Note

Visualization of Biallelic Expression of the Imprinted SNRPN Gene Induced by Inhibitors of DNA Methylation and Histone Deacetylation

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We have adapted a fluorescence in situ hybridization (FISH) method to detect nascent RNA molecules of the imprinted SNRPN gene at the initial transcription sites in nuclei of human HL60 and W138 cells. Simultaneous detection of RNA and DNA of SNRPN by FISH using the cosmid probe confirmed its monoallelic expression in these cell lines. Treatment of both cells by inhibitors of DNA methylation and histone deacetylation resulted in time-dependent increase of the cell population with the biallelic expression of SNRPN.

Key words: fluorescence in situ hybridization; genomic imprinting; SNRPN; allelic expression; histone acetylation

Fluorescence in situ hybridization (FISH) is a widely used technique in cell biology for detecting specific nucleic acid (DNA and RNA) sequences. RNA-FISH is a valuable technique for gene expression studies. The high spatial resolution properties allow visualization at the one-cell level of specific intracellular distribution patterns of gene transcripts, which can be correlated with sites of gene transcription or RNA processing and transport.1-3

Genomic imprinting is a marking process of the parental origin of the chromosome, resulting in allele-specific expression, chromatin structure, and replication.4,5 DNA methylation is involved in epigenetic marking of imprinted genes, and the methylated allele is usually transcriptionally silenced.6 Recent studies have revealed a close association of DNA methylation and histone deacetylation in transcriptional silencing.7 Involvement of these chromatin modifications in silencing of imprinted genes has been demonstrated by drug treatment of cells and a reverse transcription-polymerase chain reaction (RT-PCR) method.8-10 In this study, we adapted RNA-FISH to delineate monoallelic or biallelic expression states of human SNRPN (small nuclear ribonucleoprotein polypeptide N) imprinted gene. SNRPN is on human chromosome 15q11-q13, a region subject to genomic imprinting, and this is one candidate gene for Prader-Willi syndrome (PWS).11 RNA-FISH to detect the nascent RNA molecules provides a simple method and more direct information on the expression regulation at the transcriptional level than the RT-PCR method that detects the processed RNA. Here, we show that the expression pattern of the nascent SNRPN RNA changes from monoallelic to biallelic in a time-dependent manner upon treatment with DNA methylation and histone deacetylase inhibitors.

Human myeloid leukemia HL60 cells (Riken Cell Bank, Japan) were exponentially cultured in RPMI1640 with 10% fetal bovine serum (FBS) and fixed with paraformaldehyde onto a poly-d-lysine-coated coverslip (Matsunami, Japan) by a cytospin centrifuge. W138 fibroblast cells (Riken Cell Bank, Japan) were grown on a coverslip in DMEM with 10% FBS and fixed by the same procedure. Basic techniques for cell preparation followed the method described in the reference.12 We first examined whether the cosmid probe can detect only primary transcripts in nuclei. The cosmid c93 including SNRPN gene13 was labeled with biotin-dUTP, and the labeled probe was hybridized onto non-denatured nuclei and visualized by FITC-avidin (Roche Molecular Biochemicals, Germany). As shown in Figs. 1A and B, most nuclei had only one RNA signal (79% and 74% in HL60 and W138, respectively), suggesting that the SNRPN gene shows monoallelic expression in both cell lines through the cell cycle. We mainly observed the RNA signal in three patterns; a clear single spot (Fig. 1C), a fat or associated-twin spot (Fig. 1D), and two separated spots (Fig. 1E). To demonstrate whether these RNA signals reflect their initial transcription sites in the nuclei, we tried simultaneous detection of RNA and DNA of SNRPN by the method of Nutt et al.14 As shown in Figs. 1F-H, most RNA signals (green) were associated with DNA

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Abbreviations: FISH, fluorescence in situ hybridization; SNRPN, small nuclear ribonucleoprotein polypeptide N; RT-PCR, reverse transcription-polymerase chain reaction; PWS, Prader-Willi syndrome; FBS, fetal bovine serum; Igf2, insulin-like growth factor II gene; azaC, 5-aza-cytidine; NaB, sodium butyrate; TSA, trichostatin A
signals visualized by rhodamine (red). Since these RNA signals were not detected when the fixed cells were treated by RNase beforehand, these results confirm that the cosmid c93 probe can detect nascent RNA molecules of the imprinted SNRPN gene. Although SNRPN showed biallelic expression in a small number of cells (Fig. 1H: HL60, 5%; WI38, 5%), most nuclei revealed the patterns of Figs. 1F and 1G (HL60, 91%; WI38, 86%), suggesting the imprinted monoallelic expression of this gene in both cell lines.

We next examined the effects of the drugs, that inhibit DNA methylation and histone deacetylation, on the imprinted expression of the SNRPN gene. HL60 and WI38 cells were cultured in the presence of a DNA methylation inhibitor, 5-aza-cytidine (azaC, 100 nM for both cells), or histone deacetylase inhibitors, sodium butyrate (NaB, 1 mM and 5 mM, respectively) and trichostatin A (TSA, 33 nM and 50 nM, respectively). We treated HL60 cells with these concentrations according to the reference that had demonstrated the effects of these drugs on replication timing of imprinted genes. W138 cells were treated with higher concentrations of deacetylase inhibitors, because this cell type was less sensitive to these drugs. In the preliminary experiments, treatment of both cells with these drugs at a much higher concentration (500 nM for TSA and 10 mM for NaB) resulted in their growth inhibition and cell death (data not shown). We did not test any other concentration of azaC, because treatment with 100 nM had similar effects with deacetylase inhibitors. Other groups treated cells with a methylation inhibitor at 10 nM to 1 μM. Every 24 hours, cells were harvested and treated with RNA-FISH, and the expression patterns of SNRPN were classified as the number of the intranuclear RNA spots and chased for 120 hours. As shown HL60 cells in Figs. 2A-C as an example, in proportion to the exposure time of these drugs, cells with two signals increased, while those with one spot decreased. After 72 hours of treatment with these inhibitors, about a half of the nuclei had biallelic expression. Similar results were obtained in WI38 cells (data not shown). These results strongly suggest that DNA methylation and/or histone acetylation are important in regulation and maintenance of the monoallelic expression of the SNRPN gene. Longer exposure to the drugs increased the cells with more than three RNA signals (Fig. 2, 120 hours). This may be causally related to the appearance of abnormal cells during the drug treatment. When the cells were exposed to each drug for 24 hours and released by washing it out, the cells showing biallelic expression also increased in proportion to the decrease of the cells with one RNA signal (dotted lines in Fig. 2). Although their slopes in the figure are smaller, these results suggest that the memory of the drug treatments is maintained at least for a couple of cell cycles. Some cells still had a monoallelic expression pattern after 120 hours of treatment in all cases. This might be because demethylation or deacetylation was not perfect under our experimental conditions, which did not arrest the cell cycle. Other possibilities might be that the silent allele of these cells was activated, but its expression level is too low to detect by RNA-FISH, or that cells resistant to these drugs appeared during culture. Treatment of cells with these drugs at higher concentration results in their growth inhibition and cell death. Longer exposure increases abnormal cells. It will be difficult to induce biallelic expression efficiently in more cells in this experiment.

The effects of these drugs on the expression of the imprinted genes have been reported by using the RT-PCR method. Expression of the mouse insulin-like growth factor II gene (Igf2) in cells maternally disomic for chromosome 7 was derepressed by treatment with a DNA methylation inhibitor, but not

![Fig. 1. Visualization of Expression of SNRPN Gene by RNA-FISH.](image-url)
NaB.\textsuperscript{9} On the other hand, it has been demonstrated that treatment with histone deacetylase inhibitors activated the silent \textit{Igf2} allele in human and mouse fibroblast cells.\textsuperscript{9,10} Unlike \textit{Igf2}, derepression of the silent H19 allele required combined inhibition of DNA methylation and histone deacetylation.\textsuperscript{16} These studies done by the RT-PCR method suggest that the effects of these inhibitors depend on cell types and genes.

Despite the RNA-FISH method being very simple and convenient, it provides direct evidence reflecting the transcriptional regulation.\textsuperscript{17} A cosmid, (or other genomic) clone can be used to show monoaecytic expression of a given gene without any other information, although the RT-PCR method requires a heteromorphic sequence within the gene coding region. This enables us to analyze various types of cells. When a heteromorphic marker that distinguishes the parental origin of the alleles is available, RNA-FISH is much more powerful. In the case of human \textit{SNRPN}, a large polymorphic repeat in the pericentric region of human chromosome 15q is available as a marker to identify the parental alleles.\textsuperscript{18} This repeat may also work in the cells used here. A FISH-based replication assay may be an alternative way to identify the parental origin of the expressed allele.\textsuperscript{19} The paternal allele of \textit{SNRPN} replicates earlier than its maternal allele. In RNA-DNA-FISH, if the monoaecytic RNA signal is associated with the replicated DNA signal (a double dot) and the other DNA signal is unreplicated (a single dot), the transcription of the \textit{SNRPN} gene can be regarded as expression on the paternal allele. In fact, such a pattern was observed, although it was difficult to distinguish the duplicated DNA signal in three-dimensionally-fixed nuclei (data not shown).

We have detected the primary transcripts of \textit{SNRPN} by RNA-FISH, demonstrating derepression of this gene by the drug treatment of the cells. Using RT-PCR, Saito and Wada reported that treatment of PWS cells with a DNA methyltransferase inhibitor induced restoration of the \textit{SNRPN} expression on the silent allele.\textsuperscript{12} Our results are consistent with their observation, which was obtained by analyzing the processed RNA, indicating that expression of \textit{SNRPN} on the maternal allele is regulated by histone acetylation at a transcriptional level.

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