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Location of Ribosomal DNA in the Human Chromosome Complement

(rRNA/satellite regions/acrocentric chromosomes)

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ABSTRACT Hybridization of ^{3}H-labeled ribosomal RNA to human chromosomes on slides resulted in specific labeling of the satellite regions of chromosomes 13, 14, 15, 21, and 22, with an over-all efficiency of about 5%. Differences between D and G chromosomes, and between associated and unassociated satellites, were not significant. Labeling of all other parts of the preparations was nonspecific, and increased in the order: extrachromosomal regions < chromosome arms < centric regions.

Three lines of evidence bear on the localization of rDNA in the human genome. First, the satellited chromosomes tend to be associated with the nucleolus (1, 2). Second, when isolated chromosomes are fractionated, enrichment for small chromosomes enriches for rDNA (3). Third, with DNA from cell lines having different ratios of acrocentric to total chromosomes, rRNA–DNA hybridization increases with the relative number of acrocentrics (4). Such results are consistent with the presence of rDNA in some combination of chromosomes 13, 14, 15, 21, and 22, but do not resolve whether all of these chromosomes, are uniquely involved.

We have used the technique of *in situ* hybridization (5) of [^{3}H]rRNA to human chromosomes. The results indicate that all five acrocentric pairs are labeled in the satellite regions, while no specific rRNA binding occurs elsewhere.

MATERIALS AND METHODS

Preparation of [^{3}H]rRNA. Fragments of human ovary were explanted to GIBCO diploid growth medium. First-transfer cells were grown to semiconfluence, the medium was replaced, and 5 mCi of [^{3}H]uridine (40 Ci/mmol) was added in balanced salt solution; total volume was 4 ml per T25 flask. After 12 hr, the [^{3}H]uridine was replaced with fresh growth medium. Cells harvested 5 hr later were lysed in 1% SDS–0.1 M NaCl, and extracted four times with phenol. Nucleic acids were adsorbed to a methylated albumin Keiselguhr (MAK) column and eluted with a salt gradient yielding 18S and 28S rRNA in a single peak at 0.7 M NaCl. *Escherichia coli* rRNA was used as carrier; hence, the final preparations contained excess unlabeled bacterial RNA, possibly beneficial in competing with the [^{3}H]rRNA for nonspecific binding. The RNA was dialyzed against 2 × SSC overnight and precipitated with two volumes of 95% ethanol. The precipitate was dissolved in 8 × SSC, and formamide (pH 7.2) was added to 50% by volume. The specific activity of the human rRNA varied from 3.5×10^6 to 5×10^6 dpm/μg.

Preparation of Slides. Slides made from human lymphocyte cultures by means of standard colcemid and hypotonic treatments were placed in 2 × SSC with 100 μg of pancreatic ribonuclease per ml for 2 hr at 38°, then washed three times in 2 × SSC, twice in 70% ethanol, and twice in 95% ethanol. For denaturation, slides were then immersed for 3 min in 0.01 × SSC at 90°, quickly cooled in 0.01 × SSC, held for 2 hr in 95% formamide–1 × SSC at 65°, washed in ethanol as before, and air dried. For hybridization, slides were flooded with RNA solution, about 2 μg of [^{3}H]rRNA per ml of 50% formamide under coverslips, held 12 hr at 38°, washed three times with 2 × SSC, and treated with 20 μg of ribonuclease per ml of 2 × SSC for 1 hr at 38°. After washes with SSC and ethanol as before, slides were dried, coated with Kodak NTB 2 emulsion, and exposed for 1 month or more. After development, the preparations were stained with Giemsa.

Abbreviations: SDS, sodium dodecyl sulfate; SSC, 0.15 M sodium chloride–0.015 M sodium citrate.

RESULTS AND DISCUSSION

Interphase nuclei were labeled preferentially in nucleoli, suggesting that most of the labeled material persisting on the slides was rRNA, and that a major mode of enzyme-resistant fixation of RNA was rRNA–DNA hybridization. Metaphase plates had variable grain densities with clusters frequently occurring over satellites, as shown in Figs. 1 and 2. Some cells had grains over all ten acrocentric chromosomes, but satellite associations precluded a one to one assignment of grains to chromosomes. Since we could not directly determine whether each acrocentric chromosome was labeled, nor exclude the possibility that other chromosomes were involved, a statistical approach was used to describe the main features of the labeling pattern with minimal chromosome identification.

Equal area grain counts

Figures were selected for minimal distortion of chromosomes and completeness of the D and G groups, but not for grain distribution, and prints were prepared at a magnification of 2700. Equal areas over chromosomes were demarcated on the prints by means of a glass cylinder 1 cm in diameter, corresponding to a circle of about 3.7-μm original diameter; and the number of grains per area was recorded. This procedure yields distributions that can be tested for homogeneity. A heterogeneous grain distribution caused by specific labeling becomes homogeneous upon separation of specific regions from the data. Heterogeneity from other causes persists, however, in different subsets.

Labeling of centric regions

In 19 figures, the demarcator was centered on the kinetochore of each chromosome, with the results shown in Table 1. Of the 190 acrocentric chromosomes in these plates, 40 were involved in satellite associations that caused the areas to be partially superimposed. In such cases, grains in a common region were assigned alternately to the different overlapping areas, so that no grain was counted more than once. This does not affect

TABLE 1. *Distributions of equal areas over chromosomes, by grain count; centric regions only*

No. of grains	Plate no. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
0	19,1	17,1	22	22,1	15	19,5	15,2	16	17	15	18,1	30,2	13	15	13,1	12	15	16	11,1
1	9,3	12,1	10,1	8	14	11,1	13,4	9	13	14,2	10,2	4,1	14	8,1	17	19,1	11	14,1	16,1
2	5,1	5,2	2,3	3,1	4	3	2	7,1	5,1	4,1	6,2	1,2	4,1	9,3	3,2	4,5	8,2		8,1
3		1,2	2,1	1	1,1	3,1	2,1	1	1,1	2	1	2	2	3,4	3,1	1,3	1	1,2	1
4	2,3	2	3	3	1	1		4,2	4	1	1	2	1,3	1	1	1	1,1	2	2
5	1	1	1,1	1,1	1,1		1	2	2	4			1,2		3		2	1,3	2
6	1		1	2	4	1	1	1	1	1	1	1	2				2	2,3	1
7					1				1	1	1		2		2		2		1
8		1		1	1			2			1			1					
9					1	1	1											1	
>9								1			1			1					1

Numbers underlined are areas over acrocentric chromosomes.

the comparison of grain densities over acrocentrics and other chromosomes, but it increases the homogeneity of grain distribution for acrocentrics in the plates (Nos. 1, 3, 4, 5, 6, 7, 9, 10, 13, 15, 16, 17, 18) that had satellite associations. The totals from Table 1 are shown in the first two columns of Table 3. The difference between the grain densities over acrocentrics and nonacrocentrics is indeed significant: 2×2 contingency table, $\chi^2 = 266$.

To compare the heterogeneity of grain density in different sets of areas, a χ^2 is obtained for each set as $\chi^2 = \Sigma(x - \bar{x})^2/\bar{x}$, where x is the number of grains in an area and $\bar{x}$ is the average number of grains per area. It is assumed that $d = (2\chi^2)^{1/2} - (2n - 1)^{1/2}$ would be distributed normally around zero with unit standard deviation, if the grain distributions were random. Normal distribution of d is closely approximated for $n > 30$. To see whether it could be used where $n = 10$, we calculated d from a table of χ^2 at various P values for $n = 30$ and $n = 10$; the differences are negligible for the present purpose. We adopt the convention that a departure from randomness is significant if d is outside the range of ± 2, positive values indicating heterogeneity. By this criterion, the total centric regions are heterogeneous in all but one plate, as shown in Table 4. When acrocentric and nonacrocentric chromosomes are considered separately, however, they are mainly homogeneous. Although the d values show a positive trend, the three instances in which nonacrocentrics are significantly heterogeneous can be ascribed to a few grain clusters in different locations, or to an excess of unlabeled areas. We conclude that the centric regions of the different nonacrocentric chromosomes were closely similar in labeling potential. The same may hold for acrocentrics, but this is uncertain because of the method used in dealing with satellite associations. These results, combined with prior evidence (1–4), justify a distinction between background labeling of nonacrocentric and specific labeling of acrocentric chromosomes.

The number of specifically labeled chromosomes

The question of whether all the acrocentric chromosomes are specifically labeled can be answered by comparing the number of chromosomes having less than two grains with the number expected if one or more pairs had the same labeling potential as nonacrocentric chromosomes. Only those not in associations are useful for this comparison; they comprise 59 G and 91 D chromosomes. Of these, 15 in G and 16 in D had less than two grains, versus 546 of the 671 nonacrocentric chromosomes. The hypothesis that two pairs are not specifically labeled was

TABLE 2. *Distributions of equal areas over chromosomes, by grain count; all regions*

No. of grains	Plate no. 1	2	3	4	5	6	7	8
0	41,6	47,3	48,2	59,3	31,1	61,9	44,6	28,6
1	24,2	18,3	16,3	13,2	17,1	20,2	18,5	22
2	6	8,2	8,3	6,2	7,2	4	6,1	11,2
3		1,2	2,3	1,4	4,3	2,1	1	4,2
4		2	3	1	1		1,1	1,2
5	1	1	1	1		1		2
6	2			3	2	1		1
7	1	1	1		2	1		1
8				1				1
9				1				
>9					1		2	1

Numbers underlined are areas over acrocentric chromosomes.

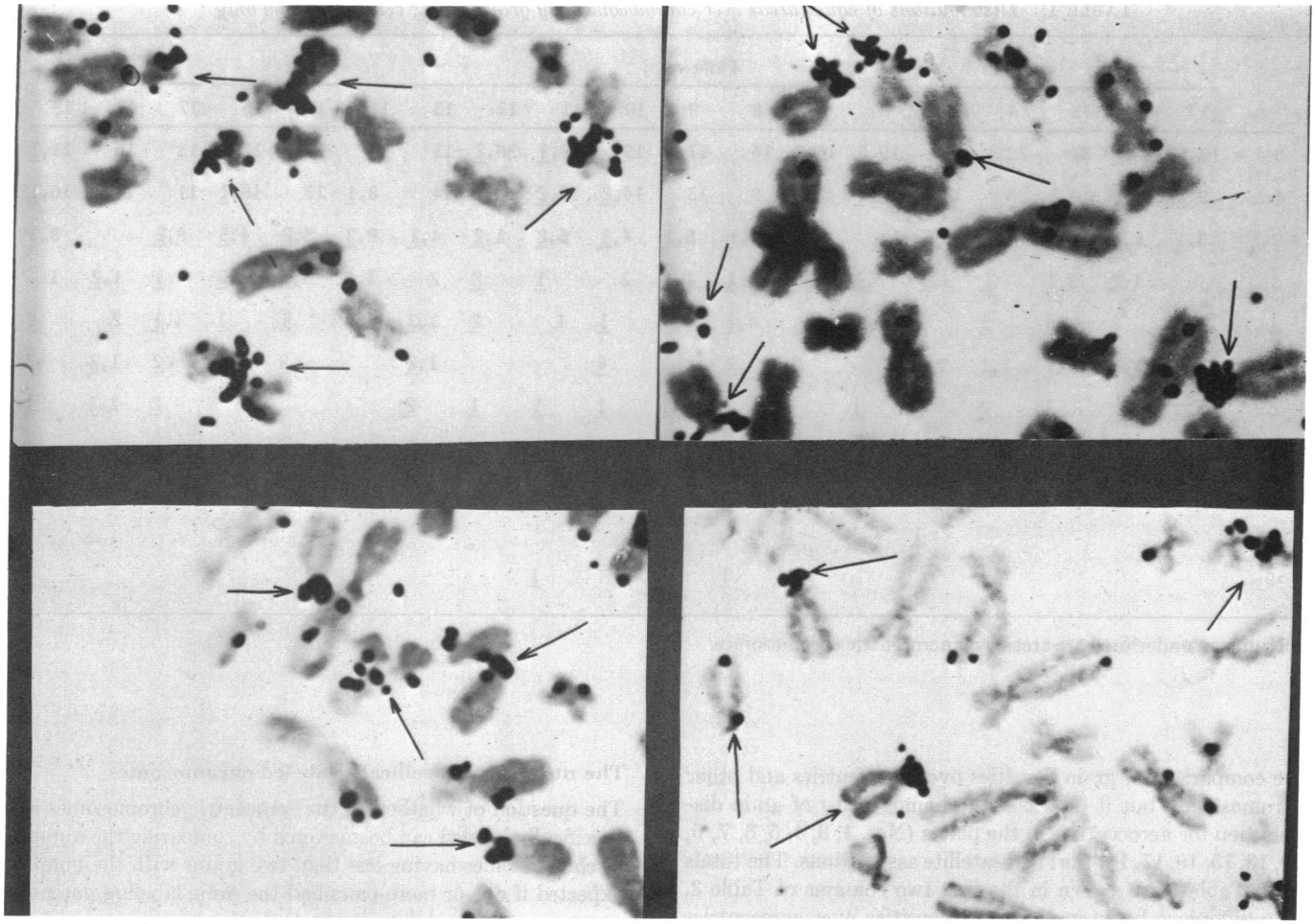

FIG. 1. Portions of metaphase plates with grain clusters. *Arrows* indicate chromosomes of groups D and G.

tested on the assumptions: (*a*) They comprise 40% of those not in associations. (*b*) The other 90 are all labeled with two or more grains. (*c*) The number of nonacrocentric chromosomes with less than two grains is two standard deviations below that observed, i.e., 526. These assumptions favor the hypothesis, within reason. A test of goodness of fit of the observed, 31:119, to the expected, 47:103, gives $\chi^2 = 7.9$, $P = 0.005$, indicating that at least four pairs of acrocentric chromosomes are specifically labeled.

TABLE 3. *Grain densities in different regions of metaphase plates*

	Plates 1-19			Plates 1-8			
	Centric regions only			Chromosomes, all regions		Centric regions only	
	Non-acro.	Acro.	Extra-chromosomal	Non-acro.	Acro.	Non-acro.	Acro.
Areas	671	190	3239	581	120	280	80
Grains	546	743	198	318	325	214	300
Grains/area	0.81	3.91	0.061	0.55	2.71	0.76	3.75

Acro., acrocentric.

A pair not specifically labeled must be in either the G or the D group; hence, each possibility was tested as before, except that the hypothetical pair comprises 1/3 of the D, or 1/2 of the G chromosomes; and assumption (*b*), proven false, was discarded. Instead, the proportion with less than two grains was assumed, for all but one pair in the group under consideration, to be the same as that in the other group, but adjusted downward by two standard deviations to favor the hypothesis. For example, if a D pair were not specifically labeled, the expected number for D chromosomes would be: $(1/3)\cdot 91\cdot(546 - 2\sqrt{546\cdot 125/671})/671/671 + (2/3)\cdot 91\cdot(15 - 2\sqrt{15\cdot 44/59})/59 = 32$. The observed, 16:75, does not fit the expected, 32:59; $\chi^2 = 12$, $P < 0.001$. Similarly for a G pair, the observed, 15:44, and expected, 26:33, numbers are discrepant; $\chi^2 = 8.3$, $P = 0.004$. We conclude that all the acrocentric chromosomes are specifically labeled.

Labeling of all chromosomal regions

On the first eight plates the demarcator was moved along each chromosome in steps of one diameter until all regions were sampled. Such samples included most of the grains previously counted over centromeres, but these were usually

subdivided into two areas. The results are in Table 2, and the totals in the fourth and fifth columns of Table 3. The last two columns of Table 3 give the totals for centric regions of the same plates, for comparison. The average grain density over the total regions is significantly lower than over centric regions. The d values (Table 4) for the total areas indicate an extremely heterogeneous grain distribution in each plate. Acrocentric chromosomes taken separately remain mostly heterogeneous because of the higher grain density over the satellite ends than over the arms. Areas over nonacrocentric chromosomes were homogeneous, except in one plate, although a positive trend of d values is apparent, exceeding that noted for centric regions. This effect may represent an excess of unlabeled areas, or of clusters. In view of the latter possibility, the locations of the 18 clusters of three or more grains in Table 2, removal of which overcompensates for the trend, were identified as closely as practicable. Results* show no regular localization of grain clusters; hence, specific labeling was not discernible except over satellites.

TABLE 4. *Average grain density over chromosomes, and a criterion of homogeneity, $d = (2\chi^2)^{1/2} - (2n-1)^{1/2}$, from the distributions in Tables 1 and 2*

	Centric regions only					
	Grains/area			d		
Plate no.	Tot.	Acro.	Non-acro.	Tot.	Acro.	Non-acro.
1	1.22	2.8	0.77	4.0*	0.82	1.90
2	1.27	3.2	0.71	4.2*	0.97	-0.26
3	1.26	3.3	0.69	4.3*	-0.70	2.9*
4	1.39	4.2	0.56	6.9*	0.30	2.9*
5	2.00	6.0	0.86	7.1*	-1.30	1.15
6	1.07	2.3	0.72	6.7*	4.5*	0.87
7	1.19	2.7	0.72	2.00*	3.4*	-0.03
8	2.04	5.5	1.08	6.7*	0.17	1.99
9	1.57	4.4	0.76	4.1*	-0.18	-0.45
10	1.53	4.1	0.80	4.5*	0.00	-0.31
11	1.61	4.5	0.74	8.8*	3.1*	0.78
12	0.69	2.5	0.17	6.0*	0.74	0.71
13	1.89	5.0	1.06	4.7*	-1.75	1.41
14	1.65	3.7	1.08	5.0*	2.05*	0.66
15	1.57	4.0	0.89	4.3*	0.44	-0.55
16	1.17	2.4	0.83	-0.82	-2.05*	-1.67
17	1.73	4.7	0.86	4.8*	-0.66	0.13
18	1.98	4.9	1.17	6.5*	-0.17	4.9*
19	1.72	4.4	0.97	5.5*	1.61	-1.55
	All regions					
	Grains/area			d		
	Tot.	Acro.	Non-acro.	Tot.	Acro.	Non-acro.
1	0.75	2.17	0.51	7.0*	4.4*	-1.04
2	0.80	2.36	0.50	4.9*	1.77	0.60
3	0.89	2.47	0.57	5.5*	0.84	3.0*
4	0.84	3.12	0.39	9.8*	2.01*	1.74
5	1.45	4.50	0.73	9.3*	2.09*	0.84
6	0.56	1.53	0.39	8.4*	5.2*	1.27
7	0.80	2.07	0.53	11.4*	7.1*	1.61
8	1.42	3.28	0.91	7.8*	4.1*	0.36

* Departure from random distribution considered significant. Tot., total; Acro., acrocentric.

TABLE 5. *Labeling of extrachromosomal areas*

Plate no.	N Areas	Grains	m Grains/area	E $N(1-e^{-m}-me^{-m})$	O
1	423	33	0.078	1.25	6
2	418	22	0.053	0.84	6
3	416	25	0.060	0.75	5
4	374	23	0.061	0.68	3
5	422	27	0.064	0.88	2
6	343	17	0.050	0.46	2
7	421	29	0.069	1.06	1
8	422	22	0.052	0.61	3
			Total: 7		28

Extrachromosomal areas

Grain densities in vacant portions of the first eight plates were estimated from the total counts within a square enclosing the chromosomes, corresponding to about 5000 μm^2, minus the envelope of the demarcated chromosomal areas and any nuclei or debris. To facilitate comparisons, the vacant areas are expressed in demarcator units. The lowest grain density is extrachromosomal, as shown in Table 3. Since the method used does not yield distributions, the question of homogeneity was approached in a different way. The column in Table 5 headed "E" gives the numbers of areas expected to have more than one grain on the hypothesis that they are homogeneous. The column headed "O" gives the corresponding observed numbers obtained by centering the demarcator on each grain and scoring any included pair or cluster as a unit. The difference between the observed and expected totals is significant; it indicates a tendency for grains to be associated even when they are not close to a visible structure.

Associated versus unassociated, and D versus G chromosomes

Grain densities over associations may be compared with those over unassociated satellites in the same plates. Associations involved 23 D and 17 G chromosomes, and a total of 178 grains, 4.45 per chromosome. Grains per unassociated D chromosome were 213/55 = 3.87, and per unassociated G were 118/35 = 3.37. None of the differences is significant. Comparison of D with G on all plates, with only mixed associations excluded, gave 466/108 = 4.31 grains per chromosome for the D group and 268/72 = 3.72 for the G group; the difference is not significant.

Efficiency of specific labeling

Neither the proportion of rDNA hybridized nor grains per disintegration is known separately, but their product can be

* The number of grain clusters in each of the regions 1p, 3 centric, Bp, B centric, Eq, Ep, Fq, and Xp was one; in regions 1Q, Cq, C centric, and F centric, it was 2, 3, 3, and 2, respectively.

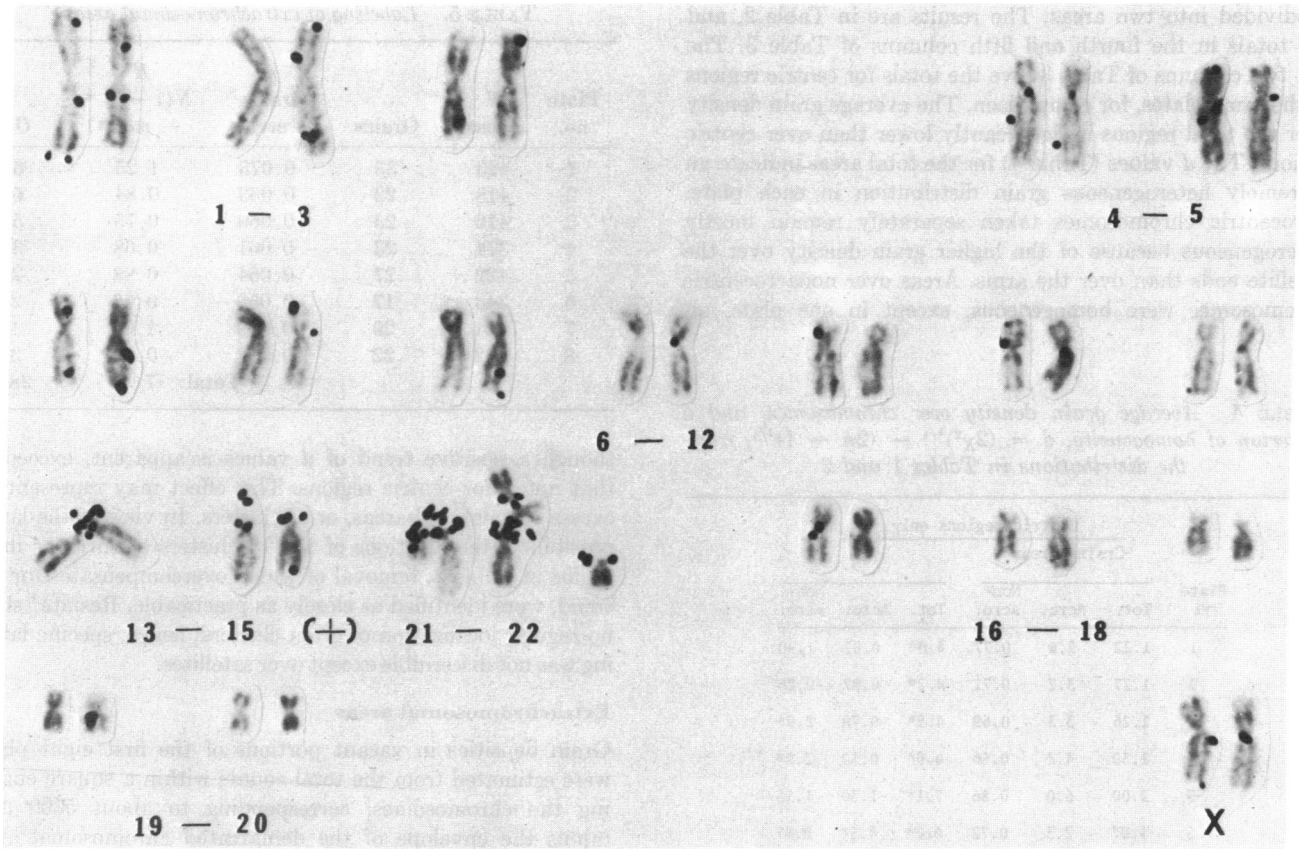

Fig. 2. Ideogram with labeled DD, DG, and DGG satellite associations.

estimated. If the ratio rRNA/DNA at saturation is 3×10^{-4}, as reported for filter-bound DNA from normal subjects (6), and the total DNA per diploid nucleus in metaphase is 1.2×10^{-5} μg (7), the average amount of rDNA per acrocentric would be 3.6×10^{-10} μg (about 44 rRNA genes per chromatid). With a specific activity of 4×10^{6} dpm/μg and 1 month exposure, the rRNA would yield 1.75×10^{11} disintegrations per μg. Thus, 63 grains per acrocentric chromosome would be expected if both hybridization and grain production were fully efficient. Only 3.1 grains per acrocentric were seen (Table 3, centric background subtracted); hence, the overall efficiency of specific labeling was roughly 5%.

Speculations on the human rDNA loci

While consistent with cytological observations, the presence of rDNA in five loci is not expected on the simplest assumptions concerning maintenance of normal multiplicity. We surmise that the optimal number of rRNA genes is maintained by selection against significant departures. When the rDNA is divided into several nucleolus organizers, their separate multiplicities may vary in supplementary fashion, without harmful changes in total multiplicity. This sets the stage for irreversible losses; that is, the multilocus system should be unstable. An explanation is required, therefore, for the dispersal of human rDNA into different loci. It would be useful to know how polymorphic the condition may be, and the extent to which it occurs in other primates, since the explanation may be merely historical. On the other hand, a multilocus distribution would be stabilized if more than one repetitive sequence in rDNA were required, or if a system had evolved to regulate rRNA production according to the number of loci inactivated. In any case, the presence of long homologous segments may facilitate exchanges or other interactions among different satellited chromosomes, considered a major cause of human chromosome abnormalities (1, 2).

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