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Flow Cytometric Characterization of a Chinese Hamster × Man Hybrid Cell Line Retaining the Human Y Chromosome

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Summary. A Chinese hamster × man hybrid cell line (CH-Y-VII) was established which retains a free human Y chromosome. Exponentially growing CH-Y-VII cells were arrested with colcemid; metaphase chromosomes were isolated and stained with 33258 Hoechst (HO) plus Chromomycin A3 (CA3), or with ethidium bromide (EB). The HO/CA3-stained chromosomes were measured in a dual beam flow cytometer, and bivariate HO/CA3 flow karyotypes and univariate HO and CA3 flow karyotypes were established. EB-stained chromosomes were analyzed in a modified Becton Dickinson FACS-Sorter. For all three stains used, the human Y chromosome forms a separate peak in univariate flow karyotypes; the optimum resolution was obtained for the HO distribution. In the bivariate HO/CA3 flow karyotype, the peak for the human Y chromosome is completely separated from the Chinese hamster chromosomes.

Introduction

The advent of flow cytometry and sorting (for review see Horan and Wheeless 1977; Melamed et al. 1979) has made it possible to distinguish and hence to purify isolated chromosomes in suspension. In principle, chromosomes isolated from mitotic cells are stained with a DNA-specific stain such as 33258 Hoechst (HO), Chromomycin A3 (CA3), or ethidium bromide (EB). During flow cytometry, they move one-by-one through an intense laser beam that excites the chromosome dye to fluoresce; the fluorescence from each chromosome is measured; and with a special electronic device, chromosomes differing in their stain fluorescence intensity can be separated from each other. Purified chromosomes may be used for a wide range of investigations, such as template activity (Sawin et al. 1979), in vitro gene mapping (Lebo et al. 1979, 1982), or molecular cloning (Davies et al. 1981; Disteche et al. 1981). The cloning of a representative genomic library of the human Y chromosome is of interest in several respects. Such a genomic library may allow the identification of coding sequences on this largely inert chromosome, and eventually homologies between the X and the Y. Moreover, unique Y chromosome-specific DNA probes can be used for monitoring presence or absence of genetic information relevant to disorders of sexual development. This approach requires the identification of clones of unique Y chromosome-specific DNA probes.

In principle, such DNA probes may be obtained from a complete library of the human genome. However, a very large number of clones must be screened before a small number of clones can be identified. A much more direct and simple approach is to establish such a gene library from Y chromosomes purified by flow cytometric sorting. Recently, it has been shown that the human Y chromosome can be discriminated in human lymphocytes by single parameter (Young et al. 1981) and dual parameter flow cytometry (Gray et al. 1982).

The flow rates (total number of chromosomes analyzed per second) compatible with a sufficient resolution, however, are in the order of 500/s (Davies et al. 1981). From this, it can be estimated that sorting times in the order of 130 h are necessary to sort a sufficient amount (~ 500 ng) of Y chromosomal DNA. Such long sorting times may be regarded as impractical. One possibility to reduce the sorting time might be to use, e.g., suitable 47 (X,YY) cells where the Y chromosomes can be discriminated as a separate peak. Because of the polymorphism of the DNA content and hence variation in fluorescence intensity (Young et al. 1981), a number of different cell strains would have to be screened until a strain is found where the fluorescence intensity of the Y differs sufficiently from that of all other chromosomes. Furthermore, human fibroblast strains have a long generation time and low mitotic index; short-time cultures of peripheral lymphocytes, on the other hand, yield large numbers of mitotic cells, however, a suitable donor may not be continually available.

Here, we show that the human Y chromosome can be discriminated flow cytometrically in Chinese hamster × man hybrid cells retaining a free human Y chromosome. This may allow to shorten considerably the time required for sorting. The hybrid cells have a short generation time, a high mitotic index may be obtained, and they are continually available.

Materials and Methods

Cell Lines

Chinese hamster × man hybrid cells established by fusion of human cells with an HPRT-deficient Chinese hamster cell line (CHW-1102; Gee et al. 1974) were kindly provided by Dr. Harold P. Klinger (New York); by subcloning, a hybrid line (CH-Y-VII) was obtained which had retained the 22 chromo-

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somes of the CHW-1102 line plus a free human Y chromosome. Q-banding analysis (K. Sperling, private communication) revealed an additional marker chromosome M3 (relative length 46% of chromosome 1), probably a hamster × man translocation chromosome. In some experiments, a hybrid line (CH-XII) was used which had lost the human Y chromosome (obtained by further subcloning of CH-Y-VII).

These hybrid cell lines were maintained under standard conditions (minimum essential medium, 10% fetal calf serum) and grown in monolayer at 37° C, 5% CO_2. The doubling time was approximately 14 h.

Compared with normal diploid Chinese hamster cells, the following karyotype alterations were observed in CHW-1102 cells (Gee et al. 1974): No chromosomes 6 are present. Chromosomes 5 appear to differ slightly in their size and G-banding pattern; the X chromosome is deleted (Xp−q−), and the deleted segment of the short arm of the X chromosome has been translocated to the long arm of chromsome 8 [t(8q;Xp)]; the two chromosomes 8, however, are similar in length. Two marker chromosomes M1 and M2 are present; the large M1 chromosome has approximately 68% of the length of chromosome 1, while M2 has about 11% of the length of chromosome 1; the Y chromosome (Y_{CH}) appears to be considerably shorter than in normal Chinese hamster cells, being about the size of a chromosome 8.

Chromosome Isolation

Cells were arrested in metaphase by adding colcemid (0.03 µg/ml) to exponentially growing cultures 5 h prior to chromosome isolation. Mitotic cells were detached by mechanical shaking. The mitotic index in the detached cell population was about 90%. Chromosomes were isolated as described previously (Gray et al. 1979; Langlois et al. 1980). Briefly, the detached cells were resuspended in hypotonic solution (0.075 *M* KCl) for 30 min and then resuspended in a Tris buffered isolation medium (25 m*M* Tris-HCl, pH 7.5, 0.75 *M* hexylene glycol, 0.5 m*M* $CaCl_2$, and 1 m*M* $MgCl_2$). This isolation buffer allows Q-banding identification at least of part of the chromosomes after sorting (Carrano et al. 1979). The cells were mechanically ruptured in a Virtis "60K" homogenizer (40 s at 21500 rpm) to release the chromosomes.

Chromosomes were stained in suspension with HO and CA3 (final concentrations HO 2 µg/ml and CA 16 µg/ml) (Gray et al. 1979) or with EB (final concentration 40 µg/ml) (Langlois et al. 1980).

Flow Cytometry

Bivariate fluorescence distributions of HO/CA3-stained chromosomes were measured with the Livermore Dual Beam Flow Sorter (Dean and Pinkel 1978; Dean 1980). In this procedure, two fluorescence measurements were made for each chromosome; in one, each chromosome was illuminated in the ultraviolet (UV, $\lambda_{ex} = 337 + 356$ nm) to excite HO fluorescence, and in the other each was illuminated with blue light ($\lambda_{ex} = 458$ nm) to excite CA3 fluorescence. Fluorescence emission was measured using an LP450 filter (passing fluorescence with wavelengths longer than 450 nm) for fluorescence resulting from the UV excitation, and a Corning 3-71 filter (passing fluorescence with a wavelength longer than 500 nm) for fluorescence resulting from 458 nm excitation.

The fluorescence measured following UV excitation of HO/CA3-stained chromosomes is very similar to that which would result from excitation of chromosomes stained only with HO since CA3 excites very poorly in the UV (Gray et al. 1979). The UV-stimulated fluorescence will be referred to as HO fluorescence. The fluorescence measured following 458 nm excitation of HO/CA3-stained chromosomes is very similar to the fluorescence that would result from excitation of chromosomes stained only with CA3 since HO excites very poorly at 458 nm (Gray et al. 1979). This 458 nm-stimulated fluorescence will be referred to as CA3 fluorescence. Dual parameter distributions were measured and stored in a 64 × 64 channel array. Simultaneously, single parameter distributions (HO fluorescence, CA3 fluorescence) were recorded in 256 channel arrays.

The fluorescence frequency distributions (flow karyotypes) of the EB-stained chromosome suspensions were measured with a modified Becton Dickinson FACS-Sorter utilizing a Spectra Physics argon-ion-laser (171-051) operating at $\lambda_{ex} = 514.5$ nm.

Fluorescence was collected through a Corning 2-60 long wavelength pass filter (fluorescence ≥ 630 nm) and stored in a 256 channel array.

In some experiments, chromosomes were sorted into frozen petri dishes (−70° C), transferred to microscope slides, and Q-banded.

Results

Dual Parameter Analysis

Figure 1 shows the bivariate distribution of HO/CA3-stained chromosomes of CH-Y-VII cells. The data are presented as a contour plot (Gray et al. 1979), i.e., the contour lines represent positions with the same frequency of particles. Twelve peaks, A–L, are observed. Peak L was assigned to the human Y chromosome for the following reasons:

1) Its relative peak position (Table 1) corresponds to the peak position observed for human Y chromosomes in bivariate flow karyotypes of human fibroblasts (Gray et al. 1979) and human lymphocytes (Gray et al. 1982; Langlois et al., in preparation).

2) A comparison with chromosomes isolated from human lymphocytes, stained and measured under the same conditions as the CH-Y-VII chromosomes, showed that also the absolute fluorescence intensities correspond to that of the human Y chromosome (data not shown).

3) The ratio of HO to CA3 fluorescence of the chromosomes of peak L is in the order of 1.9. Such high HO/CA3 ratios, which are related to the AT/GC ratio (Langlois et al. 1980), have been observed only for the human Y chromosome (Langlois et al., in preparation).

4) In CH-XII-hybrid cells which had lost the human Y chromosome, peak L was not observed.

5) Finally, the assignment of peak L to the human Y chromosome was confirmed directly by dual parameter sorting (Dean 1980) of chromosomes in this peak on microscope slides and Q-banding analysis (purity of the Y chromosome about 80%).

The provisional chromosome assignment of peaks A–K was performed in the following way: By comparison of the relative HO fluorescence, the relative CA3 fluorescence, and the estimat-

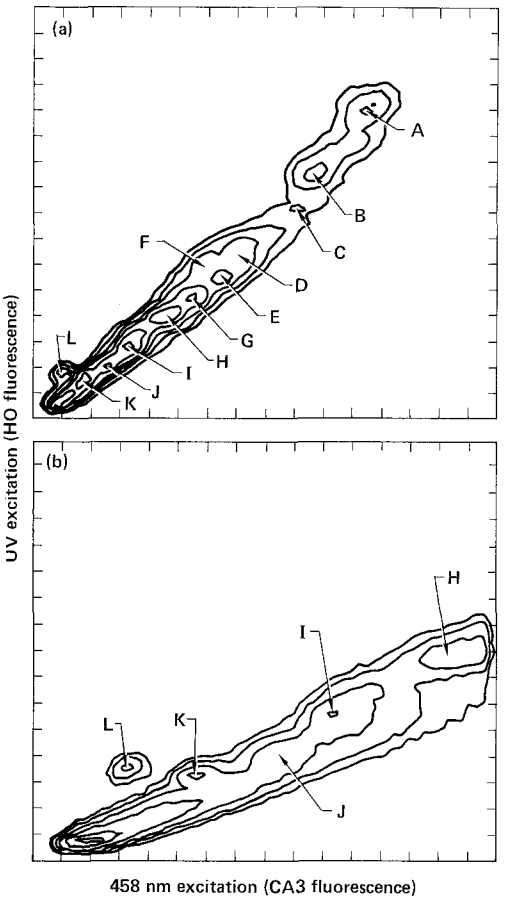

Fig. 1 a and b. Contour plot of the HO/CA3 two parameter fluorescence distribution of Chinese hamster × man hybrid (CH-Y-VII) chromosomes. **a** All chromosomes; **b** small chromosomes only (increased gains)

Table 1. Peak identification and parameters determined from the HO vs CA3 fluorescence distribution[a] for Chinese hamster × man hybrid (CH-Y-VII) chromosomes

Peak designation	HO_{rel}[b]	$CA3_{rel}$[b]	TDNA[c]	Chromosome assignment[d]	TDNA expected
A	9.73	8.90	9.4	1,1	9.8[e]
B	7.87	7.61	7.8	2,2	8.5[e]
C	6.62	7.02	6.9	M1	7.3[f]
D	5.38	5.59	5.5	4,4	5.6[e]
E	4.50	5.07	4.7	5	4.7[e]
F	5.00	4.59	4.8	5	4.7[e]
G	3.88	4.28	4.0	7,7,M3	4.0[e,f]
H	3.24	3.34	3.3	8,t(8q;Xp),Y_{CH}	3.73[f]
I	2.36	2.52	2.4	9,9,X_{del}	2.4[e,f]
J	1.71	2.02	1.8	10,10,11,11	1.8[e]
K	1.23	1.37	1.3	M2	1.7[f]
L	1.42	0.75	1.2	Y_{HUM}	0.9[e]

[a] Fig. 1

[b] Relative HO and CA3 fluorescence as obtained from the evaluation of bivariate HO/CA3 contour plots (Gray et al. 1979). Given are the means of three independent measurements. The data were normalized so that the sum of the means times the expected number of chromosomes equals 100

[c] Total DNA content estimated as $TDNA = 0.6\,HO_{rel} + 0.4\,CA3_{rel}$ (Gray et al. 1979)

[d] Peak L was identified to represent the Y chromosome by dual parameter sorting (Dean 1980) of particles in peak L on microscope slides and Q-banding analysis. For assignment of the other chromosomes see text

[e] TDNA expected according to Gray et al. (1979)

[f] TDNA content estimated from chromosome length (see text)

ed total DNA content (TDNA = 0.6 HO + 0.4 CA3; Gray et al. 1979) with the bivariate HO/CA3 flow karyotype of Chinese hamster M3-1 cells (Gray et al. 1979), peaks A, B, D, E + F, G, I, and J were assigned to chromosomes 1, 2, 4, 5, 7, 9, 10, and 11, respectively.

The remaining chromosomes were assigned to individual peaks by the following reasoning: A comparison between chromosome length (Gray et al. 1975) and TDNA content for M3-1 cells (Gray et al. 1979) revealed a strong linear correlation (coefficient of correlation 0.997). When the TDNA values of peaks A, B, E + F, G, I, and J as obtained from Table 1 were plotted as a function of the relative length of CH^{w}-1102 chromosomes (Gee et al. 1974), again an excellent linear correlation was found (coefficient of correlation 0.99). It seems reasonable to assume that the linear relationship holds also true for chromosomes 8, t(8q;Xp), Y_{CH}, X_{del}, M1, M2, and M3. According to this, the TDNA values for these chromosomes were estimated from their length using the linear regression curve determined for the Chinese hamster chromosome already identified. By this method, the hamster chromosomes Y_{CH} and 8 were assigned to peak H; X_{del} was assigned to peak I, and M1 and M2 were assigned to peaks C and K, respectively. The marker chromosome M3 was assigned to peak G (together with chromosomes 7).

Univariate Analysis

To investigate whether the human Y chromosome can be discriminated not only in bivariate flow karyotypes of CH-Y-VII cells but also in univariate distributions, the single parameter distributions for UV excitation (HO fluorescence), 458 nm excitation (CA3 fluorescence), and 514 nm excitation (EB fluorescence) were analyzed. Figure 2, a and b shows the univariate HO and CA3 distributions. The peak assignments were made by comparison with the bivariate flow karyotype (Fig. 1, Table 1). The human Y chromosome (peak L) can clearly be distinguished both in the HO (Fig. 2a) and in the CA3 distribution (Fig. 2b).

In Fig. 2c, the univariate flow karyotype of EB-stained CH-Y-VII chromosomes is presented. In contrast to HO, which is AT specific (Hilwig and Gropp 1972; Latt and Wohlleb 1975), and to CA3, which is GC-specific, (Weisblum and Haenssler 1974), EB has been reported to exhibit no base-pair specificity (LePecq and Paoletti 1967); thus, its fluorescence was assumed to be proportional to total DNA content. The chromosome assignments were made (Table 2) by comparing the relative EB fluorescence values with the TDNA values as obtained from the bivariate distributions (Table 1). Under the assignment made, the correlation between relative EB fluorescence and TDNA values is excellent (coefficient of linear correlation 0.998). The assignment of peak K* to the human Y chromosome was confirmed by Q-banding analysis of sorted chromosomes (purity about 40%).

Table 2. Peak identification and parameters determined from the single parameter EB fluorescence distribution[a] for Chinese hamster × man hybrid (CH-Y-VII) chromosomes

Peak (Fig. 2c)	EB_{rel}[b]	Assignment[c]
A*	9.03	A (1,1)
B*	7.62	B (2,2)
C*	6.42	C (M1)
D*	5.27	D (4,4)
E*	4.61	E, F (5,5)
F*	4.14	G (7,7,M3)
G*	3.49	H (8,t(8q;Xp),Y_{CH})
H*	2.54	I (9,9,X_{del})
I*	1.87	J (10,10,11,11)
J*	1.43	K (M2)
K*	1.09	L (Y_{HUM})

[a] Figure 2c

[b] Normalized so that the sum of the means times the expected number of chromosomes equals 100. The peak means were determined by a computer program, fitting the distribution with the sum of several normal distributions plus a smooth function (Gray et al. 1979)

[c] A, B, etc., corresponding peaks in the HO/CA3 distributions (Fig. 1; Fig. 2a, b); the chromosome assignment according to Table 1 is given in parentheses. The assignment was made by comparing EB_{rel} with TDNA content (Table 1). The assignment of peak K* to the human Y-chromosome was confirmed by sorting on microscope slides and Q-banding

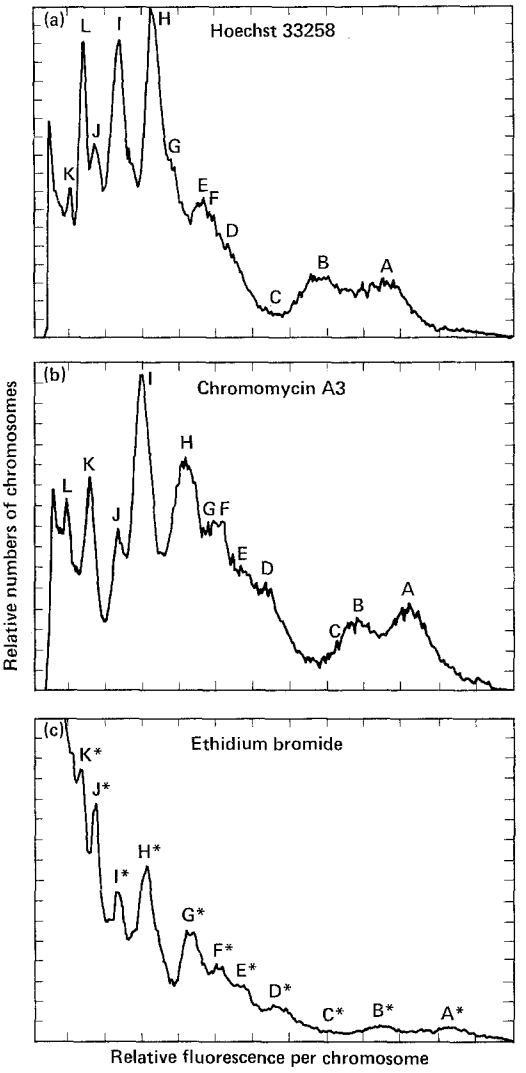

Fig. 2a–c. Single parameter distributions for Chinese hamster × man hybrid (CH-Y-VII) chromosomes. **a** HO distribution; **b** CA3 distribution; **c** EB distribution. Distributions **a** and **b** were obtained by single parameter measurements of HO/CA3-stained chromosomes, using the dual beam flow cytometer. The peak designations are the same as in Fig. 1. The distribution shown in **c** was measured with a Becton Dickinson FACS-sorter (λ_{ex} = 514.5 nm) of EB-stained chromosomes. For assignment of peaks A*, B*, etc., see Table 2

Discussion

In the present paper, we analyzed the bivariate HO-CA3 flow karyotypes of a Chinese hamster × man hybrid cell line (CH-Y-VII) which had retained a free human Y chromosome. In addition, single parameter distributions were measured for CA3 (GC specific), HO (AT specific), EB (no base-pair specificity). In bivariate flow karyotypes of CH-Y-VII cells the peak of the human Y chromosome was well separated from all other chromosomes (Fig. 1). A comparison with the bivariate HO/CA3 flow karyotypes of human fibroblasts (Gray et al. 1979) or human lymphocytes (Gray et al. 1982; Langlois et al., in preparation) shows that the relative distance r_{rel} ($r_{rel} = \sqrt{\Delta HO_{rel}^2 + \Delta CA3_{rel}^2}$, ΔHO_{rel} and Δ CA3 being the differences in HO and CA3 peak means between the Y chromosome and the next neighboring chromosome) of the Y chromosome to neighboring chromosomes is considerably increased (r_{rel} in CH-Y-VII cells, 0.67; mean r_{rel} in lymphocytes, 0.25).

Thus, CH-Y-VII cells are particularly well suited for sorting on a preparative scale, since variations in the quality of the chromosome preparation and fluctuations in laser power or adjustment resulting in small fluorescence shifts should effect the purity of the sort to a much lesser extent.

So far, however, dual beam chromosome sorters are not generally available, and sorting for extended periods of time is difficult to achieve. Therefore, we investigated the question of whether in CH-Y-VII cells the human Y chromosome might be discriminated also in an univariate flow karyotype. This would allow sorting using commercially available single beam sorters.

The results show (Fig. 2) that in the hybrid cells used, the human Y chromosome can also be discriminated in CA3, HO, and EB flow karyotypes, the best resolution being obtained in HO distributions. It is interesting to note that under the chromosome isolation conditions used here (hexylene glycol procedure; Gray et al. 1979; Langlois et al. 1980), so far no discrimination of the human Y has been routinely possible in single parameter flow karyotypes from lymphocytes (Yu et al. 1981) or fibroblasts (J. W. Gray, private communication). Young et al. (1981) used a different, polyamine-based isolation technique (Sillar and Young 1981) and reported a single parameter discrimination of the human Y chromosome in peripheral lymphocytes; however the discrimination was not verified by banding analysis of sorted chromosomes.

When human Y chromosomes are sorted on a preparative scale, the Chinese hamster × man hybrids have the important advantage that they contain only 24 chromosomes, instead of 46 as in normal human lymphocytes and fibroblasts.

Thus, the time required to sort the human Y chromosome may be reduced by a factor of 2 (estimated sorting time approximately 140 h/μg DNA in CH-Y-VII cells compared with

270 h/μg DNA in 46,X,Y cells, assuming a flow rate of 500 chromosomes/s). Another advantage of hybrid cells is that they are fast growing and continually available. Furthermore, the contamination of the sort (Gray et al. 1975; Carrano et al. 1979) with DNA from human chromosomes other than the desired one is zero (if the hybrid cell contains only one human chromosome) or negligeably small (if the hybrid cell contains a few human chromosomes differing sufficiently in their fluorescence intensities).

Chinese hamster × man hybrid cells might be used to purify also other human chromosomes. A comparison of bivariate HO/CA3 flow karyotypes of human lymphocytes and CH-Y-VII cells, stained and measured under identical conditions (unpublished observations) suggests that in appropriate hybrid lines, the HO/CA3 flow cytometric discrimination of all chromosomes except 1, 14, and 15 may be considerably improved. Thus, almost all human chromosomes might eventually be purified, including chromosomes 9, 10, 11, 12, and the active and inactive X, which cannot be discriminated on the basis of their DNA content or their DNA base composition (Gray et al. 1979, 1982; Young et al. 1981; Yu et al. 1981).

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