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Chromosome Heteromorphism Quantified by High-Resolution Bivariate Flow Karyotyping

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Summary

Maternal and paternal homologues of many chromosome types can be differentiated on the basis of their peak position in Hoechst 33258 versus chromomycin A3 bivariate flow karyotypes. We demonstrate here the magnitude of DNA content differences among normal chromosomes of the same type. Significant peak-position differences between homologues were observed for an average of four chromosome types in each of the karyotypes of 98 different individuals. The frequency of individuals with differences in homologue peak positions varied among chromosome types: e.g., chromosome 15, 61%; chromosome 3, 4%. Flow karyotypes of 33 unrelated individuals were compared to determine the range of peak position among normal chromosomes. Chromosomes Y, 21, 22, 15, 16, 13, 14, and 19 were most heteromorphic, and chromosomes 2–8 and X were least heteromorphic. The largest chromosome 21 was 45% larger than the smallest 21 chromosome observed. The base composition of the variable regions differed among chromosome types. DNA contents of chromosome variants determined from flow karyotypes were closely correlated to measurements of DNA content made of gallocyanin chrome alum–stained metaphase chromosomes on slides. Fluorescence in situ hybridization with chromosome-specific repetitive sequences indicated that variability in their copy number is partly responsible for peak-position variability in some chromosomes. Heteromorphic chromosomes are identified for which parental flow karyotype information will be essential if de novo rearrangements resulting in small DNA content changes are to be detected with flow karyotyping.

Introduction

Variability in the length and intensity of certain chromosome regions has long been noted by cytogeneticists studying metaphase cells stained to produce chromosome banding patterns. Although heteromorphisms (variants) have been described for virtually all human chromosome types, the most variable are the Y and 13, 3, 21, 22, 15, 14, 9, 1, and 16 (Geraedts and Pearson 1974; McKenzie and Lubs 1975). The variants are thought to reflect individual differences in the nature and copy number of tandemly repeated, transcriptionally inactive DNA sequences associated with heterochromatin or with the satellites of acrocentric chromosomes (see Jacobs 1977 for review; McKay et al. 1978; Gosden et al. 1981; Higgins et al. 1985; Jabs and Persico 1987; Jabs and Carpenter 1988). No clinical significance has been associated with these heteromorphisms, although reduced reproductive fitness in individuals with large variants has been noted (Jacobs et al. 1975). Variants identified by chromosome banding appear to be inherited according to Mendelian segregation (McKenzie et al. 1972; Craig-Holmes et al. 1975; McKenzie and Lubs 1975; Robinson et al. 1976; Verma and Lubs 1976; Kurnit 1979). The frequency of some chromosome variants also differs among ethnic populations (Lubs and Ruddle 1971; Lubs et al. 1977) and may represent an equilibrium between the rates of generation and elimination in various populations. Further study of the range and nature of heteromorphisms and of population differences by quantitative slide-based image cytophotometry has been limited. Identification of

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chromosomes is time-consuming, and quantification of small DNA content differences is affected by variability in staining among cells.

Flow cytometry can be used to identify mitotic chromosomes and to quantify DNA content differences among them (reviewed in Gray and Langlois 1986). The chromosomes from thousands of cells can be analyzed in a few minutes. Flow cytometry has been used previously to study chromosome variability (Green et al. 1984; Harris et al. 1985, 1986, 1987). In those studies, a single fluorochrome, ethidium bromide, was used. It binds stoichiometrically to DNA. Quantitative measurements of both relative base composition and DNA content can be made using a dual-beam flow cytometer (Gray et al. 1979). In this case, chromosomes are stained with two DNA-specific fluorochromes, either Hoechst 33258 (HO) or DAPI and chromomycin A3 (CA). Chromosomes are passed singly through the beams of two lasers. At the first beam, HO is excited, and the fluorescence intensity of each chromosome is quantified. At the second beam, the CA is excited and quantified. The HO and CA intensities are correlated for 30–50,000 chromosomes and are plotted to yield bivariate distributions called flow karyotypes. HO and CA are known to bind preferentially to short runs of adenine-thymidine or cytosine-guanine basepairs, respectively (Ward et al. 1965; Latt and Wohlleb 1975; Lin et al. 1977). Measured HO and CA fluorescence is also influenced by the degree of resonance energy transfer between these two dyes (Langlois and Jensen 1979; Langlois et al. 1980; Latt et al. 1980; van den Engh et al. 1986). This depends on the proximity of the dyes in chromosomes, which in turn depends on DNA sequence.

Flow karyotypes of all normal human cells show a characteristic pattern of peaks, each produced by one or a few chromosome types. Chromosomes can be identified in the flow karyotype on the basis of their peak position. Distance from the origin reflects relative DNA content, and the slope of a line through the origin and a peak is a measure of the relative base composition of a chromosome. The number of events in a peak is a measure of the relative frequency of a chromosome in the sample of mitotic cells. With current sample preparation and measurement accuracy (1%–4% peak coefficients of variation), differences in peak position between maternally and paternally derived homologues of some chromosomes are observed in flow karyotypes of normal individuals (van den Engh et al. 1988).

Peaks that deviate from the normal human pattern can signify DNA content changes associated with deletions, insertions, or translocations. Flow karyotyping has proved useful for detecting chromosome abnormalities in individuals with specific diseases or genetic defects (Lebo et al. 1986; Wilcox et al. 1986; Patterson et al. 1987; Arkesteijn et al. 1988; Cooke et al. 1988; van den Engh et al. 1988; Gray et al. 1988; Martin et al. 1988; Carter et al., in press; Trask et al., in press). Flow karyotyping has proved particularly useful in detecting loss or abnormal DNA content of human chromosomes in rodent × human somatic cell hybrids (Van Dilla et al. 1986; B. Trask, unpublished results). Quantification of subtle chromosome abnormalities is limited, however, by the degree of peak-position variability in flow karyotypes of normal individuals.

In the present paper, we present the results of the use of high-resolution bivariate flow karyotyping to quantify normal chromosome heteromorphism among individuals. Variability in DNA content and in base composition is addressed. We define the capabilities of flow karyotyping to determine whether and by how much a chromosome is abnormal on the basis of measured population variability. In the accompanying paper (Trask et al. 1989), we use flow karyotyping to demonstrate the fidelity of inheritance of heteromorphisms in families. Together these studies illustrate the potential of comparing parental and proband flow karyotypes for detection of de novo chromosome abnormalities (Harris et al. 1987; Martin et al. 1988).

Material and Methods

Cell Lines and Culture

Chromosomes were isolated from mitotic cells from 98 individuals. Only data concerning the normal homologues in these individuals as judged by conventional cytogenetics and available probe analyses were used for this study. The cells were from various sources: PHA-stimulated peripheral blood lymphocytes from normal donors and karyotypically normal and abnormal lymphoblast and fibroblast cell lines obtained from C. Schwartz, J. Wasmuth, W. Brown, R. Nussbaum, M. Golbus, and the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). Cell lines were cultured using conventional techniques (van den Engh et al. 1986).

Chromosome Isolation

Chromosomes were prepared, according to a published procedure (van den Engh et al. 1984, 1985, 1988), from cells that were blocked in mitosis with colcemid (12 h; 0.1 μg/ml colcemid). Cells were resuspended after centrifugation at 3×10^5 cells/ml in 50 mM KCl, 10 mM $MgSO_4$, 3 mM dithiothreitol, 5 mM HEPES,

pH 8.0 for 10 min at 22°C. Triton X-100 was added to 0.25% (v/v) final concentration, and the cell suspension was incubated on ice for 10 min. Chromosomes were released by mechanical shearing: syringing in the case of fibroblasts and rapid vortexing for 10 s for lymphoblasts and cultured lymphocytes. The chromosomes were stained with 3.8 μM HO or 1.4 μM DAPI and 17 μM CA from 50–100 × stock solutions made in H_2O. Chromosomes were stored at 4°C for ≥2 h to allow equilibration with the stain. Sodium citrate and sodium sulfite from a 10 × stock solution (100 mM sodium citrate, 250 mM sodium sulfite) were added to chromosomes 15–30 min before measurement, to improve karyotype resolution (van den Engh et al. 1985, 1988).

Flow Analysis

Stained chromosomes were analyzed in a dual-beam flow cytometer (Dean and Pinkel 1978). One laser was tuned to emit light in the UV (351–364 nm, 1.5 W), and the second laser was tuned to emit light at 458 nm (600 mW). HO intensity resulting from excitation by the first beam was quantified after passing through a spectral filter transmitting wavelengths >425 nm (Ditric Optics). Chromomycin fluorescence resulting from excitation by the second beam was quantified after passing through a filter transmitting wavelengths >520 nm filter (Corning 3–71). Chromosome analysis rates for high resolution averaged 200/s. The fluorescence pulses from the individual chromosomes were integrated by a data acquisition system, and the measurements were stored as a list by a Hewlett-Packard 9000 computer (van den Engh and Stokdijk 1989). The bivariate flow karyotypes are displayed as contour plots at 256 × 256 channel resolution. Contour lines indicate the frequency of events in each channel and are usually chosen at 10%, 20%, 40%, and 80% of the number of events in the highest peak of the distribution.

Data Analysis

To measure chromosome variability in terms of peak position, flow karyotypes of different individuals were compared. Karyotypes were chosen to meet two criteria: (1) that they be of unrelated individuals and (2) that they be unaffected by variation in measurement conditions. The staining and measurement conditions of chromosome preparations are not fully under control. As a result, replicate flow karyotypes of the same individual measured on different days can be slightly different. This difference is characterized most prominently by a rotation toward the diagonal of the line through the peaks representing chromosomes 13–17. We consider this rotation to be artifactual for two reasons: (1) no difference in the position of chromosome 17 was observed when samples of as many as 20 individuals were run on the same day, and (2) only two of 93 individuals with normal chromosome 17's showed peak-position differences between homologues of this chromosome. Therefore, we have restricted our analyses to karyotypes in which the line through the 13–17 ridge has approximately the same slope. Approximately 50% of the 98 karyotypes discussed above fall in this set. Of these, 33 karyotypes represent analyses of unrelated individuals. This set was used to evaluate normal variability in chromosome peak position. Ten of the 33 karyotypes were derived from short-term cultures of peripheral blood lymphocytes from normal human donors, and 23 were derived from Epstein-Barr virus–transformed lymphoblast cultures. Nineteen of the latter have known karyotypic abnormalities involving one or two chromosomes detected by conventional cytogenetics and probe analyses. Only the positions of the chromosome types judged normal by these methods are included in our study of normal chromosome variability. As the majority of abnormalities in this set involved the X, peak positions of 37 normal X chromosomes were obtained, as compared with 62–66 positions for each of the other chromosome types.

An interactive mouse-controlled cursor was used to mark manually on the karyotypes the coordinates of the peak representing each chromosome homologue in karyotypes of 33 unrelated individuals. Tabulated values were normalized by setting the average HO and average CA intensities of all autosomes except 9–12 at 100 a.u. in each karyotype and leaving the position of the origin fixed.

The relative distance, D_n, between the origin and the projection of the peak for each chromosome on the line running through the origin and chromosome 4 was calculated using equation (1) as a measure of the DNA content of the chromosome.

$$D_n = HO_n \times \sin \alpha + CA_n \times \cos \alpha , \quad (1)$$

where α is the angle between the x-axis and the projection line ($\tan \alpha = HO_4/CA_4$), and HO_n and CA_n are HO and CA fluorescence intensities of chromosome n (Trask et al., in press).

Fluorescence in Situ Hybridization

Metaphase slides of individuals 7-1 and 14-1 were prepared from cultured lymphocytes and were hybridized according to a method described elsewhere (Pinkel

et al. 1986; Trask et al. 1988) with biotinylated chromosome-specific repetitive sequence probes: puC1.77 (from H. Cooke), which labels 1qh (Cooke and Hindley 1979; Gosden et al. 1981), or pd15Z1 (from B. White), which labels chromosome 15 (Higgins et al. 1985). Sites of hybridization were fluorescently labeled with fluorescein (FITC)-conjugated avidin. Chromosomes were counterstained with propidium iodide (PI). Photomicrographs were made using a Zeiss Axiophot fluorescence microscope (100 ×, 1.3 N.A. objective; PI/FITC—BP485 excitation filter, FT510 and LP520 emission filters; PI only—G546 excitation, FT580 and LP590 emission filters) on Kodak color slide film (400 ASA, 120-s exposures).

Quantitative Image Cytophotometry

The relative DNA contents of chromosomes of individuals 4-1 and 16-1 were determined using quantitative image cytophotometry as described by Mayall et al. (1984). Metaphase cells from PHA-stimulated peripheral blood lymphocyte cultures of these normal donors were Q-banded first with quinacrine dihydrochloride, and chromosomes in suitable metaphase cells were identified. The slides were treated with ribonuclease and were restained for DNA using gallocyanin chrome alum. This compound stains phosphate groups in DNA stoichiometrically (Sandritter et al. 1966). The selected metaphase cells were scanned and digitized on the CYDAC flying-spot cytometric microscope system (Mendelsohn et al. 1973; Mayall et al. 1984). The digitized images were analyzed by computer to determine the boundaries of each chromosome and to determine its integrated optical density, or DNA content. Mean chromosomal DNA content, normalized so that the sum of DNA content for all autosomes equaled 100, was calculated from measurements of 5–10 cells.

Results

Homologue Discrimination

Figure 1 shows the HO and CA fluorescence intensities of chromosomes isolated from a short-term peripheral blood lymphocyte culture of a healthy female donor. In this flow karyotype, differences between the maternal and paternal homologues for chromosomes 13, 16, 18, 19, and 21 can be distinguished on the basis of their peak position. Each of the 98 cell lines analyzed recently had a unique set of variants and thus a unique flow karyotype. Visual examination of flow karyotypes on a 256 × 256 channel display revealed homologue differences for a mean ± SD of 4.0 ± 1.7

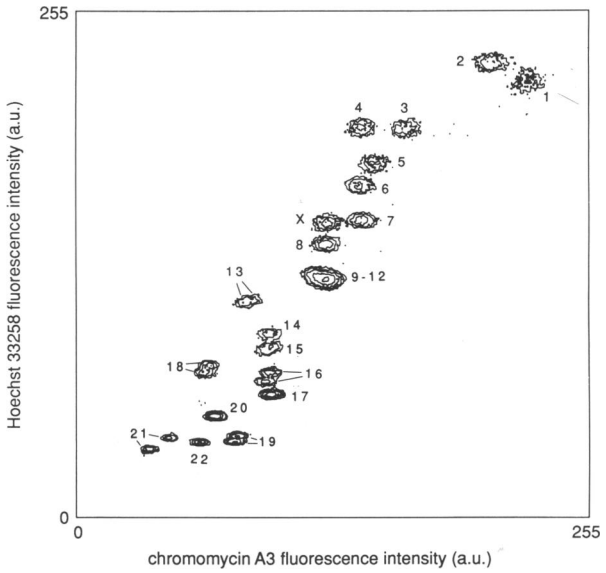

Figure 1 HO and CA fluorescence intensities of isolated mitotic chromosomes quantified using a dual-beam flow cytometer. Peaks in the flow karyotype are labeled to identify the chromosome type that each represents. Identification was made by sorting chromosomes into fractions and identifying them by quinacrine banding (Gray et al. 1979). Note the peak position differences between maternal and paternal homologues of chromosomes 16, 18, 19, and 21 in this individual.

chromosomes/karyotype. The number of chromosomes showing clear homologue differences in karyotypes of short-term peripheral lymphocyte cultures (4.6 ± 1.6; n = 43) was not significantly different from that of long-term lymphoblast or fibroblast cultures (3.5 ± 1.7; n = 55). The frequency of individuals for which homologue differences were detected differed among chromosome types (table 1). For example, 50%–60% of individuals showed peak-position differences between homologues of chromosomes 14, 15, and 16. No peak-position differences were observed for chromosomes 2, 5, 6, 7, or 8.

Range of Chromosome Variability

To measure chromosome heteromorphism in terms of peak position, flow karyotypes of 33 unrelated individuals were compared. The range of observed peak positions is shown in figure 2. Each dot represents the peak position of one chromosome homologue from one of the individuals. Only in cases where the 9–12 peak was resolved into subpopulations were these marked and plotted in addition to the position of the main 9–12 peak. It is apparent from this figure that chromosomes 2, 5, 6, X, 8, and 17 are relatively invariant in this group

Table 1

Frequency of Individuals Heterozygous for Peak-Position Heteromorphism

Chromosome	Frequency (%)
1	19
2	0
3	4
4	4
5	0
6	0
7	0
8	0
9–12	ND
13	43
14	54
15	61
16	53
17	2
18	19
19	16
20	5
21	46
22	43
X	NA
Y	NA

NOTE.—Peak-position differences between homologues carried by an individual of each listed chromosome type were detected by visual examination of the normal chromosomes in flow karyotypes of 98 different cell lines/individuals. ND = not determined; NA = not applicable.

of individuals. Chromosomes 3, 4, 18, 19, and 20 and those in the 9–12 group are more variable. A large variation in chromosome peak position was observed for chromosomes 1, 13, 14, 15, 16, 21, 22, and Y.

Figure 2*B* is an expanded view of the variability in chromosomes 13–22 and Y. It shows that the variability in HO and CA binding among normally occurring heteromorphisms is not random. Instead, the peak means seem to vary along well-defined paths whose slopes differ depending on chromosome type. For example, polymorphic variability in chromosomes 16 and Y is primarily in HO content. In contrast, chromosome 14 varies more in CA content than in HO content.

Figure 3 illustrates that the distributions of normal chromosome peak positions are of different types. The normalized HO and CA intensities of homologues for chromosomes X, 14, 21 , and 22 in the 33 individuals are plotted as univariate histograms (1.0 = population mean). As noted above, chromosome X is relatively invariant. Variability in chromosome 14 is greater in CA intensity than in HO intensity and, with the exception of five variants at high CA intensity, approximates a normal distribution. Variants for chromosomes 22 appear to fall into two HO-intensity subpopulations.

Homologue Differences in Amount of Repeat Sequences

The relationship between homologue differences in the copy number of particular chromosome-specific re-

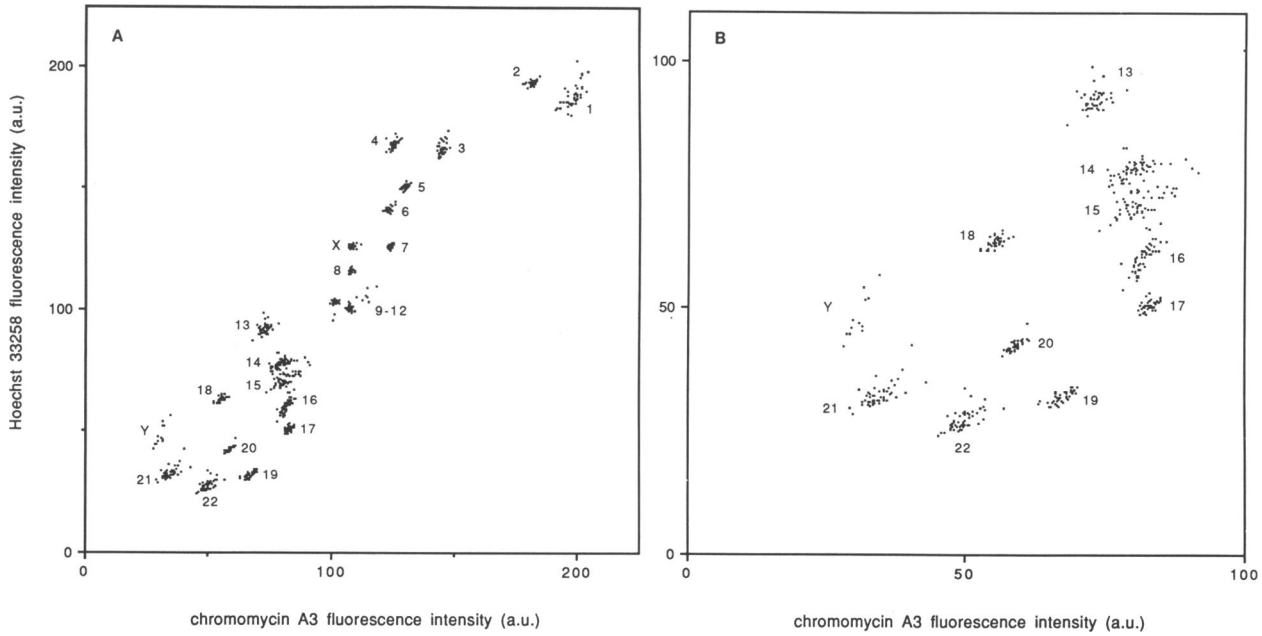

Figure 2 Flow-karyotype peak-position variability in normal chromosomes of 33 unrelated individuals. Each point represents the position of one measured homologue. Peak positions were determined from HO vs. CA flow karyotypes and were normalized to place the average HO and CA intensities of all autosomes except 9–12 at 100 a.u. in each karyotype. *A*, All chromosomes. *B*, Chromosomes 13–22 and Y only.

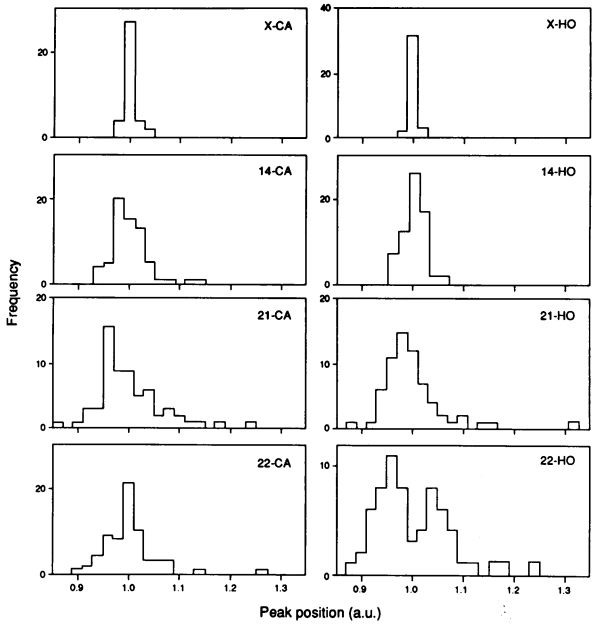

Figure 3 Univariate histograms of the distribution of HO and CA fluorescence intensities among normal human chromosomes from 33 unrelated individuals. Measured intensities are expressed as a fraction of the mean intensity of all individuals.

peat sequences and in flow karyotype peak position was studied in two individuals. Their flow karyotypes are shown in figure 4. Individual 7-1 shows distinct polymorphic variants of chromosomes 1 and 15 (Δ DNA content ≈8 megabase pairs [Mbp] and ≈9 Mbp, respectively). The homologues of chromosome 1 in individual 14-1 are approximately the same size. The absolute difference in DNA content between chromosome 15 homologues in 14-1 was less than that in 7-1 (Δ DNA content ≈5 Mbp). The presence of a variant chromosome in the 9–12 group (probably chromosome 9; see below) was also indicated in the flow karyotype of 7-1.

In situ hybridization was used to fluorescently label repetitive sequences in the heterochromatin of chromosomes 1 and 15 in metaphase cells fixed to slides. Representative photomicrographs of the hybridization results are shown in figure 5. Individual 7-1 showed significant homologue differences in the size of the region that hybridized to the chromosome 1–specific probe (puC1.77) (fig. 5*B*). Individual 14-1 did not (fig. 5*A*). One of 7-1's chromosome 9 homologues is labeled slightly at its centromere with this probe. This suggests that the peak-position difference between chromosome 1 homologues and chromosome 9 homologues in individual 7-1 are at least in part due to homologue differences in copy number of this sequence.

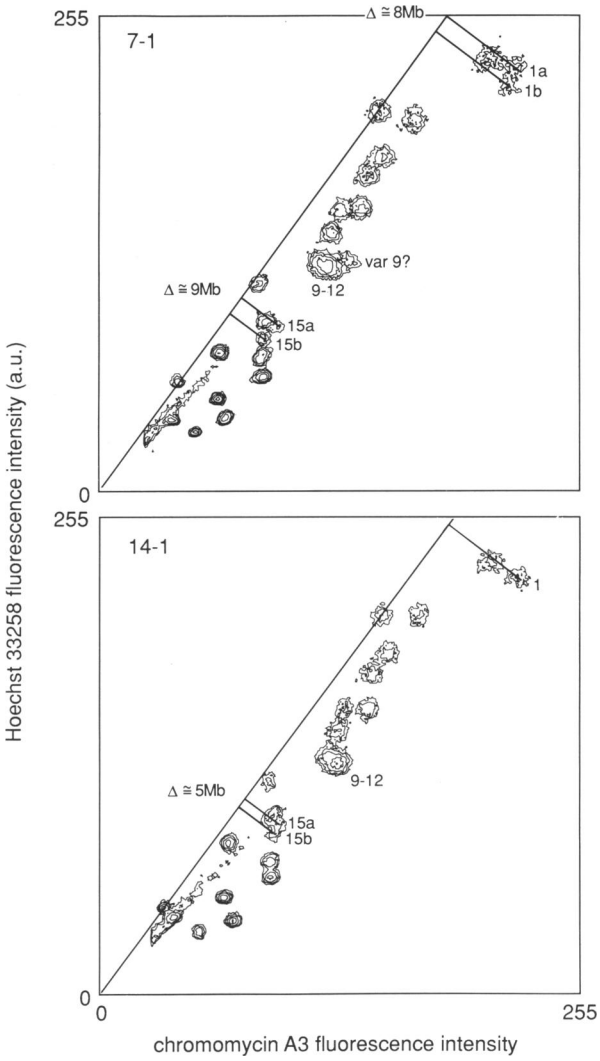

Figure 4 HO vs. CA bivariate flow karyotypes of individuals 7-1 and 14-1, whose fluorescence in situ hybridization results are shown in fig. 5. The absolute DNA content difference, at G_1, between homologues of chromosome 1 and 15 are indicated.

Hybridization of the probe pd15Z1 was used to fluorescently label heterochromatic regions of chromosome 15 in both individuals (figs. 5*C*–5*E*). A striking difference in the size of the satellite region distal to the hybridized region was observed in both individuals. Although hybridization intensities varied from cell to cell, the homologue with the larger satellite region in 7-1 tended to be labeled more intensely with the heterochromatic probe than the other homologue. This suggests that the peak-position difference between chromosome 15 homologues in 7-1 may be a result of a combination of satellite- and heterochromatic-region size differences.

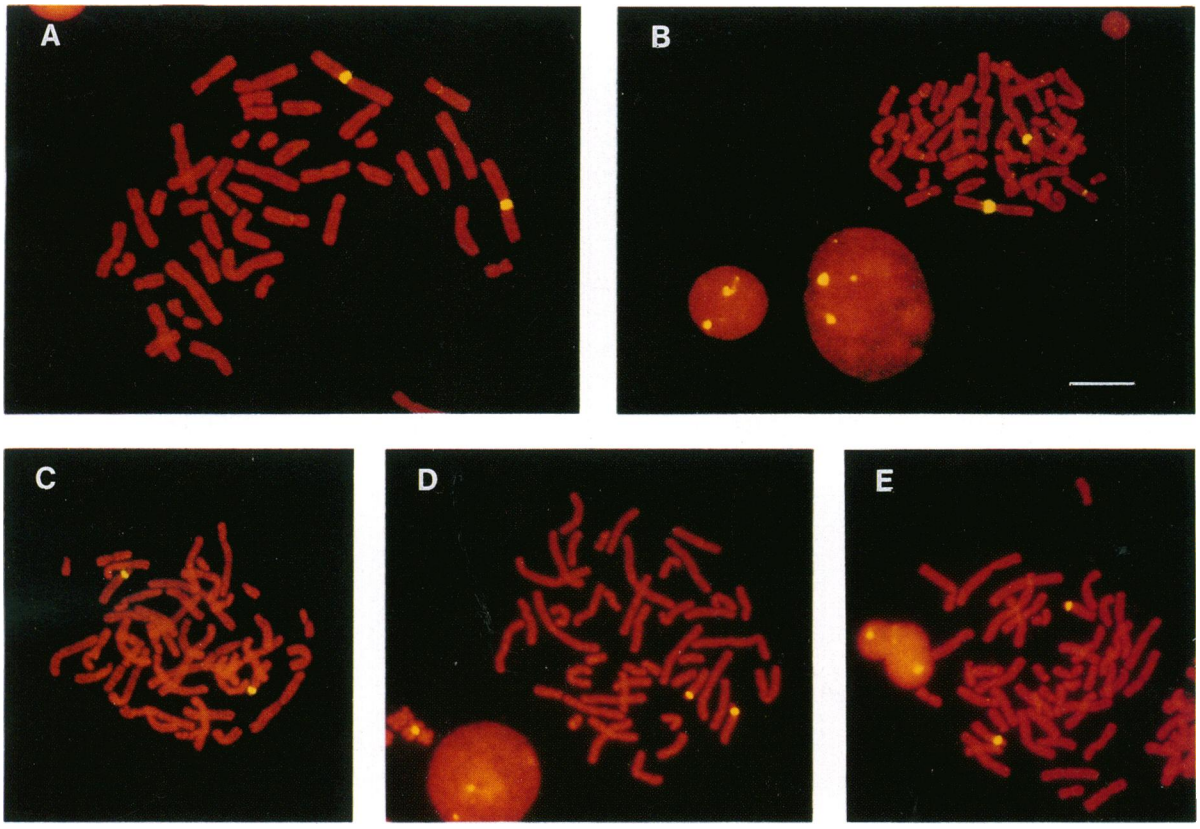

Figure 5 Fluorescence in situ hybridization with chromosome-specific repetitive sequences to metaphase cells on slides. The flow karyotypes of individuals 7-1 and 14-1, whose cells are shown here, are shown in fig. 4. Sites of probe hybridization are yellow; chromosomes fluoresce red because of propidium iodide counterstaining. The probe in panels *A* and *B* was puC1.77, a repetitive sequence specific to the heterochromatic region of chromosome 1 (Cooke and Hindley 1979). The probe in panels *C–E* was pd15Z1, a repetitive sequence specific to the heterochromatic region of chromosome 15 (Higgins et al. 1985). *A* and *C*, individual 14-1. *B*, *D*, and *E*, individual 7-1.

In contrast, the homologue with the large satellite region tended to have equal or less probe fluorescence than did its counterpart in 14-1. Thus, satellite differences may be offset by heterochromatin differences, resulting in a smaller peak-position difference between chromosome 15 homologues in 14-1 than that observed in 7-1.

Peak Position Correlates with DNA Content

To determine whether heteromorphic differences in flow karyotype peak position can be interpreted as differences in the DNA content of variant chromosomes, we compared DNA content estimates from flow karyotype peak position with those made by slide-based scanning cytophotometry for two individuals (4-1 and 16-1). These individuals were chosen because they carry highly heteromorphic chromosomes. Individual 4-1 showed the most fluorescent homologue of chromosome 21 observed to date. A heteromorphic difference in chromosome 13 homologues could also be detected. Individual 16-1 was chosen because of his relatively large Y chromosome and homologue discrimination of chromosome 16. A microscope-based quantitative image analysis system, CYDAC, was used to quantify the amount of gallocyanin chrome alum staining of metaphase chromosomes on slides. Gallocyanin chrome alum binds stoichiometrically to phosphate groups in DNA. CYDAC analysis identified a significant ($P < .05$) difference in DNA content between homologues only for chromosome 21 in individual 4-1. From the HO versus CA flow karyotypes (not shown), the distance from the origin of the projection of a chromosome peak onto a line running through both the origin and chromosome 4 was taken as a measure of relative DNA content (eq. [1]). We have recently shown that for each chromosome type, mean DNA content calcu-

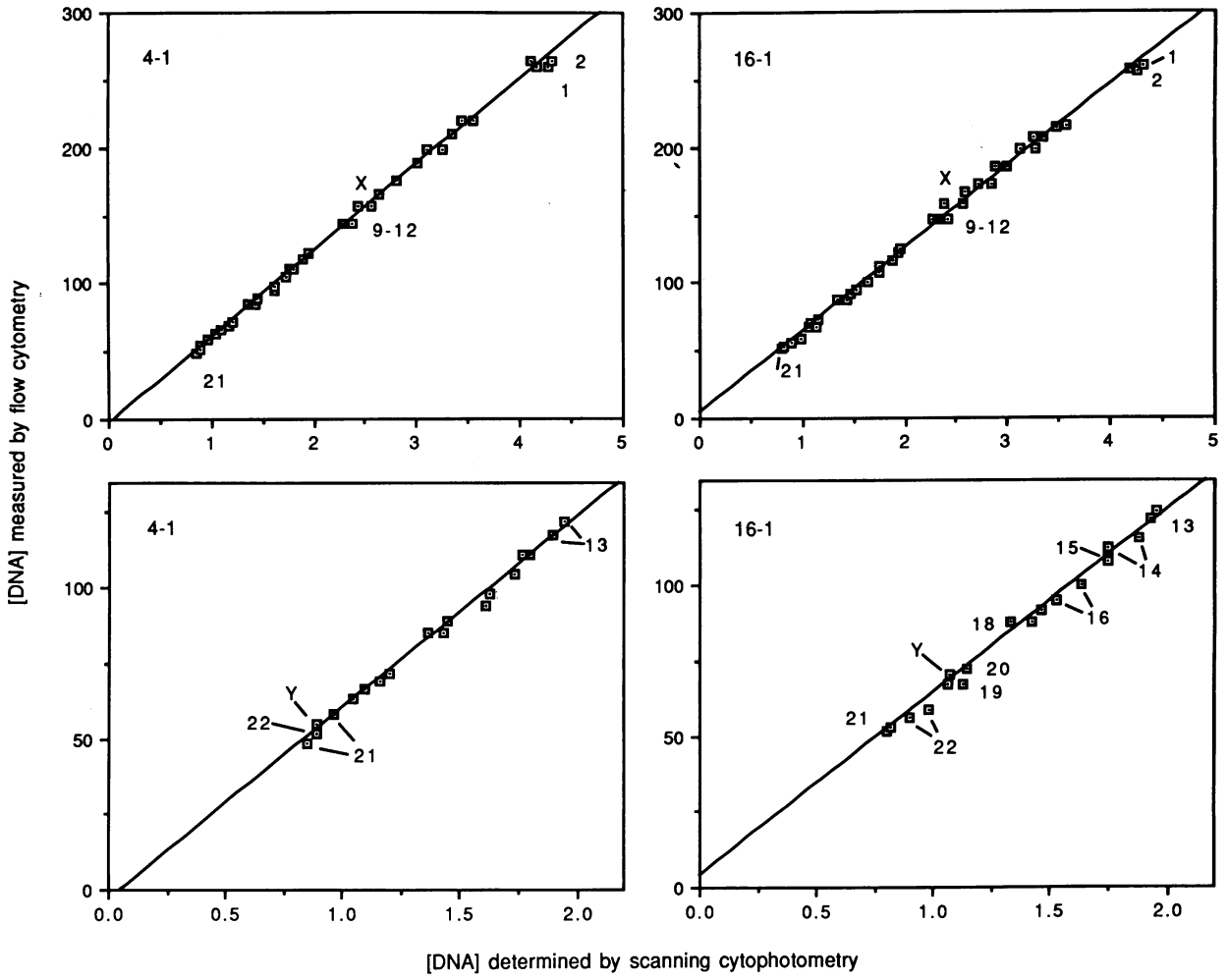

Figure 6 Relative DNA content of 46 mitotic chromosomes determined from HO vs. CA bivariate flow karyotypes vs. their DNA content estimated by gallocyanin chrome alum staining and quantitative microscopy (CYDAC). Data are shown for two individuals (4-1 and 16-1).

lated from karyotypes of all 33 individuals is linearly related to mean DNA content calculated from CYDAC measurements of 10 individuals (Mayall et al. 1984; Trask et al., in press). Figure 6 shows that this relationship holds for extremely heteromorphic chromosomes as well. Flow DNA content estimates for all homologues in both individuals correlate closely with those measured by CYDAC.

Population Variability Expressed as DNA Content

In table 2, variability among individuals in the HO and CA intensities of each chromosome type is translated into variability in DNA content. The mean HO and CA intensities and mean G_1 DNA content determined from the set of 33 unrelated individuals is listed for each chromosome type. The range between the largest and smallest variant observed for each type is given as a percentage of the population mean. Although SDs are statistically unsound descriptors of the spread of distributions not described by a Gauss function, they are listed in table 2 to indicate the relative degree of population variability observed for each chromosome type. The Mbp equivalent of 1 SD and the observed range are also given. Normal variants of chromosome 21, for example, ranged in DNA content from 87% to 126% of the population mean. This range is equivalent to a difference of 19 Mbp of DNA.

This table can serve as an aid to determine the limits that the degree of normal variability imposes on the capacity of flow karyotyping to detect chromosomes

Table 2

Variation of Normal Chromosome Peak Position in Flow Karyotypes of 33 Unrelated Individuals

	CA			HO			[DNA][a]				
								SD		Range	
Chromosome	Mean (a.u.)	SD (%)	Range (%)	Mean (a.u.)	SD (%)	Range (%)	Mean (Mbp)	%	Mbp	%	Mbp
1	198.3	1.5	96–103	188.4	2.5	95–108	256	1.9	4.8	97–105	21
2	181.6	.9	98–102	193.9	.6	99–101	250	.6	1.4	99–102	7
3	144.9	.9	99–102	166.3	1.3	98–104	209	1.0	2.1	98–103	10
4	125.6	1.1	97–102	168.2	.9	98–103	200	.9	1.7	98–102	8
5	130.3	.8	98–102	150.6	.6	98–101	190	.6	1.2	99–101	5
6	123.4	.8	98–102	141.4	.7	99–102	179	.6	1.1	99–102	6
7	124.1	.6	99–101	126.1	.6	98–101	168	.5	.9	99–101	3
8	108.1	.6	99–101	116.0	.6	99–102	152	.5	.7	99–101	3
9–12	107.7	.7	99–102	100.8	.9	98–102	140	.5	.7	99–101	3
13	72.9	2.2	94–108	92.4	2.2	94–107	114	1.8	2.1	94–104	12
14	80.1	3.6	94–113	78.0	2.2	95–106	108	2.4	2.6	95–107	12
15	80.8	3.9	92–113	71.2	3.3	92–109	103	3.3	3.4	92–111	19
16	81.7	2.0	94–105	60.2	4.9	89–113	95	3.1	2.9	93–108	14
17	82.8	1.2	98–103	50.6	2.0	96–105	89	1.3	1.2	97–103	5
18	55.4	1.9	95–106	63.4	1.4	97–103	83	1.5	1.2	96–103	6
19	67.3	2.2	94–104	32.0	3.7	94–107	66	2.6	1.7	96–105	6
20	58.7	1.7	97–104	42.3	2.2	95–111	69	1.8	1.2	96–107	8
21	34.3	6.8	85–125	32.1	6.4	88–133	48	6.0	2.9	87–126	19
22	49.9	5.3	91–126	27.4	6.8	88–123	53	5.3	2.8	90–120	16
X	108.7	1.2	98–103	126.0	.6	99–101	159	.7	1.1	99–102	4
Y	30.8	5.8	91–112	47.9	8.9	88–118	58	7.6	4.4	89–116	16

[a] The distance from the origin of the projection of a chromosome peak onto a line running through the origin and chromosome 4 was calculated as a measure of the DNA content of each chromosome type (eq. [1]). These values were converted to absolute DNA content (in Mbp) using the linear relationship between them and CYDAC estimates of chromosome DNA content ($y = 1.076x - 5.55$, where y = flow estimate of chromosome DNA content and x = CYDAC estimate of DNA content [in Mbp], and assuming a haploid genome size of 3×10^9 bp; Trask et al., in press). The Mbp estimates refer to the DNA content of chromosomes in G_1.

with truly abnormal DNA content. For example, for some purposes, one might define a chromosome as abnormal if its DNA content falls outside the following range: (mean − 2 SD) to (mean + 2 SD). Even then, 5% of normal chromosomes would be falsely classified as abnormal if the DNA contents of variants were normally distributed. For an invariant chromosome such as the X, this range is ±1.4% of the mean, or ±2.2 Mbp. For chromosome 21, this range is 88%–112% of the mean, or ± 6 Mbp.

Additional Heteromorphisms Identified by DAPI Staining

DAPI stains some chromosomal regions more intensely than does HO (Schnedl et al. 1981). It may be used to identify and characterize additional heteromorphisms. DAPI versus CA flow karyotypes differ from HO versus CA karyotypes (Lebo et al. 1984). The most notable differences are in the relative positions of chromosomes 1, 9, 15, 16, and Y, the chromosomes with large heterochromatic regions. Figure 7 compares flow karyotypes made with DAPI and HO for one individual (7-1). In this individual, chromosome Y was 1.3-fold more intense on UV excitation with DAPI staining than with HO staining. CA fluorescence was 0.8-fold lower with DAPI than with HO. In 7-1, the relative positions of both chromosome 1's remain unchanged upon DAPI staining. Both homologues of chromosomes 9, 15, and 16 show significant shifts to relatively higher UV-excitable fluorescence when DAPI is used instead of HO. For one chromosome 15 homologue and both chromosome 9's, this shift is accompanied by a relative reduction in CA fluorescence. In other individuals, these chromosome types can respond differently to DAPI staining (table 3). For example, individual 2-2 showed significant shifts in both chromosome 1 positions and in only one chromosome 16 position with DAPI staining. The relative increase in UV-excitable fluorescence of the Y chromosome of individual 2-1 is half that of individual 7-1.

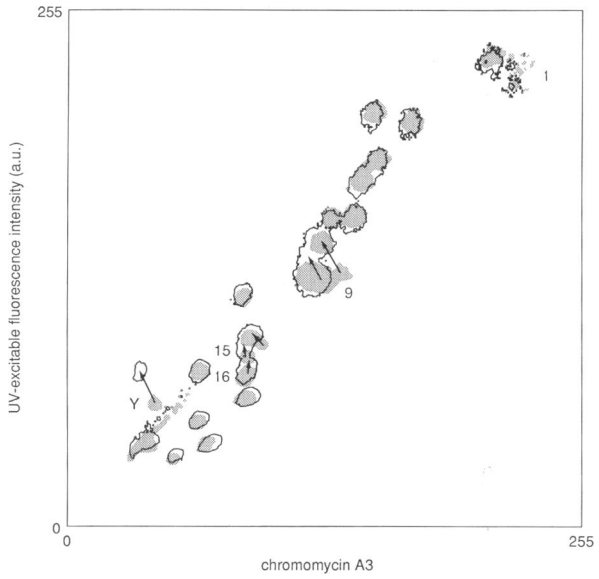

Figure 7 DAPI vs. CA (solid line) and HO vs. CA (shaded area) bivariate flow karyotypes of individual 7-1. The two karyotypes were normalized so that the position of chromosome 8 and the origin are at the same position in both plots. For simplicity, only one contour line at ≈10% of the number of events in the highest peak of the distribution is shown. Arrows point to chromosome homologues that show significant peak-position shifts depending on the staining combination.

Table 3

Ratio of DNA Contents Derived from DAPI and HO Flow Karyotypes

	Chromosome[a]			
Individual	1	15	16	Y
2-1[b]	1.01	1.00	1.03	1.06
	1.01	1.01	1.03	
2-2[b]	1.02	1.00	1.04	...
	1.02	.99	1.01	
4-1	1.02	1.01	1.03	0.95
	1.02	1.01	1.03	
7-1	1.00	1.03	1.03	1.12
	.99	1.00	1.03	
AB7929	.99	1.02	1.02	1.06
	.99	1.02	1.02	
GM130B ...	1.01	1.02	1.02	1.09
	1.01	1.00	1.02	

[a] The relative shifts of chromosome 9 upon DAPI staining observed in some individuals (see fig. 7) were not determined because the position of this chromosome within the 9–12 peak of HO vs. CA flow karyotypes is not known with certainty.

[b] Individuals 2-1 and 2-2 are, respectively, the father and mother of the family described by Trask et al. (1989; figs. 1, 4).

For the chromosomes that do shift upon DAPI staining, DNA contents derived from DAPI versus CA bivariate flow karyotypes can differ by as much as 10% from those estimated from HO versus CA karyotypes. These values do not match as closely to CYDAC DNA content estimates as do the values derived from HO versus CA karyotypes.

Discussion

In the present paper, we have demonstrated that bivariate flow karyotyping can be used to measure the incidence and to quantify the magnitude of polymorphic variability among normal chromosomes. The range in peak position in bivariate flow karyotypes has been addressed previously (Gray et al. 1988). There, ellipses were drawn to encompass the positions of 95% of the chromosomes in 50 normal amniotic cell cultures. These positions were affected by the measurement accuracy available at that time. For example, the large variability observed for chromosome X in that study can be ascribed to difficulty in defining, in karyotypes with broad peaks, its peak position between chromosomes 7 and 8. The low observed variability in chromosome 21 may have been due to difficulty in identifying homologues of this chromosome when small fluorescent debris is present in a sample. For the present study, we have improved measurement accuracy considerably in terms of both sample preparation and display capabilities. Peak coefficients of variation of 1%–4% were usual, debris levels were low (thus avoiding peak obscuration), and intensities were measured with 256 × 256 channel resolution rather than with the previously used 64 × 64. In contrast to our earlier work, here we have observed that chromosome X peak position is invariant and that chromosome 21 peak position is highly variable among normal individuals.

Homologue differences were detected by visual examination of flow karyotypes for an average of 4 chromosomes/individual in the individuals studied. This level of variation heterozygosity is comparable to the 4 variants/individual and 5.1 variants/individual found by banding analysis by McKenzie and Lubs (1975) and Geraedts and Pearson (1974), respectively, and to 4.8 variants/individual found by flow analysis of chromosomes stained with a single fluorochrome (Harris et al. 1987). In the present study, homologue differences for chromosomes 13–16 were most often observed.

Quantitative flow analysis demonstrates that variability, as a fraction of chromosome DNA content, is differ-

ent for different chromosome types. The ranges between extreme variants were greatest for chromosomes 21, 22, and Y. Chromosomes 9, 13–16, and 1 showed considerable variability. Differences among homologues of chromosomes 18 and 19 were small but significant, as demonstrated by their resolution in the karyotype of the individual shown in figure 1. These findings concur in general with data from other studies. Chromosomes 1, 9, 16, and Y were most variable in univariate flow karyotypes (Harris et al. 1986). Cytogenetic analyses have shown that the acrocentrics (13–15, 21, and 22) and chromosomes with large heterochromatic regions (1, 9, 16, and Y) are frequently variable among individuals (Geraedts and Pearson 1974; McKenzie and Lubs 1975; Lubs et al. 1977). Chromosome 3 is an exception. It has been identified frequently as heteromorphic on slides (idem), while we observed relatively little variability in flow karyotypes of different individuals.

We show in figure 6 that peak-position differences between homologues can be translated into differences in their DNA content. Estimates of DNA content match closely with relative DNA contents determined by staining phosphate groups (gallocyanin chrome alum) in the chromosomes of the same individuals. The use of DAPI instead of HO as the A-T stain results in increased UV-excitable fluorescence for some chromosome types. This increase is often accompanied by a reduction in CA fluorescence (fig. 7). Despite this, DAPI staining results in different DNA content estimates of some chromosome types (1, 9, 16, and Y) in some individuals, giving values that correlate less closely with gallocyanin chrome alum measurements (table 3) than do estimates based on HO staining.

The heteromorphism we have quantified may underestimate the extent of heteromorphism among normal chromosomes. For example, chromosomes 1, 16, and Y varied by 8%, 16%, and 30%, respectively, in bivariate flow karyotypes of the individuals studied. In contrast, univariate flow karyotypes of individuals selected for extreme heteromorphisms revealed differences in chromosomes 1, 16, and Y of 20%, 46%, and 86%, respectively (Harris et al. 1986). Chromosome Y also has been shown to vary by 60% in length (McKenzie et al. 1972). On the other hand, the observed differences of 45% and 30%, respectively, between the largest and smallest variants of chromosomes 21 and 22 are significantly greater than has been reported elsewhere (20%–21%; Harris et al. 1986).

Observed variability appears to cluster along lines of defined slope, or HO:CA fluorescence ratio. The slopes differ among chromosome types. In addition, the effects of DAPI staining on chromosomes 1, 9, 16, and Y differ among individuals. We have begun to correlate the slope of the line between variant chromosomes in bivariate flow karyotypes to the size and base sequence of certain chromosomal regions, such as heterochromatin or the satellites. This approach may help us to understand the complex binding and spectral interactions of HO, DAPI, and CA in restricted chromosomal regions.

Several observations lead to the conclusion that the HO:CA fluorescence characteristics of heterochromatin are described by the slope of the line through observed variants of chromosomes 1, 16, or Y. (1) Conventional banding analyses show that chromosomes 1, 9, 16, and Y vary primarily in the size of heterochromatic regions. (2) The homologues of chromosome 1 in individual 7-1, which differ in the amount of binding of a repetitive sequence specific for the heterochromatic C-band of chromosome 1 (Gosden et al. 1981) (fig. 5), differ in peak position in his flow karyotype (fig. 4). (3) Sequences associated with heterochromatin are AT rich (Nakahori et al. 1986), and flow karyotype variability of these chromosomes is predominantly in their HO intensity.

For chromosome 15, homologue differences in satellite and heterochromatic regions may be additive as in individual 7-1 or may offset each other as in individual 14-1. The line between chromosome 15 homologues in both individuals must reflect, at least in part, the combined HO:CA fluorescence characteristics of two regions. Individuals who differ in chromosome 15 heterochromatin band size and in binding to pd15Z1 have been described elsewhere (Higgins et al. 1985). Flow karyotype analyses of those individuals should help to characterize the HO and CA fluorescence characteristics of this subregion.

The capacity of flow karyotyping to quantify small differences in chromosome DNA content may allow revisitation of earlier studies in which heteromorphisms of reproductively isolated populations were compared (Lubs and Ruddle 1971; Lubs et al. 1977). These studies are limited both by cell-to-cell variability and by the subjective nature of heteromorphism classification using chromosome banding techniques. If flow data confirm population differences in variant frequency, it may be possible to apply quantitative flow karyotyping to the study of the origin, migration, and kinship of different ethnic groups. Further, little is known about the stability of chromosome variants during long-term culture. Our sample is presently too small to exclude the possibility that rearrangement in vitro may contribute to ei-

ther the extent of variability or the shape of the distributions we have observed. For example, 21 of the 27 variants of chromosome 22 with HO fluorescence intensities greater than the mean were from lymphoblast cultures. On the other hand, no significant difference in the frequency of heterozygosity for chromosome variants was observed between these two groups.

Finally, the extent of chromosome variability determines the sensitivity of flow karyotyping to detect submicroscopic deletions or rearrangements both in chromosomes of individuals with genetic disorders or in human chromosomes in somatic cell hybrids. For invariant chromosomes such as the X, DNA content changes involving ≥2 Mbp exceed the 95% confidence intervals of normal variation. In contrast, deviations in DNA content by as much as 10% fall well within the bounds of normal variation for chromosome 21. The loss of smaller amounts of DNA—but of sequences that result in an overall HO:CA ratio change of the chromosome—may prove detectable now that the HO:CA ratio of normal variants has been defined. In the accompanying paper (Trask et al. 1989), we show that variants are passed from parent to child without undergoing significant DNA content change. A comparison of the flow karyotypes of parent and child should increase the detection sensitivity of de novo chromosome rearrangements despite the magnitude of normal chromosome variability.

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