Measurement and purification of human chromosomes by flow cytometry and sorting

(isolated chromosomes/DNA cytophotometry/flow microfluorometer)

A. V. CARRANO, J. W. GRAY, R. G. LANGLOIS, K. J. BURKHART-SCHULTZ, AND M. A. VAN DILLA

Biomedical Sciences Division, L-452, Lawrence Livermore Laboratory, Livermore, California 94550

ABSTRACT   The 24 human chromosome types of a normal diploid fibroblast cell strain were classified into 15 groups by high-resolution flow cytometry on the basis of 33258 Hoechst fluorescence. Chromosomes associated with each group were flow sorted onto microscope slides and identified by quinacrine banding analysis. DNA cytophotometry of metaphase chromosomes from the same cell strain supported and extended this identification. Four of the groups purified were due to chromosomes of a single type—namely, chromosomes 5, 6, 13, and 17. Eight additional groups were also separated and found to contain the following chromosomes: 1 and 2; 3 and 4; 7, 8, and X; 9–12; 14 and 15; 16 and 18; 20 and Y; and 19, 21, and 22. The average purity for the 12 sorted fractions was 78%.

Flow cytometry of isolated chromosomes is a new approach to cytogenetics that provides rapid measurement of individual metaphase chromosomes. In this approach, chromosomes that are stained in aqueous suspension with an appropriate fluorochrome are constrained to flow at high speed through a narrow laser beam that excites the stain. The emitted fluorescence is measured photometrically and the accumulated data form a frequency distribution of chromosome fluorescence. The peaks of this frequency distribution are due to individual chromosomes or groups of chromosomes of similar fluorescence; the peak mean is proportional to chromosome fluorescence and the peak area is proportional to the chromosome frequency of occurrence. Thus, the frequency distribution serves as a karyotype (1, 2). In addition, flow sorting can be used to separate chromosomes on the basis of their staining properties (3, 4), in contrast to conventional methods for purifying metaphase chromosomes that rely upon velocity or isopycnic sedimentation, zonal centrifugation, or selective filtration (5). Purification of individual metaphase chromosomes is important for several reasons. Enriched or pure chromosome fractions have been analyzed biochemically to provide information on the structure of DNA or protein (6), to transfer genetic information to whole cells (7–9), or to map genes by in vitro hybridization (10). In general, however, conventional techniques have not been able to provide chromosomes of sufficient purity for high-resolution biological or biochemical studies.

By flow sorting on the basis of ethidium bromide fluorescence, we have separated, with a purity of 90%, each chromosome of the male deer Muntiacus muntjak (2n = 7) (4) and the 14 chromosome types of the Chinese hamster M3-1 cell line into eight chromosome groups (1, 3). In our previous studies with ethidium bromide-stained human chromosomes, we resolved only eight chromosome groups from the 24 chromosome types of the male (2n = 46) (2, 3). In the present study, using the DNA fluorochrome 33258 Hoechst and improved instrumentation,

we resolved 15 chromosome groups in the human male and, by sorting, purified chromosomes 5, 6, 13, and 17 and eight additional groups composed of chromosomes of more than one type.

MATERIALS AND METHODS

Chromosome Preparation. A human diploid fibroblast culture, LLL (Lawrence Livermore Laboratory) strain 592, was established from newborn foreskin tissue. Giemsa and quinacrine banding analysis showed the cells to be karyotypically normal. Early passage cells from this strain were grown in minimal essential medium with Earle's salts supplemented with gentamycin and 15% fetal calf serum. Doubling time was about 24 hr. In order to collect a large number of mitotic cells, the cultures were grown in 650-cm² glass roller bottles and Colcemid (0.32 μg/ml, final concentration) was added for the last 16 hr of growth. The rounded mitotic cells were detached from the glass surface by rotating the bottles at 300 rpm for 6 min. Aliquots (2 × 10⁶ mitotic cells; mitotic index, ≈80–90%) were swollen with 75 mM KCl at 4°C for 30 min. The cells were sedimented by centrifugation, resuspended, and maintained for 10 min at 37°C in 0.5 ml of isolation buffer (25 mM Tris·HCl, pH 7.5/0.75 M hexylene glycol/0.5 mM CaCl₂/1.0 mM MgCl₂). The suspension was then kept at 4°C for about 1 hr prior to shearing. The cells were mechanically ruptured by homogenization for about 1.5 min at 4°C by using the lowest speed setting of a VirTis "45" homogenizer. Isolated chromosomes were stained with 0.5 ml of 33258 Hoechst (final concentration, 2 μg/ml). The suspension, containing about 10⁸ chromosomes per ml, was then ready for flow cytometry or sorting.

Flow Cytometry and Sorting. High-resolution flow cytometry was accomplished on the LLL dual laser cytometer (11) utilizing a Spectra Physics 171-05 argon ion laser operating at 351–364 nm with a power of 0.8 W. Flow rate was approximately 1000 chromosomes per sec; typically, 500,000 chromosomes were analyzed. Flow sorting was performed with the LLL flow sorter (12) also equipped with a Spectra Physics 171-05 argon ion laser. About 20,000 chromosomes from each peak were sorted onto a cooled (−30°C) aluminum plate and frozen. The frozen bead containing the chromosomes was placed in the well of a modified Leif bucket (13) with fixative [absolute methanol/glacial acetic acid, 3:1 (vol/vol)] and spun onto glass slides. After two further washings in fixative, the chromosomes were stained with quinacrine dihydrochloride for banding analysis.

DNA Cytophotometry. The DNA stain content of chromosomes in metaphase cells of strain 592 was measured by using CYDAC, a scanning cytophotometer (14). The procedures have
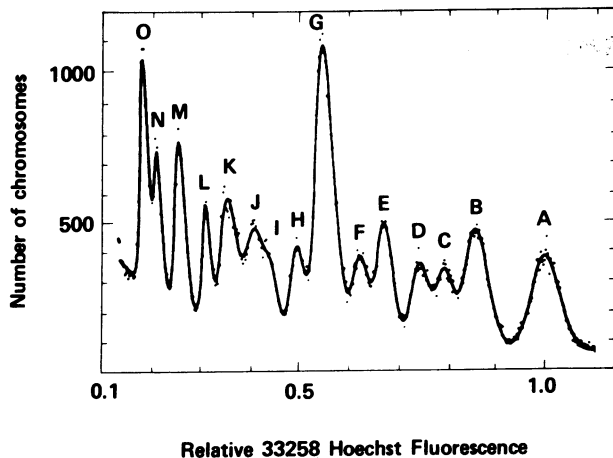
Relative 33258 Hoechst Fluorescence

FIG. 1. 33258 Hoechst fluorescence distribution of human chromosomes isolated from diploid strain 592 as measured by high-resolution flow cytometry. Data were collected into 512 channels of a pulse-height analyzer and subjected to an iterative least-squares method that fits a sum of Gaussian distributions to each peak plus a power function to the underlying continuum (3). This continuum is the area under the Gaussian distribution that we attribute to fluorescence debris, clumping, and nonspecific staining. The computer-generated line is drawn through the data above and clearly defines 15 peaks, A through O. Coefficients of variation (standard deviation of the Gaussian expressed as percentage of the mean) range from 2.1 to 4.9% (mean, 3.2%). The relative fluorescence is normalized to the mean of peak A = 1.0. The actual number of chromosomes counted is given on the ordinate.

been described (15). Briefly, the chromosomes are stained with gallocyanin chrome alum under conditions that are specific for DNA, the stained chromosomes are scanned on a flying spot microscope, and the scans are digitized and processed by computer. The end results are measurements of the integrated optical density (DNA stain content) for each chromosome.

RESULTS AND DISCUSSION

Fig. 1 shows the relative 33258 Hoechst fluorescence distribution of human chromosomes from strain 592. Each of the 15 peaks represents chromosomes of similar fluorescence. The peak means (proportional to the 33258 Hoechst fluorescence) and the peak areas (proportional to the frequency of occurrence of the chromosomes of that group) were determined by fitting each peak with a normal distribution and the underlying continuum with a power function (3). The results are shown in Table 1.

We used two methods to identify the chromosomes associated with each peak in the flow distribution: flow sorting and DNA cytophotometry. Flow sorting was accomplished at lower resolution than the flow cytometry so that chromosomes were sorted together from peaks E and F, I and J, and N and O. The regions of each peak from which chromosomes were collected encompassed approximately the central three-fourths of the peak. Because the cells were exposed to Colcemid for an extended period to accumulate cells in mitosis, the chromosomes were generally highly contracted and only 10–15% of the sorted chromosomes revealed clear quinacrine banding patterns. Clumps of smaller chromosomes were present in each aliquot of sorted chromosomes; 20% of the sorted material from peaks A–D, 10% from peaks E–G, and only a few percent from the remaining peaks were due to clumps. The sorting purity ranged from 56 to 98% (Table 1).

The identification of the sorted chromosomes was supported and extended by DNA cytophotometry of chromosomes from metaphase cells of this strain together with the relative area and mode of each peak. There is good agreement between the observed and expected numbers of chromosomes for each peak. We attribute the low relative area of peaks N and O to the inaccuracy of the mathematical fit which overestimates the continuum underlying the small chromosomes and thereby

Table 1. Analysis of normal human chromosomes (strain 592) by flow cytometry and sorting

Peak[*]	Chromo- some	Sorting purity, %	Number of chromosomes Observed[†]	Number of chromosomes Expected	Relative Hoechst fluorescence[‡]	Relative DNA content[§]
A	1,2	78	4.1	4	1.00	1.0
B	3,4	80	4.4	4	0.86	0.82, 0.78 (0.81)
C	5	56	1.9	2	0.80	0.78
D	6	65	2.5	2	0.75	0.72
E			3.0		0.68	
	7,8,X	81		5		0.67, 0.59, 0.64 (0.63)
F			2.7		0.62	
G	9,10,11,12	63	8.5	8	0.56	0.54, 0.56, 0.55, 0.56 (0.55)
H	13	84	2.1	2	0.50	0.46
I	14		1.4	1	0.44	0.45
		75				
J	14,15		2.7	3	0.41	0.40, 0.42 (0.41)
K	16,18	89	3.7	4	0.36	0.39, 0.33 (0.36)
L	17	71	1.6	2	0.31	0.35
M	20, Y	92	2.9	3	0.26	0.28, 0.22 (0.26)
N			1.9		0.22	
	19,21,22	98		6		0.26, 0.19, 0.22, (0.22)
O			2.8		0.19	

* Peaks connected by brackets could not be unequivocally resolved by the flow sorter and hence were sorted together. Assignment of the two homologs of chromosome 14 to peaks I and J is tentatively made on the basis of peak areas and the measured homolog difference in DNA content.

† Calculated by determining the relative area of the Gaussian distribution fit to each peak. Total area was normalized to 46 chromosomes.

‡ Normalized to peak A = 1.0.

§ Determined independently by scanning cytophotometry of 10 metaphase spreads stained with gallocyanin chrome alum and normalized to chromosomes 1 + 2 = 1.0. The values in parentheses are the weighted means for the individual chromosomes within that peak. The individual chromosome relative DNA values are given in the same order as in the second column.

subtracts from the area of these peaks. DNA cytophotometry indicates a 10% homolog difference between the two chromosomes 14. This is consistent with the relative fluorescence and number of chromosomes in peaks I and J.

The relative Hoechst fluorescence generally paralleled the amount of DNA in each chromosome but some differences were evident. Chromosomes 4, 8, 13, 18, and Y had greater Hoechst fluorescence and chromosomes 16, 17, and 19 had lower fluorescence than predicted on the basis of their DNA content. Chromosomes 4 and 5, which had nearly identical amounts of DNA, showed an 8% differences in Hoechst fluorescence. These disparities between DNA content and Hoechst fluorescence are not entirely unexpected because it is known that 33258 Hoechst has increased affinity for DNA rich in A·T base sequences (16, 17). In addition, the Hoechst fluorescent banding patterns on fixed metaphase chromosomes are similar to those of quinacrine (18), a fluorochrome whose fluorescence is quenched by G·C base pairs (19). Thus, the Y chromosome, which possesses an A·T rich satellite DNA (20, 21) in the distal region of the long arm, is quinacrine bright in fixed metaphase chromosomes and also shows enhanced Hoechst fluorescence in unfixed chromosomes in suspension. Similarly, in fixed metaphase cells, chromosome 13 is relatively bright whereas chromosome 19 has dull quinacrine fluorescence. The Hoechst fluorescence therefore probably reflects some internal structure of the chromosomal DNA that is different from total DNA content alone. Because interindividual chromosomal heterogeneity in chromosomal DNA and banding patterns is documented (22, 23), each human diploid cell strain might be expected to possess a slightly different flow distribution of chromosomal 33258 Hoechst fluorescence. Preliminary results in our laboratory with four additional strains confirm this. It should now be possible to select cell strains with known chromosomal variants or translocations such that these chromosomes form a unique peak in the flow distribution and are thus easily purified for further biological or biochemical characterization.

This work documents cytogenetic, cytochemical, and instrumental methods to isolate, classify, and purify human metaphase chromosomes. Sufficient quantities of highly purified chromosomes are readily obtainable for cytological studies. A limitation to this technique is the present difficulty in providing large quantities of chromosomes for some biochemical and biological studies. For example, human chromosomes are sorted (two types simultaneously) at a rate of about 100/sec. If a biochemical study requires 1 μg of DNA or protein, about 10 hr of sorting would be necessary for a single analysis—possible but inconvenient. Methods are needed to narrow the gap between analytical biochemistry and instrumental throughput. These could take the form of increasing the chromosome sort rate by increasing sorting efficiency, by using presorted chromosomes (24), and by miniaturizing the procedures for DNA and protein analysis. We visualize that chromosome analysis and purification by flow methods will contribute greatly to increased understanding of the structure and function of the chromosome and may provide a means for rapid karyotype analysis.

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