Studies on Intranuclear Arrangement of Human Chromosome 12 and Relative Locations of Chromosome 15 Centromeres and of *SNRPN* Genes in Interphase Nuclei of HL60 Cells

Masahiro Nogami

Doctor of Philosophy

Department of Genetics
School of Life Science
The Graduate University for Advanced Studies

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Contents

Chapter I

Summary

Two sets of approximately $3x10^9$ bp of human DNA are compacted within a small volume of the cell nucleus. A number of genes on the genome express timely and play each function under precise regulation. The whole genome duplicates in numerous numbers of human cells under the perfect order. To accomplish such sophisticated biological processes, the mammalian genome is organized by various levels of domain structures that essentially associate with biological functions. An understanding of chromosome organization and its relation to intranuclear arrangement is important for models of nuclear structure and function. Extensive efforts to obtain information about the primary structure of complex genomes. However, much less is understood about the higher order organization of the genome within the nucleus, in particular the chromosome organization and its relation to intranuclear arrangement.

Application of the fluorescence *in situ* hybridization (FISH) technique using specific DNA probes lead to direct visualization of the genome organization in the nucleus as well as contribute to diagnostic analysis. Various types of probes for FISH are going to be available with the progress of the genome projects. Chromosome specific painting probes demonstrated territorial organization of chromosomes in the nucleus. Interphase chromosomes in mammalian cells are generally considered to be less condensed than their mitotic counterparts, but individual chromosomes appear to occupy restricted subcompartments in the interphase nucleus, *i.e.*, chromosome territories. Many observations have been reported for temporally and spatially ordered organization of the genome within the nucleus. However, little is known about the relationship among specific gene locations, chromosome territories, and

various intracellular processes.

For understanding of the arrangement of specific genome sequences in the interphase nucleus of mammalian cells, I adopted two approaches. Firstly, I analyzed the relative positioning of specific DNA segments in the nucleus by multicolor FISH. In this approach, thousands of nuclei can be surveyed in a short time, and an intranuclear position of one specific DNA segment is inferred from comparison with those of other DNA segments from known genomic sites. The detailed information about each clone will be available, such as chromosomal localization, DNA replication timing, GC content, and gene or repetitive sequences. As an example, intranuclear arrangement of human chromosome 12 in G0(G1) nuclei of human myeloid leukemia HL60 cells was analyzed by using band-specific cosmid clones as probes. Each set of two cosmids was detected in different colors to the HL60 nuclei fixed by paraformaldehyde, and their relative positioning, internal or periphery, in the individual nucleus was scored. The results suggest that the intranuclear arrangement of human chromosome 12 is not random. Some chromosomal domains, including centromeric region, localized in the nuclear periphery, while other parts, including telomeric regions, positioned in the internal parts of the nucleus in G0(G1) cells. Based on the replication banding pattern of metaphase spreads, human chromosome 12 was divided roughly into five large domains. Interestingly, the clones in later replicating domains preferentially localized in the nuclear periphery, whereas those in earlier replicating domains were arranged in the internal position of the nuclei. DNA replication timing of each cosmid determined by FISH-based assay was consistent with the replication R-banding profile of chromosome 12. These results suggest that a topological arrangement of a human chromosome correlates to large-scale replication

domains of the genome, and possibly to the chromosome bands, even before the DNA replication stage of cell cycle.

Next, I adopted an approach to simultaneously visualize four targets including a specific gene, a centromere, a whole chromosome, and a nucleus, and to three-dimensionally analyze their relative positioning in the nucleus during cell cycle by using a deconvolution system. I selected an imprinted gene, *SNRPN* (small nuclear ribonucleoprotein polypeptide N), as an example, because this gene are well-analyzed on its imprinted expression, replication timing, *etc.*, and also homologous association of this gene region at late S phase has been reported. The relative positions of chromosome 15 centromeres and *SNRPN* genes in the interphase nuclei of HL60 cells and their cell cycle dependency were analyzed with respect to the territories occupied by the whole chromosome 15 in which these DNAs are localized. Chromosome 15 territory, its centromere, *SNRPN* gene, and the nucleus were simultaneously visualized in three-dimensionally preserved nuclei by multicolor FISH. Spatial distribution of DNAs analyzed by a cooled CCD camera deconvolution system revealed that the *SNRPN* gene relatively localizes on the periphery of the chromosome territories and that preferentially faces to the nuclear membrane in late S and G2 phases. The chromosome 15 centromere and *SNRPN* gene come close to each other in late S phase, suggesting that both DNA segments replicate together within the same intranuclear domains, since they replicate in later half of S phase and DNA replication occurs in large foci at this stage of cell cycle. Preferential association of *SNRPN* does not occur in HL60 cells through the cell cycle. This contrasts with a report of homologous association of this imprinted chromosomal domain found in lymphocytes and lymphoblasts with the imprinting expression. RNA-FISH using an intron probe within *SNRPN* gene

and the methylation status of this imprinted domain demonstrate that *SNRPN* gene is also imprinted in HL60 cells and allelically expressed through the cell cycle. This gene region in HL60 cells exhibits asynchronous replication which is a general feature of imprinted genome domains, similar with lymphocytes. These results suggest that factors other than imprinted expression and replication timing may be important determinants for the homologous association of imprinted genes.

Chapter II

Introduction

It is surprising that two sets of approximately $3x10^9$ bp of human DNA are compacted within a small volume of the cell nucleus. A number of genes on the genome express timely and play each function under precise regulation. The whole genome duplicates in numerous numbers of human cells under the perfect order. To accomplish such sophisticated biological processes, the mammalian genome is organized by various levels of domain structures that essentially associate with biological functions. The human genome consists of discrete units of chromosomes. There are a number of studies of the genome structure that focused on the metaphase chromosomes, which have characteristic patterns of chromosome bands. The banding patterns have been used in clinical cytogenetics. Chromosome bands are related to long-range GC% mosaic structure, DNA replication timing, gene and CpG island density, and chromosome condensation (Bernardi *et al.* 1985; Ikemura 1985; Holmquist 1992; Craig and Bickmore 1993; Bird 1987; Korenberg and Rykowski 1988), suggesting that the banding pattern is of functional significance. However, in spite of extensive efforts to obtain information about the primary structure of complex genomes, much less is understood about the higher order organization of the genome within the nucleus, in particular the chromosome organization and its relation to intranuclear arrangement.

Application of the fluorescence *in situ* hybridization (FISH) technique using specific DNA probes lead to direct visualization of the genome organization in the nucleus as well as contribute to diagnostic analysis (Lichter and Ward 1990). A convenient and efficient method was developed to determine the DNA replication timing by using FISH to interphase nuclei (Selig *et al.*

1992). This approach has been successfully applied to the demonstration of allele specific replication timing of imprinted (Kitsberg *et al.* 1993; Knoll *et al.* 1994) and X-linked genes (Torchia *et al.* 1994; Boggs and Chinault 1994). An knowledge of chromosome organization and its relation to intranuclear arrangement are important for understanding of nuclear structure and function.

Various types of probes for FISH are going to be available with the progress of the genome projects. Chromosome specific painting probes demonstrated territorial organization of chromosomes in the nucleus (Cremer *et al.* 1988; Lichter *et al.* 1988; Pinkel *et al.* 1988). The three-dimensional (3D) analysis of the centromere and telomere distribution of individual chromosomes in G1 and G2 lymphocytes showed that dynamic chromosome rearrangements occur in non-mitotic nuclei during cell cycle (Ferguson and Ward 1992). Many observations have been reported for temporally and spatially ordered organization of the genome within the nucleus (for review, see Cremer *et al.* 1993). Interphase chromosomes in mammalian cells are generally considered to be less condensed than their mitotic counterparts, but individual chromosomes appear to occupy restricted sub-compartments in the interphase nucleus, *i.e.*, chromosome territories, which do not intermingle with each other (Cremer, *et al.* 1993; Zink, *et al.* 1998). Sub-chromosomal regions, such as chromosome bands, are also in their own sub-compartments at interphase (Visser, *et al.* 1998; Jackson and Pombo 1998), suggesting that the structures of condensed metaphase chromosomes are reflected to their organization in interphase nuclear space. It has been suggested that the spatial position of chromatin segments within a chromosome territory follows a random distribution (van den Engh *et al.* 1992; Yokota *et al.* 1995). Recent advances in digital imaging technology made it possible to analyze the intranuclear organization of specific genome

sequences. It is important to understand how the chromosome territories relate to gene sequences and intranuclear structures. The gene coding sequences were demonstrated to be located at the surface of their respective chromosome territories, independently of their transcriptional status (Kurz *et al.* 1996). The relationship between gene activities and 3D morphology of chromosome territories was examined with a set of active and inactive human X interphase chromosomes as an example, and active X territories were found to reveal a flatter shape and exhibit a larger and more irregular surface when it was compared with the smoother surface and rounder shape of inactive X territories (Eils *et al.* 1996). Although the number of examples examined is small, these observations support the idea that transcription, RNA processing and transport occur in a space between chromosome territories called the interchromosomal domain (ICD) (Cremer *et al.* 1993; Zirbel *et al.* 1993). For understanding how complex and how variable the shapes of chromosome territories are, it is necessary to clarify the spatial relationship between the territories and splicing machineries and the localization of sites of DNA replication in the territories. Components of splicing machinery are concentrated in between the chromosome territories and DNA replication foci are observed within the interior of territories (Clemson *et al.* 1996; Zink *et al.* 1998; Visser *et al.* 1998). On one hand, patterns of DNA replication foci change in cell cycle dependent manner (Fox *et al.* 1991; O'Keefe *et al.* 1992). On the other hand, intranuclear positioning of genes and chromosomes may be also important for their biological functions. There have been reported some observations on the dynamics of intranuclear genome organization and its implication to biological phenomena. Movements of centromeres and telomeres during cell cycle have been reported (Ferguson and Ward 1992). The human acrocentric chromosomes containing rDNA

sequences are intrinsic components of nucleoli. Modulations of nuclear architecture might be associated with the change in tumorigenesis (Linares-Cruz *et al.* 1998). Homologous association of oppositely imprinted chromosomal domains has been observed in late S phase of mammalian cells (LaSalle and Lalande 1996). Therefore, information about nuclear architecture is accumulating gradually. However, there are quite few observations and little is known about the relationship among specific gene locations, chromosome territories, and various intracellular processes.

I adopted two approaches for understanding of the arrangement of specific genome sequences in the interphase nucleus of mammalian cells. Firstly, I analyzed the relative positioning of specific DNA segments in the nucleus by multicolor FISH. In this approach, thousands of nuclei can be surveyed in a short time, and an intranuclear position of one specific DNA segment is inferred from comparison with those of other DNA segments from known genomic sites. The detailed information about each clone will be available, such as chromosomal localization, DNA replication timing, GC content, and gene or repetitive sequences. Each set of two cosmids was hybridized onto large numbers of nuclei fixed with paraformaldehyde to better preserve nuclear organization. Each cosmid was detected by a different color, and then their relative positions in the individual nucleus, internal or periphery, were scored. I demonstrate that human chromosome 12, a metacentric chromosome, is divided into five large-scale replication domains and these domains are arranged in the nucleus according to their replication timing. These results suggest that specific replication domains are closely correlated to their intranuclear positionings before the DNA replication stage.

Next, I adopted an approach to simultaneously visualize four targets including a specific gene, a centromere, a whole chromosome, and a nucleus, and to three-dimensionally analyze their relative positioning in the nucleus during cell cycle by using a deconvolution system. I selected an imprinted gene, *SNRPN* (small nuclear ribonucleoprotein polypeptide N), as an example, because this gene are well-analyzed on its imprinted expression, replication timing, *etc.* (Gunaratne *et al.* 1995), and also homologous association of this gene region at late S phase has been reported (LaSalle and Lalande 1996). Firstly, I tried to pick up the general information about relative positioning of the two genomic sequences in the territory or the nucleus through this multicolor 3D analysis. Further, I tried to confirm whether homologous association of this imprinted region found in lymphocytes occurs in human myeloid leukemia HL60 cells. RNA-FISH detecting the primary transcript was also tried to demonstrate monoallelic expression of the *SNRPN* gene.

In the course of pursuing this work, I determined the chromosomal localization of many gene sequences. The information of these results are important for not only constructing of the genome map, but also intranuclear dynamics of each gene in future works. In this point of view, I mention the list of publications in which I involved as supplement.

Chapter III

Materials and Methods

1. Chemicals & DNA probes.

Phytohemagglutinin, RPMI1640, fetal bovine serum (FBS), and human Cot-1 DNA (Gibco-BRL), bromodeoxyuridine (BrdU), 4',6-diamidino-2-phenylindole (DAPI), diazacyclooctan (DABCO), and propidium iodide (PI) (Sigma), and anti-BrdU monoclonal antibody and anti mouse antibodies conjugated with either FITC or rhodamine (MBL) were purchased from the indicated sources. Reagents used for probe labeling and signal detection for FISH were purchased from Boehringer Mannheim. Other chemicals were from nacalai tesque unless otherwise stated. The band-specific cosmid clones of human chromosome 12 were obtained from the Japanese Cancer Resources Bank (JCRB) in National Institute of Health, Japan (Takahashi *et al.* 1993). The cosmid 93 (c93) spanning the *SNRPN* region was kindly gifted from Dr. Chinault, Baylor College of Medicine (Gunaratne *et al.* 1995). The centromere probes, pBR12 and pCM15, were kindly provided from Dr. Baldini, Baylor College of Medicine (Baldini *et al.* 1990). The Cy-3 conjugated chromosome 15 painting probe was obtained from Cambio (Cambridge, UK).

2. Cell culture and preparation of specimens.

Two alternative methods were used in this study. For the analysis of the intranuclear arrangement of human chromosome 12, human myeloid leukemia HL60 cells were cultured with RPMI1640 containing 10% FBS at 37°C in an atmosphere of 5% CO_2. FBS was decreased to 0.5% and cutured for 48 hours to obtain G0(G1)-rich cell population. More than 80% of the cells were in G0(G1) by the flow cytometer analysis (data not shown). For the FISH experiments, the

nuclei were isolated and fixed by the similar method described (Ferguson and Ward 1992). The cells were resuspended in isolation buffer [5mM Hepes (pH8.0), 50mM KCl, 10mM $MgSO_4$, 3mM dithiothreitol] with 0.1mg/ml RNase and incubated for 10 min at room temperature. Triton X-100 was added to give a final concentration of 0.25% (v/v), and the cell suspension was kept on ice for 10 min, then the suspension was vortexed at high speed for 10 sec and incubated for 30 min at 37°C. The isolated nuclei were fixed with 4% paraformaldehyde in PBS (phosphate buffered saline) (pH7.5) for 20 min at room temperature with gentle shaking, and added 4 volume of 0.1M Tris-HCl (pH7.4). After 10 min, the nuclei were spun down for 15 min at 200x *g*. The nuclei were washed twice with PBS and stored at 4°C until use for FISH. For the replication R-banding, human peripheral blood from a healthy male donor or HL60 cells were cultured with RPMI1640 containing 10% FBS. The R-banded chromosomes from human lymphocytes were prepared by the method described (Takahashi *et al.* 1990), which is based on the steps of the phytohemaggultinin stimulation, the excess thymidine synchronization, BrdU incorporation, and UV-irradiation. The same conditions without the stimulation step were used for randomly cultured HL60 cells. For the replication timing analysis, cells were randomly cultured and labeled by BrdU (25µg/ml) for 10 min just before harvest. Then the cells were treated with hypotonic solution (0.075M KCl) at 37°C for 20 min, and fixed three times with methanol-acetic acid. The fixed cells were dropped onto the microscope slides for FISH.

For the analysis of the *SNRPN* gene on chromosome 15, HL60 cells were fractionated by a centrifugal elutriation. Randomly cultured cells ($3x10^8$ cells) were loaded onto a Beckman elutriator rotor at 2,000 rpm at 4°C with flow of 0.3% gelatin in PBS. The flow rate was increased from 11 ml/min to 26 ml/min

at every 2.5 ml/min interval. In each flow rate, the first 100 ml were collected and aliquots of each fraction was provided for the cell cycle analysis by flow cytometry. Fractionated cells were immediately fixed by the method of Kurz *et al.* (1996) with slightly modification. Briefly, cells were suspended in PBS and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After treating with 1% paraformaldehyde in 0.1M HCl for 10 min, the fixed cells were permeabilized with 0.5% Triton X-100 containing 0.5% saponin, and 1% paraformaldehyde in PBS for 10 min. Before each treatment, cells were harvested at 200x g at 4°C for 5 min and removed the supernatant. The permeabilized cells were equilibrated with 20% glycerol in PBS for 20 min and stored in liquid nitrogen until use.

3. Fluorescence in situ hybridization.

Fluorescence *in situ* hybridization (FISH) for the specimen fixed by methanol-acetic acid was basically performed by following the method described (Lichter *et al.* 1988) except for using Cot-1 DNA as a competitor in a preannealing step. For the analysis of intranuclear arrangement of the chromosome 12 cosmids, the three-dimensionally fixed nuclei were placed on poly-d-lysine-coated slides and stored in 2x SSC (standard citrate saline). The slides were treated with the blocking solution of 3% BSA (bovine serum albumin) in 2x SSC for 30 min at 37°C and washed in 2x SSC for 10 min and subsequently equilibrated with the denaturing solution (50% formamide in 2x SSC) for 15 min prior to denaturation. The slides were drained carefully and the hybridization solution [50% formamide (v/v), 10% dextran sulfate in 2x SSC] was applied to the nuclei and sealed under a coverslip with paper bond and equilibrated for 30 min at room temperature, and then denatured for 6 min at 87°C on a heat block. The

denatured and preannealed DNA probes, prepared by the same method as described above, were further applied to the denatured nuclei after removing a coverslip, and the slides were re-sealed and incubated overnight at 37°C. Post hybridization washing was done with 50% formamide in 2x SSC at 42°C (3x 5 min) and with 2x SSC at 65°C (3x 5 min). Treating with a blocking solution (3% BSA and 0.1% Tween 20 in 4x SSC) for 30 min at 37°C, the biotinylated and digoxigenin-labeled probes were detected using fluorescein-avidin and rhodamine-anti digoxigenin Fab fragments, respectively, and then washed 3 times with 0.1% Tween 20 in 4x SSC at 45°C. The specimen were counterstained with 200 ng/ml DAPI in the antifade mounting solution [2.3% DABCO in 90% glycerol, 0.1M Tris-HCl (pH7.5)].

For the analysis of the *SNRPN* gene, a similar method was used. In this case, frozen cells in liquid nitrogen were washed twice with PBS. The cells were mounted onto a poly-d-lysine-coated coverslip and placed for 15 min for the fixation. The cells were equilibrated with 2x SSC for 10 min, and further equilibrated with the denaturing solution for 15 min in and then equilibrated with the hybridization solution at least for 20 min in prior to denaturation. The nick-translated cosmid and centromere probes with 3 μg of Cot-1 DNA in the hybridization solution were denatured at 80°C for 10 min and preannealed at 37°C for 30 min. The chromosome 15 painting probe was treated at 42°C for 20 min and mixed with the above preannealed probes just before hybridization. The cells on the coverslip was denatured for 10 min at 80°C on a heat block, and then the above hybridization probe mixture was mounted. Hybridization was carried out over night at 37°C in a moist chamber. Each coverslip was washed three times with 50% formamide in 2x SSC for 5 min at 42°C, and then three times with 2x SSC at 60°C. The coverslips were then incubated for 30 min in a

blocking solution [3% block Ace (Yukijirushi), 0.1% Tween-20 in 2x SSC] at 37°C and detected Cy-5 conjugated avidin (in 1% block Ace , 2x SSC, 0.1% Tween-20) for 30 min at 37°C. Then, coverslips were washed three times with 4x SSC, 0.1% Tween-20 for 5 min at 42°C and counterstained using 0.2 μg/ml DAPI in 2x SSC for 5 min. Finally, the coverslips were mounted on hole slide glasses with an antifade solution (Vectashield; Vector Laboratories)and sealed with nail polish.

4. RNA-FISH.

The intron probes for the RNA-FISH analysis were generated by PCR. PCR was carried out using c93 as template and the following primer sets corresponding to approximately 3 kb sequences on the upstream of exon alpha of the *SNRPN* gene.

5'-AGGAAAAGGAACAGGGTAAGGGAAA-3'

5'-GCAGACGCAGCAGAGGTGACAG-3'

5'-ACGCAACACAGACCCCCAGG-3'

5'-GGAAGGGCGGTGGTGACTGG-3'

PCR products were labeled by nick translation using digoxigenin-11-dUTP. The RNA-FISH protocol was using previously described (Dirks *et al.* 1995), with modifications. HL60 cells were fixed with 4% paraformaldehyde and 5% acetic acid for 20 min at room temperature. The cells were washed three times for 10 min with PBS and stored in 70% ethanol at -20°C.

Cells were fixed onto a poly-D-lysine-coated coverslip and treated by 0.01% pepsin in 0.01% HCl for 5 min at 37°C, followed by a short wash in water and fixed in 4% paraformaldehyde for 5 min at room temperature, then washed with PBS, dehydrated in 70, 90 and 100% ethanol series and air-dried.

The hybridization mixture containing labeled intron probe and salmon sperm DNA (10μg) in hybridization solution was mounted on the coverslip. Hybridization was carried out at 37°C over night in a moist chamber. The coverslips were washed four times with 50% formamide in 2x SSC for 3 min at 37°C, and once in 2x SSC for 3 min at room temperature. The hybridized probe was detected with rhodamine conjugated anti digoxigenin Fab fragment and followed by blocking step. Finally, the coverslip was washed three times with 4x SSC for 2 min at 37°C, then counterstained with DAPI.

5. *FISH-based replication assay.*

Replication timing of cosmid clones was determined by a FISH method (Selig *et al.* 1992) with slight modification. Each biotinylated or digoxigenin-labeled probe was hybridized onto the methanol-acetic acid fixed nuclei, and detected fluorescein-avidin or rhodamine-conjugated antibodies against digoxigenin, respectively. The slides were scanned to systematically identify S phase nuclei by detecting incorporated BrdU, through anti BrdU monoclonal antibody and subsequent anti mouse antibodies conjugated with either FITC or rhodamine, which was selected a different color combination with the detection of DNA probe. Slides were counterstained with DAPI, and examined with an oil x 63 objective on a Zeiss Axioskop fluorescence microscope fitted with a Zeiss filter set for DAPI, FITC, and rhodamine. At least one hundred nuclei in only S phase were scored their signal patterns as singlet-singlet (SS), singlet-doublet (SD), or doublet-doublet (DD).

6. Optical sectioning and 3D image analysis.

For the 3D image of the intranuclear arrangement of chromosome 12, a

deconvolution system (Delta Vision, Applied Precision) was used (Agard *et al.* 1989). The sectioning images of 60-70 layers to each nucleus were captured by a Delta Vision system for each fluorescence including DAPI and the 3D images were constructed using 20 layers detected FISH signals. The projection images were printed by Fuji Pictrography 3000.

For the analysis of the *SNRPN* gene, series of light optical sections of nuclei were recorded with a cooled CCD camera (PentaMax 1317K-1, Princeton Instruments) mounted on Zeiss Axioplan 2 MOT with a Plan-APOCHROMAT 63 x oil objective, under the control by a software IPLab Spectrum (Signal Analytics) running on a Macintosh 9600/200MP computer. Each sectioning image was deconvoluted by a software HazeBuster 2.0 (VayTek) to remove the haze and 3D images were reconstructed by one of a extension utility of IPLab Spectrum and VoxBlast 2.01(VayTek),which allow the analysis of the distance between FISH signals and the relationship between theprobes and chromosome territories. Surface model images were rendered using a version of the STRATA STUDIO Pro (STRATA) based on outlines of light optical sections of the nucleus using Adobe Photoshop 4.0 (Adobe) and Adobe Streamline (Adobe) on the market softwares.

Chapter IV

Results

1. Intranuclear Arrangement of Human Chromosome 12 in HL60 Cells.

To understand the relationship among intranuclear arrangement, chromosomal positioning, and DNA replication timing of the genome, human chromosome 12 was selected as a representative because this is a typical metacentric chromosome with a relatively clear replication-banding pattern and available band-specific cosmid clones (Takahashi *et al.* 1993). Human myeloid leukemia HL60 cells are used in this paper. This cell line is diploid with relatively stable chromosome number, and its DNA replication timing has been reported to switch in the GC content transition area in this cell (Tenzen *et al.* 1997). It also has a potential future advantage because this cell can be analyzed the changes of intranuclear genome organization with the progress in cell differentiation. In this paper, G0(G1) nuclei were extensively analyzed to obtain the information about how the human genome arranges in the nucleus before accomplishing one of the most dynamic intranuclear events of DNA replication.

1-1. Intranuclear arrangement of human chromosome 12 in G0(G1) stage.

Twenty cosmids were reassigned their chromosomal localization by FISH. As shown in Fig.1(A), among them fifteen cosmids with relatively strong FISH signals and a centromeric probe, pBR12 (Baldini *et al.* 1990), were selected as band-specific probes for this experiment. Human myeloid leukemia HL60 cells in G0(G1) stage were fixed with paraformaldehyde, to better preserve 3D structure, and placed onto a polylysine-coated slide. Each set of two cosmids was labeled with either biotin or digoxigenin and hybridized onto

denatured nuclear specimen. Each probe was simultaneously detected in either fluorescein or rhodamine and their relative positions, internal or periphery, in the individual nucleus was scored. At least 100 nuclei were observed in each set of two probes in three independent experiments. As shown in the top of Fig. 1B, each set of the clones was scored according to the signal patterns and sorted into each group dependent on the ratio of the relative positionings in the nucleus. For example, centromeric probe pBR12 detected in red is arranged in more periphery than the clones, cCI12-156, -71, and -77, detected in green (vertical row of "cen." in the Fig. 1B bottom). On the other hand, the probe pBR12 detected in green is arranged in more peripheral parts in the nucleus than the clones, cCI12-140, -108, -82, -206, -97, and -116, but shows unclear patterns with the clones cCI12-190, -197, -189, -103, and -73 of red signals (horizontal row of "cen." in the Fig. 1B bottom). The clone cCI12-103 detected in red is arranged in more periphery than all the clones in this row except for cCI12-185, -189, and centromere, and this clone detected in green is arranged in the nuclear periphery, compared with all other clones tested. Since the centromeric probe is detected in the nuclear periphery and associated with the nuclear membrane to almost all the nuclei in this stage of cell cycle, the region on which the cCI12-189 and -103 clones locate is also arranged near the nuclear membrane. Conversely, both the distal parts of this chromosome are relatively positioned in the internal parts of nuclei, as shown clearly in Fig. 1B (upper three columns of the horizontal row are almost black, and right four columns of the vertical row are almost white). The region on which the cCI12-140, -108, and -197 clones locate is arranged in the position between the distal regions and the centromeric region.

1-2. Putative arrangement of human chromosome 12 and its correlation to large-scale replication domains.

The informations described above allowed us to presume and depict the rough positioning of chromosome 12 in the HL60 nucleus. A putative arrangement of this chromosome in the G0(G1) nucleus was illustrated as a line structure by using the information from Fig. 1 (Fig. 2A). Both the telomeric regions relatively localize in the most internal parts of the nuclear volume, whereas the centromeric and q21 regions arrange near the nuclear membrane. The region between the centromere and q 21 localizes in relatively internal volume of the nucleus. On the other hand, replication R-banded chromosomes were prepared from human lymphocytes and HL60 cells, and compared with the putative arrangement of chromosome 12 (Fig. 2B and 2C). Since the banding pattern of chromosome 12 from HL60 cells is ambiguous, the same chromosome prepared from human lymphocytes was shown as a comparison (see discussion). Based on the replication banding pattern of the metaphase chromosomes, human chromosome 12 was roughly divided into five large domains which were designated as "E" or "L" in Fig. 2. In this figure, "E" means an earlier replicating domain composed of R-band rich regions, and "L" means its reverse. Interestingly, a correlation is clearly shown between these large domains and the intranuclear positioning of chromosome 12. The zones "L", including centromere and q21 regions, positioned in the nuclear periphery, two distal "E" zones arranged in internal regions of the nucleus, and the other proximal "E" zone somehow localized in internal parts of the nucleus between the two positionings. Thus, these results suggest that a topological arrangement of human chromosome, even before the DNA replication stage of cell cycle, correlates to large-scale replication domains of the genome.

1-3. Replication timing profiles of the band specific cosmid clones derived from chromosome 12.

One advantage of my approach is that information of individual clones is available. The replication timing in HL60 cells of the band specific cosmid clones were determined by a slightly modified FISH method (Selig *et al.* 1992). HL60 cells were randomly cultured and labeled with BrdU for 15 min to identify S phase nuclei. Incorporated BrdU was simultaneously viewed by rhodamine-labeled anti-BrdU antibody in the FISH detection step. FISH signal profiles of each clone, two singlets (SS), singlet-doublet (SD), and two doublets (DD), were examined under a fluorescence microscope (Fig. 3). In this method, since only the nuclei in S phase are positive and selectable by BrdU detection, DNA replication timing of each clone can be compared in various cells without considering temporal differences of the cell cycle. In addition, the SS% (or SS% + SD%) reflects the point of approximate time of DNA replication in S phase for each clone examined; a smaller SS% of the clone means earlier replication. As shown in Fig. 3, the clones mapped on R-positive bands had earlier replication timing, whereas those mapped on R-negative bands replicated relatively late except for a few clones. Since R-bands generally replicate earlier than G-bands, my observations in Fig. 3 reflect this cytogenetic evidence. Hence, these results mean that the randomly selected clones used here may be considered as representative of each band. These profiles were also consistent with large-scale replication domains distinguished in Fig. 2. A putative topological arrangement of human chromosome 12 in the nucleus was also compared with these replication timing profiles. Although there are some exceptions, each replication profile correlated to the intranuclear arrangement

(Figs. 2 and 3).

1-4. Demonstration of intranuclear arrangement of human chromosome 12 by 3D imaging.

To confirm the putative topological arrangement of chromosome 12 suggested in Fig. 2, several probes from each "E" and "L" zone were co-hybridized and visualized three dimensionally by a deconvolution system. The probes from p (including a centromeric probe) and q arms were detected in fluorescein and rhodamine, respectively. The sectioning images of 60-70 layers were captured by a DeltaVision system for each fluorescence including DAPI and 3D images were constructed. Figure 4 shows the typical projection images constructed by using 20 layers with FISH signals. Since the centromeric probes are detected as large green signals, they are easily distinguished from the other probes. One of the centromeric signals associates with the nuclear membrane in Fig. 4A, the other one is also viewed in the nuclear periphery when the left 3D image was rotated 30° clockwise for *z*-axis in Fig. 4B. The most distal probes of p arm with relatively smaller green signals are shown to locate in the internal volume of the nucleus. As the distal probes in q arm are detected by relatively weaker FISH signals than those of the proximal ones, I can presume which red signals are attributed to either proximal or distal parts of the chromosome. The probes from distal q arm were detected in the internal volume of the nucleus, whereas one of the red signals (possibly from 12q21 band) localized near the nuclear membrane (see Fig. 4B). According to these results, topological arrangement of the backbone of chromosome 12 was traced and illustrated in Fig. 4 for better understanding of these images. Hence, these images support the putative arrangement of human chromosome 12 in the nucleus.

2. Relative Locations of Chromosome 15 Centromeres and of SNRPN Genes in Interphase Nuclei of HL60 Cells.

2-1. The experimental basis of the cell fractionation and of FISH.

To investigate the cell cycle dependent locations of the genomic sequences in the nucleus, randomly cultured HL60 cells were fractionated by a centrifugal elutriation. Four fractions were pooled and aliquots of each fraction were analyzed its cell cycle profile by a flow cytometry. As shown in Fig 5, the fractions A to D were rich in G1, S, late S, and G2/M phase cells, respectively. Cells in M phase contained in the G2/M rich fraction were excluded from the analysis. So, each fraction was designated a G1, S, late S, and G2 fraction, respectively. I chose a centrifugal elutriation rather than a cell synchronization method for the fractionation of the cells, to avoid transient expression of some genes and effects of a drug to control the cell cycle. After the elutriation, cells were immediately fixed by paraformaldehyde described (Kurz *et al.* 1996). To better preserve intranuclear organization of the genomic sequences, I chose the protocol that did not include alcoholic and protease treatments.

To study the spatial arrangement of specific genome sequences in the nucleus, *SNRPN* genes, chromosome 15 centromeres, and the whole chromosome 15 were selected as targets and simultaneously detected by multicolor FISH. The cosmid coding a *SNRPN* gene was labeled with biotin-dUTP and detected by Cy-5 conjugated avidin. The centromere probe was directly labeled with fluorescein-dUTP. For the detection of whole chromosome 15, the Cy-3 conjugated painting probe was commercially available. As shown in Fig. 6, the q arm of chromosome 15 was stained homogeneously by this painting probe, with simultaneous detection of *SNRPN*, indicating that this

probe is suitable for the delineation of chromosome territories in the nucleus.

These three DNA probes were hybridized to 3D fixed nuclei and detected by the respective color, with the nucleus stained by DAPI. Every 0.1 um optical section for each nucleus (total 80 layers) was imaged through each fluorescence filter by a cooled CCD camera system under computer control. After the deconvolution step, each fluorescent image captured at the same focal plane was pseudocolored and merged as shown in Fig. 7. Although 3D reconstruction of FISH images for the analysis of intranuclear locations of each fluorescent signal was performed by a software VoxBlast, a 3D image reconstructed by a software STRATA STUDIO Pro is shown in Fig. 8, for the overall view of the topological relationship of each probe in the nucleus. The picture in the left of the figure is a typical image of the whole nucleus with four fluorescence color. Nucleoli are also included in the picture by coloring the intranuclear regions that are not stained with DAPI, although they were not detected specifically. It is interesting to delineate nucleoli, because human chromosome 15 is an acrocentric one on which rDNA genes are located. Parts of magnified views of each territorial organization are shown in the right of the figure. In the upper two images, a *SNRPN* gene faces to the nuclear membrane on the periphery of the chromosome territory, while a centromere signal faces to the inside of the nucleus. In the bottom two images, a *SNRPN* gene locates on the periphery of the chromosome territory, while a centromere is observed in the internal part of the nucleus.

The results described subsequently are based on the analysis of 74 to 94 nuclei in each fraction of the cell cycle.

2-2. Distribution of chromosome 15 centromeres and SNRPN genes within the chromosome territories.

Individual chromosomes occupy discrete compartments in interphase nuclei. However, the shape of the territories are complex and variable, and there are some genomic regions that cannot be detected by the chromosome painting probe in principle. So, it is difficult to define the precise boundary of the chromosome territories. In this thesis, only the intranuclear parts which show strong FISH signals were considered as the chromosome territories. The human *SNRPN* gene localizes on chromosome 15q11-13, which is very adjacent to the centromere. Since the centromeric probe does not contain any gene sequences and both of DNAs are detected closely by FISH even in most nuclei, they are good references for each other.

As shown in Fig. 9, distribution of chromosome 15 centromeres and *SNRPN* genes within the chromosome territories were examined in each fraction of the cell cycle by analyzing 3D reconstructed nuclei. The positions of *SNRPN* or the centromere within individual chromosome territory were analyzed and the number of the territory which has each pattern of the upper three images in the figure was scored. The image shown in the upper left is a typical example that the FISH signal of *SNRPN* (or the centromere) probe locates on the periphery of the chromosome territory and faces to the nuclear membrane. The percentage of this pattern out of the total number scored is calculated and shown as a red color bar for each cell fraction in this figure. The middle image is the case that the FISH signal of the probe locates on the periphery of the territory and faces to the interior of the nucleus. This percentage is shown as an orange color bar in the figure. The upper right image is a pattern that the FISH signal is detected in the territorial volume. This percentage is shown as a blue color bar in the figure. As

clearly shown, the centromere probe is preferentially localizes in the chromosome territory through the cell cycle (the average is 79%). The *SNRPN* gene localizes on more peripheral part of the chromosome territory than the centromere independent of the cell cycle (the average is 56% for *SNRPN*, but 21% for the centromere). These results are consistent with the finding of Kurz *et al.* (1996). There could not be noticed the allelic differences in the positions within the territories (data not shown). Furthermore, the *SNRPN* gene preferentially faces to the nuclear membrane particularly in late S and G2 phases. For *SNRPN*, 58% and 71% out of the FISH signals which locate on the peripheral part of the chromosome territories face to the nuclear membrane in late S and G2 phases, respectively, while 32% and 37% for the centromere.

Which localizes near the nuclear membrane, *SNRPN* and the centromere? Relative positions of both FISH signals in the nucleus were compared. As shown in Fig. 10, there were no significant differences in the intranuclear locations between *SNRPN* and the centromere.

Intranuclear distribution of both probes was also analyzed. The average percentages of the FISH signals observed in very close to the nuclear membrane were 57% and 63% for *SNRPN* and the centromere, respectively. There were no significant differences between both the probes. The ratio of the FISH signal which associates with the nuclear membrane slightly increased in late S and G2 phases in both cases (Fig. 11).

2-3. Cell cycle dependent association of SNRPN and chromosome 15 centromere in HL60 cells.

The distance of the FISH signals between *SNRPN* and the centromere was compared through the cell cycle. As shown in Fig. 12, the following FISH signal

patterns of *SNRPN* and the centromere were scored; the patterns that both signals colocalize, both signals overlap, both signals are separate each other but within one signal distance, and both signals are separate more than one signal distance. Both the FISH signals come close to each other in late S and G2 phase fractions (Fig. 12). This result suggests that the association of both the probes occurs particularly in late S phase, because the late S fraction was not well-fractionated and the G2 fraction contains many cells in late S phase (see Fig. 5).

2-4. Preferential association of SNRPN homologues does not occur in HL60 cells.

Since LaSalle and Lalande reported the homologous association of the oppositely imprinted human chromosome 15q11-13 region in late S phase (1996), the distance of the *SNRPN* homologues was compared with that of the centromere in HL60 cells. The number of nuclei which the distance between a pair of FISH signals is closer than the other set in the same nucleus was scored (Fig. 13). There were no significant differences observed in their relative distances through the cell cycle.

Imprinting status of the *SNRPN* gene was examined with RNA-FISH using the intron probes generated by PCR. As a typical example is shown in Fig. 14, most of the nuclei showed a single spot (73%), indicating that monoallelic expression of the *SNRPN* gene is maintained in HL60 cells and this gene expresses through the cell cycle. Allelic methylation and asynchronous replication of this gene region were also confirmed by the methods of Kubota *et al.* (1997) and Selig *et al.* (1992), respectively (data not shown). These results suggest that factors other than imprinted expression and replication timing may be important for the homologous association of imprinted genes. The

abnormality of the cells such as tumorigenesis is likely to affect the intranuclear genome organization.

Chapter V

Discussion

1. Intranuclear Arrangement of Human Chromosome 12 in HL60 Cells.

I have determined the topological arrangement of human chromosome 12 in the G0(G1) nucleus of HL60 cells by comparing the relative positions of sets of two band-specific cosmids, and I found a correlation of its intranuclear positioning to the large-scale replication domains. My results support the previous report that centromeric regions of the chromosomes in human T-lymphocyte nuclei are localized on the nuclear periphery, while telomeric domains are consistently localized internal of the nuclei in G0(G1) cells (Ferguson and Ward 1992). Furthermore, use of band-specific cosmid probes gave us an advantage to find peripheral localization of a given chromosomal domain, i.e., the region including 12q21 (Fig. 2). Compared with centromere or chromosome painting probes, a cosmid clone provides site-specific information and characteristics of the clone itself, such as replication timing, gene, and sequence information. For instance, I determined the replication timing of the individual clones by FISH analysis, and found a correlation between the intranuclear positioning of the clone and its replication timing (Fig. 3). The information of genes and specific sequences may also provide an understanding of their relation to the intranuclear arrangement of the genome. On the contrary, it is uncertain whether a cosmid can be considered as a representative of each band because of its smaller size, compared with that of chromosome bands. However, in this experiment, neighboring clones were always detected adjacent to each other in the nucleus (data not shown), suggesting that the clones on the same band occupy a similar area in the nucleus, inferring that to some extent the subchromosomal domains are folded into given compartments.

In order to confirm the validity of my approach, several probes from p and q arms of chromosome 12 were detected in different colors and the intranuclear positioning of its whole chromosome was visualized by a 3D imaging system (Fig. 4). Although I did not take many images, most imaged nuclei supported the topological model for the intranuclear arrangement of the chromosome presumed in Fig. 2. It could be traces of the mitotic cell division that centromeres and telomeres localize in nuclear peripheries and interiors, respectively, in G1 cells. However, another G-band rich domain also occupied peripheral compartments, suggesting that the human genome rearranges in the G1 stage of cell cycle and earlier replication domains localize inner compartments of the nucleus, while later replicating ones were arranged in nuclear peripheries. My results indicate that the G-banded regions do not always occupy the nuclear peripheries (Fig. 2). It is unlikely that each R- or G-band domain clearly distributes in the interiors or in the peripheries of the nucleus. Compared with the banding pattern of chromosome 12 from lymphocytes, that from HL60 cells was not clear, although large-scale replication domains are similar (Fig. 2). This may be because the same conditions as those of lymphocytes were employed for HL60 cells to prepare the replication banded chromosome spreads, even though these cells may have a slightly different span of S phase, and so not have large differences in replication timing of each cosmid (Fig. 3). Since the chromosomal bands correlate to the replication timing of the genome, the arrangement of subchromosomal domains may play an important role in various biological processes in the nucleus, particularly DNA replication. It might be possible that each genome subdomain occupies its specific compartment in the nucleus before the DNA replication event to perfectly perform this important process. In this point, it is interesting that DNA

replication foci are detected as various patterns in a cell cycle specific manner (Nakamura et al 1986; Ferreira *et al.* 1997). Since DNA replicating sites of the early S phase cells distribute as smaller foci scattered in all over the nucleus, it is difficult to imagine that only the DNA replication machinery is responsible for the intranuclear positioning of the genome subdomains. However, the DNA replication event may be the most important function of the arrangement of human chromosomes because the internal or peripheral arrangement of the chromosome correlates to large-scale replication domains. Various cellular events, such as DNA replication, gene transcription, recombination, and intranuclear structures other than the genome ought to be taken into consideration to understand the cell cycle specific behaviors of the genome. In fact, the centromeres have been shown to localize in the internal compartments in G2 phase (Ferguson and Ward 1992), suggesting that intranuclear arrangement of some genome subdomains may be expected to change dynamically through the cell cycle.

Individual chromosomes in interphase nuclei distribute to discrete entities, which have been widely adopted as chromosome territories (Cremer *et al.*1993). Kurz *et al.* showed that genes were located at the surface of their respective chromosome territories, independently of their transcriptional status (Kurz *et al.* 1996). This is likely, if genes are considered to lie on the surface of chromosome territories for easier accessibility to transcription factors. However, the DNA density in the territories is not uniform and the shape of the territorial domains is irregular and variable. So, genes might be arranged in less condensed parts of the territories. In this report, I did not take into consideration chromosome territory because I would like to use this simple approach and clarify the relationship between the chromosomal bands and their positionings in

the nucleus. Although my results suggested that each band does not always have its specific position, it may be possible that smaller band domains have their specific positionings in the chromosome territories. To clarify the distribution of the individual cosmids in the chromosome territories and their information, such as genes and their expression status, must be important for an understanding how the intranuclear genome structures are associated with gene functions and various biological phenomena.

2. Relative Locations of Chromosome 15 Centromeres and of SNRPN Genes in Interphase Nuclei of HL60 Cells.

I have analyzed the cell cycle dependent spatial arrangement of the *SNRPN* genes on human chromosome 15 in HL60 cell nuclei in relation to the chromosome territories. One purpose of this study is to characterize the intranuclear behavior of any gene on any chromosome precisely through simultaneous detection of DNA sequences by multicolor FISH. Firstly, I found that the *SNRPN* gene relatively localizes in the periphery of the chromosome territories and that preferentially faces to the nuclear membrane in late S and G2 phases (Fig. 9). The peripheral localization of *SNRPN* genes in the chromosome territories did not contradict the previous finding of Kurz *et al.* (1996) that the genes locate at the surface of their respective chromosome territories independently of their transcriptional status. Moreover, judging from the model proposed by the former chapter (large-scale replication domains of human chromosome 12), both the *SNRPN* and the centromere replicate late in S phase and they should localize in the same intranuclear domain. About 80% of the centromere signals localize in the interior of the territory (Fig. 9), and more than 60% of them are distributed to the nuclear periphery (Fig. 11). These results do

not contradict the model of chromosome 12, although a chromosome 15 is acrocentric.

The boundary of the territories is one of the most important points, when the chromosomes are painted by FISH. The shape of the territories is complex and variable, and the surface of the territories is not smooth. Even if a gene was observed within the territories, some factors could be accessible to it through a hollow of the territories. On the contrary, even though a non-coding sequences was found at the surface of the territories, it might be covered with repetitive sequences suppressed with Cot-1 DNA. The difference of the sensitivity of detection between the cosmid and the painting probe must be also taken into consideration. Kurz *et al.* (1996) characterized active and inactive genes by considering a visible border of the territory exterior region. Eils *et al.* (1996) analyzed the shape and surface structures of the active and inactive X chromosome territories by using 3D Vorunoi tessellation procedure (one of algorism to tessellate iteratively into polyhendra). In addition to the various shapes of the chromosome territories, the shape of the nuclei in HL60 cells are variable, although the cell itself is a spherical shape. Therefore, it is thought to be more accurate to compare with the relative positions of two signals than to determine the absolute distance of the FISH signals from the center or the edge of the chromosome territory (or the nucleus).

As compared in the result section, the *SNRPN* preferentially faces to the nuclear membrane in late S and G2 phases. The localization of the primary transcript near the nuclear membrane may be responsible for prompt transport of the processed RNA to the nuclear pores. DNA replication may be another possibility, because the *SNRPN* replicates late half of S phase. However, the differences are not so large, and further investigation is necessary.

I observed that the *SNRPN* and the centromere come close to each other particularly in late S phase (Fig. 12). Since the *SNRPN* maps to the chromosome 15 at band q11-q13, it is physically close to the centromere. So, even in the interphase nuclei, it is natural that both FISH signals are detected adjacently (More than 90% of their signals were detected within one signal distance; see the green bars of Fig. 12). However, as shown in Fig. 12, 49% of both probes were associated and overlapped each other in late S phase. This is very high, because the late S fraction contains lots of cells in the G2 stage. Studies on the patterns of DNA replication foci with the progress of S phase have been reported. For instance, Nakamura *et al.* (1986) observed ring-like structures as replicating domains in a rat cell line and proposed a replicon cluster model. Fox et al. reported the programmed control of replication sites in the spatial domain in mouse 3T3 cells. In most cases, the large number of small foci are observed in the early S phase, while the relatively small number of assembled foci in middle and late S phase. I observed similar patterns of replication foci in HL60 cells (data not shown). Since SNRPN and the centromere replicate in the late half of S phase, they migth replicate in the same foci (Fig. 15B).

In this study, the association of the homologous alleles of the *SNRPN* was not observed through the cell cycle in HL60 cells (Fig. 13). The RNA-FISH confirmed the monoallelic expression of the *SNRPN* through the cell cycle (Fig. 14). Most of the RNA signal were observed at the periphery of the nuclei. This corresponds to the location of the *SNRPN* gene, and also the report that a DNase-hypersensitive domain, detected in situ nick translation, was present at the nuclear periphery in human cell lines (Park *et al.*, 1998). Unfortunately, although the RNA-FISH and methylation analysis suggest the imprinted expression of the *SNRPN* in HL60 cells, the observation in this study contrasts

with a report of homologous association of this imprinted chromosomal domain found in lymphocytes and lymphoblasts with the imprinting expression (LaSalle and Lalande 1996). This may be a cell type specific phenomenon. Autosomal pairs of homologous chromosome was observed in non-dividing cell type such as Sertoli cell (Chandley *et al.* 1996) and cerebellum (Arnoldus *et al.* 1989).

The results obtained in this study are simply illustrated in Fig. 15. The *SNRPN* is an imprinted gene. Genomic imprinting is a marking process of the parental origin of chromosomes, resulting in allele-specific changes in expression, chromatin structure, and replication. Human chromosome 15q11-q13, a region subject to genomic imprinting, is a Prader-Will syndrome or Angelman syndrome critical region. Therefore, it is interesting how the intranuclear genome organization relates to this biological phenomenon. My present study is the first step to clarify this important biological problem.

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Fig. 1

Reassignment of band-specific cosmid clones (A) and analysis of their relative positionings in the nucleus of HL60 cells (B).

(A) Fifteen cosmids out of twenty examined are selected and indicated their map positions with a centromere probe after reassignment by FISH. Some of them were assigned more precisely than the previous report (Takahashi et al. 1992).

(B) Each set of two cosmids was labeled with either biotin or digoxigenin and hybridized onto denatured nuclear specimen. Each probe was simultaneously detected in either fluorescein or rhodamine and their relative positioning, internal or periphery, in the individual nucleus was scored. According to the percentage of the signal profiles illustrated in the top of (B), the relative positioning of each cosmid was determined; an empty white circle was put in the column, when the clone detected in red is observed exclusively inner parts of the nucleus, and vise versa (bottom). At least 100 nuclei were observed in each set of two probes in three independent experiments.

Fig. 1

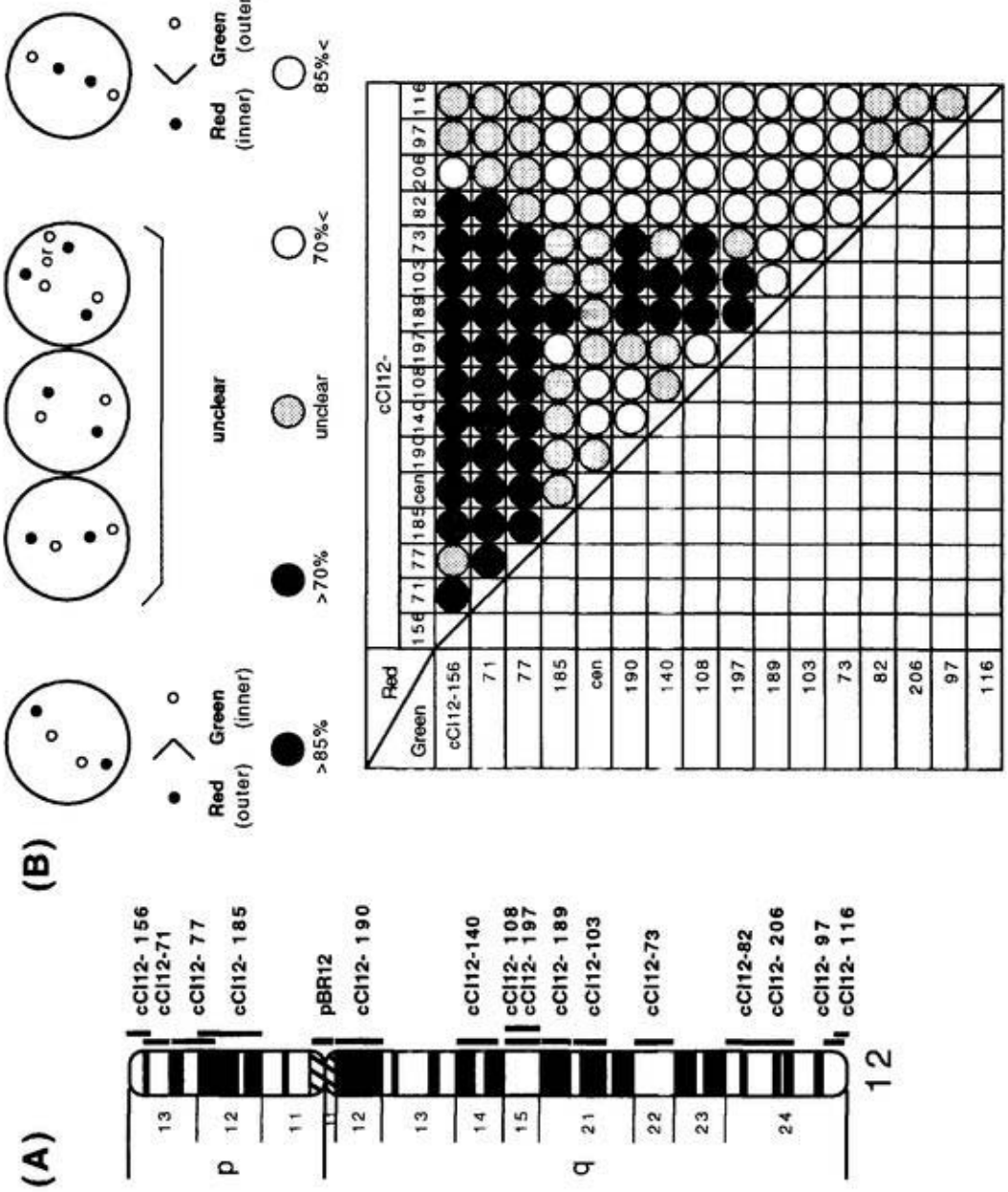

(A)
cCl12- 156
cCl12-71
cCl12- 77
cCl12- 185
pBR12
cCl12- 190
cCl12-140
cCl12- 108
cCl12- 197
cCl12- 189
cCl12-103
cCl12-73
cCl12-82
cCl12- 206
cCl12- 97
cCl12- 116
p
q
12
(B)
Red
Green (inner)
(outer)
>85%
>70%
unclear
70%<
85%<
cCl12-
Red
Green
cCl12-156
71
77
185
cen
190
140
108
197
189
103
73
82
206
97
116

Fig. 2

Putative arrangement of human chromosome 12 in the G1(G0) nucleus deduced from Fig. 1B, (A) , and replication banding patterns of this chromosome from human lymphocytes (B) and HL60 cells (C).

(A) Based on the informations of Fig. 1B, putative arrangement of the chromosome 12 was roughly illustrated. The chromosome backbone was drawn in a black line. Gray boxes show large R-band domains. A vertical line drawn at the right side is the position of nuclear membrane, and the other line at the left means only interior of the nucleus because quantitative analysis cannot be done. The hatched line in the middle is merely drawn for easier comparison of the positioning. The "cen." is centromere. (B) and (C) are the typical images of replication R-banded chromosome 12 stained by propidium iodide from human lymphocytes and HL60 cells, respectively. As indicated as "E" or "L" zones between (A) and (B), human chromosome 12 was divided roughly into five large domains, depending on the banding patterns of both images. Intranuclear arrangement of this chromosome closely correlates to these zones.

Fig. 2

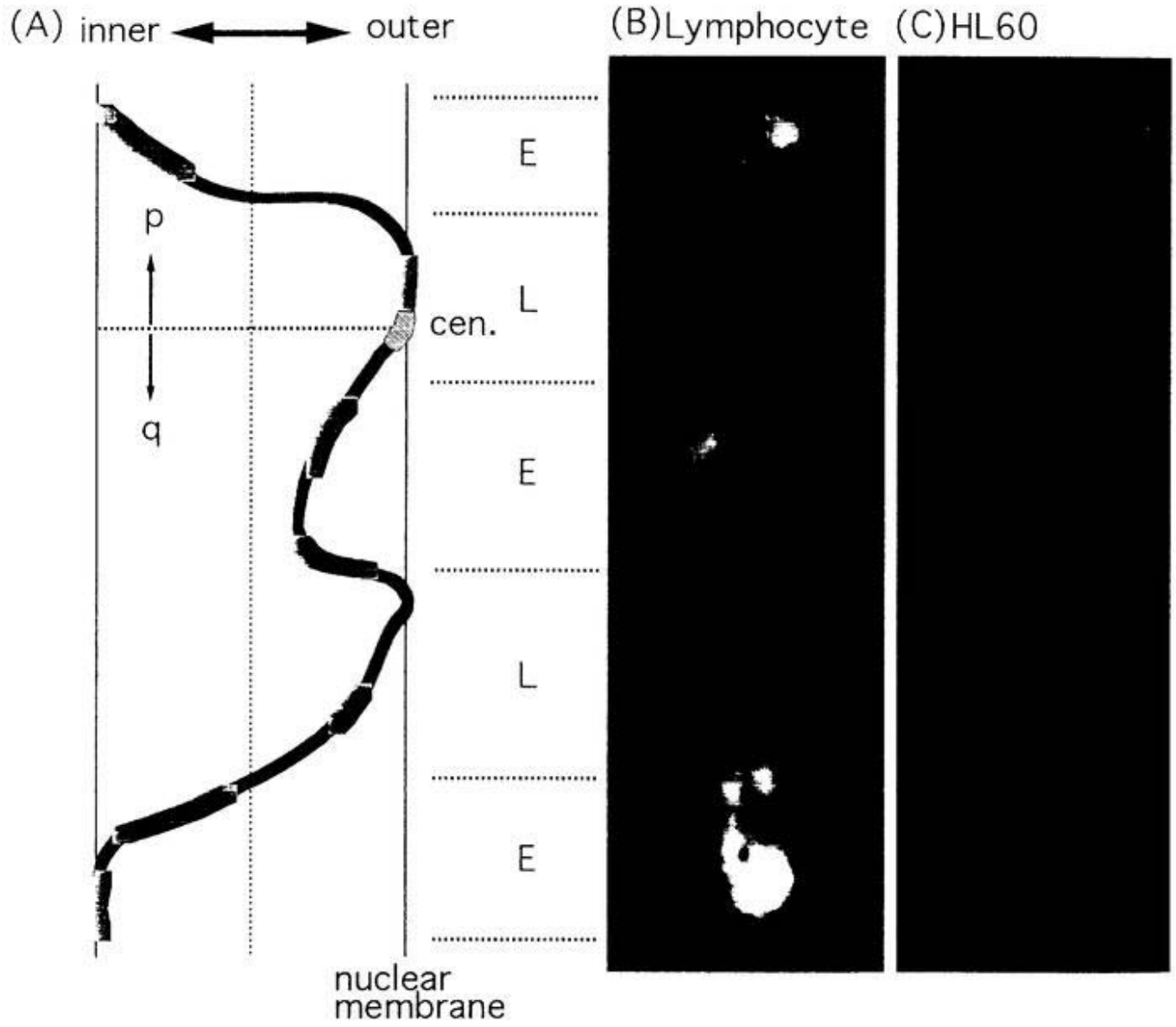

(A) inner
outer
(B)Lymphocyte
(C)HL60
p
q
cen.
E
L
E
L
E
nuclear
membrane

Fig. 3

Replication timing profiles of the cosmid clones analyzed by FISH.

For each probe over one hundred BrdU-positive nuclei (S phase) were screened. The signal patterns of SS, SD, and DD were scored and represented by dark gray, hatched, and light gray bars, respectively. Large-scale replication domains determined in Fig. 2 are added in the right side of the figure.

Fig. 3

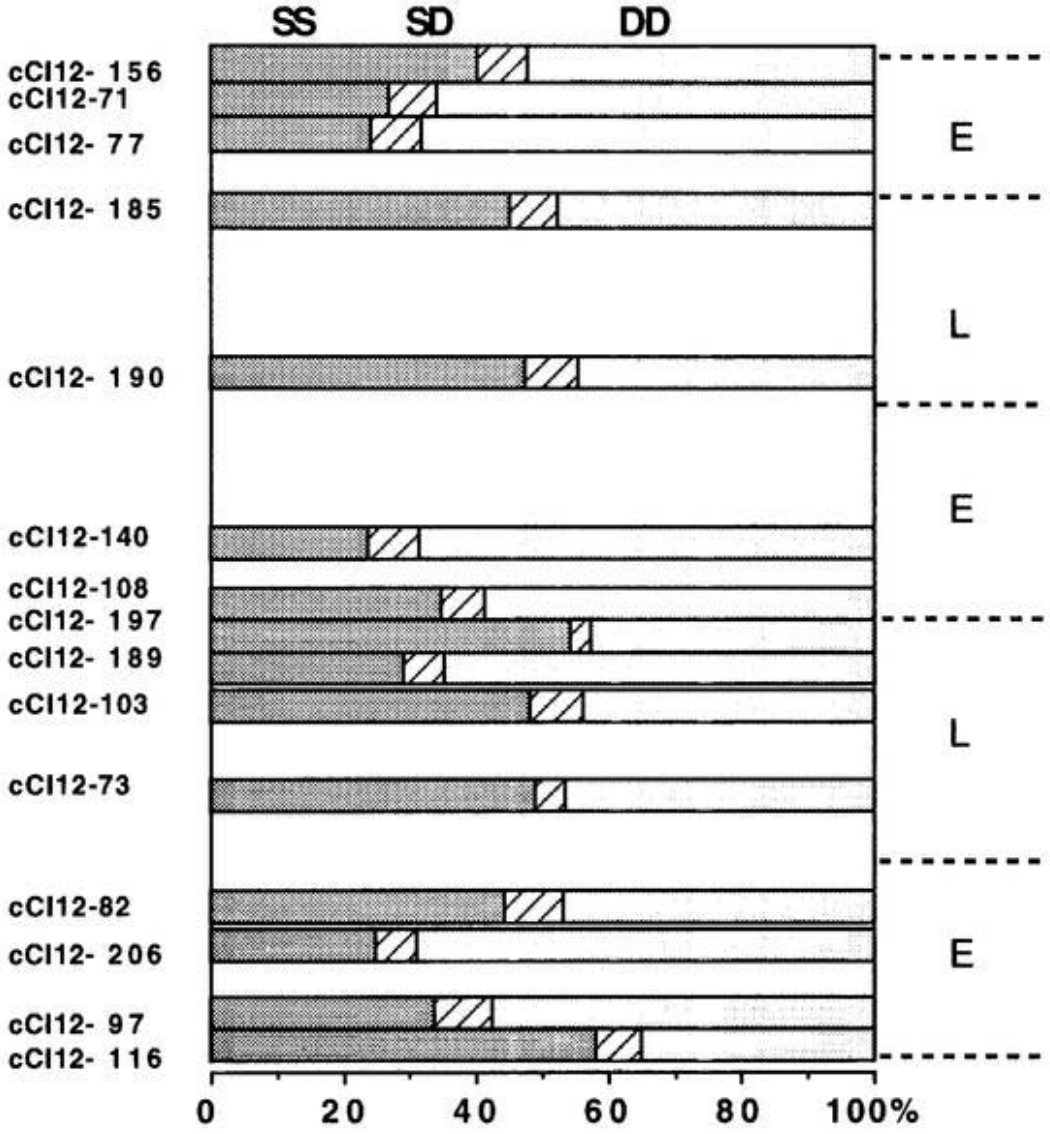
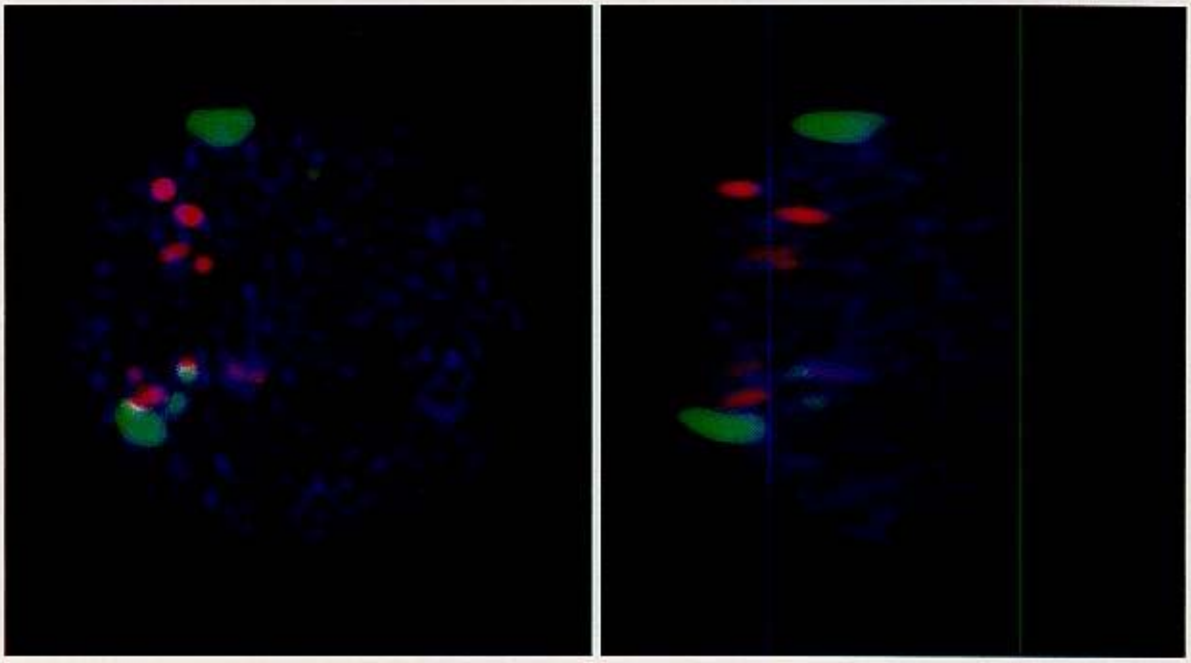

SS
SD
DD
cCI12- 156
cCI12-71
cCI12- 77
cCI12- 185
cCI12- 190
cCI12-140
cCI12-108
cCI12- 197
cCI12- 189
cCI12-103
cCI12-73
cCI12-82
cCI12- 206
cCI12- 97
cCI12- 116
E
L
E
L
E
0
20
40
60
80
100%

Fig. 4

Typical example of the projection image by a deconvolution system.

The probes from p (including a centromeric probe) and q arms were detected in fluorescein and rhodamine, respectively. The sectioning images of 60-70 layers were captured by a DeltaVision system for each fluorescence including DAPI. The typical projection image was constructed by using 20 layers with FISH signals (A). The left image was rotated 30° clockwise for z-axis (B). The topological arrangement of the backbone of chromosome 12 was traced and simply illustrated in the bottom of the figure.

Fig. 4



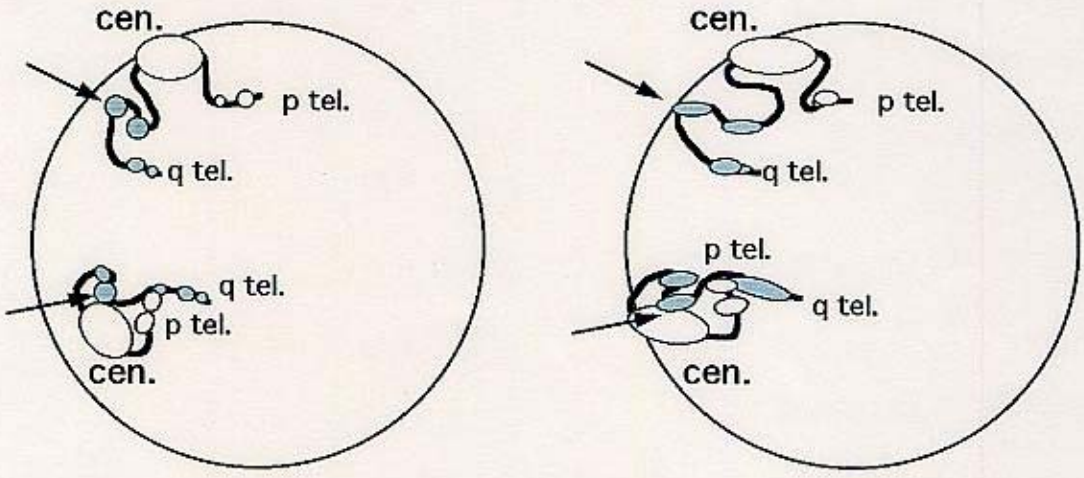


Fig. 5

The cell cycle profiles of HL60 cells fractionated by the centrifugal elutriation. Human myeloid leukemia HL60 cells were randomly cultured in RPMI1640 containing 10% FBS. 1-5 x 10^8 cells were elutriated with 0.3% gelatin in PBS. Aliquots of each fraction were analyzed by flow cytometry. (A-D) Cell cycle profiles of each fraction. The percentage of each cell cycle stage, G1, S, and G2/M, are also shown. These were regarded as G1, S, late S, and G2 rich fraction, respectively.

Fig. 5

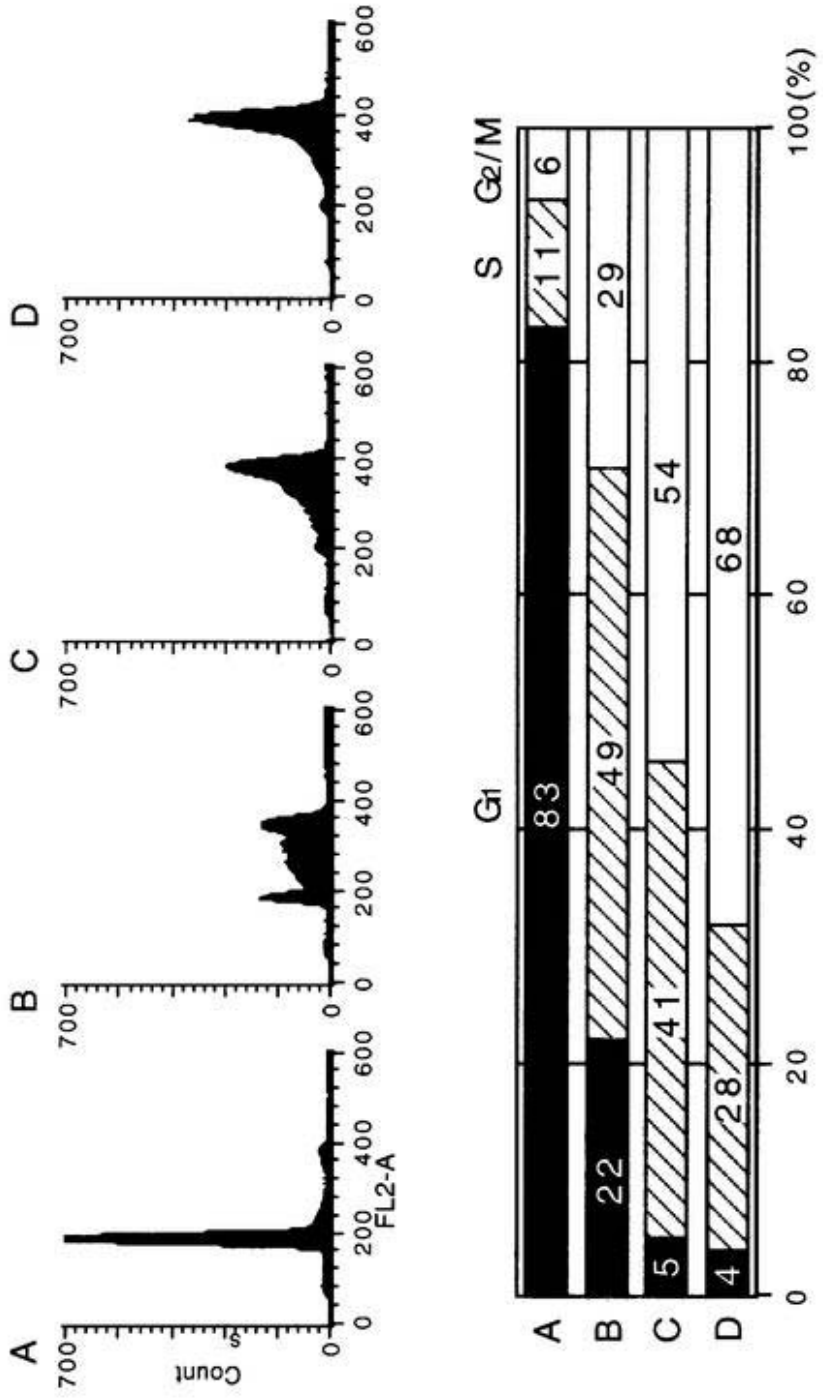

A
B
C
D
Count
FL2-A
0
200
400
600
700
G1
S
G2/M
83
11
6
22
49
29
5
41
54
4
28
68
0
20
40
60
80
100(%)

Fig. 6

FISH image of the chromosome 15 painting probe and cosmid 93 (c93; *SNRPN*) on a G-banded metaphase spread. The c93 was labeled with biotin-dUTP and detected with fluorescein-conjugated avidin (green). The chromosome 15 was detected with directly labeled Cy-3 (red). G-banding patterns of chromosomes were detected with DAPI (light blue). Upper images are the magnified view of chromosome 15 stained with Cy-3 (left) and its respective G-bands (right). The q arm of chromosome 14 was stained homogeneously except for the p arm.

Fig. 6

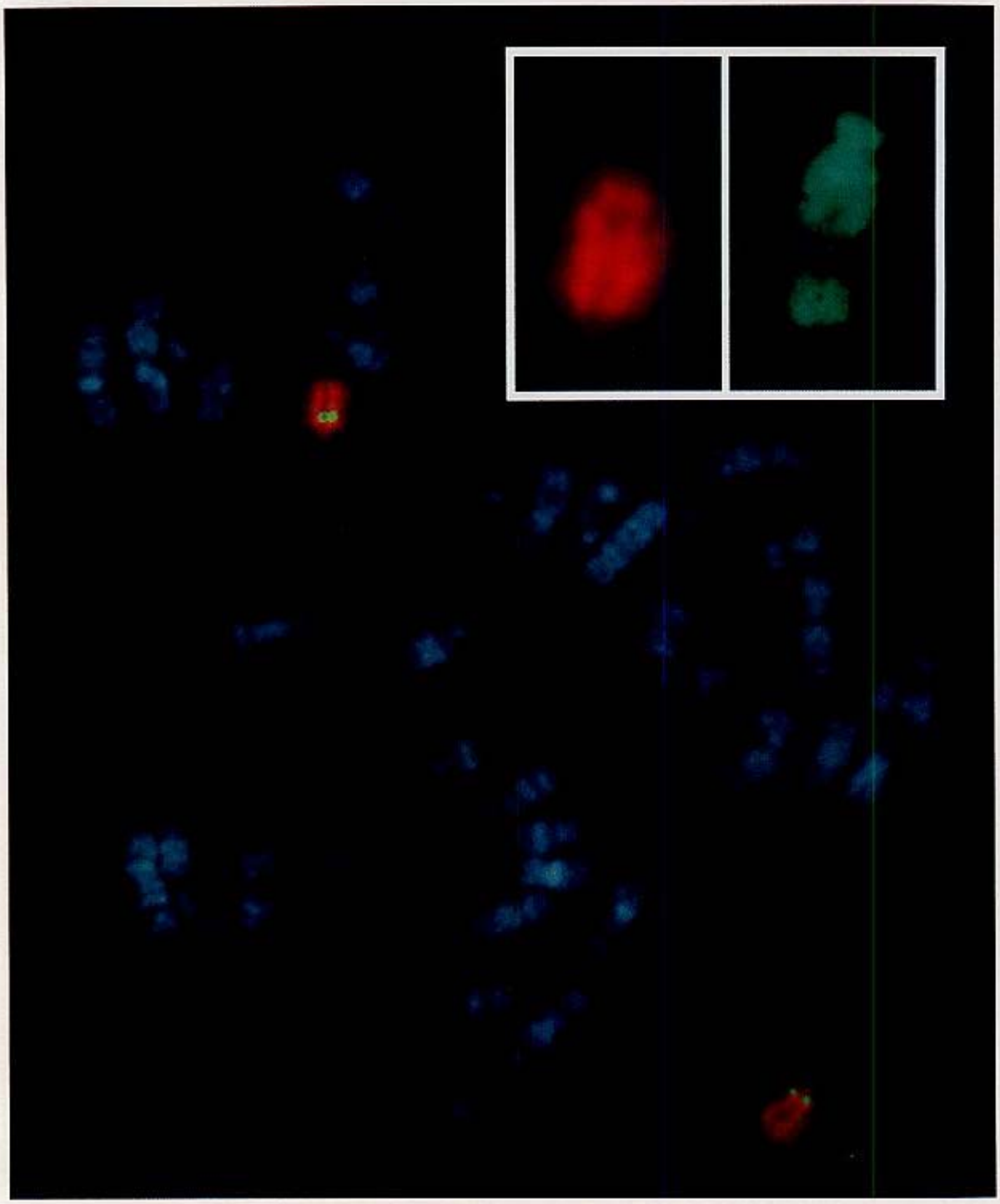


Fig. 7

Typical examples of the optical section images by a deconvolution system. Three probes and DAPI staining (gray) in HL60 nucleus were imaged in each section; chromosome 15 painting probe (red), c93 *SNRPN* probe (blue), and pCM15 centromere probe (green). 60-80 focal planes were imaged in each color. The deconvoluted images on the same focal plane were merged and pseudocolored. Only 15 images are indicated in the figure.

Fig. 7

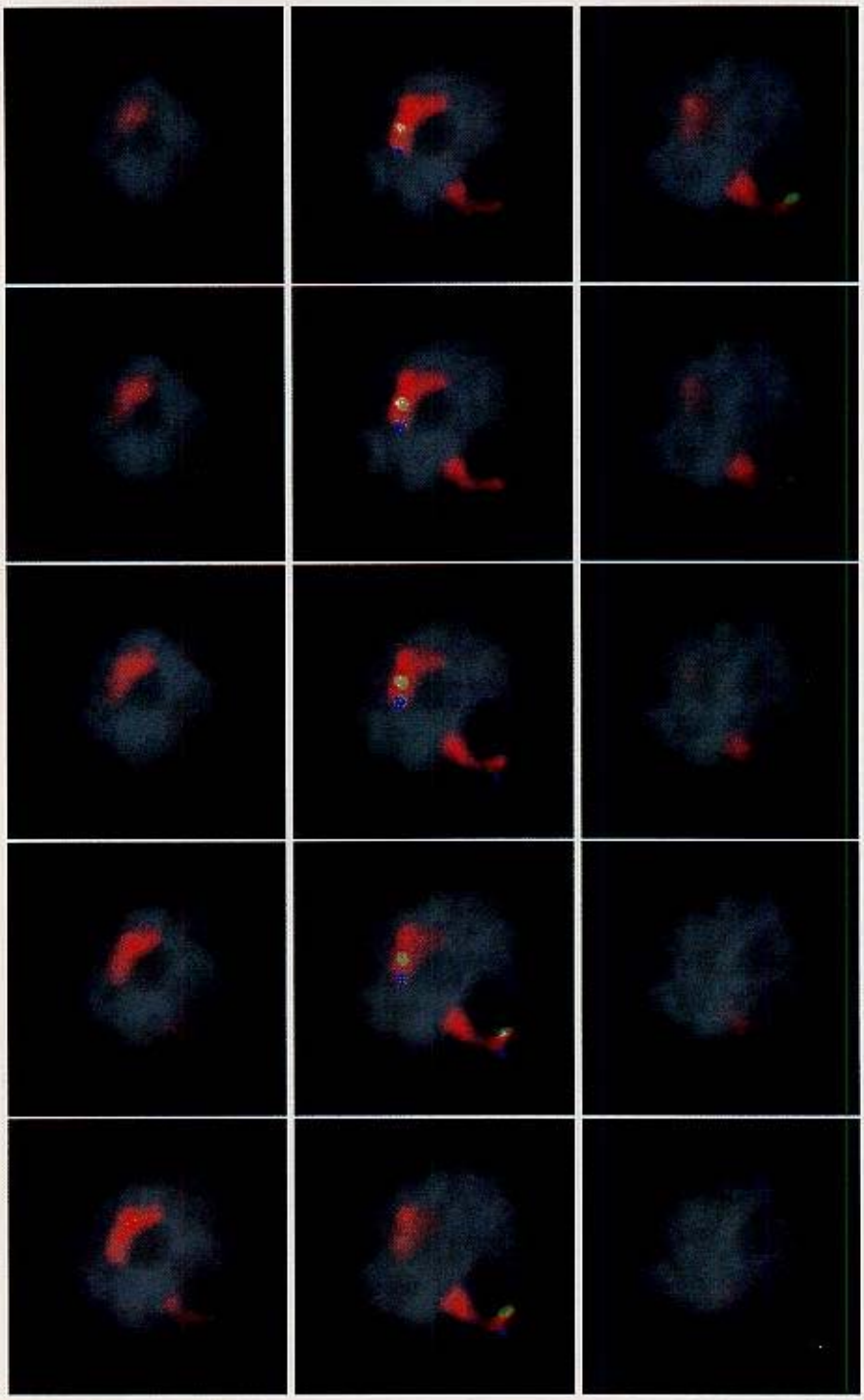


Fig. 8

The 3D reconstructed image which shows the locations of FISH signals in the HL60 cell nucleus. The image in the left is a overall view of one nucleus. Each territorial part is magnified and rotated along with the arrow (right four images). Each component was pseudocolored as indicated; the *SNRPN* genes (purple), the centromeres (green), the chromosome territories (red), and nucleus (light blue). The nucleoli (blue) were also depicted by coloring the parts which were not stained by DAPI.

Fig. 8

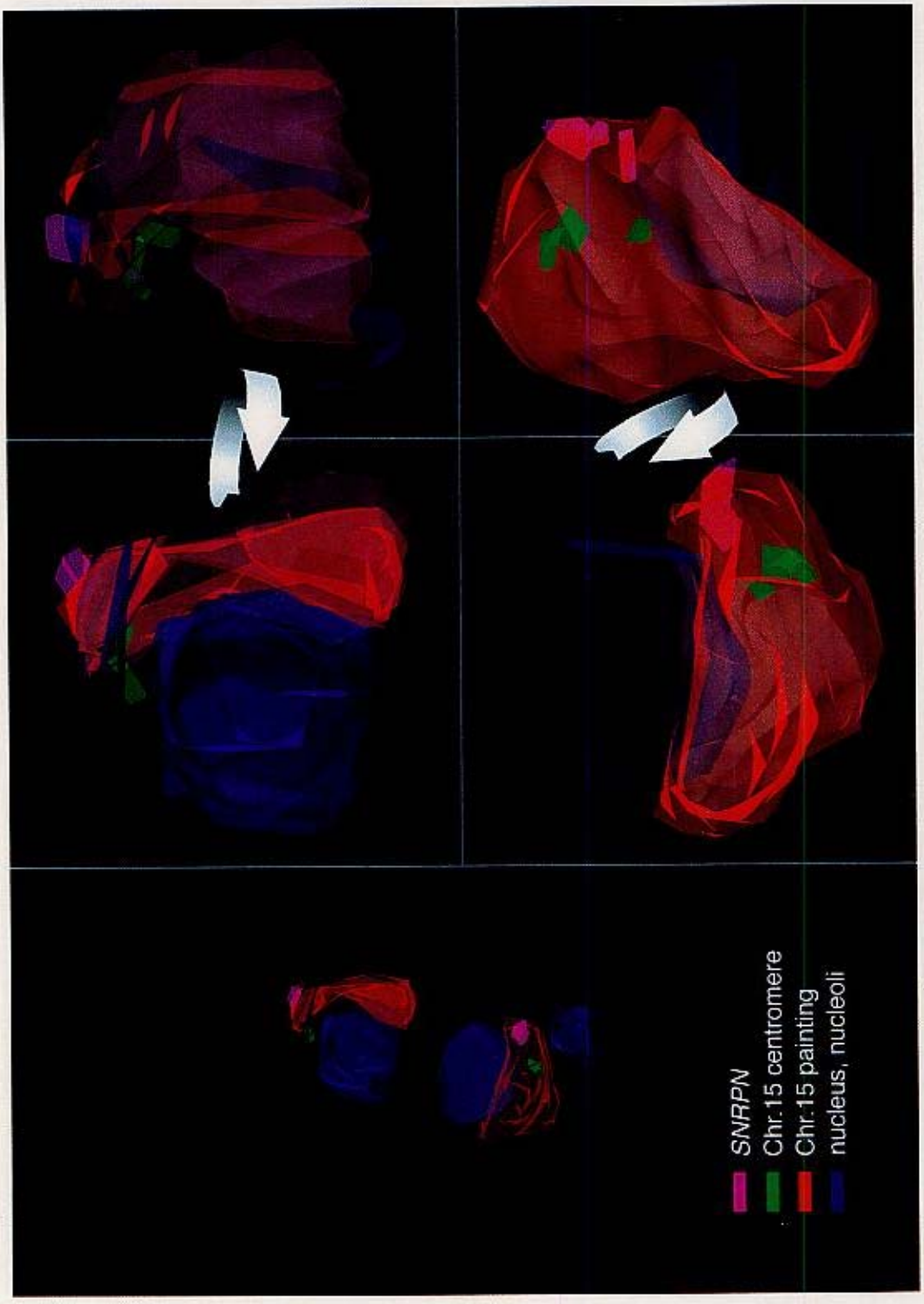


Fig. 9

Distribution of the *SNRPN* and the centromere within the chromosome territories in HL60 cells. The upper three images show the typical patterns of locations of FISH signals; the red box (left) is an example that the probe (either *SNRPN* or centromere) (blue) locates on the periphery of the chromosome territory (red) and that faces to the nuclear membrane; the orange box (middle) is an example that the probe locates on the periphery of the territory, but faces to the interior of the nucleus; the blue box is an example that the probe locates in the territorial volume. The percentage of each pattern in the individual cell fractions was scored and shown in the figure by the respective color bars.

Fig. 9

SNRPN
G1 (n=174)
S (n=148)
late S (n=158)
G2 (n=188)
28
29
43
37
27
36
31
22
47
36
15
49
centromere
G1 (n=174)
S (n=148)
late S (n=158)
G2 (n=188)
8
9
83
14
12
74
7
15
78
7
12
81
0
20
40
60
80
100 (%)

Fig. 10

The relative locations of the *SNRPN* and the centromere in the nucleus. The patterns shown in the top of the figure were scored and each percentage is indicated. The black bar is the ratio of the pattern which the *SNRPN* locates on more peripheral part than the centromere, and the white bar is its opposite. The hatched bar corresponds to the indistinguishable pattern. There are no differences in the intranuclear locations on both probes.

Fig. 10

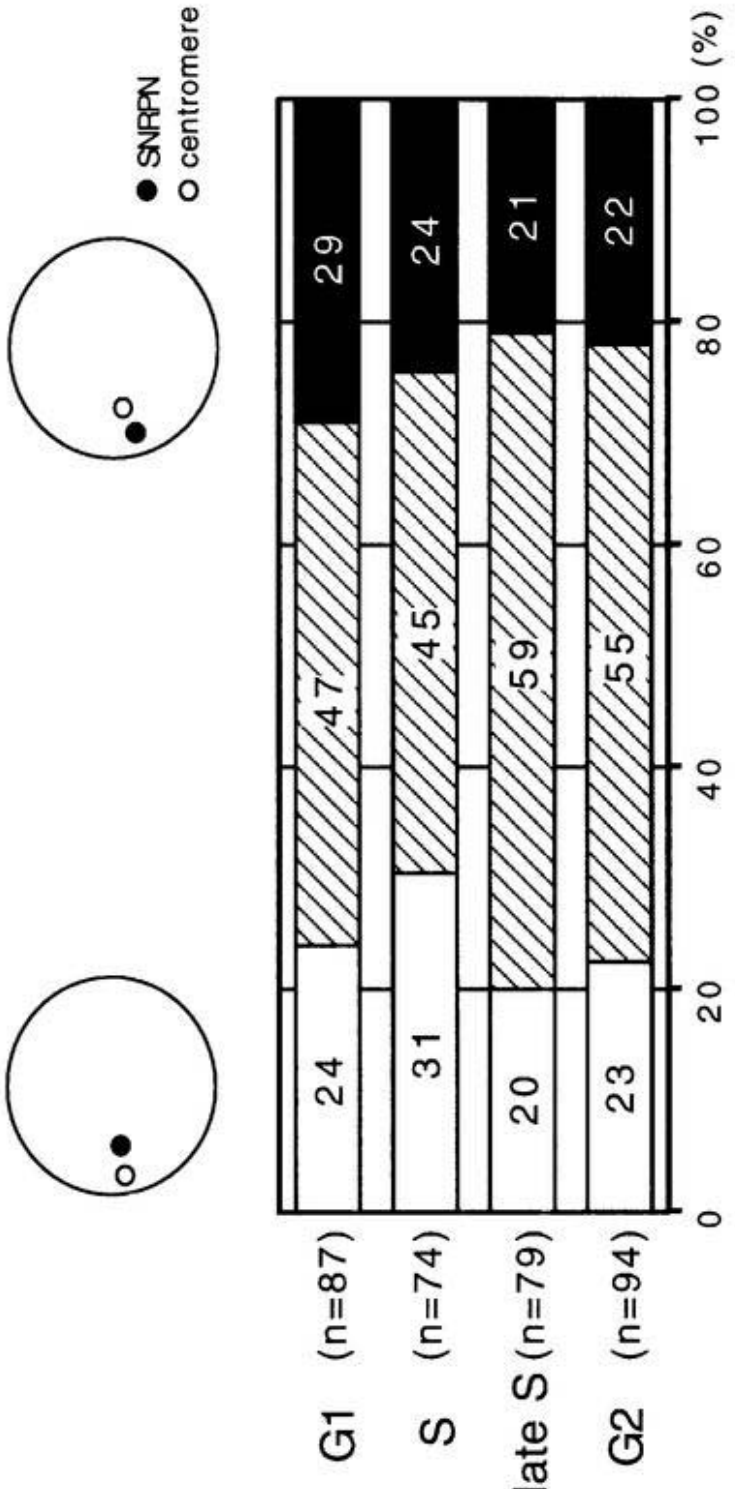

● SNRPN
○ centromere
24
47
29
31
45
24
20
59
21
23
55
22
G1 (n=87)
S (n=74)
late S (n=79)
G2 (n=94)
0
20
40
60
80
100 (%)

Fig. 11

Distribution of the *SNRPN* or the centromere in the nucleus. The patterns shown in the top of the figure were scored and each percentage is indicated. The black bar is the ratio of the pattern which the probe localizes in the nuclear periphery. The hatched bar is the pattern that the probe locates within one signal distance from the nuclear membrane. The white bar is the pattern that the probe localizes in the interior.

Fig. 11

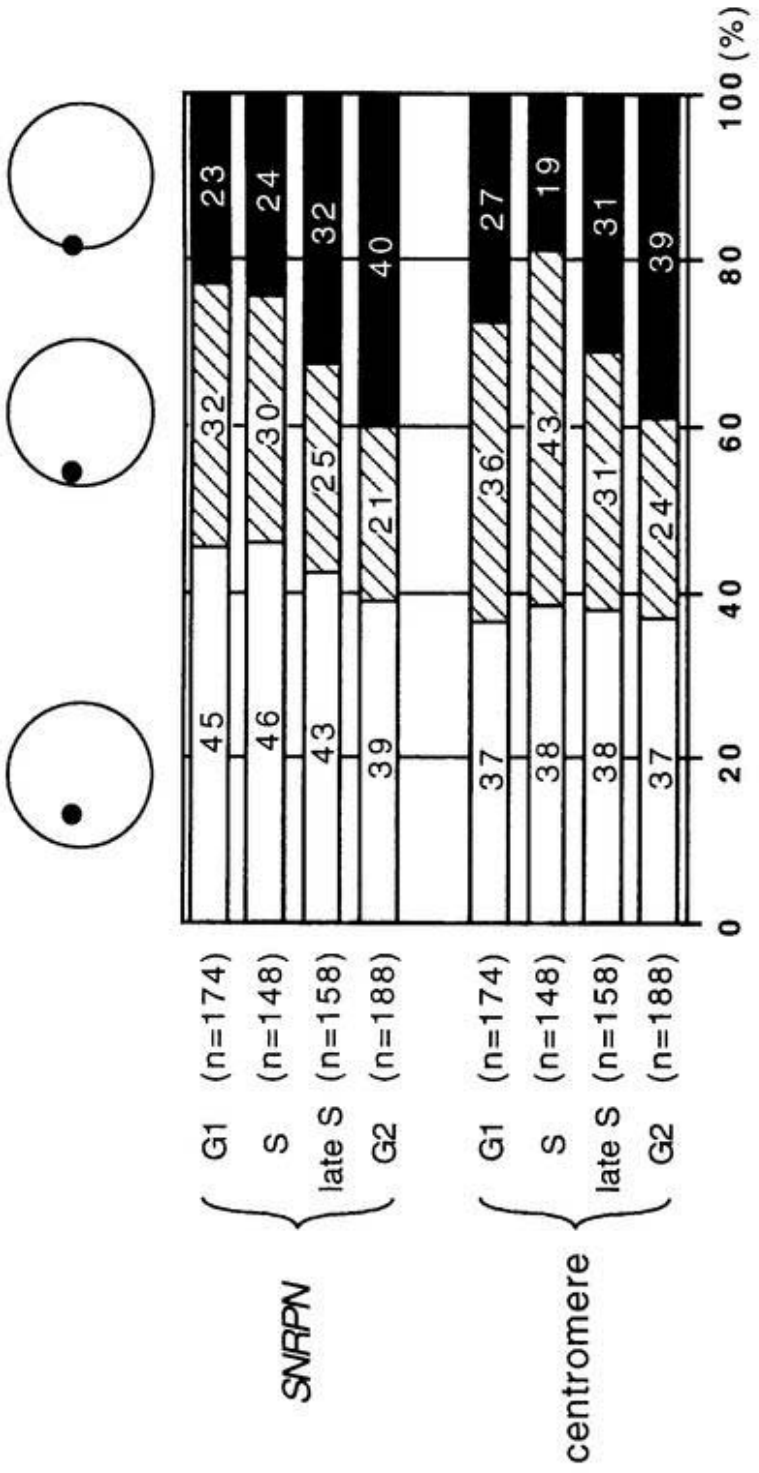

SNRPN
centromere
G1 (n=174)
S (n=148)
late S (n=158)
G2 (n=188)
45
32
23
46
30
24
43
25
32
39
21
40
37
36
27
38
43
19
38
31
31
37
24
39
0
20
40
60
80
100 (%)

Fig. 12

The cell cycle dependent changes of the distance between the *SNRPN* and the centromere on the same chromosome. The patterns shown in the top of the figure were scored and each percentage is indicated. The image with purple box is the pattern in which both the signals co-localize. The orange box is the pattern in which both the signals overlap. The yellow box is the pattern in which both the signals are separate but position within one signal distance. The green box is the pattern in which both the signals are separate each other.

The *SNRPN* and the centromere are blue and green, respectively.

Fig. 12

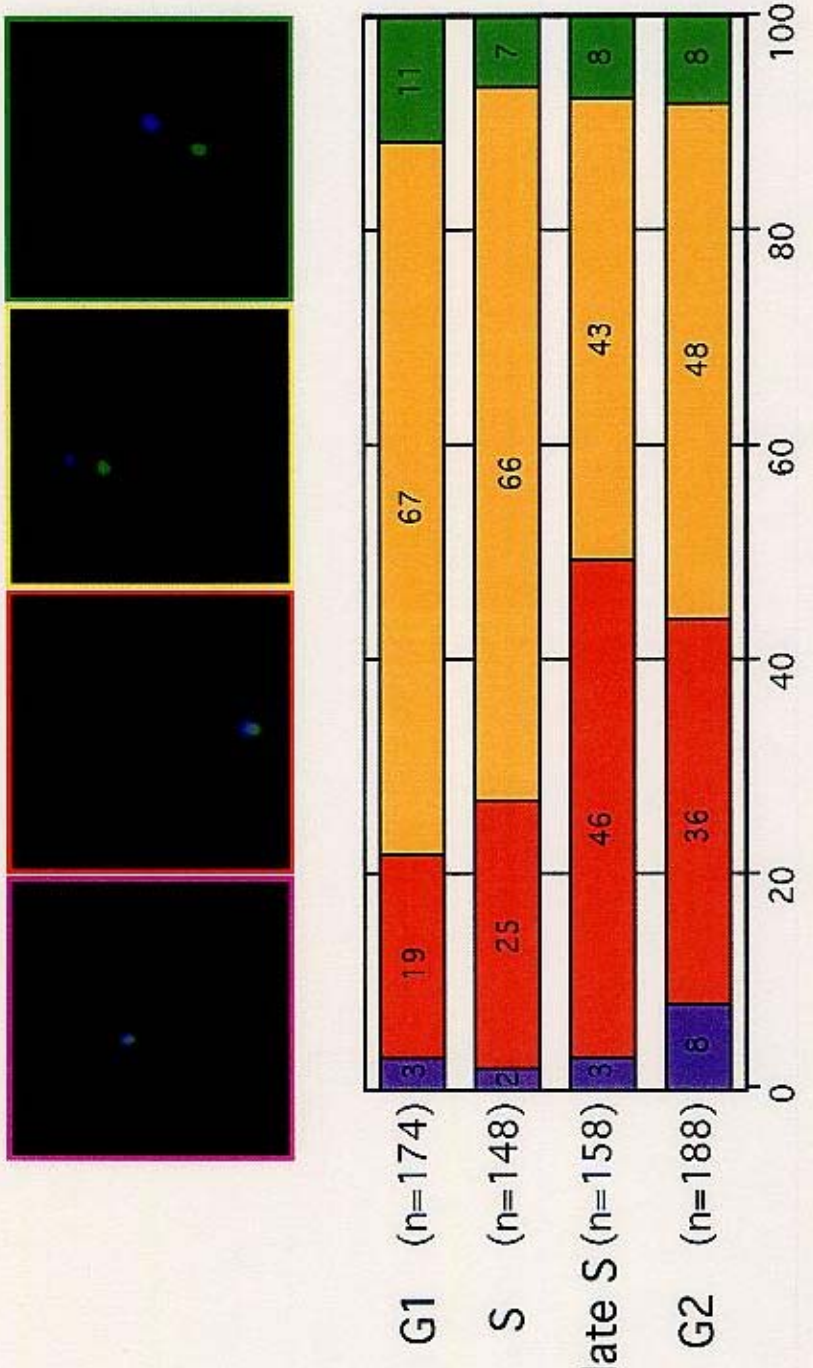

G1 (n=174)
S (n=148)
late S (n=158)
G2 (n=188)
3
19
67
11
2
25
66
7
3
46
43
8
8
36
48
8
0
20
40
60
80
100 (%)

Fig. 13

Comparison of the relative distances of the homologous alleles between the *SNRPN* and the centromere. The patterns shown in the top of the figure were scored and each percentage is indicated. The black bar is the ratio of the pattern that the distance of the *SNRPN* homologues is shorter than that of the centromere ones. The white bar is the opposite pattern. The hatched bar is the ambiguous pattern.

Fig. 13

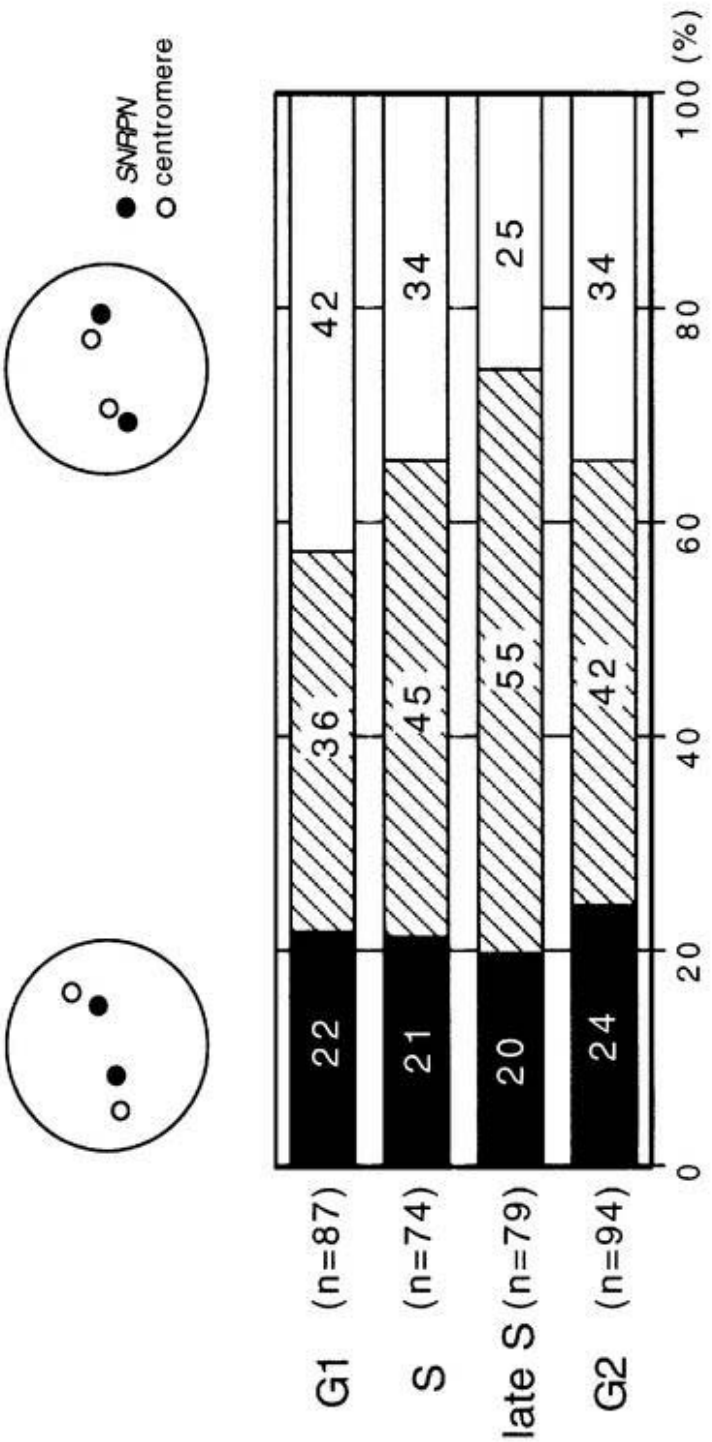

● SNRPN
○ centromere
G1 (n=87)
S (n=74)
late S (n=79)
G2 (n=94)
22
36
42
21
45
34
20
55
25
24
42
34
0
20
40
60
80
100 (%)

Fig. 14

RNA-FISH detecting the primary transcript of the *SNRPN* gene. A PCR-generated intron probe was labeled with digoxigenin-dUTP and detected with rhodamine. Most nuclei has only one spot of the signal (red). The nuclei was counterstained with DAPI (blue).

Fig. 14

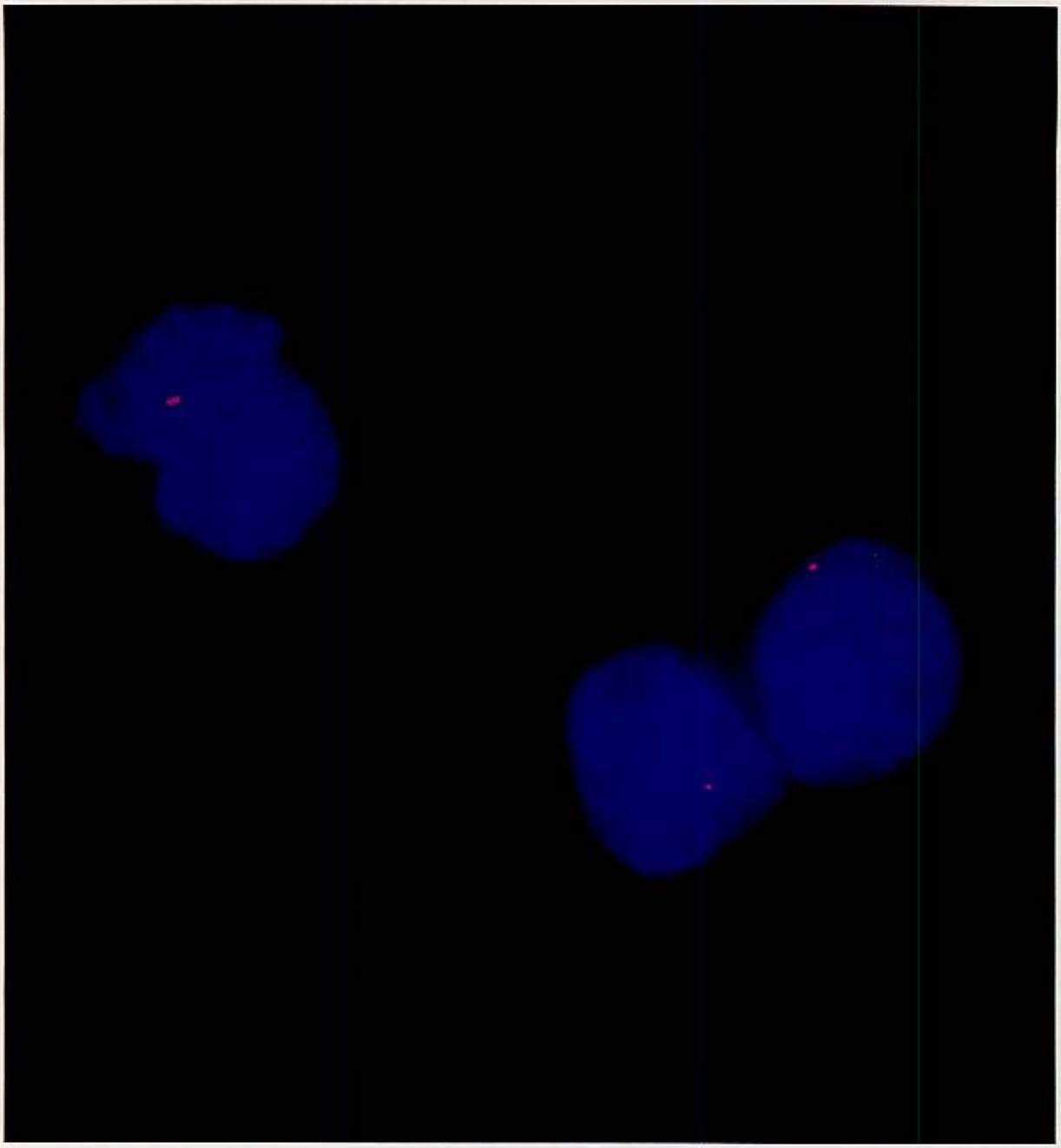
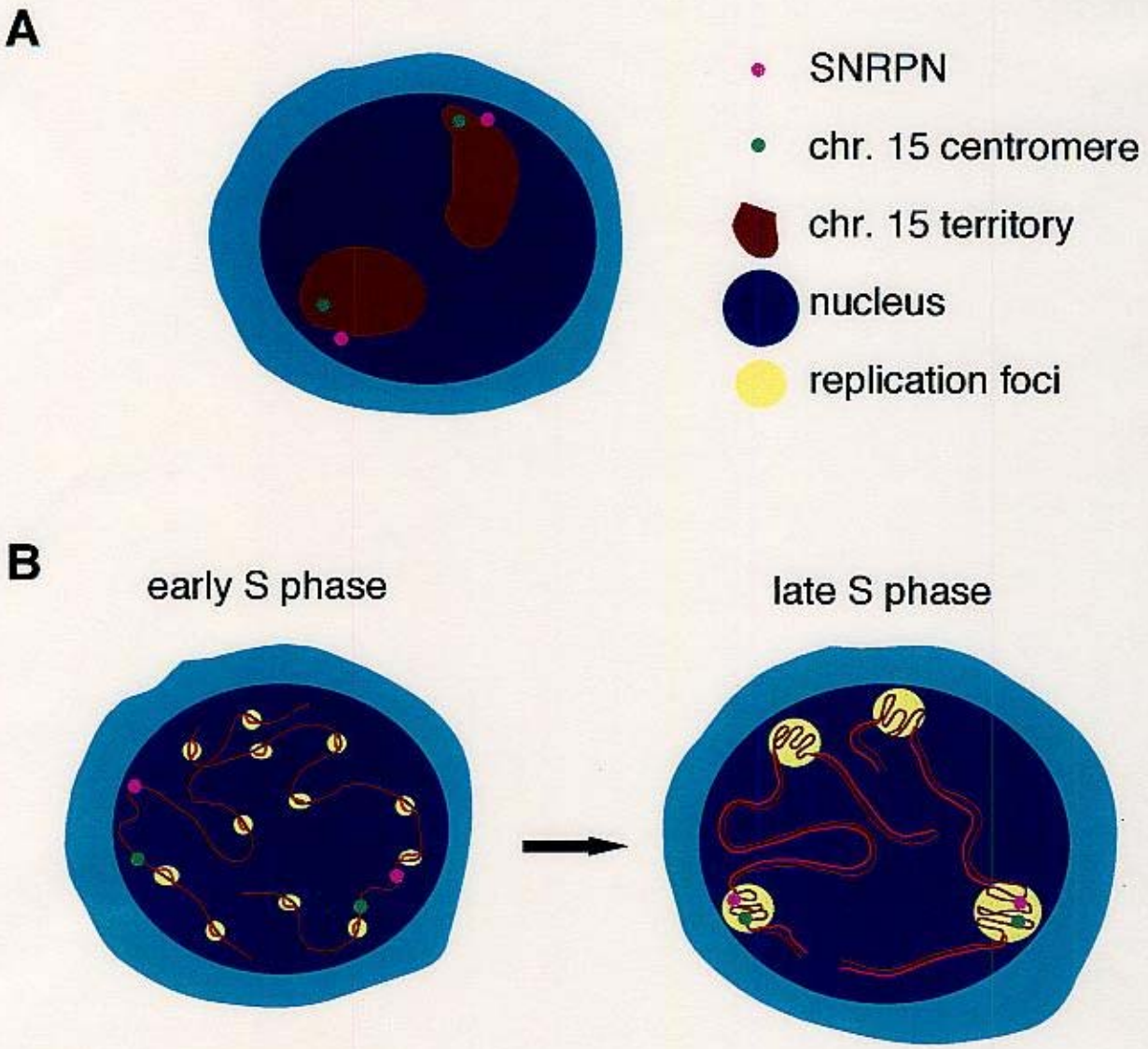


Fig. 15

Models of the intranuclear organization in HL60 cells obtained in this study. (A) Relative locations of the *SNRPN*s and the centromeres within the chromosome territories. (B) The model for the changes of the signal distance between the *SNRPN* and the centromere. In early S phase, both segments are separate each other, but in late S phase, they are assembled in the same replication foci.

Fig. 15


A
SNRPN
chr. 15 centromere
chr. 15 territory
nucleus
replication foci
B
early S phase
late S phase

Supplement.

In the course of pursuing this work, I determined the chromosomal localization of many gene sequences. The information of these results is important for not only constructing of the genome map, but also intranuclear dynamics of each gene in future works. In this point of view, I mention the list of publications in which I involved as follows:

1. Mizuki, N., Kimura, M., Ohno, S., Miyata, S., Sato, M., Ando, H., Ishihara, M., Goto, K., Watanabe, S., Yamazaki, M., Ono, A., Taguchi, S., Okumura, K., Nogami, M., Taguchi H., Ando, A. and Inoko, H., (1996). Isolation of cDNA and Genomic Clones for a Human Ras-related GTP-Binding Protein and Its Chromosomal Localization to the Long Arm of Chromosome 7 , 7q36, *Genomics,* **34**: 114-118.
2. Eki,T., Okumura, K., Amin, A., Ishiai, M., Abe, M., Nogami, M.,Taguchi, H., Hurwitz, J., Murakami, Y., and Hanaoka, F., (1996). Mapping of the Human Homologue (ORC1L) of the Yeast Origin Recognition Complex Subunit 1 Gene to Chromosome Band 1p32.2-p32.3, *Genomics,* **36**: 559-561.
3. EkiT., Okumura, K., Shiratori, A., Abe, M., Nogami, M., Taguchi, H., Shibata, T., Murakami, Y., and Hanaoka, F., (1996). Assignment of the Closest Human Homologue (DNA2L; KIAA0083) of the Yeast Dna2 Helicase Gene to Chromosome Band 10q21.3-q22.1, *Genomics*, **37**: 408-410.
4. Fujiwara, Y., Miwa, M., Nogami, M., Okumura, K., Nobori, T., Suzuki, T., and Ueda, M., (1997). Genomic Organization and Chromosomal Localization of the Human Casein Gene Family, *Hum. Genet.*, **99**: 368-373.
5. Kitanaka, S., Takeyama, K., Murayama, A., Sato, T., Okumura, K., Nogami, M., Hasagawa, Y., Niimi, H., Yanagisawa, J., Tanaka, T., and Kato, S., (1998)

Inactivating Mutations in the 25-Hydroxyvitamin D3 1a-Hydroxylase gene in Patients with Pseudovitamin D-Deficiency Rickets, *New England J. of Med.*, **338**(10): 653-661.

6. Okumara, K., Nogami, M., Matsushima, Y., Matsumura, K., Nakamura, K., Taguchi, H., and Kitagawa, Y. (1998). Mapping of human DNA-binding nuclear protein (NP220) to chromosome band 2p13.1-p13.2 and its relation to matrin 3, *Biosci. Biotech. Biochem.*, **62**: 1640-1642.

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