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Abbreviations in parenthesis refer to the following sections: (E) Editorial; (R) Review article; (O) Original investigation; (SC) Short communication; (CC) Clinical case report; (CO) Case observed; (RG) Rare genetic variant register; (DV) DNA variants; (L) Letter to the editors

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Original investigations

Sorting of chromosomes by magnetic separation

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Summary. Chromosomes were isolated from Chinese hamster × human hybrid cell lines containing four and nine human chromosomes. Human genomic DNA was biotinylated by nick translation and used to label the human chromosomes by in situ hybridization in suspension. Streptavidin was covalently coupled to the surface of magnetic beads and these were incubated with the hybridized chromosomes. The human chromosomes were bound to the magnetic beads through the strong biotin-streptavidin complex and then rapidly separated from nonlabeled Chinese hamster chromosomes by a simple permanent magnet. The hybridization was visualized by additional binding of avidin-FITC (fluorescein) to the unoccupied biotinylated human DNA bound to the human chromosomes. After magnetic separation, up to 98% of the individual chromosomes attached to magnetic beads were classified as human chromosomes by fluorescence microscopy.

Introduction

Magnetic solid supports with specific affinity couples (Oberteufer 1974) have become a commonly used method ("magnetic sorting") for separating cells, cell organelles, and microorganisms (Molday et al. 1977; Owen 1983). One partner of the affinity couple, normally an antibody, is covalently bound or physically absorbed to magnetic microspheres. Magnetic, polymeric microspheres, designed for this purpose by John Ugelstad, are polysterene beads containing iron oxide (Fe_3O_4) particles (see review by Lea et al. 1985; Howell et al. 1988). By binding specific cells to these "magnetic beads" through an antigen-antibody bridge, large quantities of the specific cells can be sorted in a very short time. We have covalently coupled streptavidin to the magnetic beads to isolate biotinylated chromosomes.

Human chromosomes in hamster × human hybrid cells can be selectively hybridized with human genomic DNA due to sufficient sequence differences of most repetitive DNA sequences in the genome of hamster and human (Durnam et al. 1985; Manuelidis 1985; Schardin et al. 1985; Pinkel et al. 1986a, b). Nucleic acid hybridization of biotinylated human genomic DNA to isolated metaphase chromosomes in suspension offers the possibility of labeling specifically the human chromosomes of a hamster × human hybrid cell line with biotin (Dudin et al. 1987).

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In this paper we describe a new approach to separating the human from the hamster chromosomes in a hamster × human hybrid cell line by use of the streptavidin-biotin affinity couple, magnetic beads, and a simple permanent magnet.

Material and methods

Metaphase chromosomes of the Chinese hamster × human hybrid cell lines Alwbf2 and ADA13SC3 (kindly provided by P. Pearson, Leiden) were isolated in a hexylene glycol buffer (Dudin et al. 1987). Most interphase nuclei were separated from the isolated chromosomes by centrifugation at 30 *g* for 4 min. Human genomic DNA was biotinylated by nick translation (Rigby et al. 1977) using the nick translation reagent kit of BRL (Eggenstein-Leopoldshafen, FRG). The isolated metaphase chromosomes were hybridized in suspension (Dudin et al. 1987) using a hybridization mixture of 40% (v/v) formamide, 2 × SSC, 1 µg biotinylated human genomic DNA, and 7.5% (v/v) dextran sulfate (1 × SSC is 0.15 *M* NaCl, 0.015 *M* sodium citrate). After the last step in 0.1 × SSC, the chromosome suspension was centrifuged at 350 *g* for 15 min and the pellet resuspended in 1 ml IB + M buffer (50 m*M* KCl, 5 m*M* Hepes, 10 m*M* $MgSO_4$, pH 8.0; Trask et al. 1985) containing 0.05% (v/v) Tween 20.

Magnetic beads, 4 µm in diameter (Dynabeads M-450), were purchased from Dynal, Oslo. The free hydroxyl groups on the polymer surface were activated with *p*-toluene sulfonyl chloride (Nustad et al. 1984). Briefly, 100 mg of uncoated magnetic beads were transferred into acetone with sequential washings in 10 ml aliquots (water to acetone (v/v) 7:3, then (v/v) 6:4, then (v/v) 2:8, then 3× (v/v) 0:10, and resuspended in 1 ml acetone). At each step the magnetic beads were collected with a permanent magnet (MPC 1, Dynal), which was held at the outside of the tube. The supernatant was simply poured off. The magnetic beads were incubated with 4.5 m*M* pyridine and 2.2 m*M* *p*-toluene sulfonyl chloride in acetone for 20 h at room temperature with end-over-end rotation. The beads were again collected with the magnet, the supernatant was discarded, and the beads were washed three times in acetone. They were transferred back to water by reversing the washing steps 1–3. The activated magnetic beads were stored in 1 m*M* HCl + 0.02% (v/w) NaN_3 at 4°C. To couple streptavidin to the activated beads, the storage solution was removed and the beads were washed twice in IB + M containing 0.05% (v/v) Tween 20. Then the magnetic beads were resuspended in

0.1 *M* borate buffer (pH 9.5) containing 1 µg streptavidin/mg beads. The suspension was mixed overnight by end-over-end rotation at room temperature. After addition of 0.02% NaN_3 to this solution, the magnetic beads were stored at 4°C. Before use, the beads were washed three times in 1 ml IB + M buffer containing 0.05% (v/v) Tween 20.

The binding of streptavidin to the magnetic beads was determined prior to further experiments. A sample of 50 µl coated beads was washed twice with API buffer (0.1 *M* Tris/HCl pH 7.5, 0.1 *M* NaCl, 2 m*M* $MgCl_2$, 0.05% (v/v) Triton X-100) and sequentially incubated in 1 ml of API buffer containing 1 µl of biotin-AP solution of the alkaline phosphatase detection reaction system (BRL, Eggenstein-Leopoldshafen, FRG). After 20-min incubation at room temperature, the beads were collected by the magnet. The beads were washed in API and three times in APIII buffer (0.1 *M* Tris/HCl, 0.1 *M* NaCl, 5 m*M* $MgCl_2$, pH 9.5); resuspended in 1 ml of staining solution containing 9 µl NBT (nitroblue tetrazolium), 6.5 µl BCIP (5-bromo-4-chloro-3-indolylphosphatate), and 1 ml of APIII buffer; and incubated in the dark for 4 h at room temperature. The reaction was terminated by 1 ml 20 m*M* Tris of pH 7.5 and 5 m*M* K-EDTA. A dark blue precipitate indicated the presence of streptavidin on the surface of the magnetic beads.

Magnetic beads coated with streptavidin were used for isolating the hybridized chromosomes (Alwbf2 and ADA13SC3) or control, Chinese hamster lung (CHL), chromosomes. Coated beads (0.1 mg, 1.4×10^6 beads) were suspended with about 10^6 chromosomes in 100 µl IB + M buffer containing 0.05% (v/v) Tween 20 and 5% (v/v) nonfat dry milk and incubated at 37°C for 2 h by slightly shaking the sample.

The beads were collected with the magnet and resuspended in IB + M containing 0.05% (v/v) Tween 20. The supernatant was transferred to a separate tube for further analysis. The binding of the chromosomes to the beads was checked microscopically. For this purpose 5 µl of the bead suspension was dropped on a slide, stained by adding 5 µl of a DAPI staining solution (5 µ*M* 4,6-diamidino-2-phenylindole 2 HCl) for 5 min, and covered by a coverglass.

To distinguish human chromosomes labeld with biotin from nonlabeled Chinese hamster chromosomes, the bead suspension and the supernatant were incubated with avidin-FITC (Enzo, Neckargemünd, FRG) in a 1:100 dilution in IB + M buffer containing 5% (v/v) nonfat dry milk and 0.05% (v/v) Tween 20. After an incubation period of 90 min at 37°C, the buffer was removed by centrifugation (300 *g*/15 min) or with the aid of the magnet. The pellets were washed twice in IB + M buffer containing 0.05% (v/v) Tween 20.

For microscopy, 5 µl samples from all fractions (including the original fraction after fluorescence hybridization) were dropped on slides, counterstained with propidium iodide by adding 10 µl of a 150 µ*M* staining solution or 5 µl of a 5 µ*M* DAPI solution to each sample, and incubated for 20 min at room temperature. Five microliters of a fluorescence antifading buffer (1 mg *p*-phenylenediamine in 1 ml glycerine buffer, pH 8) was added. The sample was enclosed in a sealed cover slide. Photographs were taken with a Leitz Vario Orthomat 2 and a Kodak Ektachrome 200 or 400 color slide film type R. Fluorescein (FITC) and propidium iodide (PI) were excited at 450–490 nm and photographed with a 515-nm long pass filter. Propidium iodide alone was excited at 530–560 nm and photographed with a 580-nm long pass filter. DAPI was excited at 270–380 nm and photographed with a 430-nm long pass filter.

To establish the percentage of human chromosomes in the different fractions, the following procedure was applied: Chromosomes were identified under the microscope by their PI-fluorescence with the 580-nm long pass filter. Then they were analyzed for their yellow-green and for their red fluorescence with the 515-nm filter. To quantify the difference in yellow-green and red fluorescences of the labeled and nonlabeled chromosomes, digital image analysis was applied using diapositives of randomly selected chromosomes of the supernatant fraction. The images were digitized using a drum scanning densitometer (Scandig 2605; Joyce Loebl). The measurements were done with a green filter in transmission mode, and 256 gray levels were distinguished. The higher the green fluroescence, the higher was the transmission and the lower was consequently the gray level registered. Evaluation was carried out on a VAX 11/780 computer. For each chromosome, the size (pixel number A), the maximum gray level MAX of the individual pixels, the maximum gray level R-MAX of the sum of gray level values on any line perpendicular to the chromosome axis, and the mean gray level density (sum I of all gray values of a chromosome divided by the size A) was determined.

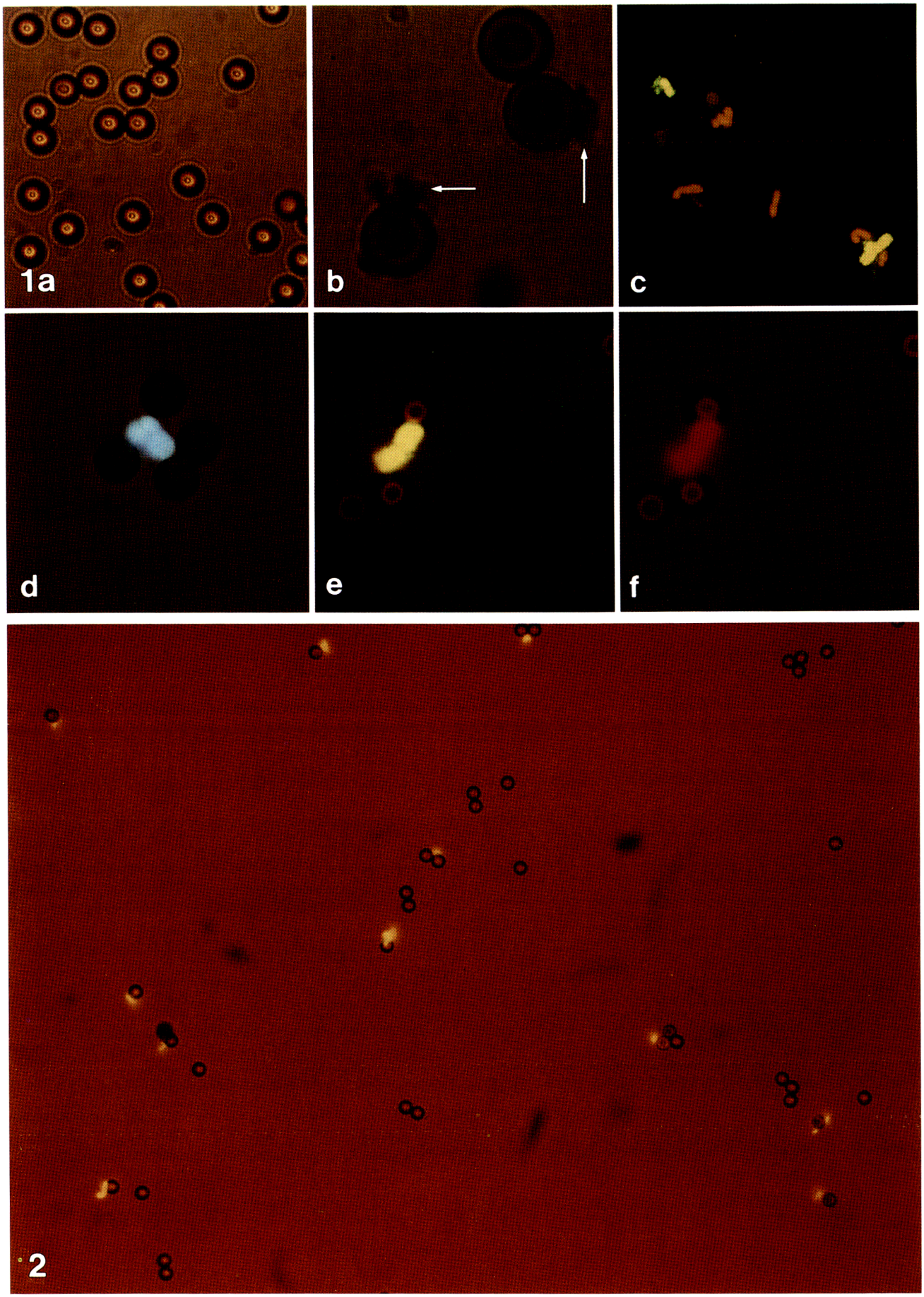
Slot blotting of DNA samples was done in a Minifold II apparatus from Schleicher & Schüll (Dassel, FRG) according to the manufacturer's protocols. Equal amounts (110 ng–0.55 ng) of hamster and human DNA were slotted in parallel on two slot filters. A parallel hybridization of both slot filters with the first to hamster DNA, the second to human DNA, was performed. Any quantitative estimates of DNA concentration were done only on the same experiment. The specific activity of both radiolabeled (^{32}P)-DNA probes was the same. Only autoradiographic signals in between the linear response range were evaluated.

Results and discussion

Isolated metaphase chromosomes of the Chinese hamster × human hybrid cell lines A1wbf2 and ADA13SC3 were hybridized with biotinylated human genomic DNA. The human biotin-labeled chromosomes were then isolated with streptavidin-coated magnetic beads. Binding of streptavidin to the magnetic beads was demonstrated prior to the experiment by the alkaline phosphatase reaction, which resulted in a dark blue precipitate on the surface of the beads (Fig. 1a, b).

Fig. 1. a Magnetic beads before streptavidin binding. **b** Magnetic beads with streptavidin covalently bound. Streptavidin is visualized on the surface of the magnetic beads as dark blue precipitate *(arrows)* after the alkaline phosphatase reaction (see text). **c** Metaphase chromosomes of the Chinese hamster × human hybrid cell line ADA13SC3 after fluorescence hybridization with biotinylated human genomic DNA and counterstaining with propidium iodide. The nonhybridized chromosomes show red and the hybridized chromosomes, yellow-green fluorescence in a 515-nm long pass filter. **d** Magnetic beads bound with streptavidin have isolated a biotinylated human chromosome by streptavidin-biotin bridges. DAPI stain. **e** Magnetic beads bound with a yellow-green fluorescing human chromosome (biotin labeled), photographed with a 515-nm long pass filter. **f** The same chromosome photographed with a 580-nm long pass filter. The diameter of the beads is 4 µm

Fig. 2. Metaphase chromosomes of the Chinese hamster × human hybrid cell line A1wbf2 after magnetic isolation. All chromosomes bound to the magnetic beads show yellow-green fluorescence (details in the text). The diameter of the beads is 4 µm


1a
b
c
d
e
f
2

Labeled human chromosomes became attached to one or more magnetic beads (Fig. 1d–f) through the strong biotin-streptavidin complex. They were distinguished from nonlabeled Chinese hamster chromosomes by their specific fluorescence following binding of avidin-FITC to free biotin molecules (Figs. 1c, e, 2). Counterstaining of the chromosomes with propidium iodide resulted in yellow-green fluorescence (FITC + propidium iodide) of labeled human chromosomes and red fluorescence of Chinese hamster chromosomes (propidium iodide) (Fig. 1c), observed microscopically using a 515-nm long pass filter. Both types of chromosomes exhibited red fluorescence when the 580-nm band pass filter was used (Fig. 1f).

The difference in the fluorescence intensity of human (yellow-green) and hamster (red) chromosomes was quantified by digital image analysis from diapositives photographed with a 515-nm long pass filter. Table 1 shows the results of the evaluation of ten randomly selected chromosomes with yellow-green fluorescence (human chromosomes) and ten randomly selected chromosomes with red fluorescence. All three modes of evaluation show a significant difference between yellow-green (human) and red (hamster) fluorescing chromosomes. The range of variation of the maximum gray level values was 83–105 for yellow-green fluorescing chromosomes and 119–156 for red fluorescing chromosomes, i.e., no overlap was observed here. These data confirm that under the conditions used there is indeed a large difference in the fluorescence intensities between the two groups of chromosomes.

Table 1. Digital image analysis of chromosomes following fluorescence hybridization (ADA 13SC3 line). For digital image analysis, the chromosomes were selected visually (yellow-green or red fluorescence). Ten randomly chosen chromosomes of each fluorescence mode were photographed under identical conditions; the images were digitized and evaluated as described in Material and methods. The means ($N = 10$) ± SD are in arbitrary units. MAX, Maximum gray level of any individual pixel (image element) of a given chromosome; R-MAX, maximum of the sum of gray levels on any line perpendicular to the chromosome axis; I/A, sum (I) of all gray values of a given chromosome divided by the chromosome area A (given in number of pixels) or mean gray level

Fluorescence mode	Maximum gray level (MAX)	R-MAX	Mean gray level (I/A)
Yellow-green (human)	93.2 ± 8.15	693.1 ± 156.5	41.5 ± 5.0
Red (hamster)	136.3 ± 12.4	1133.9 ± 207.8	67.3 ± 10.4

Cells of the Chinese hamster × human hybrid cell line A1wbf2 contain about 42 chromosomes per cell, 4 of which are human chromosomes. In the original sample, 81 (8.1%) of 1000 chromosomes counted were yellow-green (human), corresponding to 3.4 human chromosomes in each set of 42 chromosomes. This number fits with the expected number (4) of human chromosomes and confirms that the yellow-green fluorescing chromosomes are indeed the human ones.

Table 2 gives the results of a microscopic evaluation of chromosomes following magnetic separation. The total number of individual chromosomes counted was 779 in the A1wbf2 line and 2626 in the ADA13SC3 line. Of these individual chromosomes, 761 (97.7%) and 2596 (98.9%), respectively, were classified as human chromosomes due to their fluorescence (for discussion of chromosome aggregates, etc., see below).

In the supernatant after magnetic separation, 23 of 506 chromosomes counted (4.5%; 5% confidence ranges 2.8%–6.7%) were classified as human. This figure is significantly lower than the expected number of 48 (9.5%; 5% confidence ranges 7.1%–12.5%) in an unfractionated chromosome suspension. This again confirms the selective binding of human chromosomes to the magnetic beads.

Cross hybridization of biotinylated human DNA to Chinese hamster chromosomes might result in indiscriminate binding of these chromosomes to the magnetic beads and thus impair the specificity of magnetic beads separation. However, our data as follows indicate that such an effect was small:

(1) Chinese hamster lung (CHL) chromosomes were hybridized to biotinylated human genomic DNA and incubated with magnetic beads coated with streptavidin. The binding of CHL chromosomes was found to be very rare: 5 µl suspension in each sample contained about 35000 beads. In this control experiment, 229 CHL chromosomes were bound to 70000 beads (0.3%).

(2) In the ADA13SC3 experiment (Table 2), 30 individual hamster chromosomes were bound to 35000 beads (0.1%). For comparison, the ratio of individual human chromosomes bound to magnetic beads divided by the number of beads was about two orders of magnitude higher (see also Fig. 2).

Table 2. Microscopic evaluation of chromosomes following magnetic separation. Chromosome aggregates (several chromosomes bound together) were counted as human chromosome aggregates when all the chromosome showed yellow-green fluorescence. In case where at least one of the chromosomes exhibited red fluorescence only, the chromosome aggregate was listed as hamster. Total number of individual chromosome counted: A1wbf2-line, 779; ADA1, 2626

Cell line	Human chromosomes[a] (N)				Hamster chromosomes[b] (N)			
	Bound to magnetic beads		Free		Bound to magnetic beads		Free	
	Individual chromosomes	Chromosomes aggregates	Individual chromosomes	Aggregates	Individual chromosomes	Chromosome aggregates and interphase nuclei	Individual chromosomes	Chromosome aggregates and interphase nuclei
A1wbf2	751	–	10	–	14	7	4	–
ADA13SC3[c]	2596	181	–	–	30	7	–	32

[a] Yellow-green fluorescence
[b] Red fluorescence
[c] Evaluation of the entire suspension (containing approx. 35000 beads)

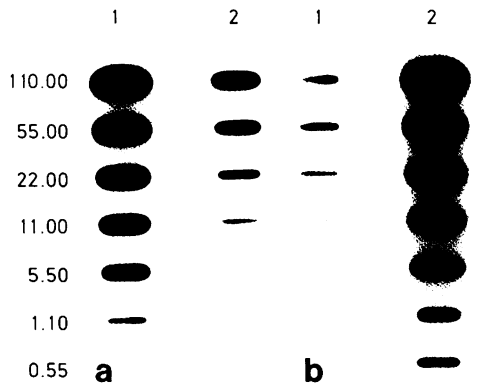


Fig. 3a, b. A concentration gradient of hamster *(lane 1)* and human *(lane 2)* genomic DNA was hybridized via DNA slot blot to nick-translated total ^{32}P-labeled DNA of hamster (**a**) and human (**b**). The *numbers* at the left indicate the amount of slotted DNA in nanograms. The percentage of cross hybridization between the DNA of both species in (**a**) and (**b**) averages between 1% (see **b**) to 10% (see **a**) under nonstringent posthybridization washing conditions (2 × SSC, 65°C)

(3) After incubation of the hybridized CHL chromosomes see (1) with avidin-FITC under the same conditions as in the separation experiments, no yellow-green chromosomes were found, but all chromosomes analyzed (1500) were stained red with propidium iodide.

(4) An independent estimate of the possible cross hybridization between the biotinylated human genomic DNA and the hamster chromosomes was done on the DNA level with the aid of a slot blot experiment. Under nonstringent washing conditions (2 × SSC, 65°C), human DNA hybridizes to Chinese hamster DNA at a level between 1%–10% (Fig. 3). This agrees well with the low percentages of individual hamster chromosomes observed after magnetic separation (18/779 = 2.3% and 30/2626 = 1.1%, respectively; see Table 2).

If individual chromosomes only are considered, the results (Table 2) of sorting human chromosomes by magnetic separation may be as good as the best results so far obtained by laser fluorescence activated flow sorting of chromosomes (Lebo et al. 1984). In these calculations, however, chromosome aggregates and interphase nuclei were not taken into consideration. Since every chromosome aggregate/interphase nucleus may contain many hamster chromosomes (up to 38), the efficacy of the sort (e.g., measured as the percentage of human DNA in the sorted fraction divided by the percentage of human DNA prior to sorting) may be considerably reduced. The figures given in Table 2 suggest that even in a "worst case" (every aggregate of chromosomes classified as human is assumed to contain two chromosomes only; every chromosome aggregate of "hamster" chromosomes is assumed to be a mitotic cell; every interphase nucleus is assumed to be in G2), the percentage of human chromosome equivalents after magnetic sorting is about 70%. This is still a reasonably high enrichment, justifying the term "sorting" (Yu et al. 1981; Cremer et al. 1984).

For high purity sorting, however, it will be important to reduce significantly or to eliminate essentially the aggregates and interphase nuclei, respectively. This may be done, e.g., by 1 *g* sedimentation (Collard et al. 1980; Blochmann et al. 1987; Schwäger et al. 1987), preferably prior to magnetic separation. The combined use of sedimentation (or other methods) and magnetic separation remains to be established. However, it has been shown that the conditions used for 1 *g* sedimentation of chromosomes are compatible with in situ hybridization (Blochmann et al. 1987).

The number of chromosomes which can be sorted in a given time by magnetic separation is, in principle, not limited. In contrast, even high-speed flow sorters (Peters et al. 1985) sort specific chromosomes at a rate not higher than about 1000 chromosomes/s, which corresponds to a few micrograms of chromosomes per hour. To realize the large sorting potential of the magnetic beads separation technique, it will be necessary, however, to overcome the severe clumping observed when larger numbers of chromosomes/ magnetic beads are used (data not shown). This problem may be resolved by different ways, e.g., the beads may be enclosed in a "magnetic bottle" and kept there in a dispersed state (Schwager 1986; Howell et al. 1988) or beads with a lower magnetic affinity may be constructed (Lea et al. 1988).

The results presented here indicate that sorting of chromosomes by magnetic beads is indeed feasible. As an application, this new approach might be used to sort a specific chromosome for library construction (Cremer et al. 1984; Fuscoe et al. 1986) and biochemical analyses. This may be achieved by using a hybrid cell line containing one human chromosome only and human genomic DNA as a probe, or by using human cell types and chromosome-specific DNA probes.

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