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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].

Fluorescence microscopy and microphotography

After the hybridization and the immunocytochemical and DAPI staining procedures, the preparations were inspected using a Dialux microscope (Leitz GmbH, Wetzlar, FRG) with epi-illumination from an HBO 200 W mercury arc (Osram GmbH, Berlin). The DAPI fluorescence emission was visualized using a combination of an UG 1 excitation filter, an LP 460 barrier filter (both Schott and Gen, Mainz) and a dichroic mirror λH 400 (Leitz). The rhodamine fluorescence was observed with LP 530 (Schott) and SP 560 (Balzers AG, Lichtenstein) filters in the excitation beam, a dichroic mirror λH 580 (Leitz) and a LP 590 (Schott) as a barrier filter.

Fluorescence photography of the metaphases was performed with the same Dialux microscope, using the oil-immersion objective (Fluoresz, 63×/1.30, Leitz) and high-speed Kodak Tri-X-Pan film (Eastman Kodak Comp., Rochester, N.Y.). The recording capabilities of the photographic emulsion were improved by pre-exposing the film to homogeneous white light [20]. Exposure times were 1 sec or less for DAPI fluorescence and up to 3 min for the rhodamine fluorescence.

RESULTS

Specificity of anti-hybrid serum

The specificity of the immunoglobulins for their respective antigens was tested in a model system consisting of Sephadex beads [21].

The Ig fractions, obtained by ammonium-sulphate precipitation of the sera from rabbits after immunization with poly(rA)–poly(dT) complexed with mBSA, were incubated in increasing dilutions with Sephadex G 10 beads to which various nucleic acids had been coupled. Sephadex beads which were inactivated following CNBr activation served as controls. In other control experiments incubations of the different types of beads were performed with either normal rabbit serum or PBS containing 0.5% Triton X-100 (conjugate control), instead of the serum tested. The immunoglobulins bound to the Sephadex beads after incubation and washing procedures are detected and quantitated by subsequent incubation of the beads with fluorochrome-labelled anti-rabbit antibodies and measuring the fluorescence intensity of the individual beads.

When using TRITC-labelled antibodies, it proved necessary to reduce non-specific background staining of Sephadex beads by the removal of unbound fluorochrome molecules from the fluorochrome-labelled anti-rabbit antibody solutions prior to use. This was accomplished by incubating the solutions with Affi-Gel 102 overnight at 4°C, according to F. T. Bosman & A. F. P M. de Goeij (personal communication). Removal of unbound TRITC molecules from the conjugated antibody solutions was confirmed by measuring the absorbance spectra (200–700 nm) of the fluorochrome-labelled antibody solutions before and after incubation with Affi-Gel 102. This procedure reduced the non-specific background staining of Sephadex G 10 beads. For FITC-conjugated antibody solutions at our disposal, incubation with Affi-Gel 102 proved unnecessary.

However, even after removal of unbound TRITC molecules with Affi-Gel 102, the batches of FITC-labelled Swine-anti-Rabbit (SwAR) antibodies proved more suitable in the experiments with the model system of Sephadex beads, than TRITC-labelled Goat-anti-Rabbit (GAR) and TRITC-labelled Sheep-anti-Rabbit (ShAR) antibodies. The latter two gave rise to considerably higher non-specific background staining of the beads. SwAR–FITC was therefore used in all experiments with the Sephadex model system.

When determining the specific fluorescence values, a correction was applied to eliminate the fluorescence value caused by non-specific binding of the immunoglobulins to the beads, using the fluorescence values of the beads treated with normal rabbit serum and SwAR–FITC. The results for

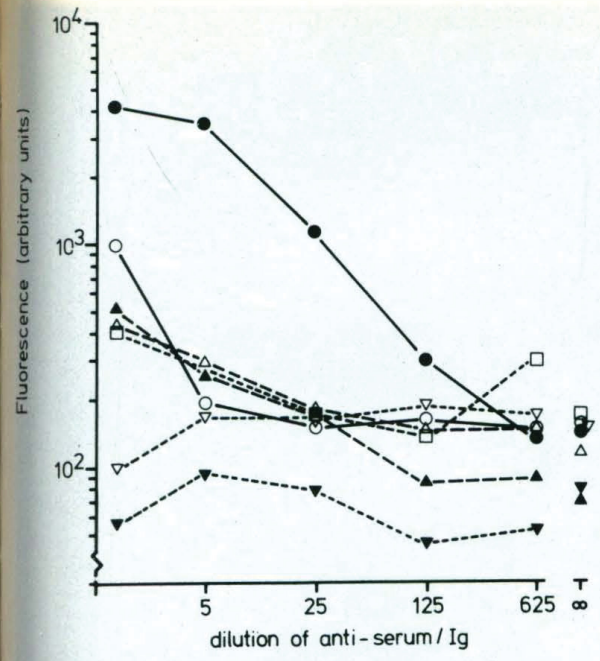

Fig. 1. Specificity test of rabbit serum against RNA–DNA hybrids. ∞ represents conjugate control, for further details see text. ●—●, Seph-poly(rA)–poly(dT); ○—○, Seph-inactivated; ▲---▲, Seph-poly(rA); △---△, Seph-poly(rA)–poly(rU); ▼---▼, Seph-ss-DNA; ▽---▽, Seph-ds-DNA; □---□, Seph-yeast RNA.

the Ig fraction from one serum are illustrated in fig. 1. The presence of IgG in the serum reacting specifically with poly(rA)–poly(dT) is evident. No significant cross-reactivity is observed with poly(rA), poly(rA)–poly(rU), single-stranded and double-stranded DNA from calf thymus, or yeast RNA [11].

Only one of the three injected rabbits produced antiserum of this specificity. This serum was used in further experiments.

In situ hybridization and immunocytochemical hybrid detection

In situ hybridization to human lymphocyte metaphase chromosomes was performed with 18S and 28S rRNA prepared from rat liver. Rat 18S and 28S rRNAs have sufficient sequence homology (98%) to human 18S and 28S rRNAs to ensure efficient hybridization of the rodent rRNA to the human ribosomal genes under the hybridization conditions used [22].

When the metaphase preparations were hybridized with 18S or 28S rRNA from rat liver and subsequently treated immunocytochemically with the specific anti-hybrid antiserum according to the procedure of Rudkin & Stollar [5], no specific chromosomal fluorescence was detectable visually or in microphotographs of individual metaphases. In addition there was a relatively high non-specific background fluorescence. Application of the method of Stuart & Porter [6] resulted in a specific fluorescence on the short arms of at best one or two acrocentric chromosomes per metaphase just visible against a high aspecific background fluorescence, which was comparable to that seen after the procedure of Rudkin & Stollar. When hybridization was performed according to the method of Bauman et al. [8] we found, after the indirect immunofluorescence procedure, specific fluorescence on the distal parts of the D and G group chromosomes. The results were evaluated both visually and via microphotographs of individual metaphases. Although the results were unmistakably specific, the fluorescence yield was relatively poor.

Since this low specific fluorescence might be due to chromosomal proteins covering the DNA and thus hampering the access of the large RNA molecules to the denatured DNA or of the immunoglobulins to the formed RNA–DNA hybrids, the following modification was evaluated to improve the procedure.

The procedures to hybridize 18S and 28S rRNA to the human metaphase chromosome preparations were varied in several ways. Proteinase K treatment was either omitted or applied before or after the hybridization procedure or applied both be-

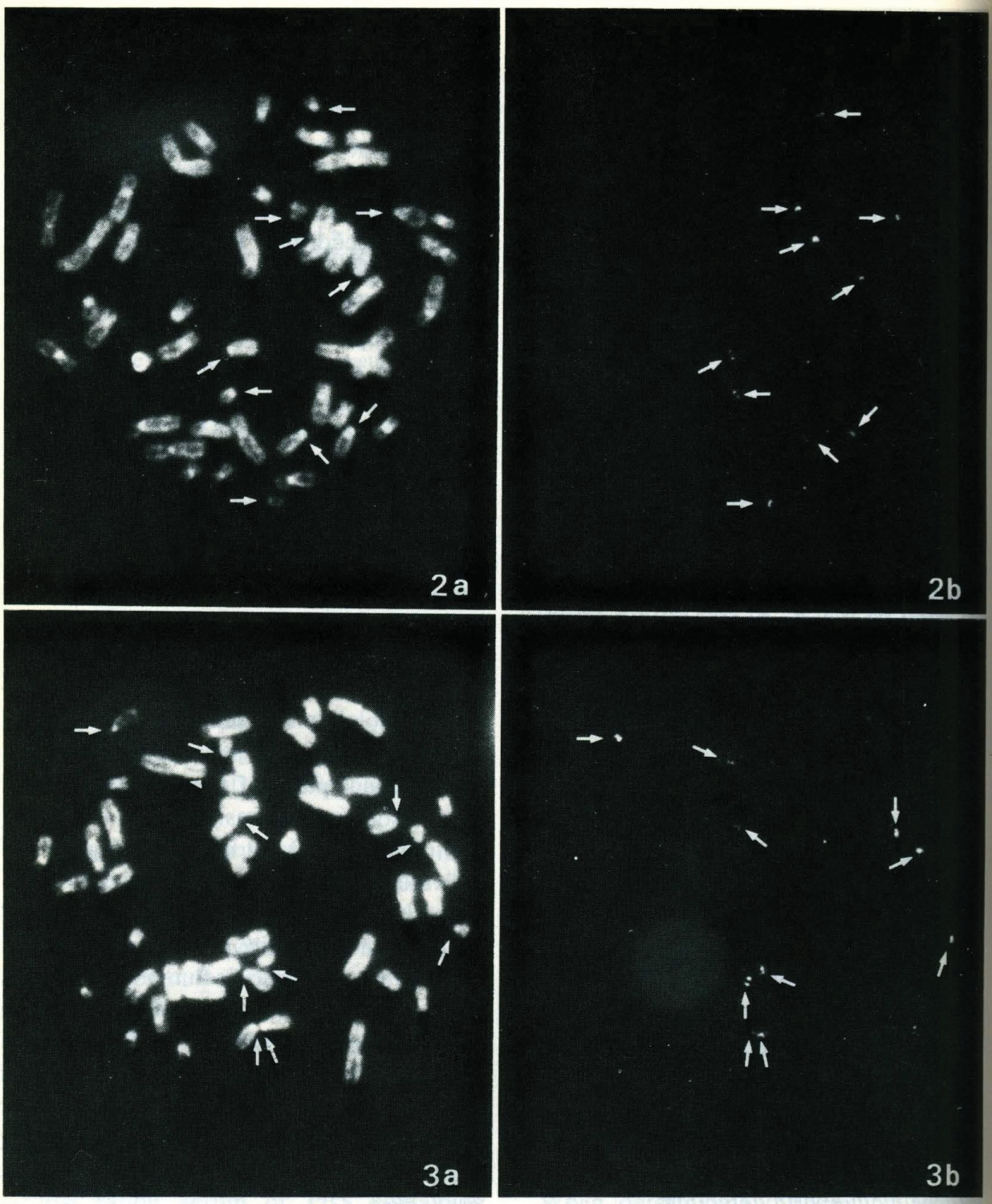

Fig. 2. Hybridization of 28S rRNA to human metaphase chromosomes. From the same field photographs were taken of (*a*) the blue DAPI fluorescence; (*b*) the red rhodamine fluorescence. Labelled chromosomes are indicated with arrows.

Fig. 3. Hybridization of 18S rRNA to human metaphase chromosomes. From the same field photographs were taken of (*a*) the blue DAPI fluorescence; (*b*) the red rhodamine fluorescence. Labelled chromosomes are indicated with arrows.

fore and after the hybridization procedure. Specific fluorescence after the immunocytochemical procedure was evaluated visually.

Compared with the results obtained after the procedure without proteinase K, treatment either before or after the hybridization reaction gave rise to an increase in specific fluorescence and a reduction of non-specific background fluorescence, the overall result being comparable for both procedures. The results from the procedure with proteinase K treatment applied before the hybridization reaction were only slightly better.

Optimal results were obtained when proteinase K was applied both before and after the hybridization reaction. A considerable increase in specific fluorescence yield was observed and the background fluorescence on the glass slides was reduced to very low values.

The optimal temperature for and duration of the hybridization reaction were determined by visual inspection of specific fluorescence for several combinations of temperatures (20°C, 34°C (i.e. T_m −25°C) and 37°C) and incubation times (18, 40 and 66 h) of hybridization with both 18S rRNA and 28S rRNA.

Hybridization for 40 h at 34°C gave optimal results, i.e. highest specific fluorescence, low non-specific fluorescence and a good preservation of chromosome morphology.

As it is known that during immunocytochemical staining IgG molecules behave like cationic dyes [23], the influence of 3-aminopropyltriethoxysilane treatment of the glass slides on the non-specific background fluorescence of the glass slides after immunocytochemistry was studied. This treatment of glass slides reduces the non-specific binding of cationic dyes to the negative groups of untreated glass [14].

It was shown that preparations on aminoalkylsilane-treated glass slides exhibit considerably less background staining of the glass slides than did preparations on routinely cleaned glass slides, when using GAR–TRITC (which produced the highest specific fluorescence).

After using the optimal procedures (as described in Materials and Methods), red fluorescent spots derived from rhodamine could be detected on the short arms of the acrocentric chromosomes, which were identified with the aid of the DAPI-staining pattern of the same metaphase (see figs 2, 3).

Fluorescence specificity was confirmed in experiments in which *E. coli* tRNA was used, instead of the 18S or 28S rRNA, for hybridization, or in which the specific antiserum was replaced by non-immune rabbit serum.

In such experiments no specific fluorescence was detectable. Chromosome preparations obtained from different blood cell donors sometimes differed in the number of chromosomes showing specific fluorescence and in the emission intensity of the fluoresceing spots. These results agree with autoradiographic grain counting data [24, 25].

DISCUSSION

The autoradiographic procedure for the detection of hybrids of radioactively labelled cRNA and DNA in chromosome preparations has several disadvantages. The procedure is time-consuming and the topological resolution is often insufficient to clearly locate hybrids within defined bands of the eukaryotic metaphase chromosomes. When fluorochrome-labelled cRNA is used for hybridization, these disadvantages are overcome. Attaching a fluorochrome molecule

to RNA does not affect the hybridization properties of the RNA and the use of fluorochrome-labelled cRNA [10] renders the procedure for detection of RNA–DNA hybrids very rapid and improves the resolving power which is now limited to that of the optical system. In addition the use of radioactive material is avoided.

The sensitivity of any microscopical method for detecting fluorescence, depends on the number of fluorochrome molecules per unit of area in the object being detectable. In our fluorescence microscope system the minimal amount of tetramethylrhodamine detectable is 250 molecules per 0.25 μm^2 against a black background [26].

It was expected that the fluorescence detection of RNA–DNA hybrids using antibodies against these hybrids in an indirect immunocytochemical technique would give rise to a high sensitivity [26]. For the immunofluorescence method it has been assumed that, when nearly all antibodies are assumed to be bound monovalently (which might be the case when high antibody concentrations are used), one antibody per 10 nucleotides can be accommodated [27], and that the indirect method, using conjugates with one fluorochrome per antibody, will result in about one fluorochrome molecule per four nucleotides.

The use of the indirect immunocytochemical method requires the availability of specific anti-RNA–DNA antibodies. Our data show that not every immunization results in the production of the desired specific antibodies but, on the other hand, this drawback may be solved by the production of monoclonal antibodies against poly(rA)–poly(dT) [28].

Specificity testing of sera

The specificity of the sera obtained from rabbits injected with poly(rA)–poly(dT) complexed to mBSA, was tested using a model system of Sephadex G 10 beads to which various nucleic acids had been coupled. This proved to be a reliable model system. The specific and non-specific reactions of nucleic acids and proteins are limited to the surface of the spheres because the exclusion limit of the Sephadex G 10 is 700 D, permitting a direct comparison of these two types of reaction, provided no free fluorochrome molecules are present [16].

It is likely that the higher non-specific background staining of the Sephadex beads in the model experiments with the TRITC-labelled GAR and ShAR antibodies used, resulted from interaction of protein-bound rhodamine with Sephadex beads and not from an inferior quality of the GAR and ShAR antibodies. The fact that the GAR-TRITC, when applied to human chromosome preparations which had been hybridized with 28S rRNA and treated with anti-RNA–DNA antibodies, gave rise to a considerably higher specific fluorescence yield than SwAR–FITC (despite the fact that the fluorochrome/antibody ratio was only 1 for GAR–TRITC compared to 3.1 for SwAR–FITC) is in accordance with this hypothesis. FITC-labelled SwAR antibodies were therefore used in all experiments with the Sephadex model system, whereas GAR-TRITC was used for chromosome preparations.

Hybridization and immunocytochemical procedures

The procedure described by Rudkin & Stollar [5] for *Drosophila* polytene chromosomes and the improved procedure described by Stuart & Porter [6] for hybridization and immunocytochemical detection of the formed hybrids, proved in our hands to be insufficient for the unequivocal identi-

fication of 18S or 28S rDNA cistrons on human chromosomes, because of the very high non-specific background fluorescence.

Therefore, the hybridization procedure, developed by Bauman & Van Duijn [10] as a part of their direct hybridocytochemical method, was adopted in the indirect method used. The specific fluorescence yield, however, was still very poor. This might be caused by poor accessibility of the single-stranded DNA to the RNAs (of different size), or of the formed hybrids to the antibodies, because of the chromosomal proteins covering the DNA. We therefore included proteinase K treatment of the preparations in the hybridization procedure. The proteinase K treatment is supposed to increase hybridization efficiency by increasing the penetration possibilities of cRNA into the specimen when applied before the hybridization reaction [17].

After proteinase K treatment a combined effect of increase in specific fluorescence and reduction of non-specific background staining was observed. It is uncertain whether the increase of specific fluorescence was due to improved hybridization efficiency, increased accessibility of the RNA–DNA hybrids for the antibodies, or both, but optimal results were observed with proteinase K being applied before as well as after the hybridization reaction. Other modifications of the hybridization procedure which proved to be of advantage were to perform hybridization at 34°C (instead of room temperature) and to reduce the incubation time to 40 h.

Non-specific background fluorescence

The success of the method depends closely on obtaining a very low background fluorescence. Experiments were carried out to improve the results by reduction of the non-specific background fluorescence to as low a level as possible.

The proteinase K treatment applied during the in situ hybridization and immunocytochemical procedures proved to be very efficient in reducing background staining. This reduction is probably caused by the removal of proteins from the glass slides. A final improvement was brought about by removal of non-protein-bound fluorochromes from the GAR–TRITC solution by pre-incubation with Affi-Gel 102 and by use of aminoalkylsilane-treated glass slides.

Background fluorescence was thus reduced to very low levels. The remaining non-specific fluorescence was very faint and homogeneous. Few non-specific fluorescent dots were observed (particularly in experiments in which the Ig solutions were centrifuged for 2 min at 10 000 g before use). More important, non-specific fluorescence was rarely confusing, since this fluorescence was usually distinct from metaphase chromosomes as determined with the aid of the DAPI staining pattern.

This low level of background staining in combination with a sufficient specific signal represents an advantage of our procedure as compared to autoradiography. This advantage is of special value in studies of interphase nuclei. In the cases where iodinated RNA has been used, the high energy of ^{125}I decay often results in autoradiographic grains outside the bounds of the chromosome bands in which the specific DNA sequences are situated [29] and, in addition, non-specific autoradiographic grains are often dispersed throughout the preparations.

Henderson et al. [30] found that the in situ hybridization and autoradiographic technology using radioactively labelled nucleic acids could hardly be relied upon to localize a gene of unknown location. Al-

though the correct chromosomal site might be identified, other sites are labelled as well, which necessitates the use of statistical evaluation of grain counts over numerous cells and chromosomes.

Localization pattern

It is known that not all D and G group chromosomes have the same number of rDNA genes. It was found [24] that in samples from three individuals, chromosome 14 had fewest genes, whereas there seemed to be most in chromosome 21. It is also evident that homologous chromosomes do not necessarily contain the same number of rDNA genes. The results of filter hybridization experiments indicate a considerable variation between individuals [31].

On the average, the G group chromosomes have been found to possess nearly twice the amount of label compared with D group chromosomes [32]. In our preparations these results are confirmed. Considerable differences in specific fluorescence intensity are found between individual chromosomes. Hybridization with 28S rRNA gives rise to a higher specific fluorescence intensity per chromosome after immunocytochemical visualization as compared with 18S rRNA, as is to be expected on the basis of their respective molecular weights.

CONCLUSIONS

A procedure has been developed which allows cytochemical localization of specific nucleotide sequences in human metaphase chromosomes and nuclei by in situ hybridization. The specific hybrids are visualized immunocytochemically with an anti-RNA–DNA hybrid serum. The feasibility of this method is demonstrated in this paper for 18S and 28S rDNA; but the method can of course be applied for the detection of any specific nucleotide sequence with a high enough local concentration. Specific fluorescence is easily visualized on the short arms of the D and G group chromosomes with high topological resolution. In our procedure background fluorescence is relatively weak and homogeneous which is advantageous in comparison to background silver grains seen in autoradiography which are sometimes located over chromosomes.

The procedure has a higher topological resolution and a lower background than in most autoradiographic procedures, which is of special advantage in interphase nuclei, where the localization of specific DNA sequences is difficult to determine in autoradiographs.

Another advantage of this method is the relative ease with which the amount of RNA hybridized can be quantified by microfluorometry or quantitative photography followed by scanning-densitometry of the photographic negatives [33]. The described procedure (when used for the detection of 18S and 28S ribosomal cistrons on human metaphase chromosomes) shows a higher sensitivity than those described earlier [5, 6, 10].

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