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Simultaneous detection of high-resolution R-banding and fluorescence in situ hybridization signals after fluorouracil-induced cellular synchronization

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A method for simultaneous detection of fluorescence in situ hybridization of DNA probes and high resolution fluorescent R banding is described. Human lymphocytes were stimulated with phytohemagglutinin and synchronized using a fluorouracil block followed by exposure to bromodeoxyuridine and Hoechst 33258 prior to harvest. Metaphase preparations were treated with Hoechst 33258 and exposed to UV light. Thereafter they were incubated in sodium phosphate buffer and dried prior to in situ hybridization with a biotin-labelled centromere-specific α-satellite DNA probe for chromosome 1 (pUC1.77) and two digoxigenin-labelled probes, i.e., a PCR-generated chromosome 8-specific alphoid probe (#8) and a cosmid probe for FLT4 gene on 5q33-qter (class III receptor tyrosine kinase). Hybridization signals were detected by an indirect immunofluorescence method using fluorescein isothiocyanate. The chromosomes were counterstained with propidium iodide and 4′,6-diamidino-2-phenylindole dihydrochloride. This simple method allows unambiguous chromosome band identification simultaneously with detection of the hybridized probes.

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Until the end of the 1980s, radioactive in situ hybridization using metaphase chromosomes was the only direct technique for assigning genes or DNA segments to specific chromosomes (HARPER and SAUNDERS 1981; ZABEL et al. 1983; FAN et al. 1989). Several disadvantages related to the use of radioisotopes have been observed. These include long exposure time, uncertain localization of signals due to low resolution, and low precision due to different focal planes of silver deposits over the chromosomes.

Recent advances in nonradioactive in situ hybridization procedures have overcome most of the aforementioned drawbacks (CREMER et al. 1988a,b; LICHTER et al. 1988, 1990a,b; LUCAS et al. 1989; PINKEL et al. 1986; TUCKER et al. 1988; VAN DEKKEN and BAUMAN 1988). Although several different techniques have been developed (KLEVER et al. 1991; LAWRENCE et al. 1988; MONTANARO et al. 1991; TAKAHASHI et al. 1989; VIEGAS-PÉQUIGNOT et al. 1989a,b, 1991), few of them allow simultaneous observation of chromosome bands and hybridization signals (CHERIFF et al. 1990; FAN et al. 1990; LEMIEUX et al. 1992; TAKAHASHI et al. 1990, 1992).

In this report we describe a method for R band induction and nonisotopic in situ hybridization after fluorouracil synchronization of human lymphocytes. Combination of these two techniques in a dual analysis system allows the detection of fluorescent signals on R-banded chromosomes.

Material and methods

Cell cultures

Whole blood (1 ml) was cultured in complete culture medium [80 % RPMI 1640 (Gibco, Grand

Island, NY, USA), 20 % fetal calf serum (Gibco), 0.29 mg/ml L-glutamine (Gibco), 100 units/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco)], and phytohemagglutinin P (PHA, 0.5 μg/ml, Gibco). The cultures were incubated in darkness at 37°C in a 5 % CO_2 atmosphere. At 60–72 hours of incubation, cells were processed according to the synchronization method of RØNNE (1984). Briefly, fluorouracil (Sigma Chemical Co., St. Louis, MO, USA) and uridine (Sigma) were added to the cultures at a final concentration of 5×10^{-7} M and 5×10^{-6} M, respectively. After 17–20 hours, bromodeoxyuridine (Sigma) and Hoechst 33258 (Sigma) were incorporated into the cultures at a final concentration of 30 μg/ml and 60 μg/ml, respectively. The cultures were then reincubated in darkness for an additional six hours until harvest, including Colcemid (0.1 μg/ml, Gibco) treatment during the last hour. Harvesting was performed using hypotonic treatment (0.075 M KCl, 15 min, 37°C) and fixation with methanol:acetic acid (3:1). The slides were air-dried for at least 16–18 hours and processed according to the combined methodology described below.

Metaphase chromosome preparations and R-banding analysis

After air-drying, the slides were processed according to the fluorescence-plus-Giemsa method of PERRY and WOLFF (1974) and KORENBERG and FREEDLANDER (1974) with minor modifications.

The chromosome spreads were treated with 1 μg/ml of Hoechst 33258 (Sigma) in 0.1 M phosphate buffer (pH 6.8) for 20 min. The slides were then mounted in Hoechst-free 0.1 M phosphate buffer and exposed to UV light (Philips TUV 15 W tube; Philips, Netherlands) for one hour. After UV exposure, the slides were rinsed in deionized water, dried, and treated according to the method of KORENBERG and FREEDLANDER (1974). The slides were incubated in 1 M Na_2HPO_4 (pH 8.2–8.4, 88°C, 5–10 min), rinsed in deionized water, and air-dried for at least 16–18 hours before in situ hybridization.

Fluorescence in situ hybridization

Probes. — The following probes were used for fluorescence in situ hybridization: a chromosome 1-specific α-satellite repetitive probe hybridizing to region 1q12 (pUC1.77; COOKE and HINDLEY 1979); chromosome 8-specific alphoid DNA probe generated by polymerase chain reaction (PCR) (#8; DUNHAM et al. 1992); cosmid probe for FLT4 receptor tyrosine kinase gene on chromosome 5q33-qter (APRELIKOVA et al. 1992; PAJUSOLA et al. 1992; WARRINGTON et al. 1992) recognizing the q35 (ARMSTRONG et al. 1992) region of chromosome 5.

Probe pUC1.77 was labelled by nick translation with biotin-11-dUTP (Sigma), and FLT4 was labelled with digoxigenin-11-dUTP (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany), both according to the instructions of the kit supplier (Nick-translation Kit, Bethesda Research Laboratories, MD, USA; DIG DNA Labeling Kit, Boehringer Mannheim). Digoxigenin-dUTP was incorporated into probe #8 during the PCR reaction.

In situ hybridization. — Probe #8 was dissolved in a hybridization buffer consisting of 65 % formamide in 2 × SSC, and probe pUC1.77 in 60 % formamide, 10 % dextran sulphate, 2 × SSC and herring sperm DNA (0.5 mg/ml). The slides were covered with 50 ng of the centromere-specific probes in 10 μl of the hybridization mixture. Denaturation of probes and chromosomes was performed simultaneously at 70°C for 3 min. The slides were incubated in a moist chamber at 42°C for 12–16 hours.

Fig. 1A–I. Detection of DNA probes hybridized to human lymphocyte chromosomes after bromodeoxyuridine-Hoechst R-banding induction. The chromosomes were hybridized with probes pUC1.77, #8 and FLT4 recognizing the centromeric regions on chromosomes 1 and 8 and the region 5q33-qter on chromosome 5. The chromosomes were counterstained with propidium iodide and DAPI, and were examined using various fluorescence filter combinations; Metaphase seen through the Omega Dualband filter (**A**). Same metaphase as in panel A seen through filter Zeiss 09 (**B**). Arrows indicate signals of probe #8 hybridized to R-banded chromosomes 8. Part of metaphase seen through Zeiss 02 filter (**C**). Arrowhead indicates late-replicating X chromosome. Metaphase seen through Zeiss filters 02 and 09 (**D and E**). Arrows indicate signals of pUC1.77. Prometaphase observed through the Zeiss 02 filter (**F**). Same prometaphase as in panel F but seen through Omega Dual band filter (**G**). Metaphases seen through filter Zeiss 09. Arrows indicate signals of probe FLT4 hybridized to telomeric region of chromosome 5 (**H and I**).

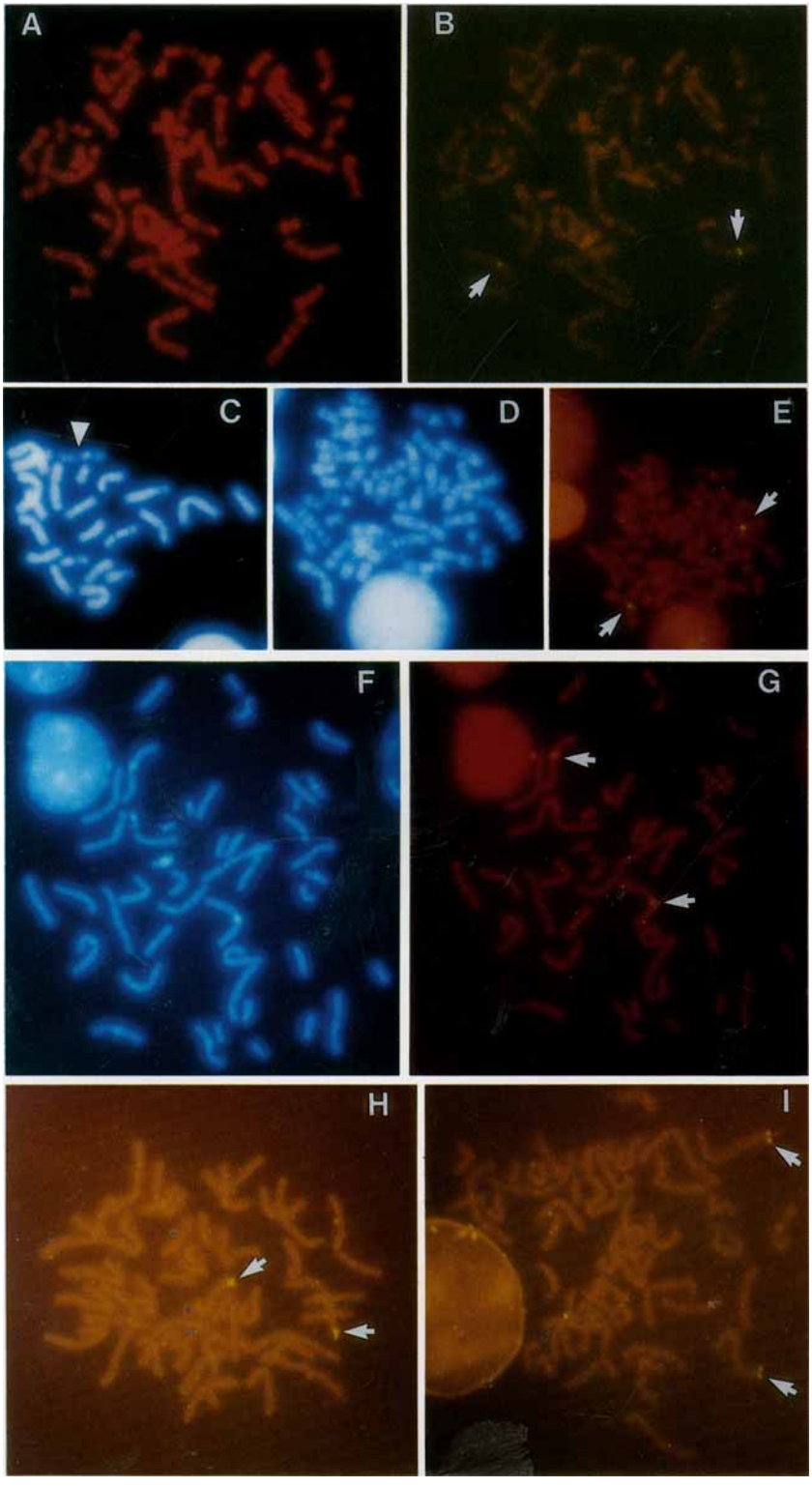

Fig. 1A–I.

100 ng of the cosmid FLT4 probe was precipitated with ethanol together with 1 μg of human Cot-1 DNA (Gibco BRL, Gaithesburg, USA) and redissolved in a solution containing 50 % formamide, 10 % dextran sulphate and 2 × SSC. The probe was denatured for 5 min at 75°C and incubated at 37°C for 30 min. The chromosomes were denatured in 70 % formamide in 2 × SSC at 70°C for 2 min and dehydrated in a series of ice-cold 70, 94 and 100 % ethanol, 5 min each. The solution containing the probe was applied to the slides, which were then incubated at 37°C for at least 12 hours.

After hybridization, slides hybridized with probes #8 and FLT4 were washed three times in 50 % formamide, 2 × SSC (pH 7.0), 0.1 × SSC (pH 7.0) at 45°C for 5 min each time, followed by one wash in 4 × SSC, 0.05 % Tween20 at room temperature.

For detection of hybridization of the digoxigenin-labelled probes, an indirect immunofluorescence technique using monoclonal mouse anti-digoxigenin antibody (1:1000, Sigma), followed by incubations with fluorescein isothiocyanate (FITC) conjugated sheep anti-mouse antibody (1:200, Sigma) and FITC conjugated donkey anti-sheep antibody (1:200, Sigma), was used.

The hybridization reaction with probe pUC1.77 was revealed using sequential incubation with avidin-FITC (1:200, Vector Laboratories Inc., Burlingame, CA, USA), biotinylated goat anti-avidin (1:200, Vector), and avidin-FITC, as described elsewhere (PINKEL et al. 1986).

Microscopy. — The slides were briefly air-dried, counterstained with 1 μg/ml propidium iodide (Sigma) and 0.2 μg/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) and then mounted in fluorescence antifading buffer (JOHNSON and DE NOGUEIRA-ARAUJO 1981). Fluorescence microscope analysis was carried out with a Zeiss Axiophot fluorescence photomicroscope equipped with the appropriate filter combinations (Zeiss 02, 09 and Omega Dualband FITC/Rhodamin filters). Metaphases were photographed on Kodak Ektachrome 400 ASA color slide film.

Results and discussion

The reported method allows detection of the fluorescent in situ hybridization signals directly on R-banded metaphase or prometaphase chromosomes.

Banded metaphase or prometaphase chromosomes stained with propidium iodide were visible by means of an Omega filter and an excitation wavelength of 530–560 nm. The chromosomes appear orange-red with a characteristic R-banding pattern (Fig. 1A,G). When a Zeiss 02 filter was used with an excitation wavelength of 340–380 nm, chromosomes stained with DAPI appeared blue with a less resolved R-banding pattern than that revealed by propidium iodide (Fig. 1C,D,F). Fluorescein-stained hybridization signals appear yellow-green on a background of orange-red chromosomes showing a fuzzy banding pattern when observed using Zeiss 09 filter with an excitation wavelength of 450–490 nm (Fig. 1E,H,I). Finally, the same mitoses when observed with an Omega filter show characteristic R-banding pattern simultaneously with green-yellow signals of the hybridized probe (Fig. G). Thus, this procedure allows unambiguous assignment of the probe to a specific chromosome band.

In the in situ hybridization with the centromere specific probes pUC1.77 and #8, 97 % of the examined metaphases showed hybridization signals in both homologous chromosomes. In the case of cosmid probe FLT4, 96 % of the metaphases exhibited hybridization signals; 62 % showed signals on both chromosomes, while 34 % showed signals on only one chromosome.

Recently, other studies have demonstrated the usefulness of different methods in the simultaneous detection of chromosome banding and hybridization signals (CHERIFF et al. 1990; FAN et al. 1990; LEMIEUX et al. 1992; TAKAHASHI et al. 1990, 1992). These procedures are based on the incorporation of bromodeoxyuridine and thymidine into DNA, followed either by chromosomal counterstaining with DAPI and/or propidium iodide, inducing Q and R bands (FAN et al. 1990), or the induction of G or R bands with the use of an alkaline antifade solution at pH 11 after propidium iodide counterstaining (LEMIEUX et al. 1992). In a different approach, CHERIFF et al. (1990) and TAKAHASHI et al. (1990, 1992) used methotrexate and bromodeoxyuridine to achieve cellular synchronization during the culture period, followed by chromosome R-banding with Hoechst 33258 and propidium iodide. Furthermore, CHERIFF et al. (1990) have reported a technique in which the detection of the hybridized probe was interrupted by a Hoechst stain, followed by a second round of signal amplification.

The cell culture system used by us is simple and

does not require changing of culture media or washing of the cells during the culture period as in other published experimental protocols (FAN et al. 1990; TAKAHASHI et al. 1990). In our system, cells are blocked in the S-phase by fluorouracil and uridine. It is known that fluorouracil is converted intracellularly into 5-fluorodeoxyuridine, which is a powerful inhibitor of thymidine synthetase (HARTMANN and HEIDELBERGER 1961; HEIDELBERGER 1965) and mimics the effect of methotrexate (RUECKERT and MUELLER 1960). Moreover, fluorouracil has two major advantages over fluorodeoxyuridine in culture media: it is less toxic and less expensive. Cells blocked by fluorouracil only require thymidine or a thymidine analogue (e.g., bromodeoxyuridine) for the cell cycle to proceed from the S-phase. RØNNE (1983) observed that pre-culture treatment of human lymphocytes in late S-G2 phase simultaneously with bromodeoxyuridine and Hoechst, results in R-banded chromosomes if a fluorescent-plus-Giemsa method is used. Bromodeoxyuridine-substituted chromosome segments stain light whereas unsubstituted ones stain dark. Accordingly, chromosome regions that replicate early appear as dark bands (brilliant fluorescent bands) while regions that replicate late assume a light colour (dully fluorescent bands). This provides a reliable way of detecting late-replicating X chromosomes (Fig. 1C).

In conclusion, the simple one-step culture technique in combination with in situ hybridization allows observation of hybridization signals from probes of different sizes on metaphase or prometaphase chromosomes with a high-resolution R-banding pattern, thus facilitating accurate and rapid gene assignment.

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