A SIMPLE G-BANDING TECHNIQUE ADAPTABLE FOR FLUORESCENT IN SITU HYBRIDIZATION (FISH) AND PHYSICAL ORDERING OF HUMAN RENIN (REN) AND CATHEPSIN E (CTSE) GENES BY MULTI-COLOR FISH

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We developed a simple technique for delineation of refined replication G-band in combination with fluorescent in situ hybridization (FISH). By using this system we localized three genes of pepsinogen C (PGC), renin (REN), and cathepsin E (CTSE) on 6p21.1, 1q32, and 1q32, respectively. Further, we determined the physical order of REN and CTSE on chromosome band 1q32 as cen-(1q32)-REN-CTSE-tel by multi-color FISH.

The construction of refined physical and genetic maps is an indispensable step for the eventual sequencing of the entire human chromosome as well as for the "positional cloning" of target chromosome loci responsible for inherited genetic disorders and tumor suppressor genes. The use of in situ hybridization techniques on metaphase chromosomes has contributed to the construction of high-resolution cytogenetic maps (7, 14). The technique with isotope-labeled probes is sensitive (8), although it has several disadvantages such as probe instability due to radiolysis of isotopes, long exposure times for detection of signals by autoradiographic procedures, and uncertain localization of signals captured by an emulsion overlay. The development of fluorescent in situ hybridization (FISH) has overcome some of these problems and this technique has been used as a standard tool for chromosomal mapping in recent years.

Application of replication-banding techniques to FISH has enabled researchers to determine the precise location of numerous DNA loci directly on banded chromosomes (2, 5, 9, 15). Further, the development of multi-color FISH has strongly enhanced cytogenetic resolution in the ordering of closely located DNA loci on metaphase and interphase chromosomes.

We describe here a simple high-resolution G-banding technique adaptable for FISH and further describe the physical mapping of pepsinogen C (PGC), renin (REN) and cathepsin E (CTSE) genes by this technique.

MATERIALS AND METHODS

Metaphase chromosome preparations

Metaphase chromosomes were prepared by thymidine synchronization, bromodeoxyuridine (BrdU) release technique as previously reported (7, 9). In brief, phytohemagglutinin-stimulated human peripheral blood lymphocytes from healthy donors were cultured at 37°C in RPMI1640 medium supplemented with 15% fetal calf serum (FCS). After 48 hr incubation, thymidine was added to the medium at a final concentration of 300 μg/ml and incubation was continued for 16 hr. The cells were rinsed twice in RPMI1640 and then resuspended in supplemented RPMI1640 medium containing BrdU (25 μg/ml) (Sigma) for 6.0 hr. During the last 0.5 hr of this treatment, the cells were exposed to 0.02 μg/ml colcemid (Welcome). They were then harvested and resuspended in 0.075 M KCL at room temperature for 30 min. After fixation in 3:1 methanol:acetic acid, slides were prepared by the air-dry method, and fixed at 65°C for 5 hr.

To delineate the replication G-band pattern, fluorescence staining was carried out as described by Takahashi et al., (14) with minor modifications (11,
The chromosome slides were stained with Hoechst 33258 (1 μg/ml) (Sigma) diluted with 2 × SSC (pH 7.3) for 5 min, briefly rinsed in distilled water, and then mounted in 2 × SSC under coverslips. The slides were exposed to a Mercury-vapor lamp (HB100 W/A; Toshiba) at a distance of 6 cm for 5 min. They were then rinsed in distilled water and air-dried. Before hybridization, the slides were denatured in 70% formamide/2 × SSC at 75°C for 2 min, immersed in 70% ethanol at −20°C, and dehydrated through an ethanol series.

DNA probes and labeling
Three cosmid clones of human pepsinogen C (PGC), renin (REN) and cathepsin E (CTSE), which contain about 40 kb genomic fragment of each gene, were used as probes. To identify the precise locations of each gene by conventional FISH, cosmid probes (0.5 μg of each) were labeled with biotin(bio)-16-dUTP (Boehringer) by nick translation. To determine the physical order of REN and CTSE by multicolor FISH, REN probe was labeled with digoxigenin(dig)-11-dUTP (Boehringer) and CTSE probe was labeled with bio-16-dUTP. Labeled probes were precipitated with sonicated salmon sperm DNA and E. coli tRNA, and were dissolved in 30 μl of formamide.

In situ hybridization and detection of hybridized probes
FISH was carried out for assignments of each gene as previously reported (9, 10, 11, 12). The hybridization signals of bio-labeled probes were detected with avidin-FITC (Boehriger) and the metaphase chromosomes were counterstained with propidium iodide (PI) (1 μg/ml) (Sigma).

To physically order REN and CTSE, probe solutions of bio-labeled CTSE and dig-labeled REN were mixed in a ratio of 7 : 3, and 0.7 μl sonicated human placental DNA (10 mg/ml) was added to 9.3 μl of the mixture to eliminate background noise due to repetitive sequences of Alu and L1. The final mixture was denatured at 75°C for 5 min and mixed with an equal volume of 4 × SSC with 20% dextran sulfate. The hybridization mixture was placed on denatured slides, covered with Parafilm, and incubated in a humid box at 37°C over night. After being washed in 50% formamide/2 × SSC, 2 × SSC, and 1 × SSC (37°C, 15 min, each), they were then incubated in 4 × SSC with 1% Block Ace™ (Dainippon Pharmaceutical Co., Ltd.) containing avidin-FITC and anti-dig rhodamine (Boehringer) at 37°C for 40 min, and then washed for 5 min in each of 4 × SSC, 4 × SSC/0.05% TritonX-100, and 4 × SSC. Then, the slides were counterstained with DAPI (1 μg/ml) and mounted in an anti-fade solution containing p-phenylenediamine (Sigma) and DABCO (1,4-diazabicyclo[2,2,2] octane) (Sigma).

RESULTS
Replication G-banding pattern
Replication banding pattern through UV-2A filter was delineated as two-color bands stained by two different fluorochrome dyes; Hoechst 33258 and PI (Fig. 1). The G-bands appeared white under Hoechst 33258 and G-negative bands (R-banding regions) appeared red under PI, providing rapid and easy identification of chromosome bands.

In comparing the replication G-bands with ISHB on the same metaphase chromosomes, Alu sequences were shown to be concentrated at G-negative bands of chromosomes (Fig. 1).

Localization of PGC, REN, and CTSE
We examined 50 metaphase cells for localization of PGC, REN, and CTSE; of these 43 metaphase cells (86%) showed twin-spot signals specific for PGC on both chromatids of one or both short arms of
chromosome 6. The delineation of the replication G-band pattern on the same metaphase chromosomes allowed us to determine the precise location of PGC at 6p21.1 as shown in Fig. 2. REN and CTSE were mapped on the same chromosome band of 1q32 in the same manner (data not shown).

**Ordering of REN and CTSE**

To determine the order of REN and CTSE on the same band 1q32, we performed multi-color FISH. As shown in Fig. 3A, the FITC signals of CTSE is distal to...
the rhodamine signals of REN along the longitudinal axis of chromosome 1. The order was confirmed by digitized images of hybridization signals generated with confocal laser microscopy as shown in Fig. 3B.

DISCUSSION

To determine the precise locations of probes on chromosomes by FISH, it requires not only visualiza-
tion of discrete hybridization signals specific for the probes, but also delineation of high-resolution chromosome bands. Recently, Takahashi et al., (15) developed the direct R-banding FISH system which permits the direct localization of hybridization signals on R-banded chromosomes through the same filter. In this system, FITC-detected hybridization signals appear yellow-green on a background of PI-stained red chromosomes with an R-banding pattern. Although this system is efficient for cytogenetic mapping, in FISH with reiterated DNA probes the R-band pattern of chromosomes sometimes disappear due to FITC background-noise as a result of incomplete suppression of repetitive sequences. On the other hand, the technique described here provides a discrete G-band pattern of whole chromosomes without interfering with FITC background noise (Fig. 1), facilitating rapid and precise localizations of cosmids or \( \lambda \) phage clones that contain repetitive sequences.

The chromosomal locus of PGC, reported elsewhere (6) as 6pter-p21.1, was confirmed in our study and further refined to 6p21.1 (Fig. 2). The cytogenetic locations of REN and CTSE were reported as (1q32 or 1q42) (3, 13) and 1q31 (4), respectively, by in situ hybridization with isotope-labeled probes. However, our FISH studies showed that REN and CTSE are syntenic to the same band region of 1q32. Further, we determined the order of these two genes as cen-(1q32)-REN-CTSE-tel by multi-color FISH (Fig. 3).

In the process of positional cloning of cancer breakpoints or genes responsible for unknown genetic diseases, it is important to determine high-precision localizations of DNA markers as well as the order of
them in the vicinity of the target locus. The simple G-banding technique combined with FISH and the probe-ordering system by multi-color FISH, described here, should contribute materially to construction of high-resolution cytogenetic maps, and to positional-cloning studies.

ACKNOWLEDGEMENT

We are grateful to Drs. Murakami, and Taggart for providing REN, and PGC cosmid probes. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture, Japan.

REFERENCE