Single-stranded Noncoding RNAs Mediate Local Epigenetic Alterations at Gene Promoters in Rat Cell Lines

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Background: Noncoding RNAs (ncRNAs) can alter epigenetic processes, mostly causing gene repression. In this study, promoter-associated ncRNAs (pancRNAs) were associated with active chromatin marks at Nefl and Vim promoters. Forced expression and knockdown of these pancRNAs caused DNA demethylation and methylation, respectively.

A growing number of noncoding RNAs (ncRNAs) are thought to be involved in sequence-specific alterations of epigenetic processes, mostly causing gene repression. In this study, promoter-associated ncRNAs (pancRNAs >200 nucleotides in size) that were endogenously generated from the sense strand at Map2b, antisense strand at Nefl, and both strands at Vim were investigated regarding their epigenetic potential as positive or negative regulators in rat pheochromocytoma (PC12) and fibroblast (normal rat kidney) cell lines. The respective antisense pancRNAs were associated with several active chromatin marks at the Nefl and Vim promoters. Forced expression of fragments expressing the antisense pancRNAs caused sequence-specific DNA demethylation, whereas a decrease of expression induced methylation of the same sequences. In contrast, perturbing the expression of the two sense pancRNAs did not change the DNA methylation status. These results suggest that a fraction of naturally occurring ncRNAs acts in cis as a single-stranded form and that the transcriptional orientation of pancRNA is important for the establishment of sequence-specific epigenetic modifications consistent with open chromatin structure.

During cell differentiation and development, chromatin structure is highly organized in ways that may direct the differential gene expression profile depending on the cell type. Several epigenetic modifications are involved in this process. For example, histone acetylation on gene promoter regions is generally correlated with transcriptional activation. On the other hand, DNA methylation occurring on cytosines that are parts of CG dinucleotides generally down-regulates the associated genes (2–4). Histone methylation, another epigenetic modification, confers differential effects on gene expression status according to the lysine residue modified (5). In histone proteins there are several positions that can be modified by methylation (6). For example, methylation at lysine 9 of histone H3 (H3K9) is correlated with transcriptional silencing in most cases, whereas methylation at lysine 4 of histone H3 (H3K4) is associated with gene activation (1, 5, 7). Currently one key issue is the identification of the molecules that establish/maintain these epigenetic modifications in a sequence-specific manner (8).

The number of known noncoding RNAs (ncRNAs) is now rapidly increasing. Thousands of ncRNAs have been found in addition to the classical functional ncRNAs such as transfer RNA and ribosomal RNA (9). It was revealed by the RIKEN FANTOM project that more than 60% of the genomic DNA contributes to the transcriptome (10). That project also indicated that for a fraction of the transcriptionally competent genomic regions, both DNA strands are used for RNA generation (11). This implies that the total number of functional ncRNAs will not be negligible. Indeed, ncRNAs have been shown to act to negatively regulate gene expression at the post-transcriptional level in animals via processes such as RNA editing, RNA degradation, RNA interference, splicing, and translation by forming RNA duplexes (12–14). Because only 1–2% of the genome provides templates for protein-coding gene expression (15–17), bulk RNA from most of the genome theoretically would not form perfectly matched duplexes with mRNA. Rather, such ncRNAs would have their respective functions at the genomic level, if any.

Extensive RNA-mediated gene regulation would be possible if double-stranded structures were formed between RNA/DNA...
(18–21). In addition to regulation at the posttranscriptional level, double-stranded RNAs (dsRNAs), including siRNA, microRNA and Piwi-interacting RNA, seem to be essential for chromatin-level regulation, especially for constituting a transcriptionally inert status (21, 22). For example, in yeast, dsRNA derived from transposon-like inverted repeats stabilizes the heterochromatin structure by inducing the methylation of histone H3 (23, 24). Single-stranded RNA (ssRNA) is also functional in a chromatin context. Examples include thousands of large intergenic ncRNAs, named lincRNAs, about 20% of which are physically associated with Polycomb repressive complex 2 (25–27). Similarly, Airn is required for silencing autosomal-imprinted genes via DNA methylation (20). Airn truncation leads to the complete loss of silencing of the Igf2r/Slc22a2/Slc22a3 cluster on the paternal chromosome. However, we do not yet know if ssRNA functions only to set up a closed chromatin structure. Previous reports have shown that DNA demethylation is directed by antisense promoter-associated ncRNAs (pancRNAs) in the rat Spkh1/Khps1 locus (28, 29). Therefore, additional information about the functional properties of ssRNAs is necessary to understand how such RNAs direct gene activation as well as gene repression.

Changes in cytoskeleton organization are frequently associated with cell lineage commitment during normal development and pathological processes, including malignant transformation (30). In this study we selected three genes encoding cytoskeletal proteins to test the hypothesis that ncRNA is functionally associated with certain gene promoter regions. 1) Microtubule-associated protein 2 (Map2) is a neuron-specific intermediate filament protein family that is expressed in neural cells of very early developmental origin as well as many skeletal muscles (35). 34789

Bovine serum, 5% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO₂. For induction of differentiation, PC12 cells were cultured in DMEM supplemented with 1% bovine serum, 0.5% horse serum, and 100 ng/ml nerve growth factor (NGF) for 3 days.

Intact male Wistar-Imamichi strain rats (350 ± 30 g) were housed in a controlled environment (14 h light and 10 h dark, lights on at 0500 h; 23 ± 3 °C) with food (CE-2, Clea Japan Inc., Japan) and water ad libitum. The animal experiments were approved by the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University. Brain samples were collected and immediately frozen in liquid nitrogen and stored at −80 °C until used.

**RNA Analysis**—To examine RNA expression, total RNA isolated from cells and tissues with TRIzol reagent (Invitrogen) was treated with DNase I (Invitrogen) and reverse-transcribed with random hexamer using the SuperScriptIII First-Strand Synthesis System (Invitrogen). PCR was carried out with specific primers for each transcript (see supplemental Table S1).

To determine the direction of transcripts, first-strand cDNA was synthesized from total RNA (3 μg) using each respective gene-specific primer. The specific cDNA was then directly amplified by PCR using the primer sets. The information about primer sequences and positions is given in supplemental Table S2 and Fig. S2.

5′-RACE was performed to identify the transcription start sites (TSSs) of the RNAs using a GeneRacer kit (Invitrogen). The first-strand cDNA was prepared from RNA adaptor-ligated total RNA. Subsequent PCR amplification was carried out using LA-TaqDNA polymerase (TaKaRa; Shiga, Japan) with GeneRacer 5′ primer and gene-specific primers. In parallel, 3′-RACE was done with oligo-dT primer.

**Chromatin Immunoprecipitation (ChIP) Assay**—ChIP was performed using a ChIP Assay Kit (Upstate Biotechnology Inc., Lake Placid, NY). One million cells were used per ChIP assay. Cells were treated with formaldehyde at a final concentration of 1% and incubated at room temperature for 20 min. Cells were collected and treated with lysis buffer containing proteinase inhibitors (1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml pepstatin). Cell lysates were sonicated on ice until the chromatin fragments became 200–1000 bp in size. Fragmented chromatin samples were diluted 10-fold in ChIP dilution buffer, and 10% of the total volume was stored as input sample. The rest of each sample was precleared by rotation with protein A-Sepharose beads containing salmon sperm DNA at 4 °C for 1 h followed by the addition of 1 μg of one of the following antibodies; antibody against acetylated histone H3 (Upstate Biotechnology, catalogue no. 06-599), dimethylated H3K4 (Upstate Biotechnology, catalogue no. 07-030), trimethylated histone H3K4 (Abcam, catalogue no. ab8580), trimethylated H3K9 (Abcam, catalogue no. ab8898), or 1 μg of rabbit IgG (Santa Cruz Biotechnology., catalogue no. SC-2027). Immunoprecipitation was performed at 4 °C overnight with rotation. Rabbit IgG was used as a negative control to check the immunoprecipitation specificity. Immune complexes were collected using protein A-Sepharose beads and washed with a series of wash buffers. After the immune complexes were washed, formaldehyde cross-linking in the immunoprecipitated samples and input chromatin sam-
ple was reversed by overnight incubation with 300 mM NaCl at 65 °C. DNA was further treated with proteinase K, purified by phenol/chloroform extraction, and ethanol-precipitated. DNA was resuspended in 30 μl of sterile water and used as a template for PCR with specific primers for each gene locus (see supplemental Table S1 and Fig. 3). The intensity of the signal for each PCR product was measured by using ImageJ software (Version 1.34, provided by United States National Institutes of Health), and the results from two independent experiments performed in triplicate were quantified and averaged.

A part of the ChIP samples was subjected to RNA amplification for the investigation of whether RNA was involved in chromatin architecture according to a previous report with slight modifications (36). Briefly, the nucleic acid preparations from ChIP samples were digested with DNase I followed by cDNA preparation. Semi-nested amplification was then performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Norwalk, CT) and the primers shown in supplemental Table S1. PCR products were detected using an ABI PRISM 7000 sequence detector.

Bisulfite Genomic Sequencing—The conditions for the bisulfite reaction for the determination of DNA methylation status have been described previously (37). The DNA corresponding to the region encompassing the Map2b, Nefl, or Vim promoter was amplified by PCR using AmpliTaq Gold (Applied Biosystems) and the primers listed in supplemental Table S3. The purified PCR products were cloned into pCR2.1 TOPO (Invitrogen), and three or more clones randomly picked from each of three independent PCRs were sequenced using the BigDye Terminator Ver.3 System (Applied Biosystems) and an ABI3100 sequencer (Applied Biosystems).

Construction of Plasmids for Transient Transfection—DNA fragments containing the nucleotide sequence corresponding to each novel RNA (−596 to −68, −1106 to −59, −1070 to −153, and −920 to −355 relative to the transcription start sites of the Map2b, Nefl, and Vim genes, respectively) were isolated by genomic PCR using specific primers (see supplemental Table S4). The upstream primers included a HindIII site at the 5′ end of the sequence, and the downstream primers also included an Apal site. Each fragment was inserted between the HindIII and Apal sites of the expression vector pRc-CMV (Invitrogen) to construct the expression plasmid of the respective novel RNA. For shRNA expression vector preparation, each fragment was inserted into the region of each gene (see supplemental Table S1 and Fig. 3). The intensity of the signal for each PCR product was measured by using ImageJ software (Version 1.34, provided by United States National Institutes of Health), and the results from two independent experiments performed in triplicate were quantified and averaged.

RESULTS

Identification of Sense and Antisense pancRNAs from Map2b, Nefl, and Vim Loci—To investigate if RNA species other than mRNA were expressed from the Map2b, Nefl, and Vim loci in vivo, RT-PCR primers were designed to amplify the upstream region of each gene (Fig. 1A). All of the primer sets gave positive bands in RT-PCR analysis using total RNA from the rat adult brain (Fig. 1B). These novel RNAs were also detected in NRK cells or in PC12 cells, which have differentiation competence to form neuron-like structures, suggesting that there were endogenous pancRNAs for all three genes examined. The expression patterns of these candidate pancRNAs were different depending on the cell type (Fig. 1B). RNAs derived from the upstream region of Map2b and Nefl were expressed in PC12 cells, whereas their expression was not detected in NRK cells. Conversely, RNAs derived from the upstream region of Vim were expressed in NRK cells, whereas their expression was not detected in PC12 cells. Therefore, the expression of these candidate pancRNAs was specific to one of the cell lines examined. The candidate pancRNAs and mRNA expressions showed positive correlation in the Nefl and Vim loci and negative correlation in the Map2b locus.

To determine the transcriptional direction of the candidate RNAs, we prepared cDNA using strand-specific RT primers followed by PCR amplification (Fig. 1, C–E). When the sense primer for the Map2b promoter sequence (primer a in Fig. 1C) was used for reverse transcription, the subsequent PCR did not amplify any specific products. In contrast, a specific product was amplified from the cDNA synthesized with an antisense primer (primer b in Fig. 1C), indicating that the candidate pancRNA was transcribed in the sense direction from the 5′-flanking sequence of Map2b. Similar experiments for the Nefl locus showed that the candidate pancRNA was transcribed in the antisense direction. In the case of the Vim locus, both strands seemed to be the templates for pancRNA generation. Sequence analysis showed that the resulting cDNAs completely matched the genomic sequences of the Map2b, Nefl, and Vim loci. We concluded that there were pancRNAs for these three genes and designated these pancRNAs Map2b-s, Nefl-as, Vim-s, and Vim-as.

To determine the TSSs of the Map2b-s, Nefl-as, Vim-s, and Vim-s, 5′-RACE was performed. Map2b-s and Nefl-as originated from 681 bp upstream and 1161 bp downstream of the TSS of the protein-coding transcripts, respectively (Fig. 1A). Vim-s and Vim-as originated from 1048 and 480 bp upstream of the TSS for Vim. Vim-s was not an alternative 5′ end of the Vim mRNA, as fragments containing both Vim-s and Vim mRNA sequences failed to be amplified by RT-PCR. The longest possible ORFs in cDNAs for Map2b-s, Nefl-as, Vim-as, and Vim-s were 144, 198, 564, and 411 bp, respectively. However, no recognizable amino acid motifs or domains were observed in the predicted amino acid sequences. These data strongly support the notion that all of the novel transcripts are ncRNAs.

DNA Methylation and Histone Modification Status of 5′-Flanking Regions of Map2b, Nefl, and Vim Genes in PC12...
and NRK Cells—All of the genes examined here contain a relatively higher proportion of CG dinucleotides around the TSS compared with the bulk DNA. Especially notably, the presence of CpG islands was observed in the *Nefl* and *Vim* loci (Fig. 2, A–C). Therefore, next we examined the DNA methylation status of the *Map2b*, *Nefl*, and *Vim* promoters by means of bisulfite sequencing. The 12 CG sites within the *Map2b* locus (−292 to +31) were much more highly methylated in PC12 cells than in NRK cells (Fig. 2A). The 60 CG sites in the *Vim* locus (−275 to +531) were highly methylated in PC12 cells and were mostly unmethylated in NRK cells (Fig. 2C). The 32 CG sites within the *Nefl* locus (−64 to +309) were hypermethylated in NRK cells, whereas they were almost completely unmethylated in PC12 cells (Fig. 2B). These results suggest the involvement of DNA methylation of the *Map2b*, *Nefl*, and *Vim* promoter regions in the regulation of the differential expression of these genes.

To examine the histone modification status of the *Map2b*, *Nefl*, and *Vim* upstream regions, ChIP assays were performed for the detection of acetylation of histone H3 and di- and trimethylation of H3K4 as active chromatin markers and trimethylation of H3K9 as a repressive chromatin marker (Fig. 3). At the *Map2b* locus, all the active chromatin markers were detected at higher levels in NRK cells in which *Vim* was expressed (Fig. 3B). At the *Vim* locus, all the active chromatin markers were detected at higher levels in NRK cells, where *Vim* was repressed. These results suggest that multiple, but not all, histone modifications are correlated with gene expression status.

**Effect of the Transient Expression/Repression of pancRNAs on DNA Methylation Status of Promoter Regions in PC12 and NRK Cells**—Down-regulation of the mRNAs studied here showed a stronger correlation with increased DNA methylation than with histone modification status. Therefore, to investigate if changes in pancRNA expression could modify the DNA methylation patterns, we constructed CMV promoter-controlled recombinant plasmids that directed the expression of RNAs corresponding to fragments of *Map2b*-s, *Nefl*-as, *Vim*-s, or *Vim*-as (supplemental Table S4). The DNA fragments used for these pancRNA overexpression experiments overlapped with the 5′-flanking sequence but not with the 5′-untranslated region to prevent duplex formation of these pancRNAs with the corresponding mRNAs. In parallel, we constructed CMV enhancer- and human U6 promoter-controlled shRNA expression plasmids to see the effect of knockdown of the endogenous pancRNA on epigenetic regulation (supplemental Table S5).

Transfectants were analyzed by means of bisulfite PCR with primer sets designed to anneal specifically to endogenous sequences followed by sequencing to determine the methylation levels of individual cytosines. Twenty-four hours after the
transfection of Map2b-s-expressing plasmids in NRK cells, in which the corresponding RNA was not endogenously expressed (see Fig. 1B), the genomic methylation pattern did not change in either the Map2b or the Nefl upstream region (Fig. 4, A and B). However, the genomic methylation was dramatically diminished in the Nefl locus upon overexpression of a Nefl-as fragment in NRK cells (Fig. 4B). This effect was genespecific, as the Map2b-s fragment did not affect the DNA methylation pattern of the Nefl locus. Nefl-as did not behave as a general demethylator, as the Nefl-as fragment did not reverse the DNA methylation of the Map2b. The genomic methylation was also dramatically diminished at the corresponding CG sites only when a fragment of Vim-as, not a fragment of Vim-s, was transfected into PC12 cells (Fig. 4C). These results indicate that the antisense and sense RNA did not contribute equally to regulating the DNA methylation status. There were no cases in which hypomethylated status changed to hypermethylated status as a result of pancRNA overexpression.

Similar experiments were performed with shRNA expression plasmids (Fig. 5). The results showed that knocking down the two endogenous antisense RNAs, Nefl-as and Vim-as, induced DNA methylation in sequence-specific manners (Fig. 5, B and C). When the Map2b-s was targeted by shRNA, we did not observe DNA methylation changes. Therefore, the expression of the two antisense pancRNAs was functionally involved in demethylating the corresponding genomic sequences. The function of the two sense pancRNAs remains unknown.

Histone Modification and pancRNA Association at the 5′-Flanking Region of Nefl and Vim in pancRNA-engineered PC12 and NRK Cells—The histone modification pattern was partially correlated with mRNA expression status in this study. Next we performed ChIP experiments using Vim-as- or Vim-s-overexpressing PC12 and NRK cells to see if alteration of histone modifications occurred in association with sense or antisense pancRNA expression (Fig. 6A). The results showed about two times more efficient amplification of Vim promoter fragments after pulldown of H3K4-dimethylated histone-complex in Vim-as-overexpressing PC12 cells. Conversely, shRNA-directed knockdown of Vim-as led to decreased H3K4 dimethylation. In addition, both of these cell preparations showed less H3K9 trimethylation (decreased by more than 3-fold) on the promoter fragments. When Vim-s was overexpressed, no such effects were seen. Rather, a tendency of decreased H3K4 dimethylation and increased H3K9 trimethylation was observed, indicating that the histone modification status changed differentially depending on the orientation of the pancRNA.

To see if the sense and/or antisense pancRNAs were physically associated with specific chromatin structures, RNAs recovered from ChIP samples were subjected to RT-PCR analyses to detect Vim-s or Vim-as. Fig. 6B shows that there was differential association of the pancRNA with the H3K4-dimethylated and H3K9-trimethylated chromatin structures. Vim-s and/or Vim-as RNAs were detected in the H3K4-dimethylated chromatin fraction in NRK cells. Similar results were obtained when Vim-as was overexpressed in PC12 cells. The pancRNA signal was significantly decreased after shRNA-based knockdown of Vim-s/Vim-as in NRK cells. In contrast, much lower levels of RT-PCR products were detected when Vim-s was overexpressed in PC12 cells. These results indicate the biased physical association of Vim-as with the active chromatin structure, which was in line with the DNA methylation data (Fig. 3). Much lower levels of Vim-s and Vim-as RT-PCR products were detected.
in the H3K9-trimethylated chromatin fraction of all sample preparations examined, although there seemed to be a tendency toward higher affinity of Vim-s association with H3K9 (Fig. 6B).

**Effect of pancRNA Disturbance on mRNA Expression Levels of Nefl and Vim**—Finally, to see if the observed DNA demethylation by antisense pancRNA at the Nefl and Vim promoter regions was linked with associated mRNA changes, we performed quantitative RT-PCR to detect the mRNA expression level in cells after the pancRNA overexpression or knockdown and confirmed the expected up-regulation or down-regulation of the two genes (Fig. 7, A–C). According to the above results, the phenomena of DNA demethylation/methylation by pancRNA increase/decrease

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**FIGURE 3. Histone modification status of Map2b, Nefl, and Vim 5′-flanking region in PC12 and NRK cells.** Each diagram at the top shows the location of the ChIP assay primers within the 5′-flanking regions of the Map2b (A), Nefl (B), and Vim (C) genes. The gel images show the histone modification status. PC12 cells and NRK cells were subjected to ChIP assays with antibodies against acetylated histone H3 (AcH3), dimethylated H3K4 (H3K4me2), trimethylated H3K4 (H3K4me3), or trimethylated H3K9 (H3K9me3). Normal rabbit IgG (IgG) was used as a negative control for the specificity of the immunoprecipitation. The same amount of chromatin fragments as used for each immunoprecipitation was also subjected to PCR without IP as a positive control (Input). Lower graphs show semiquantitative analysis of the relative levels of histone modifications. The intensity of each PCR band was measured as described under “Experimental Procedures.” Each bar represents the mean ± S.E. of two independent experiments performed in triplicate. Values for samples from undifferentiated PC12 cells on each gel image were set as 1 for each modification. **, p < 0.01; *, p < 0.05; Student’s t test. diff., undifferentiated; undiff., undifferentiated.
and resultant mRNA expression status are summarized in Fig. 7D.

DISCUSSION

The number of known mammalian ncRNA is rapidly increasing, necessitating evaluation of the functions of these RNAs, if any. In this study we discovered pancRNAs (Map2b-s, Nefl-as, Vim-s, and Vim-as) that were derived from GC-rich gene promoters. CpG islands often function as promoters of genes that lack a TATA-box that determines the orientation of the RNA transcription (38). These TATA-less promoters contain many TSSs in the sense and antisense directions. In this context the discovery of the sense and antisense pancRNAs in the present study was not surprising. Notably, we found that forced expression of the antisense pancRNAs (Nefl-as or Vim-as) induced sequence-specific DNA demethylation, whereas knockdown of these endogenous antisense pancRNAs led to the hypermethylation of the corresponding sequences. On the other hand, the relative expression of the pancRNA and mRNA was not consistent for Map2b, which fits with the finding that Map2b-s does not modify DNA methylation in the same way as the two antisense pancRNAs (Figs. 1, 4, and 5). Previous findings on ncRNA function have largely concerned its involvement in heterochromatin/closed chromatin formation. In contrast, antisense, but not sense pancRNA seemed to be involved in setting active chromatin marks, especially in demethylating DNA, in this study. Relatively little information is available about the molecular mechanisms mediating formation of active chromatin, including DNA demethylation. Based on our observations, the relevant molecular features are discussed below.

Antisense pancRNA Functions as a Single-stranded Form—It is not possible for the pancRNA fragment used in our forced expression study to form an RNA duplex with sense mRNA, as this fragment is complementary to the sequence of the promoter and does not overlap with the 5’-untranslated region. Therefore, this pancRNA could influence the epigenetic processes as a ssRNA. ssRNA-induced DNA methylation changes have been reported for the rat Sphk1/Khps1 locus (28, 29), and thus interaction of a promoter with a single-stranded pancRNA appears to be one type of general mechanism regulating epigenetic status in a sequence-specific manner. Nonetheless, we discovered two largely overlapping, naturally occurring pancRNAs from the Vim locus, namely, an antisense pancRNA, Vim-as, and a sense pancRNA, Vim-s. Several lines of evidence have suggested that dsRNA is associated with gene suppression (21, 24). In mammalian cultured cells, dsRNA designed based on the promoters of Argonaute-1 and Argonaute-2 suppressed the corresponding gene transcription (39). The administration of siRNA designed based on the central portion of the CpG
islands of CDH1 into human cancer cells induced gene suppression (21). However, the pancRNAs derived from the upstream region of the Vim locus were observed to be coexpressed with Vim in NRK cells (Fig. 1 B). The upstream region of the Vim locus showed active chromatin modifications in NRK cells. Opposite epigenetic modification patterns were evident in PC12 cells, in which both Vim-as and Vim-s were repressed (Fig. 2 C). Therefore, the Vim-s/Vim-as duplex may set the sequence-specific DNA hypomethylated status. However, Vim-as expression was sufficient to induce hypomethylated status of the Vim upstream region before and after forced expression of shRNA mixture for Vim-s/Vim-as in PC12 and NRK cells is shown. In parallel, the same region was examined in cells with shRNA for Vim-as overexpression. Note that hypomethylated CG sites at the Vim locus were dramatically methylated as a result of the transfection of the construct designed for Vim-s/Vim-as knockdown in NRK cells.

It is still possible that the pancRNA forms a secondary structure(s) that locally mimics dsRNA. Indeed, prediction software (CentroidFold) predicted that the identified pancRNAs could form 10-base pair-scale dsRNA structures. Nonetheless, we found no predicted long double-stranded structures that could explain the observed 100-base pair-scale DNA methylation changes, supporting the notion that the pancRNA functions as ssRNA.

Although our analysis clearly showed the involvement of antisense pancRNA in DNA demethylation, its involvement in changes of histone modifications to allow gene expression remains somewhat enigmatic. Multiple, but not all, histone modifications were correlated with gene expression status (Fig. 3). Therefore, we speculate that genomic DNA is primarily targeted by pancRNA, and the subsequent processes, including histone modification changes, later diverge according to the differential chromatin-modifying ability depending on the cell.

Possible Molecular Mechanisms of DNA Demethylation Triggered by pancRNA—The effect of the antisense pancRNA on DNA demethylation we observed here was quite different from that of reagents previously used, such as 5-azadeoxycytidine, which could not regulate this modification at specific loci but, rather, nonspecifically reacted with chromosomal components. We do not yet know the molecular mechanism inducing sequence-specific DNA demethylation in vertebrates. The RNA-directed DNA demethylation observed in this study was probably not dependent on cell cycle progression, because the sequence-specific changes due to pancRNA disturbance occurred even in differentiated PC12 cells whose survival was maintained with a minimum quantity of serum (1% fetal bovine serum and 0.5% horse serum). We counted the number of these PC12 cells at 30 min and 24 h after transfection and found no significant proliferation (2.1 ± 0.4 × 10^5 and 2.3 ± 0.6 × 10^5/dish, respectively). Several lines of evidence indicate that Gadd45 promotes deamination followed by base and nucleotide excision repair in the process of active DNA demethylation.
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**FIGURE 6. Association of the pancRNAs with the specific chromatin structures.** A, shown are ChIP analyses of the Vim 5’-flanking region in NRK and PC12 cells without and with forced expression of Vim-s or Vim-as or their shRNA in PC12 cells using antibodies to dimethylated H3K4 (H3K4me2) and trimethylated H3K9 (H3K9me3). ChIP enrichment was measured using real-time PCR and normalized by the input DNA. B, RT-PCR analyses of the Vim-as/...
Although there are no publicly available data on the transcriptomes of PC12 and NRK cells that were obtained by using RNA deep sequencing (RNA-Seq), there are some available RNA-Seq data on embryonic stem cells and their differentiated cells. For example, data on H1 human embryonic stem cells and their neurally differentiating cells are available at NCBI Gene Expression Omnibus (46). We analyzed a couple of poly(A)/H11001 RNA-enriched data sources with similar sequencing depth (1.2 × 10^8 reads for accession numbers SRR037167 and 1.3 × 10^8 reads for SRR037194) to be mapped on the hg19 version of the human genome using Tophat software (47) and found that thousands of genes bear transcripts derived from 5′-flanking sequences of RefSeq genes, as shown in Supplemental Table S7. There were 603 and 2074 candidate pancRNAs up-regulated more than 3-fold in H1 cells and their neurally differentiating cells, respectively, suggesting that differentiation of H1 cells into the neural cell fate may be associated with expression changes of a significant fraction of pancRNAs. Because RNA-Seq data that retains the information of transcriptional direction have recently emerged, it will be interesting to determine the exact orientation of these candidate pancRNAs.

Similarly, comparing mRNA/pancRNA expression profiles in proliferating and differentiated PC12 cells might also enable us to extract the gene set under the influence of epigenetic changes associated with cell differentiation in a relatively simple context. However, the model system of PC12 cell differentiation is regarded as reversible, in which removal of NGF from the culture medium causes neurite retraction (48). In fact, data about the mRNA expression levels of Map2b, Nefl, and Vim are available under the public microarray data deposit at NCBI Gene Expression Omnibus (accession numbers GSE4557 and GSE18016), and show no significant difference upon differentiation of PC12 cells, which is in line with previous reports (49, 50) as well as our data (Fig. 1). Moreover, overexpression of pancRNAs and their shRNAs did not induce obvious phenotypic abnormality in our experimental conditions. It is possible that PC12 cells have been largely directed into a unidirectional cell fate, and therefore, it may be relatively difficult to identify an epigenetically oriented pancRNA set that allows their differentiation.

**Conclusion**—In conclusion, we found that the 5′-flanking sequences of the genes examined here had the ability to be templates for pancRNAs independently of the protein-coding transcripts. Whether these pancRNAs are present or absent seems likely to be a deciding factor in assembling the transcription machinery for the expression/repression of the

![Figure 7. Transcription regulation mediated by pancRNA.](image)

A, B, and C, shown are the effects of ncRNA overexpression/knockdown on downstream gene expression of Map2b (A), Nefl (B), and Vim (C) in vitro. In each experiment the shRNA against the pancRNA corresponding to the examined gene was used. Expression levels determined by real-time PCR are the mean ± S.E. (n = 4) relative to that for mRNA in empty vector-transfected PC12 samples or for mRNA in NRK samples, where biased expressions were expected to be detected (see Fig. 1B). *, p < .01 versus empty-vector-transfected cell samples (Student's t test).

D, a model for the pancRNA-mediated epigenetic regulation of gene expression is shown. Ac and Me denote acetyl and methyl residues on histones, respectively. Open and filled lollipops denote unmethylated and methyl-cytosines, respectively. Dotted lines denote antisense pancRNAs. Upon antisense pancRNA expression, methylation at H3K9 is replaced by that at H3K4, accompanied by DNA demethylation. Antisense pancRNA expression is parallel to the expression of the associated gene, whereas whether sense pancRNA is involved in the epigenetic setting remains unknown.

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protein-coding transcripts. The orientation of the pancRNA is clearly important for the regulatory functions (if there are any) at the genomic level. The discovery of the antisense pancRNA-induced epigenetic modifications described here will be important for basic studies as well as applied biology and medicine.

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