Karyotyping of Single Human Chromosomes from Dry Mass Determined by Electron Microscopy

GUNTER F. BAHR*† AND HARVEY M. GOLOMB*

*Armed Forces Institute of Pathology, Washington, D.C. 20305; and † Department of Pathology, Georgetown University, Washington, D.C. 20007

Communicated by Curt Stern, January 21, 1971

ABSTRACT Human chromosomes from cultures of peripheral lymphocytes were prepared for electron microscopy by surface spreading and critical-point drying. Total and partial determinations of chromosomal dry mass were made from contrast in electron microscopic negatives. The distribution of dry weights was analyzed with respect to the Denver-Chicago classifications. There is considerable variability of mass within each group, but no statistically significant differences between four donors. A single chromosome can be correctly classified from its total mass and the ratio of long- to short-arm mass.

The identification and taxonomy of human chromosomes, i.e., karyotyping, has been the subject of a number of publications and scientific meetings (2-6). Widely accepted standards of nomenclature and measurement appear to have reached a final state with the proceedings of the Chicago Conference (4). Its chromosome classifications are intended for light microscopic images of stained metaphases.

Several sophisticated, computer-assisted, light-microscopic systems for automation of karyotyping have been described in which the metaphase plate is either scanned directly or in a photograph of it (7-12). Relative total length and arm ratio are the main features by which the eye and the computer classify a chromosome. Only in the system of Mendelsohn and collaborators is use also made of the stain content of individual chromosomes.

Electron microscopic studies of thin-sectioned human chromosomes have been singularly unsuccessful because of lack of specific contrast; however, surface spreading and (in particular) critical-point drying (13) renders chromosomes in what appears to be their natural configuration. More importantly, the chromosomes appear in natural contrast since no special fixatives or heavy-metal stains are required for their visualization. By means of certain operating conditions of the electron microscope, controlled exposure, and controlled photographic processing, a close proportionality of the dry mass of the object to photometric transmission in the electrographic negative can be achieved (14, 15). Since the relationship of mass and contrast (difference of transmission $\bar{T} - T_0$)† is independent of the object's shape and its chemical composition, the electron microscope constitutes a sensitive balance. Masses from 10^{-10} to 10^{-18} g may be determined. This technique of dry-mass determination is most suitable for quantita-

tive studies of individual chromosomes. It has previously been used in a rather detailed study of human chromosomal fibers, especially the relation of fiber mass to DNA content (16), and further in an analysis of chromosomes from Burkitt's lymphoma (17).

In the present study, the dry mass and related features of a large number of human chromosomes were determined in order to see whether such parameters would lead to distinct two- or three-space clusters analogous to Groups A through G of the Chicago classification. Unfortunately, the most productive technique of preparation is that of chance selection of groups of chromosomes, up to about 20. Whole metaphases were found extremely rarely.

MATERIAL AND METHODS

Peripheral leukocytes from four male donors were cultured in DIFCO (Detroit, Mich.) medium. Cell division was blocked with colchicine for 6 hr. After harvesting and washing of cells in normal Hanks solution by low-speed centrifugation, they were exposed for 10 min to hypotonic Hanks solution (1:1). After a final low-speed centrifugation, a pellet of swollen cells was obtained, portions of which were transferred onto the surface of distilled water in a Langmuir-type trough, where surface tension broke cellular and nuclear membranes. Only chromosomes, nuclei without membranes, and a few mitochondria were sampled when Formvar-coated grids for the electron microscope were lightly touched to the water surface. Grids so prepared were directly submerged in 30% methanol and brought through a graded series of aqueous methanol solutions to absolute alcohol and to amyl acetate. A batch of grids was then dried by the critical-point-drying method of Anderson (13). In order to assure contamination-free electron microscopy, a custom-built RCA-4B microscope was used with an Orb-Ion pump that differentially pumped the specimen chamber and the objective pole piece. The pressure at the specimen ranges from 5×10^{-7} to 2×10^{-7} Torr. There was no measurable contamination of specimens exposed to normal beam currents for up to 30 min. Electrography was at carefully controlled magnifications and controlled background exposure (14, 15). Plates were processed according to a standard technique. Each batch of 12 or 24 plates contained two electrographs of mass standards prepared from polystyrene latex [polystyrene spheres (14)]. Integrated transmission of chromosome images or parts of them (arms) were measured on an integrating photometer IPM-2 (Zeiss, Oberkochen, Germany). Photometric mass determinations from

† $\bar{T}$ is the integrated transmission of a defined area encompassing the image of the object; T_0 is the background transmission determined in an empty area of the same size.

electron micrographs are subject to two different types of error. The first is the error of relative determinations, which is only $\pm 1-2\%$. However, setting an absolute scale to relative measurements involves standardization, which has error of $\pm 7-9\%$. In another type of assessment, the mass of single chromosomal fibers was determined from single line scans relative to scans of polystyrene spheres. The data from both types of measurement are fed into computer-compatible incremental tape recorders. A typical printout is shown in Fig. 1, which also lists other chromosomal data that are assessed concurrently.

RESULTS

Chromosomes prepared by surface spreading and critical-point drying and photographed in an electron microscope have a general appearance closely related to that under the light microscope (Fig. 2). Bumpy, twisting loops of chromatin fibers constitute the entire chromosome (16).

The blind sampling of objects capable of attaching themselves to the electron-microscope grid (18) should give a truly random sample of chromosomes or chromosome groups from many metaphases. In Table 1, the frequency with which chromosomes of Groups A through G were measured is tabulated for 868 entries and the expected frequency is calculated. There is excellent agreement for Groups C and F, a slight disproportion for Groups A, B, and D, and a more pronounced deviation from expectation for Groups E and G. It is not clear at this time whether the relatively small discrepancies are due to assignments in wrong groups, nonrandom selection of measurable chromosomes, or preferential loss during preparation. The general representativity of the entire sample is, nevertheless, good.

```

IDENTIFICATION          = 11087012 12
01 DONOR                 = C
02 SEX                   = M
03 TENTATIVE GROUP       = E
04 TENTATIVE IDENTIFICATION
05 QUALITY                = 3
06 MASS * 10-E13 GM      = 15.17
07 LONG ARM MASS         = 9.00
08 SHORT ARM MASS        = 6.17
09 LONG/SHORT            = 1.46
10 CHROMATID A MASS      = 7.14
11 CHROMATID B MASS      = 7.68
12 A/B                   =
13 B/A                   = 1.08
14 FIBER DIAMETER ANG.   = 317
15 S.D.                  = 46
16 FIBER MASS*10-E16 GM/U
17 S.D.                  = 2.34
18 TOTAL FIBER LENGTH/CHROMATID
19 DNA PACKING RATIO     = 52.6
20 CHROMOSOME INDEX      = 1.54
21 VOLUME * 10-E12 CC    = 4.04
22 MASS/VOLUME GM/CC     = 0.38
23 TELOMERE A            = 61
24 TELOMERE B            = 87
25 CENTROMERE            = 29
26 SHORT ARM A           = 79
27 SHORT ARM B           = 69
28 LONG ARM A            = 65
29 LONG ARM B            = 64
30 METAPHASE DESIGNATION = H
31 COMPACTION INDEX (PERCENT)
32 NUMBER OF FIBERS MEASURED = 13

```

Fig. 1. Computer printout from Chromosome Register showing the type of entries normally made and some resulting calculations.

Fig. 2. Electron micrograph of a group of human chromosomes in natural contrast. Note the fibrous nature of the chromatid. Magnification $\times 6900$.

In order to study the possibility that chromosomal mass data would cluster in a two-parameter space, individual entries were made in a net of total mass versus mass of long arm over mass of short arm (Fig. 3). The more than 900 entries do not cluster visually into anything approaching the Denver-Chicago classifications. With guidance from visual impressions of electrographs and *a priori* knowledge of group characteristics, a *best-fit* partition of data from Fig. 3 into Groups A through G was performed; it is illustrated in Fig. 4. Setting of group boundaries was further supported by data from reconstituted karyotypes, i.e., karyotypes for which only 8-12 chromosomes were found, several of which could be clearly identified.

On the basis of such identifications, the remainder of a karyotype can be extrapolated. Entries to Fig. 4 with arm ratios exceeding 6.0 are shown at the 6.0 ratio. A small inserted histogram represents G-group chromosomes for which the arm ratio could not be determined, as the centromere was too telocentric. Subgroups A (1-3) and B (4, 5) are separated, with few (6) intervening entries. The common difficulty of distinguishing B5-group chromosomes from C under the light microscope is reflected in mass entries between B and C. The B4-B5 boundary was given from reconstituted karyotypes. If the lower boundary of B5 was moved to lower mass values, this would disturb the B4-B5 balance; also, the heavier, and thus larger, C-group chromosomes usually tend to be more metacentric, suggesting a B5-C boundary as drawn. A few unclassified entries are also seen between E and F; C, D, G and E; and between D and G.

The group boundaries of Fig. 4 reflect the best state of current understanding, but will probably be altered somewhat

TABLE 1. Frequency of occurrence (46,XY)

Group	Expected	Measured*
A	113	123
B	76	56
C	283	274
D	113	93
E	113	184
F	76	79
G	94	59

* A selected population was measured, since a number of fully overlapping chromosomes or those only partially visible in the electron microscopic field of view were not measured.

FIG. 3. Dry mass of individual chromosomes in units of $\text{pg} \times 10^{-1}$ is plotted against the mass ratio of long to short arms. The even scatter of values is not a consequence of instrumental error, which is only $\pm 1-2\%$ for the relative position of each entry, but is the result of actual biological variability in protein content from metaphase to metaphase.

as all data from whole metaphases and reconstituted karyotypes become available, and as further single entries are collected. In view of the variability of chromosome weights, it is also likely that more areas of overlap will be seen. Numeri-

cal definitions of group boundaries and the mean group masses are given in Table 2. The standard deviation of the mean for large chromosomes is commensurate with the error of quantitative dry-mass determination of absolute mass. Most notable

FIG. 4. Same as Fig. 3, but group boundaries drawn according to Denver-Chicago classification (1, 3). The histogram (*insert*) represents additional G-group chromosomes that were too telocentric for the arm ratio to be determined (see text).

TABLE 2. Quantitative definitions of groups A through G and subgroup boundaries by dry mass (wt.)

Group	Mass range*		Mean wt. (pg × 10 ⁻¹)	SD†	Mean of long arm (pg × 10 ⁻¹)	SD	Mean of short arm (pg × 10 ⁻¹)	SD	Mean L/S‡	SD	Max* L/S	Min* L/S
	Max	Min										
A ₁	>50	35	41.33	3.10	22.41	2.35	18.98	1.70	1.19	0.15	1.50	1.00
A ₂	39.50	30.75	34.05	2.26	22.89	2.26	11.16	1.91	2.13	0.50	3.30	1.50
A ₃	37.00	25.00	30.37	2.51	17.02	1.71	13.30	1.83	1.30	0.23	1.70	1.00
B ₄	30.00	26.75	28.51	1.03	19.52	1.79	8.51	1.40	2.37	0.52	4.00	1.70
B ₅	26.75	23.50	25.18	0.91	17.69	1.34	7.36	1.08	2.47	0.49	4.00	1.70
C(X)	27.00	13.75	19.58	2.93	12.32	1.78	7.40	1.87	1.75	0.43	2.85	1.00
D	19.00	9.00	14.11	2.39	11.53	2.13	2.73	0.75	4.56	1.59	11.00	2.85
E	16.00	5.50	11.13	2.77	7.10	1.59	4.14	1.46	1.83	4.21	2.70	1.00
F	13.00	5.50	9.37	1.58	5.13	0.86	4.36	0.81	1.19	0.12	1.50	1.00
G(Y)	10.00	3.60	6.88	1.23	5.59	0.90	1.53	0.32	3.76	0.79	6.00	2.85

* Range from Fig. 4.

† SD, standard deviation.

‡ L/S = Long-arm mass/short-arm mass.

TABLE 3. Distribution of dry mass among chromosomes of four normal male donors

C				E				S				Cu			
		weight				weight				weight				weight	
n		(pg $\times 10^{-1}$)	SD	n		(pg $\times 10^{-1}$)	SD	n		(pg $\times 10^{-1}$)	SD	n		(pg $\times 10^{-1}$)	SD
A ₁	8	41.69	4.19	1		37.40		2		40.98	0.13	14		41.45	2.43
A ₂	8	34.23	2.21	4		33.51	2.41	7		34.04	1.70	19		34.09	2.40
A ₃	14	30.86	2.44	11		29.08	1.27	10		30.94	3.64	25		30.44	2.19
B ₄	8	28.86	1.20	5		28.48	0.74	2		28.36	1.28	9		28.25	0.82
B ₅	9	25.20	0.93	6		24.65	0.74	4		24.64	0.92	13		25.58	0.73
C	71	19.54	2.88	46		19.59	2.76	60		19.77	2.90	97		19.49	3.06
D	24	14.70	2.84	20		13.74	2.21	18		13.39	1.86	31		14.31	2.25
E	51	11.26	2.86	30		10.14	3.04	34		11.16	2.45	69		11.44	2.62
F	25	9.03	1.43	21		9.23	1.58	7		9.95	1.64	26		9.66	1.61
G	14	6.86	0.79	14		7.25	1.08	13		6.70	1.31	18		6.75	1.47

is the marked spread of entries for each group. The maximum and minimum values of the ratio of long- to short-arm mass in Table 2 serve to set Groups B, D, and G numerically apart from the others. An average human male (46,XY) metaphase weighs 83.59×10^{-12} g.

A breakdown of our data with respect to the four male donors is given in Table 3. In order to analyze whether there were significant differences between donors, the null hypothesis was stated as $P = 0.01$. It was first established that the sample came from populations with equal variances, and then Student's t -test was applied. Pairs of variables were alternately correlated, and no difference was found. The null hypothesis is thus valid, and we conclude that there are no significant differences between donor populations.

When the means of chromosomal dry mass for each group from Table 3 are compared to the mean-length measurements compiled by Penrose (19), a good agreement is found (Table 4). Not only is the relative correctness of our group boundaries (Fig. 4) thereby supported, but the proportionality of mass and length reinforces the common notion that the average chromosome has a rather constant cross section.

DISCUSSION

Dry-mass determinations are developing into a means for identifying individual chromosomes under the electron micro-

scope outside of the reference frame of a complete karyotype. Current preparatory techniques give extremely few whole-metaphase plates for the electron microscope, so that the identification of normal single chromosomes at about 100

TABLE 4. Comparison of optical data* with electron microscopy measurements (haploid autosomal complement)

Group	% of total length†	% of total mass‡	Length L/S‡	Mass L/S‡
A ₁	4.39	4.94	1.08	1.19
A ₂	4.11	4.07	1.55	2.13
A ₃	3.45	3.63	1.20	1.30
B ₄	3.17	3.41	2.75	2.37
B ₅	2.96	2.01	2.64	2.47
C(X)	2.41	2.34	1.91	1.75
D	1.69	1.69	5.73	4.56
E	1.45	1.33	2.11	1.83
F	1.15	1.12	1.38	1.19
G(Y)	0.83	0.82	3.93	3.76

* From Ref. 19.

† Calculated for 46,XY karyotype.

‡ Long arm/short arm.

times better resolution than the light microscope is the prerequisite for studies of chromosomes in disease.

925 chromosomes, a relatively large number, do not sort into significant clusters, but rather into a continuum of entries, as seen in Fig. 3. As matters stand, this situation is not changed in a three-parameter space where arm masses and total mass are used as coordinates. Our earlier studies (16, 20) suggest that the variability of dry mass is a feature of cellular nucleoprotein. Only future evaluation of whole metaphases will allow judgment as to whether a single chromosome can be uniquely identified (by its number) from dry mass alone.

A preliminary presentation of this study has been made (1).

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

This study was supported by grant no. P-259-J from the American Cancer Society.

1. Golomb, H. M., W. F. Engler, and G. F. Bahr, Abstract, *Seventh International Congress of Electron Microscopy*, 247 (Grenoble, 1970).
2. Denver Classification, *Eugenics Quart.*, **7**, 96 (1960); also *Amer. J. Human Genet.*, **12**, 319 (1960).
3. London Conference, *Cytogenetics*, **2**, 264 (1963).
4. *Chicago Conference, Birth Defects*, Original Article Series (1966) vol. 2, no. 2.
5. Patau, K., *Amer. J. Hum. Genet.*, **12**, 250 (1960).
6. Levan, A., K. Fredga, and A. Sandberg, *Hereditas*, **52**, 201 (1964).
7. Neurath, P. W., G. Gallus, and W. D. Gelles, *J. Ass. Advanc. Med. Instrum.*, **4**, 6 (1970).
8. Mendelsohn, M. L., B. H. Mayall, and J. M. S. Prewitt, in *Image Processing in Biological Science* (Univ. of Calif. Press, 1969), p. 119.
9. Mendelsohn, M. L., T. J. Conway, D. A. Hungerford, W. A. Kolman, B. H. Perry, and J. M. S. Prewitt, *Cytogenetics*, **5**, 223 (1966).
10. Mendelsohn, M. L., D. A. Hungerford, B. H. Mayall, B. Perry, T. Conway, and J. M. S. Prewitt, *Ann. N.Y. Acad. Sci.*, **157**, 376 (1969).
11. Frey, H. S., *Comput. Biochem. Res.*, **2**, 274 (1969).
12. Ledley, R. S., and F. H. Ruddle, *Sci. Amer.*, **214**, 40 (1966).
13. Anderson, T. F., *Trans. N.Y. Acad. Sci. Ser. II*, **13**, 130 (1951).
14. Bahr, G. F., and E. Zeitler, *Lab. Invest.*, **14**, 217 (1965).
15. Zeitler, E., and G. F. Bahr, *J. Appl. Phys.*, **33**, 847 (1962).
16. DuPraw, E. J., and G. F. Bahr, *Acta Cytol.*, **13**, 188 (1969).
17. Lampert, F., G. F. Bahr, and E. J. DuPraw, *Cancer*, **24**, 367 (1969).
18. Bahr, G. F., *Exp. Cell Res.*, **62**, 39 (1970).
19. Penrose, L. S., *Amer. J. Human Genet.*, **24**, 319 (1960).
20. Bahr, G. F., and E. Zeitler, *J. Cell Biol.*, **21**, 175 (1964).