# Cytogenetics and Cell Genetics

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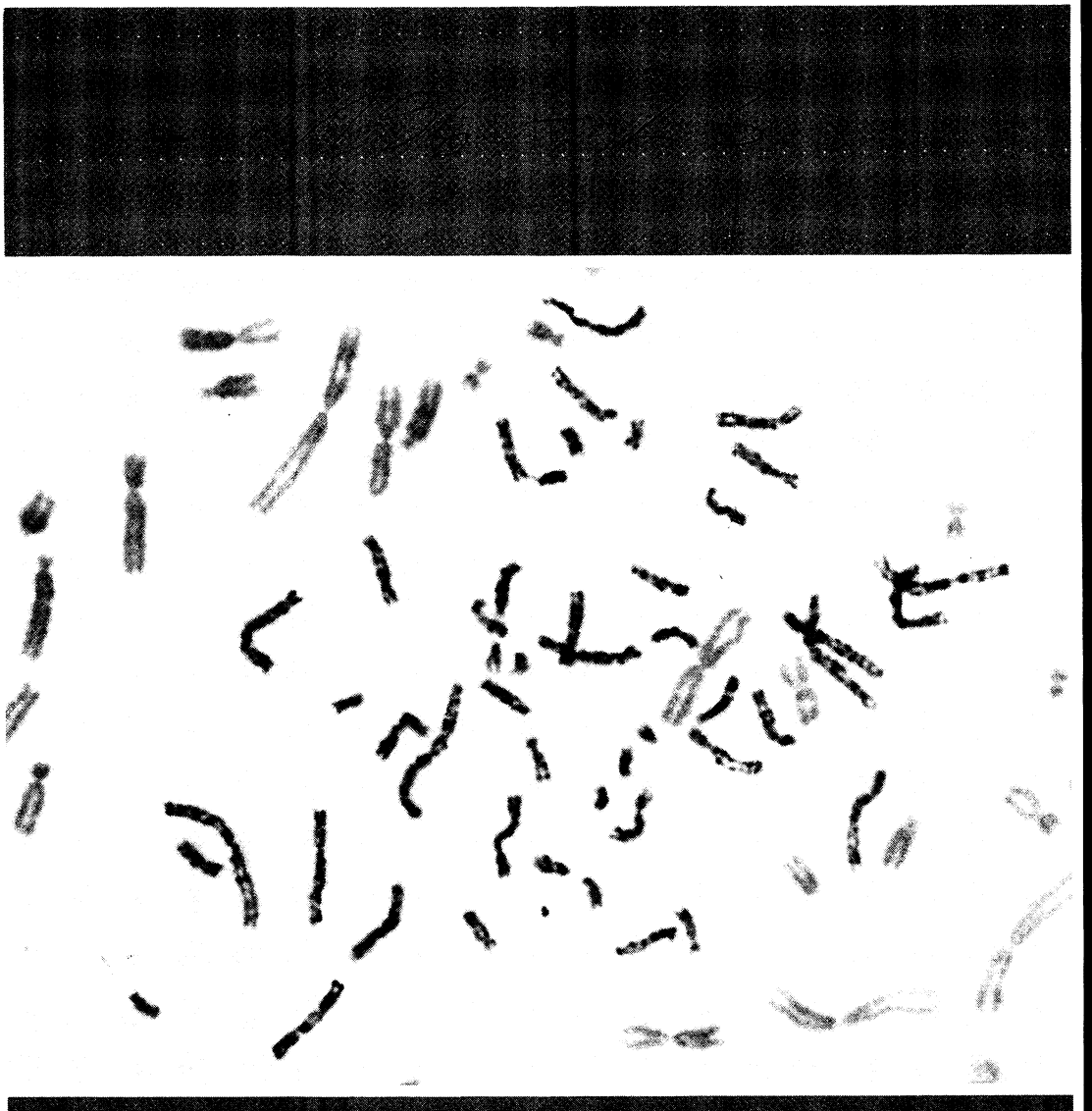

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# Cytogenetics and Cell Genetics

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Cytogenet Cell Genet
Printed in Switzerland

S. Karger AG,
Postfach, CH-4009 Basel (Schweiz)

Erscheint monatlich
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## General Information

**Publication data:** 'Cytogenetics and Cell Genetics' is published 12 times annually. Volumes 56–58 with 4 issues each appear in 1991.

**Subscription rates:** Subscriptions run for a full calendar year. Prices are given per volume, surface postage included.

*Personal subscription:* SFr. 316.40, US$ 207.20. (Must be in the name of, billed to, and paid by an individual. Order must be marked 'personal subscription'.)

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Cytogenet Cell Genet
Printed in Switzerland

S. Karger AG,
Postfach, CH-4009 Basel (Schweiz)

Erscheint monatlich;
pro Jahr 3 Bände zu 4 Heften

Cytogenet Cell Genet 56:27–30 (1991)


0301–0171/91/0561–0027 $ 2.75/0

# Painting of defined chromosomal regions by in situ suppression hybridization of libraries from laser-microdissected chromosomes

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**Abstract.** "Painting" of defined chromosomal regions provides a powerful tool for cytogenetic analyses. Here, we demonstrate that chromosomal in situ suppression (CISS)-hybridization of DNA libraries derived by microcloning laser-microdissected chromosomal regions can be applied to achieve this goal. As an example, we used unbanded metaphase spreads from a female patient carrying a balanced translocation, t(1;7)(1qter→1p36::7q11→7qter). Fragments from the long arms of 130 translocation chromosomes were microdissected. After microcloning, human inserts with an average size of about 3 kb were pooled from 400 recombinant bacteriophage DNA clones and used as a complex probe set in CISS-hybridization experiments. This resulted in painting of the translocation chromosome along the region 7q35 to 1p31. Painted chromosomal subregions in normal chromosomes 1 and 7 were consistent with this finding. This approach may be used to perform painting of any chromosome regions for which microlibraries can be established. Possible applications include the definition of marker chromosomes in clinical and tumor cytogenetics and studies of chromosomal evolution, as well as studies of nuclear chromosome topography in animal and plant species.

Chromosomal in situ suppression (CISS)-hybridization of biotinylated libraries from sorted human chromosomes, in conjunction with fluorescent or colorimetric detection procedures, has provided a new tool for the specific "painting" of complete individual chromosomes in both mitotic and interphase cells (Lichter et al., 1988a, b; Pinkel et al., 1988). CISS-hybridization has also been useful for the mapping of genomic DNA sequences (e.g., cloned in cosmid vectors) which contain interspersed repetitive sequences, such as *Alu* sequences, in addition to chromosome site-specific sequences (Landegent et al., 1987; Lichter et al., 1990a, b). Still, the generation of complex probe sets for the selective staining of desired chromosomal subregions, such as chromosome arms or chromosome bands, has remained a time-consuming task. In addition, the lack of sorted chromosome libraries from nonhuman species has, until now, constituted another limiting factor in the general use of the CISS-hybridization technique.

This study was carried out to investigate whether probe sets useful for the painting of chromosomal subregions could be established by microcloning of laser-microdissected chromosomes. As a model case, a marker chromosome (1qter→1p36::7q11→7qter) (Fig. 1) from a healthy female carrying a balanced translocation, t(1;7)(p36;q11), was microdissected using an excimer laser microbeam. Chromosomes were not stained to facilitate microcloning procedures, but the marker chromosome could be easily recognized as the largest chromosome of the complement. Laser microdissection was aimed to include the region 7q22→32 (Eckelt et al., 1989). Accordingly, two laser cuts were performed, one to remove the chromosome 1 material and the other to remove the distal region of 7q. In the absence of banding, it was difficult to reproduce the localization of the laser cuts precisely in marker chromosomes that had undergone various amounts of condensation. While a preliminary investigation of the resulting microlibrary indicated the presence of clones from the desired region (Eckelt et al., 1989), CISS-hybridization should answer the question of the actual extent of the microdissected region.

Supported by a grant (Cr 59 10-1) from the Deutsche Forschungsgemeinschaft. C.L. is a recipient of a scholarship from the Konrad-Adenauer-Stiftung; T.C. is a recipient of a Heisenberg Stipendium from the Deutsche Forschungsgemeinschaft.

Received 6 April 1990; accepted 24 July 1990.

Request reprints from Dr. T. Cremer, Institut für Humangenetik and Anthropologie, Universität Heidelberg, Im Neuenheimer Feld 328, D-6900 Heidelberg (FRG).

## Materials and methods

*Cell material.* Phytohemagglutinin (PHA)-stimulated human lymphocyte cultures were established from healthy (46,XY; 46,XX) donors. Metaphase spreads were prepared for subsequent use in CISS-hybridization experiments (see below) using standart procedures and stored at 4°C. For laser microdissection, Epstein-Barr virus-transformed cells of a female patient carrying a balanced translocation, t(1;7)(p36;q11), were kindly provided by K. Miller (Medizinische Hochschule Hannover) (Eckelt et al., 1989). Metaphase spreads were prepared and fixed as described by Martinsson et al. (1989).

*Laser microdissection.* The details of the procedure have been described previously (Monajembashi et al., 1986; Eckelt et al., 1989; Ponelies et al., 1989). Briefly, pulses of an excimer pumped dye laser (Lambda Physik, Göttingen, FRG), at a repetition rate of about 10 Hz, were directed into an inverted IM 35 microscope via the fluorescence illumination path. In the focus, power densities of up to $10^{11}$ W/cm$^2$ were obtained. Laser cutting of the translocation chromosome, t(1;7)(1qter→1p36::7q11→7qter), was achieved by moving the marker chromosome at preselected sites through the laser focus. A total of 130 fragments were collected for microcloning.

*Microcloning.* Microcloning was performed essentially as described by Scalenghe et al. (1981) and Martinsson et al. (1989), with several modifications described below. Micromanipulation work was done in a paraffin oil-filled

chamber under a phase-contrast microscope. After laser dissection, fragments were taken up by a glass needle, using a de Fonbrune micromanipulator, and transferred into a 0.6-nl aqueous droplet contaning 1 mg/ml proteinase K and 0.2% SDS. After digestion for 2 h at 55 °C, proteins in the microdrop were extracted three times with four volumes of phenol saturated with TE buffer, folloved by a wash with 0.5 µl of chloroform. Restriction digestion was carried out ina 1-nl reaction volume containing 10 mM Tris HCl (pH 7.5), 5 mM NaCl, 0.5 mM $MgCl_2$, and 280 units/µl *Eco*RI. After incubation for 2 h at 37 °C, the enzyne was removed by extraction with phenol. The digested DNA was ligated for 2( h at 12 °C to 0.56 ng *Eco*RI-cut dephosphorylated λ phage NM1149 DNA (Murray et al., 1983), using 0.1 units T4 ligase in a total volume of 2.4 nl of ligation buffer (50 mM Tris HCl [pH 7.5], 10 mM $MgCl_2$, and 1 mM ATP). The ligation product was packaged in vitro as described by Scherer et al. (1981). Phages were plated on plates with the hfl$^-$ mutant NM514 as bacterial host (Murray et al., 1983).

A total of 450 recombinant phage clones from the microlibrary (containing 478 cones) were picked onto LB-agar plates with an overlayer of 3 ml of soft agar :ontaining the NM514 bacterial host and incubated at 37 °C overnight. Plaque hybridization was carried out with $^{32}$P-labeled, sheared human genomic DNA (Church and Gilbert, 1988). After autoradiography, 230 (51%) of the 450 phages showed signals of varying intensity, indicating the presence of highly or moderately repetitive human sequences. Clones from the remaining fraction were not further tested individually but would likely contain single or low-copy human sequences. The average insert size of 22 randomly picked clones (11 "singe copy" clones and 11 clones containing highly repetitive sequences) was 3 kb range, 0.55–8.2 kb).

*DNA probes and preparation.* As a probe set for CISS-hybridization experiments, 400 recombinant phages from the microcloned phage-DNA library were pooled at random and amplified in liquid culture using *E. coli* LE 392 as bacterial host. Purification of the phages and extraction of phage DNA were carried out as described by Maniatis et al. (1982). After digestion with *Eco*RI, human inserts were recovered from a 0.8% agarose gel by electroelution into dialysis bags and concentrated with *n*-butanol (Maniatis et al., 1982).

Plasmid DNA clone pα7t1 containing the alphoid sequence specific for human chromosome 7 was established by Waye et al. (1987) and kindly provided by Peter Devilee (Leiden). Plasmid transformation and DNA preparation were carried out following standard procedures (Birnboim and Doly, 1979).

*Probe labeling, CISS-hybridization, and probe detection.* Plasmid DNA pα7t1 and human inserts from the pooled phages of the microlibrary established from the microdissected chromosome region were labeled with biotin-11 dUTP by nick translation (Langer et al., 1981). CISS-hybridization and detection of hybridized probes with fluorescein isothiocyanate (FITC)-labeled avidin were performed as described in detail by Lichter et al. (1988a). One cycle of signal ampification was performed as described by Pinkel et al. (1986). Preparations were counterstained with 0.2 µg/ml 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI) and 1 µg/ml propidium iodide and mounted in fluorescence antifading buffer (1 mg *p*-phenylenediamine in 1 ml of glycerin buffer, pH 8.0). Cells were evaluated with a Zeiss Photomicroscope III equipped for epifluorescence. Photographs were taken on Agfachrome 1000 RS color slide film. Alternatively, digitized images were obtained using a laser-scanning confocal microscope as described by Lichter et al. (1990b). In the latter case, photographs were taken from a video monitor.

## Results

Figure 2a (left side) shows the marker chromosome, t(1;7)(1qter→1p36::7q11→7qter), after painting with the probe set derived from the microlibrary. Fractional length measurements (Lichter et al., 1990b) of 10 marker chromosomes, with the telomere of the long arm and the centromere serving as boundaries, made it possible to determine the extension of the painted region from 7q35 to 1p31 (Fig. 1). This result indicates that the microcloned chromosome fragments frequently included chromosome 1p3 material in addition to 7q material. Consistent with this conclusion was the observation in normal chromosomes 1 that 1p3 was painted except for the telomeric region (Fig. 3a). This finding

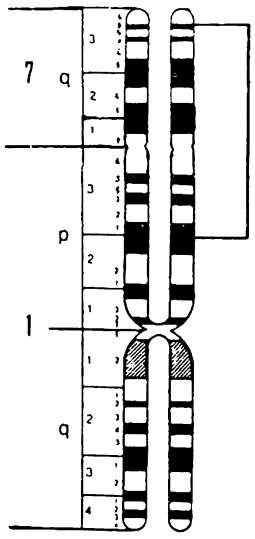


**Fig. 1.** Schematic drawing of the translocation chromosome, t(1;7)(1qter→1p36::7q11→7qter). The bracket on the right indicates the extension of the painted region, as obtained by fractional length measurements of 10 marker chromosomes (see text).

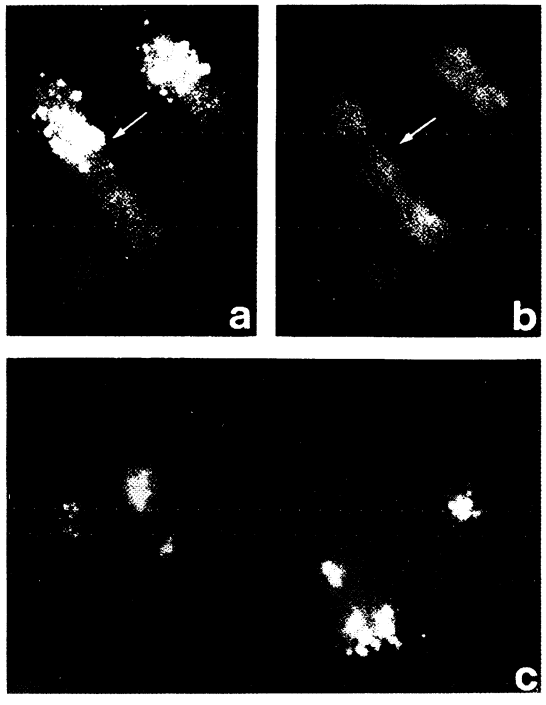


**Fig. 2.** CISS-hybridization of a biotinylated DNA library derived by microcloning of a laser-microdissected chromosomal region (see text) to a metaphase spread **(a, b)** and interphase nuclei **(c)** from PHA-stimulated lymphocytes of a female patient with a t(1;7)(p36:q11). **(a)** Translocation chromosome t(1;7)(1qter→1p36::7q11→7qter), after detection of biotinylated sequences with avidin-FITC, demonstrates the specific staining of the microcloned chromosome region. In an adjacent normal chromosome 7 (right), the long arm is specifically painted. Chromosomes are counterstained with propidium iodide. **(b)** The same chromosomes counterstained with DAPI. The arrow indicates the border between the painted and nonpainted chromosome regions. **(c)** Three distinctly stained domains in interphase nuclei from the same experiment. The largest domain likely represents the painted region of the translocation chromosome, the medium-sized domain the painted long arm of the normal chromosome 7, and the small domain the painted part of the short arm of the normal chromosome 1 contained in these nuclei.

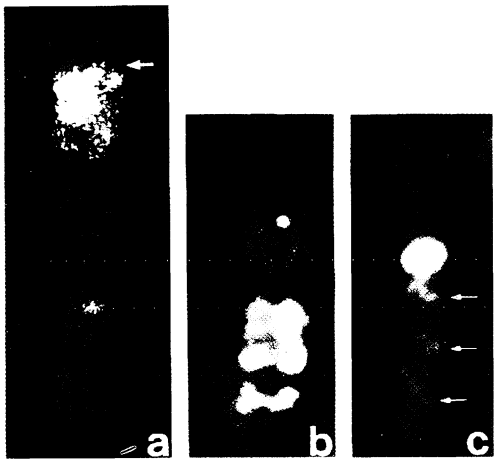


**Fig. 3.** Typical, enlarged normal chromosomes 1 **(a)** and 7 **(b, c)** after CISS-hybridization of the microcloned library, detection with avidin-FITC, and counterstaining with propidium iodide. In **a** and **b** the chromosomes were photographed directly from the video monitor after laser-scanning confocal microscopy and digital image enhancement; the photograph in **c** was taken with a Zeiss Photomicroscope III equipped for epifluorescence. In **a**, specific labeling of chromosome 1 extends from the subtelomeric region to the middle of the short arm. Note that the telomeric region is not labeled (arrow). The chromosome 7 in **b** demonstrates a banded staining pattern on 7q. Three fluorescent bands are also apparent on the prometaphase chromosome 7 in **c** (small arrows). The centromeric region of this chromosome is strongly stained by simultaneous in situ hybridization of the alphoid probe pα7t1.

is in accordance with the absence of sequences from this region in the marker chromosome. In normal chromosomes 7 (see Fig. 2a, right side, and Fig. 3b for examples) a region of the long arm (from 7q11 to 7q35, according to fractional length measurements) was specifically stained. This chromosome was further identified by simultaneous hybridization of the probe set with an alphoid probe specific for the chromosome 7 centromeric region (Fig. 3c). Painting was most prominent over the middle and proximal parts of 7q (Fig. 2a). This result is in agreement with the fact that, in the microdissected marker chromosomes, material close to the end of the long arm was cut off. Notably, three fluorescent bands were observed in more extended painted chromosomes 7 (Fig. 3b, c). Digital imaging microscopy using a laser-scanning confocal microscope facilitated the analysis of the hybridized chromosomes. In this way, the signal-to-noise ratio could be enhanced by optical filtering and image processing, while nonspecific background could be removed.

The conditions used for CISS-hybridization in the present experiments were optimized for chromosome painting in metaphase spreads but not in interphase nuclei. Still, some nuclei from cells of the translocation carrier clearly showed three distinctly stained domains (Fig. 2c), representing the painted regions of both the marker chromosome and the two normal chromosomes 1 and 7.

## Discussion

In this study we demonstrate that a microlibrary established from laser-microdissected fragments of a translocation chromosome, t(1;7)(1qter→1p36::7q11→7qter), is sufficient for specific painting of the microdissected chromosome region. In addition to most of the long arm of the normal chromosome 7, a region of the short arm of the normal chromosome 1 (1p3, except for the telomeric region) was also painted, proving that the microdissected fragments included material from both chromosomes.

The painted region is estimated to include 100–150 Mb of DNA from the marker chromosome—equivalent to the length of a medium-sized chromosome of the human complement. Provided that all 400 microlibrary clones used for probe preparation carry human inserts from different sites of the microdissected fragments, the maximum complexity of sequences contained in the probe pool could equal up to 1.2 Mb of DNA, which might possibly include some 600 kb of unique sequences. The actual complexity of the probe may be lower, however, depending on the number of clones that carry identical inserts.

The banded pattern seen on the long arms of more extended chromosomes 7 after CISS-hybridization of the microlibrary was observed, although *Alu* banding of unpainted chromosomes due to the presence of biotinylated *Alu* sequences in the probe set was efficiently suppressed. The presence of some clustered, 7q-specific, repetitive sequences may provide a possible explanation. Other explanations, such as a chance spatial clustering of the cloned sequences in the microlibrary or a nonuniform distribution of *Eco*RI restriction sites (used for digestion of the microdissected DNA) along 7q, may be taken into consideration. Notably, such a banded pattern was not obvious in CISS-hybridization experiments using a library from sorted chromosomes 7 where the DNA sequence complexity of this chromosome was represented much more completely (Cremer et al., 1988; Lichter et al., 1988a).

We expect that the approach exemplified here will open intriguing new possibilities in basic and applied cytogenetics. These possibilities include the evaluation of unidentified marker chromosomes or translocated chromosome material contained, for example, in tumor cells. Furthermore, microlibraries derived from individually collected complete or microdissected chromosomes may provide a universal tool for chromosome painting in any desired species. Recently, the elegant work of Lüdecke et al. (1989) showed that banded chromosomes can be used for microcloning. Since the collection of banded chromosomes by micromanipulation can be precisely controlled (Senger et al., 1990), impurities in a microlibrary caused by undesired chromosomes or fragments can be avoided. In contrast, libraries from sorted chromosomes may not only be difficult to achieve for many species, but even the best sorts inevitably contain a certain fraction of unwanted chromosomes or fragmented chromosome pieces.

At present, microdissection and microcloning of chromosomes are highly specialized procedures which may not be easy to establish in all clinical cytogenetic laboratories. We believe, however, that probe sets useful for the painting of chromosome arms and bands, once they are established in a few specialized laboratories, could provide an important service to the cytogenetic community in general. Furthermore, polymerase chain reaction-based methods, when used to amplify DNA from microdissected chromosome material, have the potential to facilitate or even replace elaborate microcloning procedures (Nelson et al., 1989; Ledbetter et al., 1990; Lengauer et al., 1990).

## Acknowledgements

We thank Dr. David C. Ward for his generous support, Dr. Lore Zech for helpful comments, Anna Jauch and Susanne Popp for helpful discussions of the methodological aspects of this study. Dr. Konstantin Miller for providing cell cultures from the female proband carrying the translocation, and Ulrike Bauder and Angelika Wiegenstein for excellent technical assistance and photographic work.

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