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A Unique Downregulation of h2-Calponin Gene Expression in Down Syndrome: a Possible Attenuation Mechanism for Fetal Survival by Methylation at the CpG Island in the Trisomic Chromosome 21

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To understand the effect of trisomic chromosome 21 on the cause of Down syndrome (DS), DNA methylation in the CpG island, which regulates the expression of adjacent genes, was investigated with the DNAs of chromosome 21 isolated from DS patients and their parents. A methylation-sensitive enzyme, *Bss*HII, was used to digest DNAs of chromosome 21, and the resulting DNA fragments were subjected to RLGS (restriction landmark genomic scanning). Surprisingly, the CpG island of the h2-calponin gene was shown to be specifically methylated by comparative studies with RLGS and Southern blot analysis. In association with this methylation, h2-calponin gene expression was attenuated to the normal level, although other genes in the DS region of chromosome 21 were expressed dose dependently at 1.5 times the normal level. These results and the high miscarriage rate associated with trisomy 21 embryos imply that the altered in vivo methylation that attenuates downstream gene expression, which is otherwise lethal, permits the generation of DS neonates. The h2-calponin gene detected by the RLGS procedure may be one such gene that is attenuated.

Chromosome dosage imbalance is toxic to autosomal chromosomes, and chromosomal aneuploidy in mammals interferes with normal developmental processes, often resulting in intrauterine death (3, 8, 15). In both mice and humans, monosomies resulting from dosage imbalance with any autosome are lethal in the early embryonic stage. All mouse embryos with autosomal trisomy die in utero, except for newborns having trisomies with chromosomes 16 and 19, which survive for only a few days after birth (8). In humans, almost all trisomies are lethal or allow survival for only several months. Trisomy 21 in human Down syndrome (DS) is the major exception, since it allows development to the adult stage (8, 15), which prompted us to choose DS as a model of human aneuploidy to investigate gene regulation.

DS patients usually show a set of complex abnormal phenotypes, called DS features, such as mental retardation, congenital heart disease, a characteristic set of facial and physical features, risk of leukemia and early onset of Alzheimer-like dementia (6, 17), and premature aging. Generally, all genes on the trisomic chromosome are assumed to be expressed in proportion to the gene dosage. The genes on chromosome 21 responsible for these clinical phenotypes must be expressed at the trisomic level in DS patients, even though this trisomic expression is not lethal to the DS embryo, since the embryo can develop into an adult. The genes identified in the DS region on chromosome 21, such as the genes for superoxide dismutase 1 (SOD-1) (7), liver-type phosphofructokinase (PFKL) (32), β-amyloid precursor protein (14), alpha-beta interferon receptor (9), CD18 (31), HMG14 (26), S-100β (16), and carbonyl reductase (20), are all expressed at the trisomic level.

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In this study, we investigated whether all of the other genes located on chromosome 21 in DS are indeed expressed in a dosage-dependent manner. We first studied DNA methylation in the CpG islands of chromosome 21, since methylation is correlated with the expression of downstream genes (2, 4, 21). High-resolution restriction genomic scanning (RLGS) analysis (11, 12) was applied to isolated chromosome 21 by using *Bss*HII, a methylation-sensitive enzyme that can distinguish the state of methylation at specific loci. Briefly, in the RLGS method originally developed by us for high-speed genomic DNA analysis, restriction sites in the genome are used as landmarks (25). Genomic DNA is digested with specific restriction enzymes and then subjected to isotopic end labeling. These end-labeled restriction fragments are separated by high-resolution two-dimensional electrophoresis and detected as discrete spots on an RLGS profile. Because each spot corresponds to a unique locus on the genome, the spots can be used as landmark signals. More than 2,000 loci on the genome can be analyzed in one procedure. Furthermore, most of the restriction landmarks located on the RLGS gel can be subsequently cloned and subjected to further analysis by the spot target cloning method (13, 24, 29).

We isolated chromosome 21 by flow cytometry from lymphocytes of DS families in which the profile of genomic methylation was reflected in vivo (18). Subsequently, we applied these sorted chromosome samples to RLGS to compare the methylation patterns between DS patients and their parents. Surprisingly, the intensity of 3 of 294 chromosome 21-specific *Bss*HII spots was significantly lower in the RLGS profiles of DS patients, whereas no new spots were found with the DS cells. One of the three spots was derived from the h2-calponin region at 21q11.1 (5), while another spot was from the ribosomal DNA. The remaining spot was not identified. Northern blot analysis showed that expression of the h2-calponin gene

was specifically attenuated to a level similar to the level of the genes on the diploid chromosome. These data show that the transcriptional activity of h2-calponin genes at 21q11.1 is strictly downregulated due to specific DNA methylation. This is the first report that describes a specific alteration in the profiles of DNA methylation in vivo in chromosome 21 from DS patients which apparently influences downstream gene transcription.

MATERIALS AND METHODS

Isolation of chromosome 21. Sekine (27) described the method used in this study for cultivation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells were isolated from 5 to 10 ml of heparinized venous blood from DS patients and their parents after centrifugation on Histopaque (Sigma). The complete culture medium used in primary culture contained RPMI 1640, 10% fetal bovine serum, and 700 U of recombinant human interleukin-2 (Shionogi) per ml. All cultures were incubated in an anti-CD3 antibody-coated flask at 36.7°C in a humidified atmosphere containing 5% CO_2. Cultivation continued for 12 days, and the cells were synchronized to the metaphase by extended culturing with 0.2 μg of colcemid per ml for 12 to 16 h. Metaphase cells were treated with polyamine and digitonin and stained with both Hoechst 33342 and chromomycin A3 in the presence of 10 mM $MgCl_2$ (10, 19). The stained chromosomes were sorted by an EPICS Elite ESP dual-laser flow sorter (Coulter) at a rate of about 4,000 objects per second under UV excitation at 338 and 457 nm. About 50 samples of chromosome 21 were collected for 1 s, and the purity was confirmed to be up to 90% by fluorescence in situ hybridization with a chromosome 21-specific probe and resorting.

DNA preparation and RLGS method. The DNA was extracted from 2×10^6 copies of sorted chromosome 21 isolated with a cell sorter. The sorted chromosomes were suspended in 50 μl of 20 mM Tris-HCl (pH 8.0)–50 mM EDTA–1% sodium dodecyl sulfate–1 mg of protease K per ml and incubated at 37°C for at least 5 h. After phenol extraction, 2-butanol concentration, and diethyl ether extraction, the aqueous phase was dialyzed against 1 liter of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA through a 0.05-μm-pore-size membrane disk filter (Millipore).

Okazaki et al. (25) described the precise protocols for the RLGS-M method (RLGS with a CpG methylation-sensitive enzyme). The method comprises eight steps. Briefly, (i) Chromosome 21 DNA was treated with 10 U of *Escherichia coli* DNA polymerase I (Takara) in the presence of 0.33 μM dGTPαS, 0.33 μM dCTPαS, 33 μM ddATPαS, and 33 μM ddTTPαS; (ii) the DNA was then digested with restriction enzyme A, *Bss*HII, which is sensitive to 5′ methylation of the cytosine residue; (iii) the cleavage ends were filled in with 1.3 U of Sequenase version 2.0 (U.S. Biochemical Co.) in the presence of 0.33 μM [α-^{32}P]dCTP and 0.33 μM [α-^{32}P]dGTP; (iv) the second digestion was carried out with 100 U of restriction enzyme B, *Eco*RV; (v) the DNA was electrophoresed in a 0.8% agarose gel; (vi) the disc of the gel containing the DNA was treated with 750 U of restriction enzyme C, *Mbo*I (Takara); (vii) the DNA was subjected to second-dimension polyacrylamide (6%) gel electrophoresis; and then (viii) the DNA was autoradiographed. Complete digestion of DNAs by restriction enzymes was always confirmed in a separate reaction under the same conditions, except that an equivalent amount of plasmid DNA was added. Spots which differed in the RLGS profiles among a DS family were selected visually.

Cloning of specific DNA fragments from RLGS spots and Southern blot analysis. Molecular cloning of genomic DNA fragments corresponding to RLGS-M spots was described previously (13, 24, 29). The *Bss*HII-*Mbo*I genomic DNA fragments were recovered from RLGS gels with a Gene Trapper, cloned with pBlueScript II KS(+) (Stratagene; modified by insertion of an *Asc*I linker at the *Hin*dIII site), and used for the following analysis. The Gene Trapper was provided originally by Japan Synthetic Rubber Co. Ltd. and is now available from AGENE Research Institute Co. Ltd. The total genomic DNAs used for Southern blot analysis were prepared from lymphocytes of DS patients and their parents. The DNA (5 μg) was digested with *Mbo*I and *Bss*HII, electrophoresed in a 3% NuSieve agarose gel (FMC), and subjected to Southern hybridization by using the cloned RLGS spot fragments as probes.

DNA sequencing and analysis. An ABI377 automated DNA sequencer was used for sequencing. A homology search (GenBank) was done with the BLASTN program (1).

cDNA cloning of calponin by RT-PCR. The PCR primers used for h2-calponin were 5′-GCCATGAGCTCCACGCAGTTC-3′ and 5′-GTCCATGGGGGGCA GGATATG-3′, and those used for h1-calponin were 5′-GAGGTCAAGAACA AGCTGGCCCAGAA and 5′-GGCAGAGTTGTAGTAGTTGTGCG-3′. Total RNA (5 μg) from human placenta was used for reverse transcription (RT) with a first-strand cDNA synthesis kit (Bethesda Research Laboratories). The PCR was done with 1 μl of the first-strand cDNA product: the reaction mixture included 1× PCR buffer, 5% dimethyl sulfoxide, 50 pmol of each PCR primer, each deoxynucleoside triphosphate at 200 mM, and 0.5 U of *Taq* EX polymerase (Takara) and was incubated in a thermal cycler (MJ Research). Each cycle consisted of incubation at 94°C for 1 min, 1 min of annealing at 64°C, and PCR for 1 min at 72°C. Before the first cycle, the samples were incubated at 95°C for 5 min, and after 28 cycles, they were further incubated at 72°C for 5 min. These PCR products were cloned with a TA cloning kit (Invitrogen) (GenBank accession numbers D86058 and D86059).

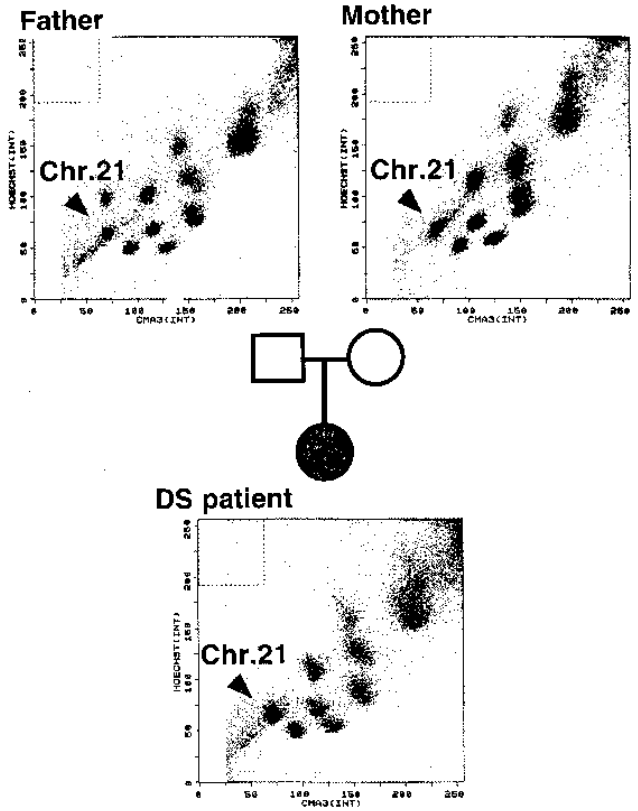

FIG. 1. Flow karyotypes of chromosomes from a family with a DS patient. Staining was done with Hoechst 33342 and chromomycin A3. Arrowheads indicate chromosome (Chr.) 21. Symbols: open, normal control; closed, DS patient; square, male; circle, female.

Northern blot analysis. Poly(A)$^+$ RNA (5 μg) from the lymphocytes of DS families was loaded onto 1.2% formaldehyde agarose gels and electrophoresed. After transfer to nylon filters, hybridization was carried out with Biodyn B (Pall BioSupport) by using the cloned cDNAs of h1-calponin and h2-calponin as probes. Calibration was performed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, PFKL cDNA (23), and an oligomer of SOD-1 (5′-ACT TCCAGCGTTTCCTGTCTTTGTACTTTCTTATTTCCACTTTGCCC AAGTCATCTGC-3′).

RESULTS

Isolation of chromosome 21 from DS families. In this study, we prepared the metaphase chromosomes from lymphocytes and sorted them. By using the fluorochromes Hoechst 33342 (with specificity for AT-rich DNA) and chromomycin A3 (with specificity for GC-rich DNA), chromosomes can be separated on a flow cytometer not only by DNA content but also by base pair ratio (10, 19). Figure 1 shows the sorting profiles of one of the DS families. Almost all chromosomes were separated clearly, except for chromosomes 9 to 12. The arrows indicate the fractions of human chromosome 21. As expected, the amount of chromosome 21 in the sorting profiles from a DS patient was approximately 1.5 times that of the parents. DNA extracted from the purified chromosome 21 was subjected to RLGS analysis.

Detection of an altered methylation profile in DS chromosome 21 by RLGS-M screening. We used RLGS-M to identify landmarks. This system has proved to be a powerful method for screening of genes that are regulated by DNA methylation

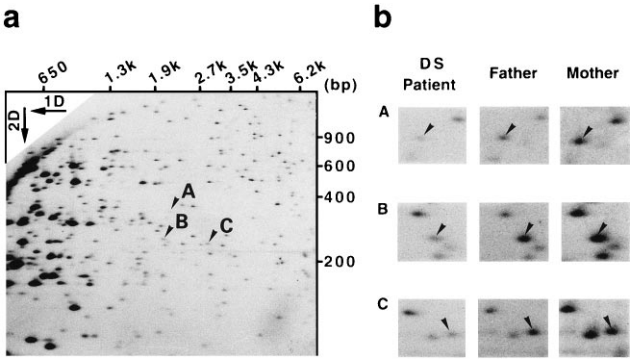

FIG. 2. Detection of altered RLGS-M spots in a DS family. (a) RLGS-M profile of a DS patient obtained with a *Bss*HII-*Eco*RV-*Mbo*I enzyme combination. Arrowheads A, B, and C indicate three spots whose intensities were lower in a DS patient than in the parents. (b) Parts of RLGS-M profiles, including the three spots in panel a (A, B, and C). 1D, first dimension; 2D, second dimension.

at upstream or intronic CpG islands (11, 12). In a recent protocol for RLGS, version 1.8 (25), 1.5 μg of total genomic DNA is required to produce an RLGS profile, leading to the calculation that at least 5×10^5 copies of chromosome 21 would be required to produce one RLGS profile. To elevate the quantitativeness of the method, we used the DNA isolated from about 2×10^6 copies of chromosome 21 to produce a single RLGS-M profile (Fig. 2a). The *Bss*HII-*Eco*RV-*Mbo*I enzyme combination was used to produce this RLGS-M profile, containing 294 spots specific to chromosome 21. The individual spots were then compared with the spots in the RLGS-M profiles of relatives of DS patients. When all of the spots were compared with the intensities of adjacent spots and then with the intensities of the spots from RLGS profiles of the parents, only three spots of the DS patient had a lower intensity (Fig. 2b). A similar alteration of these three spots was observed in two independent families with DS patients (data not shown). This change was not due to genetic variation between individuals, because polymorphic spots had been ruled out before assaying the DS patient by an initial comparison between the father and mother. Also, the alteration of the spot intensity does not seem to come from blast formation in vitro, because the RLGS profiles were completely reproducible regardless of cell culture. Although a very small population of the spots were changed after the blast formation induced by interleukin-2 and the immobilized anti-CD3 antibodies, the same spots were always altered, depending on the timing of the harvest after induction (18). We eliminated these spots from our assay. Thus, all of the RLGS spots used in this assay should reflect the in vivo condition of DNA methylation. Subsequently, we also compared the RLGS-M profiles of DS families obtained with total genomic DNA. However, none of the more than 3,000 spots examined was changed by the trisomic dosage, indicating that DNA methylation is rarely affected by the trisomic dosage of chromosome 21 (data not shown).

Characterization of reduced-intensity RLGS-M spots. The genomic DNA fragments contained in the three spots whose intensities decreased in the RLGS profiles of the DS patients were recovered and cloned into pBlueScript II KS(+) as described previously (13, 24, 29). The resulting clones were sequenced to identify the genes. The spot A clone (374 bp) contained the sequence of the h2-calponin gene located on 21q11 (5). It showed a perfect match to the 5′ region of the mouse h2-calponin gene, including the ATG initiation codon (Fig. 3), and is identical to the human h2-calponin cDNA recently described by Masuda et al. (22). The calponin gene, initially cloned from the chicken gizzard smooth muscle cDNA library, encodes a troponin-like polypeptide that may be involved in the regulation of thin filament activity (30) and is thought to regulate muscle contraction. Several murine and porcine calponin-related genes have also been reported (28). One of them, mouse h2-calponin, is expressed at a much lower level in smooth muscle cells than is h1-calponin. A recent study by Masuda et al. (22) indicated that the mouse h2-calponin gene is expressed in the hearts of both embryonic and adult mice. By contrast, our preliminary experiments showed that the mouse h2-calponin gene is differentially expressed in fetal and adult brains, the transcript existing only in the fetal brain (data not shown). The sequence of the spot B clone was identical to that of ribosomal DNA. The spot C clone, which carried the very GC-rich sequences, showed no significant homology to any known sequences.

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          10         20         30         40         50         60         70         80
gcgcgcggaagccggcgcgtcccaaccccgcaccagcccggcggtcccgtcccgtcccgtcctgtgcggccccgtcccg
BssHII

          90    1   100        110        120        130        140        150        160
ccgcccgcccgccAGCCATGAGCTCCACGCAGTTCAACAAGGGCCCCTCGTACGGGCTGTCGGCCGAGGTCAAGAACCGG - SpotA
----------cgCCATGAGCTCCACGCAGTTCAACAAGGGCCCCTCGTACGGGCTGTCGGCCGAGGTCAAGAACCGG - Mouse

 2       170        180        190        200        210        220        230        240
GTgagtgaggggcgccccttgtcccccgacagcgcggccgtcgcacgctccgaccggtgcaggagcccccaggcgcccc

          250        260        270        280        290        300        310        320
cggcacctcccgggcagggagcttggagacccggggcggaggggcccttgttctccctgacgcctggtgggggggatgtct

          330        340        350        360        370
gcgggcacccccctgaggctcccgcgaatcttggggagcacccaggtctgagatc
                                                    MboI
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FIG. 3. Sequence data of RLGS-M spot clone A. The nucleotide sequence of the spot A clone is shown, and the 374-bp *Bss*HII-*Mbo*I fragment is shown above it. The sequence matching the mouse h2-calponin exon, starting from its initiation codon, is shown below. Underlined sequences 1 and 2 are splicing consensus sequences.

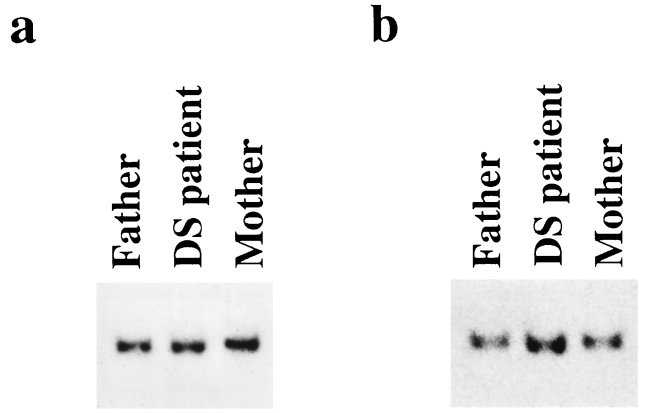

FIG. 4. Southern blot analysis of the correlation between DNA methylation at the *Bss*HII site and RLGS-M spot behavior. (a) Genomic DNAs from a DS family were digested with *Mbo*I and *Bss*HII and probed with a clone from RLGS-M spot A. (b) The filter shown in panel a was rehybridized with SOD-1, located on chromosome 21.

Confirmation of specific methylation on the CpG island of the DS h2-calponin gene by Southern blot analysis. To confirm the correlation between the changes in spot intensity and the changes in genomic copy number, we used Southern blot analysis with total genomic DNAs. Figure 4 shows the results for a family with a DS patient. The h2-calponin (spot A)-specific band from DS patient DNA has almost the same intensity as that from the parents, although the copy number in DS is 1.5 times as great. This apparent reduction of the *Bss*HII-*Mbo*I spot is consistent with the RLGS analysis data obtained previously with isolated chromosome 21 (Fig. 4a). Figure 4b shows a control experiment in which the SOD-1 gene located similarly on chromosome 21 was tested for gene dosage with the same filter. Here, as expected, the bands derived from the SOD-1 gene showed 1.5 times the control intensity specifically in the DS sample, consistent with the DS trisomic gene dosage. Thus, these data show that the intensity in the h2-calponin gene-specific spot in the RLGS profile was precisely correlated with the decreased intensity of the band detected by Southern blot analysis. These results led us to the conclusion that the *Bss*HII landmark on the h2-calponin gene is specifically methylated when chromosome 21 is trisomic.

Downregulation of human h2-calponin expression in DS cells. To test if expression of the h2-calponin gene is downregulated by this methylation in DS patients, we first cloned cDNA of human h2-calponin from human placental RNA by the RT-PCR method with 5′ and 3′ primers designed from the mouse cDNA sequence (see Materials and Methods). This cDNA clone was then used as a probe for Northern blot analysis to measure the level of expression. The cultured lymphocytes were used as the source of mRNA because it was known that the h2-calponin gene is expressed in lymphocyte cells (28) and the peripheral lymphocytes were the only mRNA source available from both patients and their parents. Figure 5a and b shows the data from a Northern blot analysis of lymphocytes from two independent patients with DS. The SOD-1 and PFKL genes on chromosome 21 were found to be expressed at higher levels in DS patients than in normal controls, consistent with the increased gene dosage in DS. However, the h2-calponin gene in DS patients was transcribed at the same level as in the normal controls, indicating that expression of the h2-calponin gene is attenuated to the normal level typical of the genes in other, disomic chromosomes. The observed differences in h2-calponin expression are real because in the same samples the level of GAPDH expression was equivalent. Here, the GAPDH gene in chromosome 12p13 was used as a control to assess the extent of gene expression, and the amount of mRNA loaded in each lane was strictly adjusted by the control gene. Also, we excluded the possibility that the h2-calponin signal in the Northern blot was derived from cross-hybridization with h1-calponin mRNA, since there was no h1-calponin mRNA in lymphocytes as determined by testing for hybridization with h1-calponin cDNA. These data show that expression of the h2-calponin gene is specifically attenuated to disomic levels in DS cells by a mechanism that is not understood. This attenuation seems to be correlated with methylation at a specific site on a CpG island.

DISCUSSION

To screen the methylation occurring on the CpG islands of DS patient DNAs, we analyzed the sorted chromosome 21 by RLGS with a methylation-sensitive restriction enzyme. In this RLGS screening, the *Bss*HII sites were used as landmarks. Bird (2) and Cedar (4) reported that GC-rich restriction sites, such as *Bss*HII in CpG islands, have an important *cis*-acting role in the transcriptional regulation of downstream genes by methylation. About 74% of all *Bss*HII sites are on CpG islands (21), suggesting that about 220 CpG-associated genes are in chromosome 21 (about 220 of the 294 *Bss*HII landmarks obtained in this study were on CpG islands). This means that we scanned about one-fifth (220 genes) of the total genes (~1,000 genes) in chromosome 21 in the 100- through 200-kbp interval.

Three distinct spots of reduced intensity were detected in the RLGS profile of the DS patient, and they were analyzed extensively by spot cloning, sequencing, cDNA cloning, and

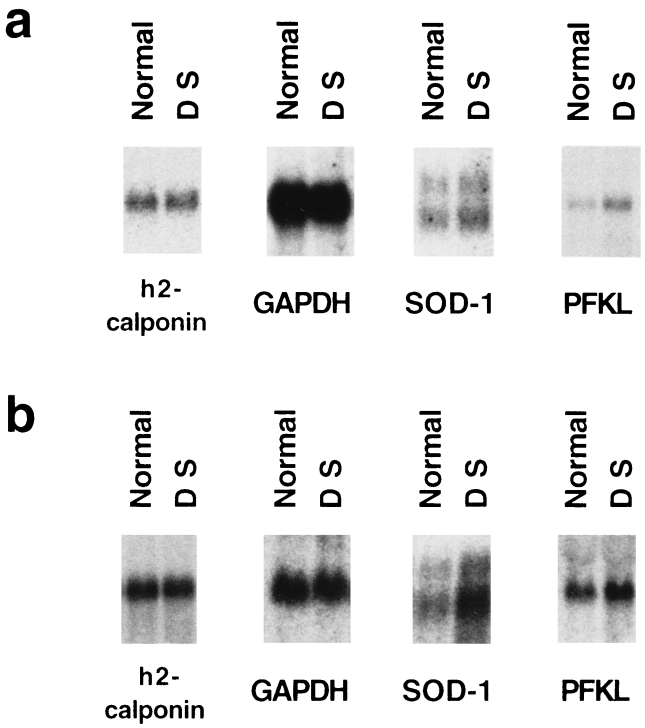

FIG. 5. Northern blot analysis of the correlation between transcript expression and RLGS-M spot behavior. (a) Poly(A)$^+$ RNAs from lymphocytes of a DS patient were probed with h2-calponin, GAPDH, SOD-1, and PFKL. (b) Another Northern blot analysis was done with h2-calponin from another DS patient.

Southern and Northern blotting. Two of the spots were derived from the h2-calponin gene and the ribosomal DNA gene on chromosomes 21q11 and 21p, respectively, and the remaining spot was from an unknown GC-rich sequence in chromosome 21. We found that expression of the h2-calponin gene in DS was attenuated to the same level as that of normal individuals with diploid chromosomes, correlating with DNA methylation at the CpG island, perhaps in one of its three alleles. In other words, the transcription of the h2-calponin gene appears to be downregulated by DNA methylation to attenuate the effect of the trisomic situation.

This study provided the first data concerning the in vivo DNA methylation status of CpG islands in chromosome 21 trisomy. In almost all CpG islands, methylation is thought to repress downstream gene transcription (2, 4). Theoretically, four transcriptional states can be envisaged for the abnormal trisomic state.

The first state is 1.5 times the normal level of expression, in proportion to the trisomic chromosome dosage in DS. Our experiment with two genes on DS chromosome 21, SOD-1 and PFKL, indeed showed that they are expressed at about 1.5 times the level of the genes on other, disomic chromosomes. In addition, the genes located in the DS region on chromosome 21, such as SOD-1 (7), PFKL (32), β-amyloid precursor protein (14), alpha-beta interferon receptor (9), CD18 (31), HMG14 (26), S-100β (16), and carbonyl reductase (20), are expressed at this 1.5-fold trisomic level. Clearly, expression of these genes at this level is not lethal, because these patients survived to the adult stage.

The second state is a specific downregulation of gene expression in which transcription of particular genes is attenuated to the normal, disomic level to evade the effect of the trisomic chromosome dosage. Such genes cause serious defects at the developmental stage, as many of embryos (70 to 95%) with trisomy 21 die in utero (3, 14). The h2-calponin gene described in this report may be one of the genes that fall into this category, as it appears to be regulated by DNA methylation. Whether the genes that are modulated by methylation are common to all DS patients or vary depending on the genetic background of each DS patient is of great interest.

The third state is a special induction of transcription by the trisomic dosage. Such genes may not usually be transcribed in normal cells but are induced by the effect of the trisomic dosage. However, no genes of this class have been described.

The fourth state is specific repression of transcription in particular genes by the trisomic dosage effect, as opposed to the third state. Assuming that the transcriptional state reflects DNA methylation, RLGS spots from genes in the third and fourth states would newly emerge or completely disappear, respectively, in trisomic cells. However, we did not detect any such spots belonging to these states.

The primary abnormality in DS cells is considered to be a change in the transcriptional level caused by the increased dosage of chromosome 21. The next problem would be if this aberrant transcription affected the DNA methylation of other chromosomes, as a secondary effect, and ultimately affected the transcription of downstream genes. To examine this possibility, we compared the entire genomic methylation patterns of the lymphocytes from a DS family by RLGS by using *Not*I and *Bss*HII as restriction landmarks but found no changes in intensity among over 3,000 spots from DS genomic DNAs (data not shown). These results suggest that the methylation profile of other chromosomes was unaffected by the trisomic dosage of chromosome 21.

The RLGS-M method provided a powerful tool for screening of the methylation status of a large number of loci. With chromosome 21 isolated by sorting, this method allowed us to investigate changes in genes that might influence the development of DS. A change in the methylation status of a *Bss*HII site in the h2-calponin gene is intriguing with respect to the development of DS patients. Although this particular methylation may not be a cause of DS, it could be an important DNA modulation that permits DS cells to survive the embryonic and neonatal stages of development owing to a specific transcriptional attenuation. The biological role of the h2-calponin gene and the reason(s) behind its obligatory downregulation in DS cells remain to be studied in the light of this unique result obtained with this new RLGS procedure.

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J.K. and H.Y. contributed equally to this work.

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