

PERIODICAL ROOM  
HEALTH SCIENCES LIBRARY

LIBRARY

ISSN 0014-4827

THIS NUMBER COMPLETES VOLUME 141

# EXPERIMENTAL CELL RESEARCH



EXECUTIVE EDITORS

T. CASPERSSON · T. GUSTAFSON · D. MAZIA · NILS R. RINGERTZ

---

ASSISTANT EDITOR

BARBARA CANNON

ADMINISTRATIVE EDITOR

VERA RUNNSTRÖM-REIO

EDITORS

R. BASERGA · D. BOOTSMA · M. BOUTEILLE · O. I. EPIFANOVA · W. W. FRANKE · P. R. HARRISON  
K. E. HELLSTRÖM · S. A. LATT · R. LEVI-MONTALCINI · G. L. NICOLSON · I. PASTAN · P. A. PETERSON  
D. M. PRESCOTT · F. H. RUDDLE · P. O. SEGLEN · K. WEBER

---

VOLUME 141 · OCTOBER 1982 · NUMBER 2

ECREAL 141 (2) 231-521 (1982)



ACADEMIC PRESS

**DOCKET**  
**A L A R M**

Find authenticated court documents without watermarks at [docketalarm.com](http://docketalarm.com).

# EXPERIMENTAL CELL RESEARCH

## EXECUTIVE EDITORS

- T. CASPERSSON, Karolinska Institutet, Department of Tumor Pathology, Karolinska Sjukhuset, S-10401 Stockholm 60, Sweden
- T. GUSTAFSON, Wenner-Gren Institute for Experimental Biology, University of Stockholm, S-11345 Stockholm, Sweden
- D. MAZIA, Hopkins Marine Station, Stanford University, Department of Biological Sciences, Pacific Grove, CA 93950, USA
- NILS R. RINGERTZ, Department of Medical Cell Genetics, Medical Nobel Institute, Karolinska Institutet, Box 60400, S-10401 Stockholm, Sweden

## ASSISTANT EDITOR

- BARBARA CANNON, Wenner-Gren Institute for Experimental Biology, University of Stockholm, S-11345 Stockholm, Sweden

## EDITORS

- R. BASERGA, Department of Pathology, Temple University, School of Medicine, 3400 North Broad Street, Philadelphia, PA 19140, USA
- D. BOOTSMA, Department of Cell Biology and Genetics, Erasmus University, Postbus 1738, Rotterdam, The Netherlands
- M. BOUTEILLE, Laboratoire de Pathologie Cellulaire, Institut National de la Santé et de la Recherche Médicale (INSERM) (U 183), 15, rue de l'Ecole de Médecine, 75270 Paris, Cedex 06, France
- O. I. EPIFANOVA, Academy of Sciences of USSR, Institute of Molecular Biology, Vavilov ul. 32, Moscow B-312, USSR
- W. W. FRANKE, Abteilung für Membranbiologie und Biochemie, Institut für Zell- und Tumorbiologie, Deutsches Krebsforschungszentrum, D-6900 Heidelberg 1, Germany
- P. R. HARRISON, Wolfson Laboratory for Molecular Pathology, The Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Barsden, Glasgow G61 1BD, UK
- KARL ERIK HELLSTRÖM, Division of Tumor Immunology, Fred Hutchinson Cancer Institute, 1124, Columbia Street, Seattle, WA 98104, USA
- S. A. LATT, Harvard Medical School, The Children's Hospital Medical Center, 300 Longwood Avenue, Boston, MA 02115, USA
- R. LEVI-MONTALCINI, CNRS, Laboratory of Cell Biology, Via G. Romagnosi 18/A, I-00196 Rome, Italy
- G. L. NICOLSON, Department of Tumor Biology, University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, Houston, TX 77030, USA
- I. PASTAN, National Institute of Health, Laboratory of Molecular Biology, National Cancer Institute, Bldg 37, Room 4B27, Bethesda, MD 20205, USA
- P. A. PETERSON, Wallenberg Laboratory, University of Uppsala, Box 562, S-751 22 Uppsala, Sweden
- D. M. PRESCOTT, University of Colorado, Department of Molecular, Cellular and Developmental Biology, Boulder, CO 80302, USA
- F. H. RUDDLE, Department of Biology, Yale University, New Haven, CT 06520, USA
- P. O. SEGLEN, Department of Tissue Culture, Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, Oslo 3, Norway
- K. WEBER, Abt. Biochemie I, Max-Planck-Institut für biophysikalische Chemie, Karl-Friedrich-Bonhoeffer-Institut, Postfach 968, D-3400 Göttingen, Germany

*Administrative Editor:* VERA RUNNSTRÖM-REIÖ, Karolinska Institutet, Box 60400, S-10401 Stockholm, Sweden

*Editorial Office:* Karolinska Institutet, Box 60400, S-10401 Stockholm, Sweden  
Tel. Nat. 08-33 93 80; Int + 46 8-33 93-80

---

Published monthly at S-75181 Uppsala, Sweden, by  
Academic Press, Inc., 111 Fifth Avenue, New York, NY 10003, USA  
1982: Volumes 137-142. Price: \$528.00 USA; \$604.50 outside USA

All prices include postage and handling.

All subscription orders should be addressed to the office of the Publishers at  
111 Fifth Avenue, New York, NY 10003, USA.

Send notices of change of address to the office of the Publishers at least 6-8 weeks in advance.

Please include both old and new addresses.

Postmaster: Send changes of address to Experimental Cell Research,  
111 Fifth Avenue, New York, NY 10003, USA.

Second class postage paid at Jamaica, NY 11431, USA. Air freight and mailing in the USA by

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

A. C. Van PROOIJEN-KNEGT,<sup>1,\*</sup> J. F. M. Van HOEK,<sup>1</sup> J. G. J. BAUMAN,\*\*  
P. Van DUIJN,<sup>1</sup> I. G. WOOL<sup>2</sup> and M. Van der PLOEG<sup>1</sup>

<sup>1</sup>Department of Histochemistry and Cytochemistry, Leiden University, 2333 AL Leiden, The Netherlands, and <sup>2</sup>Department of Biochemistry, University of Chicago, Chicago, IL 60637, USA

### 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 *Droso-*

*phila* 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,

\* To whom offprint requests should be sent.

\*\* Present address: Radiobiological Institute, REPGO-TNO, Rijswijk, The Netherlands.

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<sub>2</sub>HPO<sub>4</sub> and 1.47 mM KH<sub>2</sub>PO<sub>4</sub> (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% Na<sub>3</sub>N 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%  $\text{NaN}_3$ . 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  $\mu\text{l}$  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  $\mu\text{l}$  and 25  $\mu\text{l}$  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%  $\text{NaN}_3$  and fluorescence of the individual beads was measured microfluorometrically.

#### Microfluorometry

Fluorescence measurement of the individual Sephadex beads embedded in PBS containing 0.02%  $\text{NaN}_3$  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  $\mu\text{g}$  RNase A (from bovine pancreas, Boehringer) plus 1  $\mu\text{g}$  RNase T<sub>1</sub> (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 Pilsen, chromatographisch gereinigt, Merck) solution of 1  $\mu\text{g}/\text{ml}$  in 20 mM Tris-HCl pH 7.4 containing 2 mM  $\text{CaCl}_2$  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  $\mu\text{l}$  of the RNA solution of 20  $\mu\text{g}/\text{ml}$  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  $\mu\text{g}$  RNase A plus 0.15  $\mu\text{g}$  RNase T<sub>1</sub>/ml in 2×SSC for 120 min; (3) two rinses in 20 mM Tris-HCl pH 7.4 containing 2 mM  $\text{CaCl}_2$  for 5 min each; (4) incubation in a proteinase K solution of 1  $\mu\text{g}/\text{ml}$  in 20 mM Tris-HCl, pH 7.4 containing 2 mM  $\text{CaCl}_2$  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%  $\text{NaN}_3$  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%  $\text{NaN}_3$  for 60 min (Ig concentration 300  $\mu\text{g}/\text{ml}$  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%  $\text{NaN}_3$ . 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  $\mu\text{l}$  Affi-Gel 102 suspension/96  $\mu\text{l}$  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  $\mu\text{l}$  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].

# Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

## Real-Time Litigation Alerts



Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

## Advanced Docket Research



With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

## Analytics At Your Fingertips



Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

## API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

## LAW FIRMS

Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

## FINANCIAL INSTITUTIONS

Litigation and bankruptcy checks for companies and debtors.

## E-DISCOVERY AND LEGAL VENDORS

Sync your system to PACER to automate legal marketing.