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IN SITU HYBRIDIZATION OF DNA SEQUENCES IN HUMAN METAPHASE CHROMOSOMES VISUALIZED BY AN INDIRECT FLUORESCENT IMMUNOCYTOCHEMICAL PROCEDURE

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SUMMARY

In situ hybridization and immunocytochemical procedures are described which allow identification and localization of specific DNA sequences in human chromosomes by fluorescence microscopy. With this method the genes coding for 18S and 28S ribosomal RNA (rRNA) were localized on human metaphase chromosomes by in situ hybridization of 18S or 28S rRNA followed by an immunocytochemical incubation with specific anti-RNA–DNA hybrid antiserum. Visualization of the immunocytochemically localized RNA–DNA hybrids was achieved by indirect immunofluorescence. The antiserum against RNA–DNA hybrid molecules was raised in a rabbit injected with poly(rA)–poly(dT). The specificity of the sera was determined using a model system of Sephadex beads to which various nucleic acids had been coupled. To obtain optimal specific fluorescence and very low aspecific background staining, several modifications of the in situ hybridization and the immunocytochemical procedures were investigated. The use of aminoalkylsilane-treated glass slides, removal of unbound fluorochrome molecules from the fluorochrome-labelled antibody solutions and application of a proteinase K treatment during the hybridization procedure and the immunocytochemical procedure proved to be essential for optimal results.

In chromosomes and cell nuclei DNA can be detected cytochemically by staining methods, which are based on distinctive chemical properties of this nucleic acid. These staining procedures are, however, unable to identify specific base sequences.

Localization of such specific base sequences can be achieved by in situ hybridization with complementary nucleotide sequences. Until recently, the localization of the molecular hybrids in microscopic preparations could only be detected by autoradiography [1–4].

A first approach to the fluorescent detection of nucleic acid hybrids was described [5] for 5S rRNA genes in *Drosophila* polytene chromosomes. Visualization was achieved by an immunocytochemical procedure using an antiserum specific for RNA–DNA hybrids. Subsequently the hybridization method was improved [6] by combining the denaturation and reannealing processes.

Recently, another approach was advocated [7] in which a fluorescent marker is attached directly to the complementary RNA. This method was used to localize the kinetoplast DNA in *Crithidia luciliae* cells,

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the DNA of adenovirus type 5 in virus-infected KB cells [8] and a number of genes in giant chromosomes from salivary glands of *Drosophila hydei* and *Drosophila melanogaster* [9, 10].

The present paper describes modifications of the in situ hybridization and immunocytochemical procedures, permitting identification of specific DNA sequences in human chromosomes by fluorescence microscopy. Antisera specific for RNA–DNA hybrids were raised in rabbits by injecting complexes of poly(rA)–poly(dT) and methylated bovine serum albumin. The specificity of the obtained sera was determined using a model system of Sephadex beads to which various nucleic acids were coupled. The effects of modifications of several steps in the complete procedure on the final results were investigated and modifications are presented which increase the specific hybridization and immunocytochemical localization potentials and which results in a lower non-specific background fluorescence. This combination of improved methods allows a clear visualization of the 18S and 28S ribosomal cistrons in human metaphase chromosomes.

MATERIALS AND METHODS

Phosphate-buffered saline (PBS) contained 0.137 M NaCl, 2.68 mM KCl, 7.98 mM Na_2HPO_4 and 1.47 mM KH_2PO_4 (pH 7.2); 1×SSC (sodium saline citrate, pH 7.0) contained 0.15 M NaCl and 0.015 M sodium citrate. All chemicals used were of analytical grade.

Immunization procedure

Antibodies against poly(rA)–poly(dT) were elicited in rabbits [11]. A 300 μl solution containing poly(rA)–poly(dT) (Miles Laboratories Inc. Research Products, Stoke Poges, Slough, Bucks., UK; 1 mg/ml in PBS) was mixed with 30 μl of a solution of methylated bovine serum albumin (mBSA, Miles; 10 mg/ml in distilled water). PBS was added to the resultant turbid suspension for a final volume of 3 ml. This mixture was stored at −30°C. Immunization was carried out by mixing equal volumes of the poly(rA)–poly(dT)–mBSA solution and Freund adjuvant (Bacto; Difco Laboratories, Detroit, Mich.) and injecting the resultant water-in-oil emulsion twice intramuscularly over a 7-day period, followed by an intravenous injection of 1 vol of the polynucleotide–mBSA solution diluted with an equal volume of PBS, one week later. Each immunizing dose contained 30–50 μg of poly(rA)–poly(dT). The rabbits were bled one week after the intravenous injection. The immunoglobulins were precipitated with ammonium sulphate [12].

Chromosome preparations

Metaphase chromosome preparations were prepared from human blood cell cultures [13], using aminoalkylsilane-treated glass slides [14].

Aminoalkylsilane-treated glass slides were prepared following a procedure described [15] as a part of a coupling procedure of enzymes to porous glass beads. Microscope glass slides were cleaned by incubation overnight in a 10% solution of Extran MA 01 (alkalisch, E. Merck, Darmstadt) in deionized water. They were then rinsed with hot (60°C) tap water and with deionized water and dried at 80°C. These slides were incubated for 16 h in a 2% (v/v) solution of 3-aminopropyltriethoxysilane (Aldrich Europe, Beerse, Belgium) in dry acetone. Afterwards the slides were rinsed in acetone and two changes of deionized water and stored in 0.02% NaN_3 in deionized water. Prior to use they were again rinsed in deionized water and air-dried. Slides could be stored for up to 6 weeks without losing their properties.

Coupling of nucleic acids to Sephadex G10

Sephadex G 10 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) was activated with CNBr, nucleic acids were coupled to the activated agarose beads and the remaining active groups were blocked with ethanolamine [16]. The following nucleic acids were used: poly(rA) and yeast RNA (Boehringer Mannheim GmbH), double-stranded and denatured DNA from calf thymus (BDH Chemical Ltd, Poole).

Hybridization of poly(dT) and of poly(rU) to Sephadex–poly(rA) was performed by the following procedures. A settled volume of 120 μl Sephadex–poly(rA) was diluted with an equal volume of PBS containing 1 M NaCl. To this solution 60 μg of poly(dT) (Miles) or poly(rU) (Boehringer) in 700 μl PBS containing 1 M NaCl were added. Incubation was performed by agitating the mixture at room temperature for 2 h on a modified hematocryt microshaker (frequency 50 Hz, amplitude less than 0.5 mm). The beads were then centrifuged and the supernatant was removed and saved for UV measurements. The beads were washed three times in PBS. The amounts of poly(dT) or poly(rU) hybridized to the beads were determined by measuring the UV absorbance at 260 nm for the poly(dT) or poly(rU) solutions before and after hybridization.

Procedure for the immunocytochemical staining of Sephadex beads

Swollen Sephadex G 10 beads with coupled nucleic acids were stored diluted 1:10 in PBS containing

0.02% NaN_3. A 20-fold dilution of this suspension was made in PBS containing 1% Triton X-100 (BDH Chemicals). Twenty-five microlitre samples were transferred to 1.5 ml plastic reaction tubes (Eppendorf Geratebau Netheler+Hinz GmbH, Hamburg) and 25 μl of a 5-fold serial dilution of the Ig solution in PBS from the anti-hybrid or the normal rabbit serum (initial Ig concentration 10 mg/ml) were added. Incubation was for 90 min at room temperature, during which the tubes were constantly agitated, as described before. After incubation the beads were washed twice in 1 ml of PBS containing 0.65 M NaCl followed by subsequent sedimentation at 10000 g and once with 1 ml PBS containing 0.5% Triton X-100, for at least 10 min each. The final wash buffer was removed to a volume of 25 μl and 25 μl from a 1:15 dilution of SwAR–FITC (Swine anti-rabbit serum IgG, heavy and light chain; DAKO Immunoglobulins, Copenhagen; Ig concentration 0.48 mg/ml; fluorochrome/protein ratio 3.1) in PBS containing 0.5% Triton X-100, were added. After incubation and two more washings with 1 ml PBS containing 0.65 M NaCl as in the first step, the beads were finally washed in 1 ml PBS containing 0.02% NaN_3 and fluorescence of the individual beads was measured microfluorometrically.

Microfluorometry

Fluorescence measurement of the individual Sephadex beads embedded in PBS containing 0.02% NaN_3 was performed as described by Bauman et al. [16].

Hybridization of RNA to human metaphase chromosomes

In order to remove possibly present endogenous RNA, the slides were first treated with a solution of 100 μg RNase A (from bovine pancreas, Boehringer) plus 1 μg RNase T_1 (from *Aspergillus oryzae*, Boehringer) per ml in 2×SSC for 2 h at room temperature. A few drops of the RNase solution were layered over the preparation which was then covered with a cover glass. This incubation was performed in a moist chamber. After incubation the cover glasses were removed and the slides were washed three times in 2×SSC for 30 min and then dehydrated in 70% ethanol (twice), 90% ethanol (twice) and 100% ethanol. Each alcohol dehydration was for 5 min after which the slides were air-dried. The DNA in the preparation was then denatured with freshly prepared 0.07 N NaOH for 3 min followed by rinses in 70% ethanol (twice), 90% ethanol (twice) and 100% ethanol, for 1 min each, and air-drying.

After this pretreatment, hybridization was performed [8] with the following modifications.

The preparations were treated with a proteinase K (*aus Pilzen, chromatographisch gereinigt*, Merck) solution of 1 μg/ml in 20 mM Tris-HCl pH 7.4 containing 2 mM $CaCl_2$ for 15 min at 37°C [17] (the proteinase K solution being preincubated for 4 h at 37°C to eliminate possible traces of RNase or DNase activity) and after this treatment the preparations were dehydrated by incubation in 70% ethanol (twice), 90% ethanol (twice) and 100% ethanol, for 5 min each, and air-dried. 18S and 28S rRNA prepared from rat liver [18] using freshly distilled phenol instead of *m*-cresol were used for hybridization.

The hybridization reaction was carried out with 15 μl of the RNA solution of 20 μg/ml in 70% formamide/3×SSC (which is a mixture of 7 vol parts formamide plus 3 vol parts 10×SSC) placed between the slide and a 24×50 mm coverslip. Incubation was for 40 h at 34°C in a culture-dish containing paper tissue saturated with 70% formamide/3×SSC. Coverslips were then removed and the slides were washed for 60 min in three changes of 70% formamide/3×SSC and for 30 min in two changes of 3×SSC to remove non-hybridized RNA.

Immunocytochemistry

Immunocytochemical visualization of the RNA–DNA hybrids was performed by the following procedure, each step being carried out at room temperature unless otherwise stated: (1) two rinses in 2×SSC for 5 min each; (2) incubation in a solution of 15 μg RNase A plus 0.15 μg RNase T_1/ml in 2×SSC for 120 min; (3) two rinses in 20 mM Tris-HCl pH 7.4 containing 2 mM $CaCl_2$ for 5 min each; (4) incubation in a proteinase K solution of 1 μg/ml in 20 mM Tris-HCl, pH 7.4 containing 2 mM $CaCl_2$ for 15 min; (5) three rinses in PBS for 15 min each; (6) incubation in non-immune goat serum 2% in PBS containing 0.02% NaN_3 for 30 min; (7) incubation in Ig fraction of anti-hybrid serum diluted 1:20 in PBS containing 2% non-immune goat serum plus 0.02% NaN_3 for 60 min (Ig concentration 300 μg/ml based on absorbance measurements at 280 nm); (8) three rinses in PBS at 37°C for 2 min each; (9) one rinse in PBS for 10 min at 37°C; (10) incubation for 60 min in rhodamine-labelled goat-anti-rabbit IgG (GAR–TRITC, United States Biochemical Corp., Cleveland, Ohio) diluted 1:80 in PBS containing 2% non-immune goat serum plus 0.02% NaN_3. Free rhodamine molecules were removed from the GAR–TRITC preparations by incubation overnight at 4°C with Affi-Gel 102 (amino-agarose; BioRad Laboratories, Richmond, Calif; 1 μl Affi-Gel 102 suspension/96 μl GAR–TRITC solution) under continuous agitation; (11) three rinses in PBS at 37°C for 2 min each; (12) one rinse in PBS for 10 min at 37°C; (13) dehydration in 70% ethanol (twice), 90% ethanol (twice), both containing 300 mM ammonium acetate, followed by 100% ethanol, for 5 min each, and air-drying.

Incubation in the RNase solution, 2% non-immune goat serum, specific anti-RNA–DNA IgG solution and GAR–TRITC solution were all performed with 35 μl of the solutions layered on the preparation, that was then covered with a coverslip and kept in a moist chamber.

In some experiments the preparations were hybridized and treated immunocytochemically according to the procedures of Rudkin & Stollar [5] or of Stuart & Porter [6]. These experiments are indicated as such in the text. After the in situ hybridization and immunocytochemical procedures the human metaphase chromosomes were identified by counterstaining with 4′,6-diamidino-2-phenyl-indole (DAPI) [19].

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