beads (Amersham Biosciences). The immunoprecipitated DNA was PCR-amplified using forward primer 5'G-ATTACTTCGGTGGGCTTCT-3' and reverse primer 5'GACCACAT-GACGAAAACAG-3'. PCR conditions were 1 × 94°C for 5 min; 3× (94°C for 1 min, 57°C for 1 min, and 72°C for 1 min); 24× (94°C for 45 s, 57°C for 30 s, and 72°C for 45 s), and 1 × 72°C for 5 min.

RESULTS

Genomic Footprinting Analysis Reveals Methylation-sensitive Occupancy at the Kcnq1ot1 Promoter—By using the genomic footprinting approach, we have earlier fine mapped DNasel and DMS footprints containing several cis-acting elements (CTCF, OCT-1, CCAAT, YY1, GATA, and CREB) in the Kcnq1ot1 promoter region in unmethylated conditions (3). Given that the Kcnq1 ICR is differentially methylated on the parental alleles and that DNasel and DMS footprints were identified on the unmethylated ICR, we were interested to know whether these footprints are sensitive to CpG methylation. To this end, we used a genomic footprinting approach on episomal plasmids stably propagated in cultured cells, containing methylated and unmethylated versions of the Kcnq1 ICR in the PS4 orientation (in which the Kcnq1ot1 transcript faces toward the H19 reporter gene (see Fig. 5A)). As seen in Fig. 1, the DNasel and DMS footprints were sensitive to CpG methylation of the ICR on the upper strand, a treatment that inhibits its silencing activity. Interestingly, YY1 and all three NF-Y binding sites (CCCAAT 1–3) show sensitivity to DNA methylation, although none of them contains any CpG nucleotides in their core binding sequence (Fig. 1C). In contrast, the CREB binding site contains CpG, and it is known that the in vitro interaction of CREB with its site is inhibited by CpG methylation (18). Changes of reactivity toward DMS were also observed within the DNasel footprints regions (indicated by stars and arrowheads in Fig. 1B). We detected some of the DNasel hypersensitive sites that flank the NF-Y binding sites in the unmethylated condition (Fig. 1B, HS). Similar HS sites were also scored in in vitro footprinting analysis of the CycB1 B2 promoter with recombinant NF-Y (19). These are mostly due to DNA bending upon interaction with DNA, according to a model based on the crystallographic structure of the histone-like subunits recently presented (11). We have also carried out DNasel and DMS footprinting on the lower strand in the antisense promoter region. The footprints detected on the lower strand were similar to the upper strand (Ref. 3 and data not shown).

Selective Deletion of DNasel Footprint Containing NF-Y Binding Sites Results in Loss of Antisense Transcription and Bidirectional Silencing—As the antisense promoter lacks the TATA box, we presumed that three NF-Y binding sites upstream of the Kcnq1ot1 promoter might play a critical role in the antisense promoter activity. Using site-directed mutagenesis, we selectively deleted the antisense promoter region containing three NF-Y binding sites by introducing AgeI restriction sites into the Kcnq1ot1 promoter region (Fig. 1, B and C). To measure bidirectional silencing and antisense transcription levels from the wild type and the modified Kcnq1 ICRe, we used an episomal-based vector, PH19 (Fig. 2B, PH19), harboring the mouse H19 and hygromycin resistance gene (hygromycinR) as reporter genes. We inserted into the PH19 episomal plasmid the wild type and the modified versions of the Kcnq1 ICR between the H19 and hygromycinR reporter genes. This enabled us to simultaneously analyze the effects of the wild type and the modified Kcnq1 ICRe on the activity of H19 and hygromycinR reporter genes (see PS4 and ΔCATPS4 in Fig. 2B).

These episomal plasmids were transiently transfected in cultured cells, and the activity of both the H19 and Kcnq1ot1 genes was analyzed by RNase protection assay (RPA) 4 days after transfection, whereas the hygromycinR gene activity was analyzed by counting the number of resistant colonies obtained after selection. The reporter gene activities of each construct are represented relative to the control PH19 (H19 and hygromycinR) or PS4 (Kcnq1ot1) vectors. As shown in Fig. 2, deletion of the antisense promoter region resulted in loss of antisense expression and parallel loss of the silencing of both H19 and hygromycinR reporter genes, suggesting that the region containing NF-Y binding sites plays a critical role in the Kcnq1ot1 promoter activity as well as silencing activity mediated by the Kcnq1 ICR.

The Association of NF-Y Transcription Factor with the Kcnq1ot1 Promoter Region Regulates Antisense Transcription and Bidirectional Silencing Activity—The next logical question was whether or not NF-Y associates with the antisense promoter region in vivo. To address this issue, we carried out ChIP analysis on cross-linked chromatin obtained from the cells transfected with episomal plasmids harboring the wild type Kcnq1 ICR fragment. As shown in Fig. 3, A, NF-Y indeed associates with the Kcnq1 ICR. We addressed the question of whether association of NF-Y to the Kcnq1 ICR is determined by the CpG methylation status. We carried out ChIP analysis on episomal plasmids containing the methylated Kcnq1 ICR fragment. As can be seen from Fig. 3, A, CpG methylation of the Kcnq1 ICR did indeed inhibit NF-Y association to DNA. To check the specificity of NF-Y association with the Kcnq1 ICR, we performed ChIP assays with cells transfected with episomal plasmids containing mutations at all three NF-Y sites within the conserved CCAAT pentanucleotide. We observed hardly any association of NF-Y to the Kcnq1 ICR fragment containing mutations (Fig. 3A). Next, we performed gel shift assays to check whether all three NF-Y binding sites associate with NF-Y. As shown in Fig. 3B, NF-Y does indeed associate with all three wild type CCAAT boxes but not to sites carrying mutations in the conserved nucleotides. Taken together, these observations indicate a specific association of NF-Y with the CCAAT boxes of the Kcnq1ot1 promoter region in vivo, in a methylation-sensitive manner.

We were then interested in identifying the functional role of NF-Y in Kcnq1ot1 promoter activity. To this end, we introduced mutations into the conserved nucleotides at the three NF-Y binding sites in the antisense promoter region of the Kcnq1 ICR (see Fig. 1C). We inserted the wild type and the mutated Kcnq1 ICR fragments into the PH19 episomal plasmid, transiently transfected these plasmids into cells, and analyzed the antisense transcription using RPA as described above. The PS4 episomes with the Kcnq1 ICR carrying mutations at one NF-Y binding site (CCCAAT 1) showed a marginal decrease in antisense expression as compared with wild type PS4 plasmid (compare PS4 with PS4CAT1 in Fig. 4, A and B). However, the PS4 episomal plasmids with Kcnq1 ICR carrying mutations at either two (CCCAAT 1 and 2) or three (CCCAAT 1–3) CCAAT boxes, showed a significant decrease in antisense expression (compare PS4 with PS4CAT1&2 and PS4CAT1–3 in Fig. 4, A and B). In fact, the Kcnq1 ICR with mutations at all NF-Y binding sites showed a maximum loss of antisense expression (compare PS4CAT1–3 with PS4 in Fig. 4, A and B). A gradual loss of antisense expression from the Kcnq1 ICR corresponds to the number of mutated NF-Y binding sites, which suggests that all three NF-Y sites are critical for the Kcnq1ot1 promoter activity. More importantly, we also observed loss of antisense expression when we cotransfected the episomal plasmids with dominant negative NF-Y mutants (Fig. 4, E and F). This ob-
servation further reinforces the importance of NF-Y in Kcnq1ot1 promoter activity.

We addressed whether the Kcnq1ot1 promoter carrying NF-Y mutations supported the bidirectional silencing property of the Kcnq1 ICR. To assess this, we measured H19 and hygromycin R reporter gene activities as described previously. As shown in Fig. 4, C and D, the H19 and hygromycin R reporter genes showed maximum expression in the episome carrying the Kcnq1 ICR mutated at all three NF-Y binding sites, when compared with episomes with mutations at one

and/or two NF-Y binding sites (compare PS4CAT1–3 with PS4CAT1 and PS4CAT1&2 in Fig. 4, C and D). Taken together, these observations indicate that a gradual increase in H19 and hygromycin R reporter gene expression correlates to a gradual decrease in the Kcnq1ot1 expression in the Kcnq1 ICR carrying mutations at the NF-Y binding sites. Hence, NF-Y occupancy at the Kcnq1ot1 promoter determines the Kcnq1ot1 expression and bidirectional silencing property of the Kcnq1 ICR, proving its prominent role in the activity of the Kcnq1 ICR.
NF-Y Regulates Silencing and Epigenetic Marks of Kcnq1 ICR

**NF-Y Transcription Factor Maintains Differential Epigenetic Marks at the Kcnq1 ICR**—The Kcnq1 ICR carries differential epigenetic marks on the parental alleles, i.e. on the paternal chromosome, the Kcnq1 ICR is unmethylated at the CpGs, whereas on the maternal allele the Kcnq1 ICR is enriched with CpG methylation. It is, however, not known how these differential epigenetic marks are established and maintained at the ICR. We addressed whether NF-Y transcription factor occupancy has any role in the maintenance of differential epigenetic marks at the Kcnq1 ICR. For this purpose, we analyzed acetylation and methylation levels at the lysine 9 residue of the histone H3 and methylation at the CpG residues in the Kcnq1 ICR (Fig. 5, A and B). The histone acetylation and methylation levels were analyzed by ChIP on the cross-linked chromatin obtained from the JEG-3 cells transiently transfected for 20 days with episomal plasmids PS4 and PS4CAT1–3. As observed in Fig. 5B, the wild type Kcnq1 ICR was enriched with histone acetylation, whereas the histone methylation was present at low levels. However, the Kcnq1 ICR with mutations at the NF-Y binding sites showed low levels of histone acetylation and enhancement in the histone methylation levels.

Next, we addressed whether mutations at the NF-Y binding sites affected the CpG methylation levels in the Kcnq1 ICR. We have shown earlier that JEG-3 cells have a capacity to methylate de novo episomal sequences upon transfection and that the intensity of de novo methylation over the episomal sequences increases with time in culture (20). In that scenario, when NF-Y sites are involved in the maintenance of differential epigenetic marks, their mutations should enable rapid CpG methylation. As shown in Fig. 5C, the modified ICR was heavily methylated as compared with the wild type ICR (compare the PS4 lane with PS4CAT1–3). Taken together, these observations demonstrate that NF-Y transcription factor is involved in the maintenance of differential epigenetic marks at the Kcnq1 ICR. We have earlier documented that the Kcnq1 ICR spreads de novo methylation into the flanking H19 coding region (8) and that this property occurs as a consequence of antisense RNA-mediated silencing. Because antisense transcription is lost in the PS4CAT1–3 constructs, we tested for the methylation spreading property of the Kcnq1 ICR in the PS4CAT1–3 construct. Fig. 5D shows that CpG methylation over the H19 coding region is lost in the PS4CAT1–3 but not in the PS4 construct, suggesting that NF-Y-mediated antisense transcription is crucial for the spreading of heterochromatin blocks over the flanking H19 coding region.

**DISCUSSION**

The following are the main observations we made. 1) By genomic footprinting, we have shown that most of the transcription factors in the antisense promoter region interact in a methylation-sensitive manner. 2) By selectively deleting the antisense promoter encompassing the binding sites for NF-Y, we have demonstrated that this region is crucial for antisense promoter activity and bidirectional silencing. 3) Using ChIP assay, we have shown specific association of NF-Y with the antisense promoter. 4) Introducing mutations at the conserved nucleotides in all three NF-Y binding sites resulted in loss of NF-Y occupancy. Loss of NF-Y occupancy at the Kcnq1ot1 promoter resulted in abrogation of antisense transcription and bidirectional silencing. 5) NF-Y occupancy at the Kcnq1ot1 promoter regulates the differential epigenetic marks such as CpG methylation, histone acetylation, and methylation, within the Kcnq1 ICR.

We have documented earlier that the mouse Kcnq1 ICR
harbors a bidirectional silencer activity and that this feature involves production of an antisense RNA, \(Kcnq1ot1\) (3). In this investigation, we demonstrated that NF-Y plays an important role in the regulation of the \(Kcnq1ot1\) promoter and that the loss of NF-Y occupancy in the \(Kcnq1ot1\) promoter region resulted in the loss of both \(Kcnq1ot1\) transcription and the bidirectional silencing property of the \(Kcnq1\) ICR. This conclusion is primarily based on the observation that mutation of conserved nucleotides in all three NF-Y binding sites leads to loss of antisense transcription and parallel loss of silencing of \(H19\) and \(hygromycinR\) reporter genes in the episomal-based system. In our earlier study (3), we have implicated antisense transcription in bidirectional silencing based on the selective deletion of the antisense promoter region. However, this does not rule out the possibility that the deleted region may contain cis-acting elements that independently participate in the \(Kcnq1\) ICR-mediated silencing without interfering with antisense transcription. In this investigation, the correlation of loss of silencing with loss of antisense transcription in the NF-Y-mutated \(Kcnq1\) ICR conclusively demonstrated that the antisense promoter activity is critical in the bidirectional silencing mediated by the \(Kcnq1\) ICR. This conclusion is further corroborated by the loss of antisense transcription from the episomal plasmids cotransfected with plasmids containing a dominant negative form of NF-YA. A recent investigation in the multiple CCAAT-containing human \(KCNQ1OT1\) promoter also implicated NF-Y with \(in vitro\) binding assays, although the effects on silencing were not determined (21). Therefore, NF-Y plays a critical role in regulating an evolutionarily conserved region within the \(Kcnq1ot1\) promoter, which is crucial for the bidirectional silencing property of the \(Kcnq1\) ICR.

The tandem arrangement of conserved CCAAT boxes observed here is reminiscent of other systems, particularly cell-cycle promoters, in which NF-Y takes a prominent role (Ref. 19 and references therein). Careful dissection of the triple CCAAT of the \(Cyclin B2\) gene, for example, indicates that their precise stereo alignment is crucial for p300 recruitment and promoter function of co-activators with all CCAAT boxes, irrespective of their relative affinity for NF-Y and similarly contributing to the overall activity. Here, a gradual decrease in the antisense transcription and a corresponding increase in reporter gene expression in the PS4 mutated at the NF-Y sites suggests all three CCAAT boxes are important for the antisense promoter activity and that the levels of antisense RNA production may determine the extent of bidirectional silencing. We have proposed earlier (3) that the \(Kcnq1ot1\) transcript may silence the
NF-Y Regulates Silencing and Epigenetic Marks of Kcnq1 ICR

reporter genes by recruiting repressive complexes containing histone methyltransferases and histone deacetylases. In that scenario, we presume that recruitment of heterochromatic machinery is perhaps dependent on the levels of antisense RNA and that it is, in turn, correlated to the extent of silencing. Loss of methylation spreading in the flanking \( H19 \) coding region of \( PS4CAT1–3 \) further reinforces the suggestion that the antisense transcription is involved in the modification of chromatin.

**Fig. 4.** Loss of NF-Y transcription factor occupancy at the NF-Y binding sites in the Kcnq1ot1 promoter leads to loss of antisense transcription and bidirectional silencing. A, shows an autoradiogram displaying the Kcnq1ot1 transcript levels in the PS4, PS4CAT1, PS4CAT1&2 and PS4CAT1–3. B, a bar graph showing percentage levels of expression of the Kcnq1ot1 in the wild type and mutant PS4CAT constructs. The percentage expression levels were calculated after normalization against input RNA and episome copy number as described in the legend to Fig. 2. C and D, mutations at the conserved nucleotides in NF-Y binding sites result in loss of bidirectional silencing of the Kcnq1 ICR. C, an autoradiogram of RPA showing the mouse H19 reporter gene transcription in the wild type and mutant PS4CAT constructs. D, contains a bar graph showing the relative expression levels of the H19 and hygromycin\(^R\) reporter genes, normalized as described in the legend to Fig. 2.

E and F, loss of antisense transcription from the PS4 episomal plasmids cotransfected with constructs containing a dominant negative subunit of NF-Y. E contains an autoradiogram of RPA showing the levels of antisense transcripts in the PS4 episomal plasmids with and without cotransfection with dominant negative NF-Y constructs. F contains a bar graph showing relative expression levels of Kcnq1ot1 transcript.
NF-Y Regulates Silencing and Epigenetic Marks of Kcnq1 ICR

Fig. 5. NF-Y regulates differential epigenetic marks at the Kcnq1 ICR. A, physical map showing the PS4 episomal plasmid. Underneath the PS4 physical map are extended maps of the Kcnq1 ICR and the H19 coding region showing HhaI restriction sites (represented as H), DNA probes used for Southern hybridization, and the primers for amplifying the ChIP DNA. B, ChIP analysis in the antisense promoter region using antibodies to acetylation or methylation at the lysine 9 residue of the H3 histone. Immunopurified DNA was amplified with primers as indicated under “Experimental Procedures.” C and D, Southern hybridization of genomic DNA obtained from the cells after transient transfection for 7 and 20 days with PS4 and PS4CAT1–3 plasmids. Genomic DNA was restricted with PstI, PstI/HhaI to analyze the methylation status at the HhaI sites in the Kcnq1 ICR by probing with the ICR-specific probe (C), and BamH1, BamH1/HhaI to analyze methylation status in the H19 coding region by using the H19 coding region-specific probe (D). The methylated fragments are marked with asterisks.
sistent with this presumption, in a recent study (22), it has been documented that the RNA component also plays a crucial part in recruiting DNA methyltransferases and methyl-specific CpG binding proteins complexed with histone deacetylases.

One of the most interesting findings of our study is the loss of differential epigenetic marks in the Kcnq1 ICR with mutations in the NF-Y transcription factor binding sites. This suggests that the NF-Y transcription factor in association with other transcription factors may be involved in maintaining the differential epigenetic marks at the Kcnq1 ICR. This is an important finding from the perspective that the loss of differential epigenetic marks of the human KCNQ1 ICR is present in >40% of the sporadic Beckwith-Wiedemann syndrome patients. We presume that deregulation of the functions that maintain the differential epigenetic marks could be a cause for the loss of differential epigenetic marks at the KCNQ1 ICR in Beckwith-Wiedemann syndrome patients. Loss of antisense transcription as well as differential epigenetic marks from the Kcnq1 ICR with mutations at the NF-Y binding sites suggests that the NF-Y transcription factor plays an important role not only in keeping the antisense promoter in a transcriptionally competent state under strong silencing conditions but also keeps the antisense promoter CpG methylation free by preventing de novo methylation. One of the main questions that needs to be addressed is how the NF-Y transcription factor is losing the differential epigenetic marks at the Kcnq1 ICR. It has recently been documented that NF-Y recruits histone acetyltransferases and thereby keeps the promoter in a highly acetylated state (23). The presence of high acetylation and low methylation at the lysine 9 residue of H3 histone in the wild type Kcnq1 ICR probably suggests that NF-Y occupancy at the antisense promoter recruits histone acetyltransferases to keep the antisense promoter transcriptionally competent under strong silencing conditions.

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