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Methylation profiles of genomic DNA of mouse developmental brain detected by restriction landmark genomic scanning (RLGS) method

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ABSTRACT

Restriction landmark genomic scanning using methylation-sensitive endonucleases (RLGS-M) is a newly developed powerful method for systematic detection of DNA methylation. Using this method, we scanned mouse brain genomic DNAs from various developmental stages to detect the transcriptionally active regions. This approach is based on the assumption that CpG methylation, particularly of CpG islands, might be associated with gene transcriptional regulation. Genomic DNAs were prepared from telencephalons of 9.5-, 13.5- and 16.5-day embryos, 1- and 10-day neonates and adults, followed by subjecting them to RLGS-M and comparing their patterns with each other or with that of the adult liver. We used *NotI* as a methylation-sensitive restriction enzyme and surveyed the methylation states of 2,600 *NotI* sites, almost of which should correspond to gene loci. Although almost all RLGS spots (98%) were present constantly at every developmental stages, only a few percent of spots reproducibly appeared and disappeared at different developmental stages of the brain (44 spots, 1.7 %) and some were tissue-specific (10 spots, 0.7 %). These data suggest that DNA methylation associated with gene transcription is a well-programmed event during the central nervous system (CNS) development. Thus, RLGS-M can offer a means for detecting systematically the genes in which the state of DNA methylation changes during development of the higher organism.

INTRODUCTION

In vertebrate animals, the cytosine moieties at CpG palindromic dinucleotide sequences are heavily methylated at the 5-position (1), generating tissue-specific and embryonic development-specific methylation patterns (2, 3). DNA methylation is probably crucial for embryonic development, since targeted mutation of the DNA methyltransferase gene resulted in embryonic lethality (4). The state of methylation in some genes, such as ApoAI (5), α1(I) collagen (6), γ-crystalline (7) and γ-glutamyl transpeptidase (8), change during the development of the animal and reversely correlate with their gene activities. Although the exact function of DNA methylation is not known, most of the evidence suggests that a role of DNA methylation in transcriptional control is associated with gene inactivation and vice versa (9). Therefore, the information on the methylation status of each gene will provide useful clues for the detection of active genes in various developmental stages.

To estimate the states of cellular differentiation in greater detail, it is necessary to detect active genes systematically covering as many gene loci as possible. The methods available so far, such as Northern blotting, mRNA subtraction (10–12) and the large-scale cDNA sequencing methods (13) have a limited use for the systematic detection of active genes. All of these conventional methods depend on transcribed messages, and have problems in detecting rarely expressed messages. The key genes responsible for the determination of cellular differentiation might express in very low levels in the particular developmental periods. Thus, we tried to detect the methylation states of genomic DNA instead of transcribed messages, since DNA methylation, in most

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instances, reflects the switching-on and -off of gene transcription, and does not depend on the amount of each message.

Recently, a novel approach has been developed for the systematic detection of genomic DNA methylation that is based on Restriction Landmark Genomic Scanning using methylation-sensitive endonuclease (RLGS-M) (14, 15, 16). In RLGS-M, the restriction sites recognized by methylation-sensitive endonucleases, such as *Not*I, are systematically detected as spots on two-dimensional RLGS profiles. Based on the assumption that the methylation status of CpG island correlates with gene expression, a large amount of loci can be screened at one time by the RLGS-M method, regardless of the amount of gene expression. In this study, we applied RLGS-M to detect methylation profiles of genomic DNAs from mouse telencephalons at various developmental stages, and found that out of more than two thousand *Not*I landmarks examined, a small proportion of them showed well-reproduced changes in methylation state depending on the developmental stages, suggesting that methylation states are programmed genetically throughout the development.

MATERIALS AND METHODS

Tissue preparation and genomic DNA isolation

All tissues were prepared from C3H/HeN inbred mice purchased from Charles River Japan Inc. The telencephalons at the ages of embryonal day 9.5 (E9.5), E13.5, E16.5 were resected microscopically from 30, 18 and 42 embryos, respectively. Furthermore, the telencephalon of E16.5 was separated by micromanipulation into two parts, inner and outer layers which consist mainly of neuronal precursor cells (ventricular zones) and differentiated neurons (cortical plates), respectively. Also, the telencephalons at the ages of postnatal day 1 (P1), P10 and 8 week-old were prepared from 8, 8 and 3 male mice, respectively. From the same mice at 8 week-old, livers were also prepared. Dissociated tissues were pooled and immediately frozen in the liquid nitrogen, and genomic DNA preparations were performed as described previously (14).

Restriction landmark genomic scanning method using methylation-sensitive restriction enzyme (RLGS-M)

Details of the RLGS-M method are described in reference (15). Briefly, RLGS-M consisted of the following steps (Fig. 1A): (a) the genomic DNA was treated with 10 units of *E.coli* DNA polymerase I (TAKARA) in the presence of 0.33 μM dGTPαS, 0.33 μM [α-^{32}P]dCTP and 0.33 μM [α-^{32}P]dGTP; (d) the second digestion was performed with 100 units of restriction enzyme B, either *Bam*HI or *Eco* RV; (e) the first fractionation was carried out in 1% agarose gel electrophoresis; (f) the strip of the first dimensional gel was treated with 1500 units of restriction enzyme C , *Hin*fI; (g) the second fractionation was done in the second-dimensional acrylamide gel (6%) electrophoresis; and (h) autoradiography was performed. Complete digestion of genomic DNA was always monitored in another reaction tube with the addition of test plasmid DNA.

Cloning of DNA fragment from the RLGS-M spot

DNA fragments corresponding to RLGS-M spots were cloned following the method of Hirotsune *et al.* (17). Briefly, 500 μg of genomic DNA from the cerebrum of 8 week-old C3H/HeN mice was sequentially digested by *Not*I and *Bam*HI. DNA fragments with *Not*I ends were collected using restriction trapper (18), the latex beads, to which hair-pin looped oligolinkers with *Not*I-cleaved sites at their ends were connected covalently (kindly provided by Nippon Roche Research Center). Fragment collected

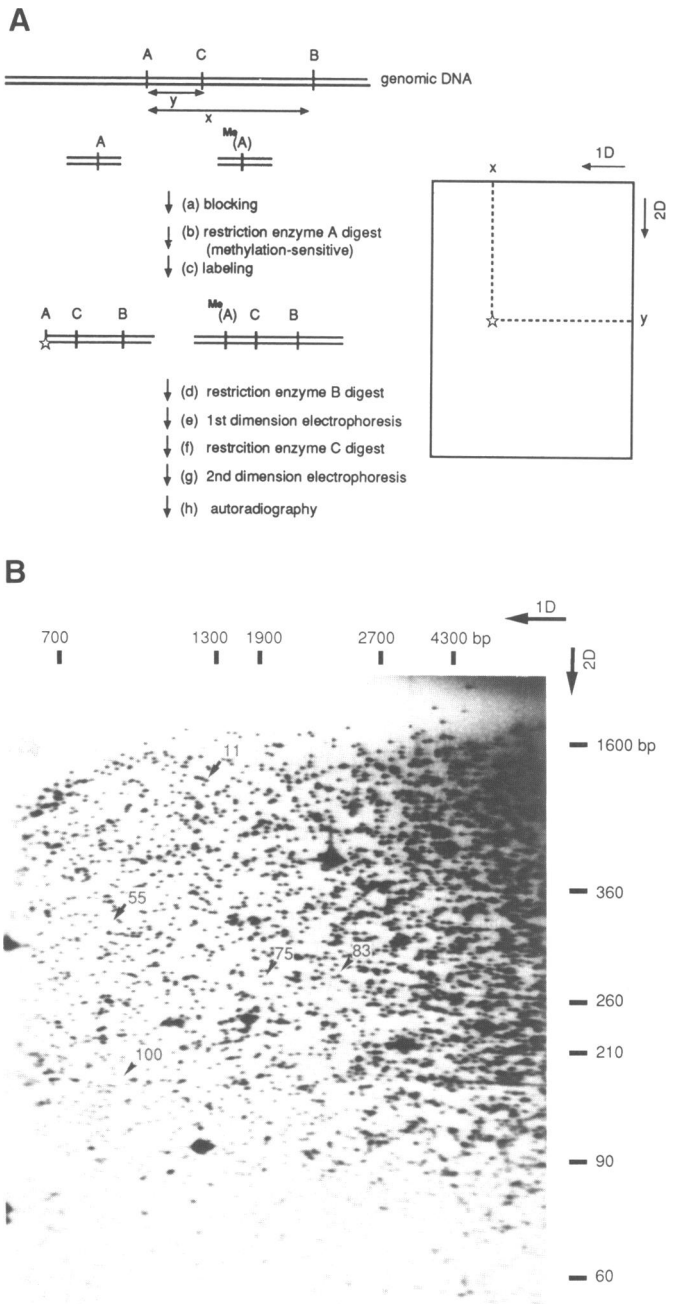

Figure 1. The RLGS-M procedure and its typical profile. **A;** Outline of RLGS-M procedure (See text and a reference (15)). A, B, and C; sites for restriction enzymes A, B, and C, respectively. ^{Me}(A); methylated sites for restriction enzyme A. x, y; distances from site A to B and C, respectively. **B;** RLGS-M pattern of mouse embryonic telencephalon. The genomic DNA was prepared from the mouse telencephalons of embryonal day 9.5, and applied to RLGS-M. *Not*I, *Bam*HI and *Hin*fI were used as the three kinds of restriction enzymes A, B and C, respectively (Fig. 1A). Three developmental stage-dependent RLGS-M spots (75, 83 and 100) and two tissue-specific spots (11 and 55) were indicated by arrowheads and arrows, respectively. Approximate sizes of fractionation were shown. 1D and 2D shows directions of first and second electrophoresis, respectively.

was separated to two aliquots. One quarter was labeled at its *Not*I 5'-protruding ends with [α-^{32}P]dCTP in the presence of dGTPαS by Sequenase Ver 2.0^{TM} . Labeled and unlabeled *Not*I fragments were mixed and subjected to first dimensional electrophoresis and subsequent steps in RLGS-M protocol (See above) to produce autoradiograms. With reference to the autoradiogram, the targeted RLGS spot, Spot 11 (See Fig. 1B), on dried acrylamide gel were punched out, from which unlabeled *Not*I fragments were eluted and cloned (NB11).

Using genomic clone NB11 covering genomic DNA from the *Not*I site to *Hin*fI as a probe, a genomic clone, pCL11, carrying the DNA fragment from *Not*I site to *Bam*HI , was isolated from a genomic library of adult C3H/HeN liver. The library was constructed from *Sau*3AI-partially digested DNAs with a phage vector, λDASHII (Stratagene) and packaging extracts, GigapackII (Stratagene).

Southern blot analysis

Restriction enzyme-digested DNAs (4 μg genomic DNAs or 40 pg phage clone), were electrophoresed in an 0.8% agarose gel and blotted onto Hybond-N filters (Amersham), following manufacturer's protocol. Southern hybridization was performed with α-^{32}P]dCTP-labeled DNA fragment as a probe in the presence of sonicated mouse genomic DNA (200 μg/ml) to prevent hybridizations of repetitive sequences.

RESULTS

Typical patterns of RLGS-M of the genomic DNA from mouse brains

The genomic DNAs were prepared from the telencephalons of C3H/HeN mice at various developmental stages (E9.5, E13.5, E16.5, P1, P10, and 8-week old). The telencephalons at E16.5 were further separated to the neuronal precursor-rich inner layers containing ventricular zones and the differentiated neuron-rich outer layers containing cortical plates. The liver from 8-week old mice was also used as a control to test tissue-specificity.

The procedure of RLGS-M is outlined in Fig. 1A. In the RLGS-M profile, methylated DNAs escape the digestion of *Not*I, resulting in disappearance of a corresponding spot on the autoradiogram. This inference is confirmed below. The typical RLGS-M profile of genomic DNA from telencephalons of E9.5 obtained using the restriction enzymes *Not*I, *Bam*HI and *Hin*fI is shown in Fig. 1B. In this figure more than 2000 spots can be observed, each of which corresponds to the *Not*I landmark in the genome. We surveyed 1500 spots in the RLGS-M profile obtained using *Not*I, *Bam*HI and *Hin*fI, and another 1100 spots from the profile obtained using a different set of restriction enzymes, *Not*I, *Eco*RV and *Hin*fI. Among a total of 2600 spots surveyed, 44 spots (1.7 %) changed depending on developmental stages and several others did tissue-specifically, although almost all spots were constantly seen regardless of the dramatic morphological change of neuron precursor cells during the telencephalon development.

Developmental changes of RLGS-M patterns for the telencephalons

The developmental patterns of three representative RLGS-M spots, 75, 83 and 100 are shown in detail in Fig. 2; their locations in the RLGS-M profile were indicated by arrowheads in Fig. 1B. Spot 75 appeared from E16.5 through adult. Interestingly, this spot was seen at the outer layer of the telencephalon on E16.5 but not at the inner layer of telencephalon on the same developmental day. This transition of Spot75 coincides with neuron differentiation. Spot 83 was not detected on E9.5, but seen after the initial stage of the differentiation from precursor cell to neuron (E13.5 through adult). Whereas, Spot 100 was observed in the adult telencephalon alone (Fig. 2). A total of 44 spots out of 2600 spots surveyed showed alteration developmentally, and they are classified in Fig. 3. The 13 spots appeared from E13.5 through adult occupied the largest population among the spots that changed during development and one to six others belonged to minor populations. The alterations in RLGS-M patterns during the brain development were reproduced quite well in repeated examinations, indicating that DNA methylation is well-programmed by genetic information (programmed methylation).

Tissue-specific alterations of RLGS-M patterns

Spots 11 and 55 indicated with the arrows in Fig. 1B are tissue-specific. Spot 11 was seen in telencephalon at every stages regardless of the developmental stages, but not in liver from 8 week-old mice, and Spot 55 vice versa. We could find only ten spots showing this type of tissue-specific pattern among the total 2600 spots surveyed. Seven out of ten spots were brain-specific and three were liver-specific (Data not shown).

Correspondence of the RLGS-M spots with DNA methylation

To examine whether the presence and absence of spots respectively reflect the demethylation and methylation of *Not*I landmarks, we analyzed Spot 11 (Fig. 1B) as a sample of those, which varied in intensity during the tissue-specific development (brain and liver). Spot 11 is one of the spots which shows a brain-specific appearance and it is expected that this *Not*I landmark is demethylated in the brain tissue. For the analysis, the DNA clone (Clone NB11) corresponding to Spot 11 was isolated from the punched out gel by the method for RLGS spot targeting (17) and cloned. Using NB11 as a probe, the genomic clone, pCL11, including the region of NB11 and its flank, was also isolated from the phage library of the liver, and we carried out Southern blot analysis of genomic DNAs of the adult telencephalon and liver. The result is shown in Fig. 4. The telencephalon DNA digested with *Bam*HI and *Not*I produced a 1.3 kb band. On the other hand, the liver produced a 2.2 kb band, although the pCL11, a genomic clone from the liver, generated a 1.3 kb band. These data clearly indicate that this *Not*I landmark is demethylated in the brain, and methylated in the liver, but not mutated. Thus, the appearance and disappearance of RLGS-M spots are associated with the methylation condition.

DISCUSSION

We systematically surveyed the DNA methylation states of more than two thousand *Not*I sites in various developmental stages of the telencephalon using the RLGS-M method, and found that DNA methylation states were controlled or programmed throughout the development, the so called programmed methylation.

To describe the cellular differentiation in greater detail, several lines of efforts have been made to develop new methods such as Northern analysis, large-scale cDNA sequencing (13) and

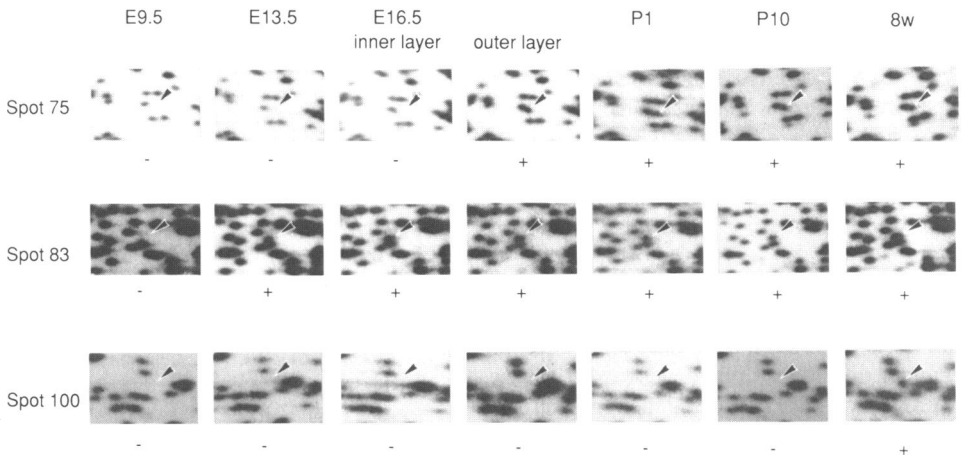

Figure 2. Detection of RLGS-M spots that change during the mouse telencephalon development. Three spots in Fig. 1B (75, 83 and 100) were followed through the developmental stages indicated. The inner and outer layers on E16.5 consist mainly of neuronal precursor cells and differentiated neurons, respectively. (−) and (+) below each photograph represent disappearance and appearance of RLGS-M spots, respectively.

E9.5	E13.5	E16.5 inner	E16.5 outer	P1	P10	8w	No. of spots
□	□	□	□	□	□	■	2
□	□	□	□	□	■	■	1
□	□	□	□	■	■	■	3
□	□	□	■	■	■	■	2
□	□	■	■	■	■	■	2
□	■	■	■	■	■	■	13
■	■	■	■	■	■	□	3
■	■	■	■	■	□	□	1
■	□	□	□	□	□	□	1
■	□	□	□	□	■	■	3
□	□	■	□	■	■	■	2
□	□	■	■	ND	ND	□	2
□	■	■	■	ND	ND	□	4
■	■	■	■	■	□	■	6
total spots 2,600							44 (1.7%)

Figure 3. Classification of RLGS-M spots that changed during the telencephalon development. White and black squares represent disappearance and appearance, respectively. ND; not done.

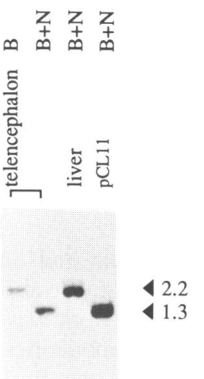

Figure 4. Southern blot analysis on the correlation between DNA methylation at the *Not*I site and the behavior of RLGS-M spot. Genomic DNAs from the adult telencephalon and liver were probed with NB11, which was cloned from RLGS-M Spot 11. Spot 11 was seen in the adult brain, not in the liver (See text and Fig. 1B). The genomic clone, pCL11, was also analyzed. pCL11 was isolated from the phage library of the adult liver, and included the region of NB11 and its flanking region. Four mg of genomic DNAs from the adult liver or brain, or 40 pg of pCL11 was digested with either *Bam*HI (B) alone or *Bam*HI+*Not*I (B+N), followed by hybridization with NB11 fragment (1100 bp) as a probe, to investigate the methylation states. Sizes of fragments were shown in kb.

reassociation method (11, 12). All these methods that depend on transcribed messages, have difficulties in detecting the usually small amount of gene messages which are responsible for the determination of cellular differentiation. Thus, it is greatly advantageous to detect DNA methylation that reflects the switching-on and -off of each gene, regardless of the amount of transcripts. However, little is known about methylation profiles of whole genomic DNA at various developmental stages because the methods currently available are very limited. Recently, we have developed a novel and powerful method (RLGS) for genomic DNA scanning with restriction sites as landmarks (14) and reported its various applications (15) such as detection of amplified DNA in cancer (19), genetic mapping (20, 21) and tissue-specific methylation patterns (15). In this study, we applied this RLGS to systematic detection of DNA methylation profiles during the telencephalon development, using a methylation-sensitive restriction enzyme, *Not*I.

In the RLGS-M method, methylated DNA which escapes the digestion of restriction enzymes is detected as a disappearance of a RLGS-M spot. Actually, the Southern blot analysis of the materials, all of which were prepared from the genetically homogeneous inbred strain, C3H/HeN, showed that the disappearance of Spot 11 in the liver was resulted from methylation in the tissue (Fig. 4). In the case of the other spots showing tissue-specific or developmental changes in intensities, we cannot exclude completely the possibility that mutation or recombination might have caused changes in spot appearance. These seem, however, quite unlikely, since the use of a CpG methylation-resistant restriction enzyme, *Pac*I, instead of the methylation-sensitive enzyme *Not*I, generated no difference in RLGS profiles among developmental stages (data not shown).

Recombination, which takes place frequently in immunogloblin genes, occurs rarely in other mammalian genomes.

Most (89%) of the *Not*I sites were calculated to be present in CpG islands and considered as useful markers for detection of transcriptional units in genomes (22). Actually, we observed earlier that almost (at least 86%) all of the unmethylated *Not*I sites *in vivo* were associated with transcriptional units (manuscript in preparation). It is also well known that CpG islands methylation correlates positively with repression of genes: for example, in the inactivation of the X chromosome in female organisms and α-actin gene as a transgene (2).

Although almost all spots were constantly seen, only a minor population (1.7%) changed during the brain development. These RLGS-M profiles were very reproducible, depending on the developmental stages and tissues. These findings strongly suggest that DNA methylation, possibly CpG island methylation, and its gene transcription, are strictly regulated by genetical information. This is the first report which indicates the percentage of genes in which methylation states change during the mammalian development, based on the screening of such a large amount of loci. The RLGS-M landmarks in which methylation states change during the brain development may correspond to the genes responsible for brain differentiation. The spot cloning method (17) that we have recently developed will be available to isolate the target genes showing programmed methylation discovered in this study.

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REFERENCES

1. Bird, A.P., (1986) *Nature*, **321**, 209–213.
2. Cedar, H. and Razin, A., (1990) *Biochimica et Biophysica Acta*, **1049**, 1–8.
3. Frank, D., Keshet, I., Shani, M., Levine, A., Razin, A. and Cedar, H., (1991) *Nature*, **351**, 239–241.
4. Li, E., Bestor, T.H. and Jaenisch, R., (1992) *Cell*, **69**, 915–926.
5. Shemer, R., Kafri, T., O'Connell, A., Eisenberg, S., Breslow, J.L. and Razin, A., (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 11300–11304.
6. Rhodes, K. and Breindl, M., (1992) *Gene Expression*, **2**, 59–69.
7. Peek, R., Niessen, R.W.L.M., Schoenmakers, J.G.G. and Lubsen, N.H., (1991) *Nucleic Acids Res.*, **19**, 77–83.
8. Baik, J, Griffiths, S., Giuili, G., Manson, M., Siegrist, S. and Guellaen, G., (1991) *Carcinogenesis*, **12**, 1035–1039.
9. Bird, A., (1992) *Cell*, **70**, 5–8.
10. Hedrick, S.M., Cohen, D.I., Nielsen, E.A. and Davis, M.M., (1984) *Nature*, **308**, 149–153.
11. Ko, M.S.H., (1990) *Nucl. Acids Res.*, **18**, 5705–5711.
12. Patanjali, S.R., Parimoo, S. and Weissman, S.M., (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 1943–1947.
13. Okubo, K., Hori, N., Matoba, R., Niiyama, T., Fukushima, A., Kojima, Y. and Matsubara, K., (1992) *Nature Genetics*, **2**, 173–179.
14. Hatada, I., Hayashizaki, Y., Hirotsune, S., Komatsubara, H. and Mukai, T., (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 9523–9527.
15. Hayashizaki, Y., Hirotsune, S., Okazaki, Y., Hatada, I., Shibata, H., Kawai, J., Hirose, K., Watanabe, S., Fushiki, S., Wada, S., Sugimoto, T., Kobayakawa, K., Kawara, T., Katsuki, M., Shibuya, T. and Mukai, T., (1993) *Electrophoresis*, **14**, 251–258.
16. Hayashizaki, Y., Shibata, H., Hirotsune, S., Sugino, H., Okazaki, Y., Sasaki, N., Hirose, K., Imoto, H., Okuizumi, H., Muramatsu, M., Komatsubara, H., Shiroishi, T., Moriwaki, K., Katsuki, M., Hatano, N., Sasaki, H., Ueda, T., Mise, N., Takagi, N., Plass, C. and Chapman, V.M., (in press) *Nature Genetics*.
17. Hirotsune, S., Shibata, H., Okazaki, Y., Sugino, H., Imoto, H., Sasaki, N., Hirose, K., Okuizumi, H., Muramatsu, M., Plass, C., Chapman, V.M., Miyamoto, C., Tamatsukuri, S., Furuichi, Y. and Hayashizaki, Y., (1993) *Biochem. Biophys. Res. Commun.*, **194**, 1406–1412.
18. Hayashizaki, Y., Hirotsune, S., Hatada, I., Tamatsukuri, S., Miyamoto, C., Furuichi, Y. and Mukai, T., (1992) *Genomics*, **14**, 733–739.
19. Hirotsune, S., Hatada, I., Komatsubara, H., Nagai, H., Kuma, K., Kobayakawa, K., Kawara, T., Nakagawara, A., Fujii, K., Mukai, T. and Hayashizaki, Y., (1992) *Cancer Res.*, **52**, 3642–3647.
20. Hayashizaki, Y., Hatada, I., Hirotsune, S., Okazaki, Y., Komatsubara, H., Mukai, T., Kawai, J., Hirasawa, T., Nishitani, Y., Watanabe, S., Manly, K., Elliott, R., Taylor, B. and Chapman, V.M., (1992) *CSH meeting on Genome Mapping and Sequencing, Abstract*, 120.
21. Chapman, V.M., Hirotsune, S., Okazaki, Y., Hatada, I., Mukai, T., Kawai, J., Hirasawa, T., Nishitani, Y., Watanabe, S., Shiroishi, T., Moriwaki, K., Matsuda, Y., Manly, K., Elliott, R. and Hayashizaki, Y., (1992) *CSH Meeting on Genome Mapping and Sequencing, Abstract*, 247.
22. Lindsay, S. and Bird, A.P., (1987) *Nature*, **327**, 336–338.