

Human nucleolus organizers on nonhomologous chromosomes can share the same ribosomal gene variants

(multigene families/concerted evolution/unequal crossing-over/nucleolar structure/mouse-human hybrid cells)

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Contributed by Frank H. Ruddle, May 8, 1981

ABSTRACT The distributions of three human ribosomal gene polymorphisms among individual chromosomes containing nucleolus organizers were analyzed by using mouse-human hybrid cells. Different nucleolus organizers can contain the same variant, suggesting the occurrence of genetic exchanges among ribosomal gene clusters on nonhomologous chromosomes. Such exchanges appear to occur less frequently in mice. This difference is discussed in terms of the nucleolar organization and chromosomal location of ribosomal gene clusters in humans and mice.

The 400 copies of the ribosomal genes (rDNA) found in humans are distributed over five acrocentric chromosomes: 13, 14, 15, 21, and 22 (1–5). The distribution of these genes is nonuniform in two respects. The number of rDNA copies is different on different chromosomes in an individual, and the number on a given chromosome varies from individual to individual in the population (6). This variability suggests that duplication and deletion of rDNA repeating units occurs frequently. Further, the entire human rDNA gene family appears to have evolved in a concerted fashion (7). That is, members of the family on nonhomologous chromosomes are far more similar to one another than would be expected if, following an ancestral duplication event, the genes on separate chromosomes had evolved independently. This similarity suggests that genetic exchanges take place among nucleolus organizers on nonhomologous chromosomes. Unequal crossing-over (8–11) would provide a mechanism for this phenomenon and also would explain the variability in rDNA gene number per chromosome.

Three polymorphisms in the human rDNA family have been revealed by restriction enzyme analysis of human genomic DNA (12–16). The model of unequal crossing-over between rDNA sequences on nonhomologous chromosomes would predict that, within sampling fluctuation, the three polymorphisms should occur uniformly on all nucleolus organizer chromosomes. To test this hypothesis, we have examined mouse-human somatic cell hybrids, in which one or a few human nucleolus organizer chromosomes are isolated on a constant background of mouse genetic material, in order to determine the distribution of the human polymorphisms among the various chromosomes. The results of this analysis are consistent with a generally uniform distribution of the polymorphisms over the chromosomes, although a few exceptions were found.

MATERIALS AND METHODS

The mouse-human hybrid cell lines used in these studies were constructed using L cells and two different human parental lines as described (17, 18). The WA series (17) was derived from the

WI38 human cell line. When the two WA lines were expanded for our studies, their content of human nucleolus organizer-containing chromosomes was evaluated by both isoenzyme and karyotypic analysis. Line WA V RdF94a was found to have chromosome 21, and line WA IVa had chromosome 22. The BDA series was derived from a different primary human fibroblast, GM 589 (18). Because of difficulties in carrying out karyotypic studies on these lines, their content of human nucleolus organizer-containing chromosomes was determined solely by isoenzyme analysis. BDA10a-4a contains chromosomes 13 and 14, and BDA10a-3a carries only chromosome 13 by these criteria. BDA14b-25 carries 14 and possibly 13 and 15. The placental DNAs used in this study have been described (13).

DNA purification, restriction enzyme digestion, agarose gel electrophoresis, Southern transfer, nick translation, hybridization, and autoradiography were carried out by described procedures (7, 13).

RESULTS

The *EcoRI* Polymorphism. The human rDNA repeating unit can contain either three or four *EcoRI* sites (ref. 15; Fig. 1). All repeating units have a site in the gene for 18S rRNA (called "18S gene"), one at the 3' end of the gene for 28S rRNA (called "28S gene"), and one 19 kilobase (kb) 3' to this 28S gene site. Most, but not all, repeating units have one additional site that is 6 kb 5' to the *EcoRI* site in the 18S gene (12, 14, 15). This polymorphism is detected when total human DNA is digested with *EcoRI* and used in a Southern transfer and hybridization experiment with an 18S gene probe. The genes with the *EcoRI* site 6 kb 5' to the site in the 18S gene produce a 6-kb fragment that hybridizes to the probe. Those genes that lack this site yield an 18-kb band. We estimated the population frequencies of these two types of rDNA through an analysis of 200 nucleolus organizers derived from 20 placental DNA samples. Each nucleolus organizer is thought to contain an average of 40 rDNA repeating units (6). Therefore, our sample represents 8000 rRNA genes. Equal amounts of DNA from each of 20 placental DNA samples were mixed and analyzed with respect to the frequencies of the two *EcoRI* types. About 80% of the rDNA repeating units had an *EcoRI* site 6 kb 5' to the site in the 18S gene; 20% of them lacked this site (Fig. 2, Table 1).

We examined this rDNA polymorphism in individual human nucleolus organizer-containing chromosomes, using hybrid cells derived from mouse L cells and either of two human parent cell lines. Mouse L cell DNA digested with *EcoRI* and hybridized to the 18S gene probe yielded neither 6-kb nor 18-kb fragments, so that the 6-kb and 18-kb human polymorphic forms could be scored unambiguously. We analyzed five hybrid lines carrying different and limited groups of human nucleolus or-

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Abbreviations: kb, kilobase; rDNA, ribosomal genes.

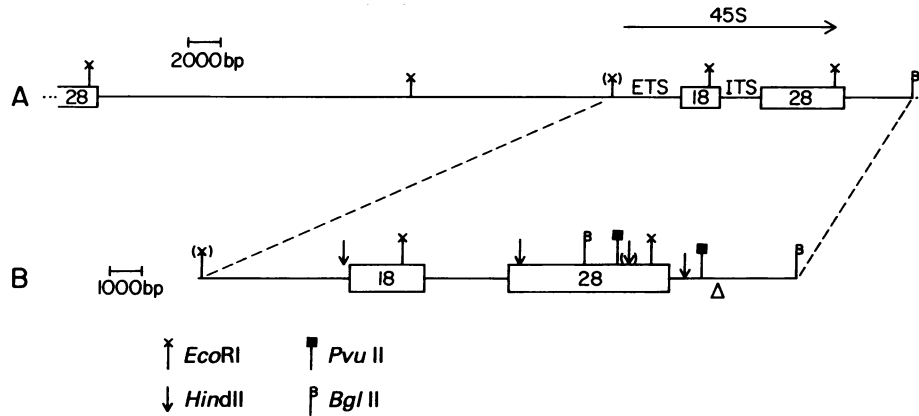


FIG. 1. (A) Structure of a human rDNA repeating unit. ETS, external transcribed spacer; 18, 18S gene; ITS, internal transcribed spacer; 28, 28S gene; bp, base pairs. The remainder of the repeat is nontranscribed spacer. The region transcribed into 45S precursor RNA is shown. (B) More detailed restriction enzyme map of portions of the rDNA repeat. Δ , Position of the nontranscribed spacer length variation as defined (13).

ganizer-containing chromosomes for the presence of this polymorphism (Fig. 3 and Table 2). In one line, only the 6-kb band was found. In another hybrid cell line, only the 18-kb band was observed. The remaining three cell lines contained human rDNA of both types.

The *Hind*II Polymorphism. Human 28S genes are cut by *Hind*II either at two positions or at only one (Fig. 1B). The *Hind*II site found in all genes is 4 kb 5' to the *Eco*RI site in the 28S gene. The polymorphic site is 750 bp 5' to this *Eco*RI site. When total human DNA is doubly digested with *Hind*II and *Pvu* II and used in a Southern transfer experiment with 28S gene probe, the fragments characteristic of both types of 28S genes are readily separable from each other (ref. 7; Fig. 1B). When we analyzed the mixture of 20 human DNA samples, we observed (Fig. 2, Table 1) that about 70% of the 28S genes in the human population are cut twice by *Hind*II (a correction was made for the fact that the smaller fragment, which is derived from genes cut twice by *Hind*II, has fewer 28S gene sequences that hybridize to the probe); the remaining 28S genes are cut once by *Hind*II.

The fragments of mouse 28S DNA generated by *Pvu* II/*Hind*II digestion are easily resolved from the polymorphic human 28S gene fragments in our five somatic cell hybrids; therefore, we were able to score them for this polymorphism (Fig. 4 and Table 2). All 28S genes in hybrid 21 were cut twice by *Hind*II. Hybrid 22 had both types of 28S genes. Two other hybrid cell lines had human 28S genes which were all cut once by *Hind*II.

The Nontranscribed Spacer Length Polymorphism. Human ribosomal genes show length heterogeneity in the nontranscribed spacer region within 4 kb of the 3' end of the 28S gene (7, 13, 16). Four length classes have been identified in the human population. *Bgl* II digestion of the mixture of placental DNA samples revealed five fragments that hybridize to a 28S gene probe (Fig. 2). The fragments labeled I-IV represent rDNA segments carrying 2 kb of the 28S gene and an additional 6.4(I), 5.8(II), 5(III), or 4.2(IV) kb of nontranscribed spacer DNA. The relative frequency of these four rDNA classes in the population sample is given in Table 1. Analysis of the four length classes in each mouse-human cell line are shown in Fig. 5 and Table 2.

The Number of rRNA Genes Within a Single Nucleolus Organizer. We determined the rDNA content of the nucleolus organizer found on chromosome 21. Five micrograms of DNA from the hybrid cell line carrying this chromosome were digested with *Bam* HI and run on an agarose gel. Known amounts

of a *Bam* HI-digested human rDNA clone (19) were added to the sample wells 1.5 hr after electrophoresis of the hybrid cell line DNA was started. This resulted in an electrophoretic separation of a 2.2-kb *Bam* HI fragment derived from the cloned rDNA from the same 2.2-kb *Bam* HI piece present in the digested hybrid cell DNA. By varying the known amount of cloned rDNA added to each 5- μ g aliquot of cell line DNA, we were able to quantitate the amount of human rDNA in the hybrid after Southern transfer and hybridization by using the cloned human fragment as probe (Fig. 6). We estimate the number of rRNA genes on this chromosome 21 to be approximately 30, after taking into consideration the fact that approximately 25% of the hybrid cells lack human chromosome 21.

Estimates of the total number of rRNA genes in the human genome have been based upon rRNA saturation hybridization experiments with nitrocellulose filter-bound human DNA (3-5). Therefore, rRNA gene number determinations depend critically on accurately estimating the saturation level, which is extremely sensitive to the purity of the rRNA used as probe. We also estimated the total number of rDNA repeating units in the human genome using the cloned rDNA fragments. Our results, using mixtures of placental DNA samples, approximate previous estimates of rDNA content per diploid genome and suggest an average of 350 copies (data not shown).

DISCUSSION

Restriction enzyme studies on the rDNA families of apes and humans revealed two types of structural features (7). On the one hand, each species was found to have evolved its own specific molecular markers in the nontranscribed spacer which vary between but not within a species. Since the rDNA of these species is distributed among many chromosomes, the homogeneity of these structural traits within each species indicates that genetic exchanges have occurred between nucleolus organizers on nonhomologous chromosomes.

A second set of structural features found in ape and human rDNA were polymorphic within an individual and between individuals in a species. The polymorphism that these markers displayed made it possible to examine their chromosomal distribution within a species. Our analysis of the distribution of three human rDNA polymorphisms among individual human nucleolus organizer-containing chromosomes is also consistent with the model of genetic exchange among rDNA sequences on nonhomologous chromosomes. Nucleolus organizers on nonhomologous chromosomes generally share the polymorphic forms found in the population as a whole. For example, chro-

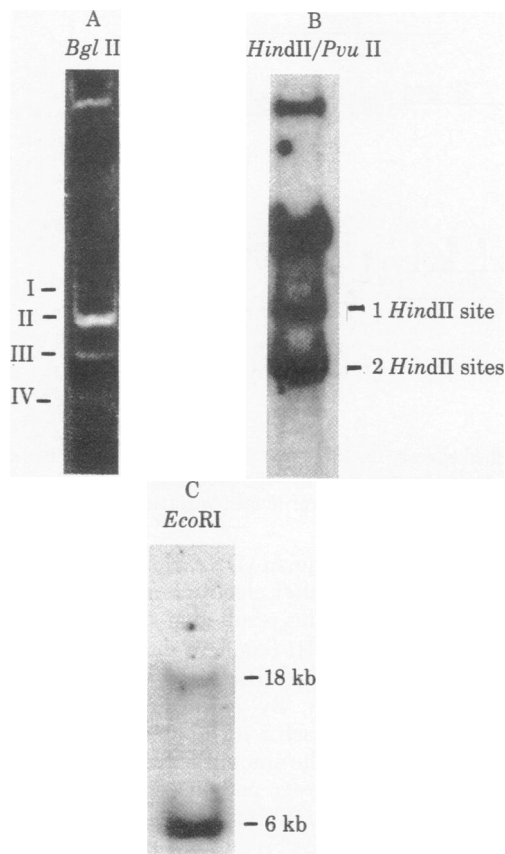


FIG. 2. Population frequencies of human rDNA polymorphisms; restriction enzyme digests of a mixture of 20 human placental DNA samples. (A) Length variation detected by *Bgl* II. Hybridization was with a 28S gene probe. The invariant 18-kb fragment extends 5' from the site in the 28S gene to another site in the nontranscribed spacer. The size of this fragment is the same in all human ribosomal genes examined. (B) *Hind*II variation detected by *Hind*II/*Pvu* II double digestion. Hybridization was carried out with a 28S gene probe. The two fragments characteristic of 28S genes cut once or twice by *Hind*II are indicated. The origin of the other fragments is explained in ref. 7. (C) *Eco*RI variation. Hybridization was carried out by using an 18S gene probe that contains the 5'-most 75% of the 18S gene. This probe does not detect the 7.0-kb human *Eco*RI fragment that contains the remainder of the 18S gene, the internal transcribed spacer, and almost all of the 28S gene (Fig. 1).

mosomes 13, 21, and 22 each carry length variant II and the 6-kb *Eco*RI fragment. Both chromosomes 21 and 22 carry 28S genes that are cut twice by *Hind*II. No rDNA variant was found

Table 1. Frequency of human rDNA variants in a sample of 8000 rDNA repeats

| Polymorphism | Frequency rDNA Repeats |
|--------------------|---------------------------|
| <i>Eco</i> RI | |
| 6-kb type | 0.80 |
| 18-kb type | 0.20 |
| <i>Hind</i> II | |
| One 28S gene site | 0.30 |
| Two 28S gene sites | 0.70 |
| Length variation | |
| I | 0.05 |
| II | 0.60 |
| III | 0.20 |
| IV | 0.15 |

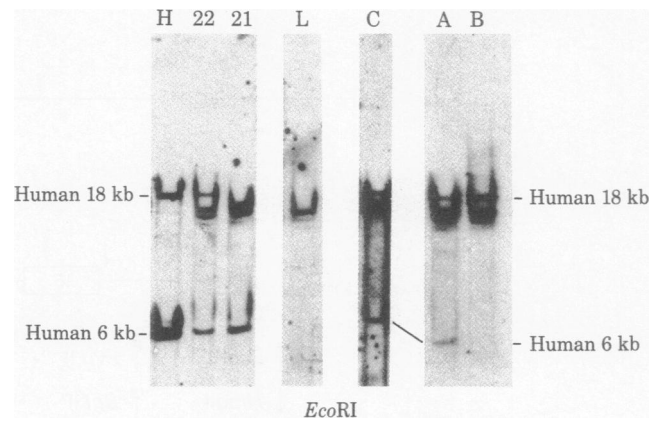


FIG. 3. *Eco*RI digestion patterns of mouse-human hybrid cell lines. Lanes: H, human DNA sample—parent of the BDA group of hybrid cells; 22, hybrid 22; 21, hybrid 21; L, L cell DNA; C, hybrid (13, 14); A, hybrid (13); B, hybrid (13?, 14, 15?). The number designation of each hybrid cell line indicates which of the human nucleolus organizer-containing chromosomes are present. When the number is in parentheses, the chromosome content was determined by isozyme analysis alone. The probe discussed in Fig. 2C was used in this experiment.

to be unique to any one of the D- or G-group chromosomes we studied. Although nucleolus organizers on nonhomologous chromosomes can share the same rDNA variants, none of the individual chromosomes we examined had representatives of all the variants present in the population. Some were completely or partially homogeneous for the three polymorphic forms. Thus, all 30 rDNA repeats on chromosome 21 have the 6-kb *Eco*RI fragment, length variant II, and 28S genes cut twice by *Hind*II. Another hybrid (13?, 14, 15?) only carries rDNA repeats with the rare 18-kb *Eco*RI fragment and 28S genes cut only once by *Hind*II.

These data are consistent with a model of concerted evolution involving unequal crossing-over both between sister chromatids and between nucleolus organizers on nonhomologous chromosomes. The first event, a relatively frequent one, would tend to cause the conversion of all members of a single cluster to the same form of any given polymorphism. The second event relatively infrequently could introduce blocks of rRNA genes from other chromosomes into the cluster, restoring heterogeneity. Both events would cause the number of rRNA genes per cluster to fluctuate randomly, without affecting the total number of these genes (6, 8–11). This model, based on our analysis of chromosomes from two human individuals, leads to the hypothesis that any given nucleolus organizer-containing chromosome, in the human population as a whole, can carry all polymorphic forms of rDNA and will do so in the proportions that these forms exist in the total human genome. No unique combination of rDNA polymorphisms would be expected to be characteristic of any one of the D- or G-group chromosomes. Direct proof of this hypothesis will require the extension of our analyses to chromosomes from many more individuals.

Our data on the chromosomal distribution of human rDNA variants contrasts with the results of similar studies in the mouse, which also has a multichromosomal distribution of the ribosomal genes (20–22). Analysis of the chromosomal location of mouse rDNA nontranscribed spacer length variants has shown that each nucleolus organizer has its own specific set of variants, which it does not share with nucleolus organizers on nonhomologous chromosomes (D. Treco, B. Taylor, and E. Eicher, personal communication). The same variants are found linked to the same chromosome in two apparently unrelated inbred strains, suggesting that the observed linkage patterns

Table 2. Distribution of human rDNA variants among human nucleolus organizer-containing chromosomes*

| | Parent 1 | Hybrid 21 | Hybrid 22 | Parent 2 | Hybrid (13) | Hybrid (13?, 14, 15?) | Hybrid (13, 14) |
|----------------------------|----------|-----------|-----------|----------|-------------|-----------------------|-----------------|
| Length variation | | | | | | | |
| I | - | - | - | - | - | - | - |
| II | + | + | + | + | + | + | + |
| III | + | - | + | + | - | + | - |
| IV | + | - | - | + | + | - | + |
| EcoRI polymorphism | | | | | | | |
| 6 kb | + | + | + | + | + | - | + |
| 18 kb | + | - | + | + | + | + | + |
| HindII polymorphism | | | | | | | |
| 1 site | + | - | + | + | ND | + | + |
| 2 sites | + | + | + | + | ND | - | - |

ND, not determined.

* Data are taken from Figs. 3-5.

may predate the origin of inbred strains. One interpretation of this difference between mouse and human rDNA is that the rate of interchromosomal exchange between rDNA clusters is lower in mice. Recent data on the structure of the nucleolus in humans and mice have a bearing on this hypothesis. A cytological study of the structure of nucleoli during meiotic prophase in human oocytes has shown that rDNA from two or three nonhomologous chromosomes often can contribute to the formation of a single nucleolus (23). The physical proximity of rDNA sequences from nonhomologous chromosomes in this organelle could provide opportunities for genetic exchange. On the contrary, the same

cytological study also reported that nucleoli in mouse oocytes do not contain rDNA sequences from more than one chromosome. This could limit the opportunity for nonhomologous exchanges. The molecular basis for the difference in the way mouse and human nucleoli are organized is unknown. However, we find it interesting that the genetic events responsible for the evolution of a multigene family might be significantly influenced by factors related to their biochemical function in the cell.

Another possibly significant difference between the mouse and human ribosomal gene families is in the chromosomal location of the nucleolus organizer region. In humans and apes, it is found on the stalks (24-27) between the short arm and satellite body of the D- and G-group chromosomes. In inbred mice, it is located very close to the centromere (20-22) on the long arm of chromosomes 12 and 15-19. The recovery of the products of genetic exchanges among rDNA clusters on nonhomologous chromosomes could be affected by the chromosomal location of the nucleolus organizer. If a reciprocal event occurred between two rDNA clusters on nonhomologous hu-

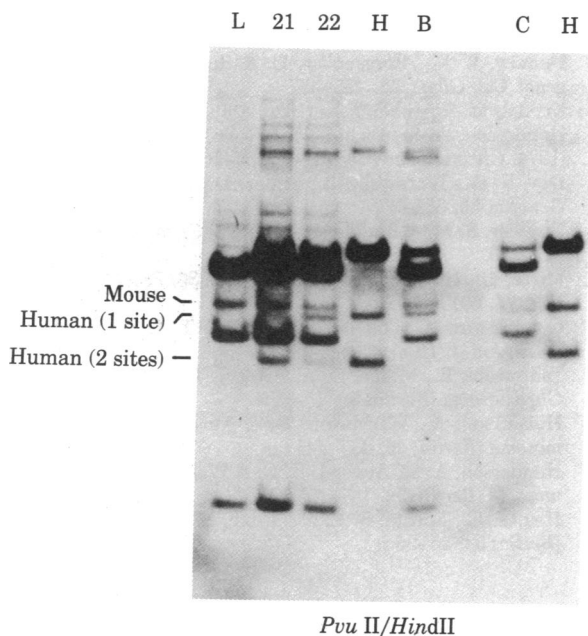


FIG. 4. Distribution of human *HindII* variants in the mouse-human hybrid cell lines. *PvuII/HindII* digestion was used to effect separation of the mouse and human bands. Lanes: L, L cell DNA; 21, hybrid 21; 22, hybrid 22; H, human DNA; B, hybrid (13?, 14, 15?); C, hybrid (13, 14). Hybridization was with a 28S gene probe. The human fragments indicative of 28S genes cut once or twice by *HindII* are shown. The human DNA sample is the parent of the BDA group of hybrid cells. Other experiments confirmed that the "band" in the position of genes cut once by *HindII* in hybrid 21 was an artifact. In all cases, the absence of bands was confirmed by longer autoradiographic exposures.

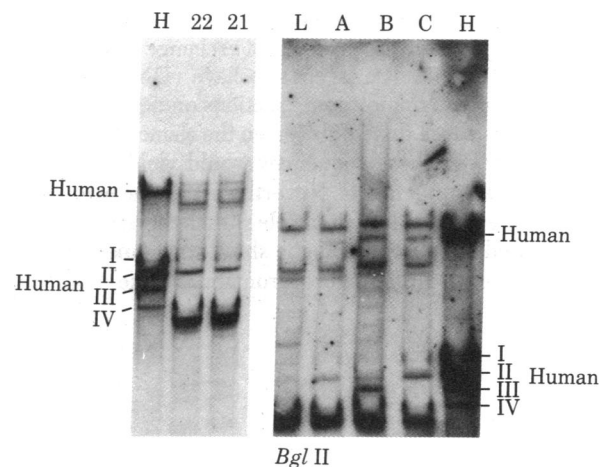


FIG. 5. Distribution of the rDNA length variation in the mouse-human hybrid lines as detected by *BglII* digestion. Lanes: H, human DNA; 22, hybrid 22; 21, hybrid 21; L, L cell DNA; A, hybrid (13); B, hybrid (13?, 14, 15?); C, hybrid (13, 14). Hybridization was with a 28S gene probe. The large human specific fragment was discussed in the legend to Fig. 3.

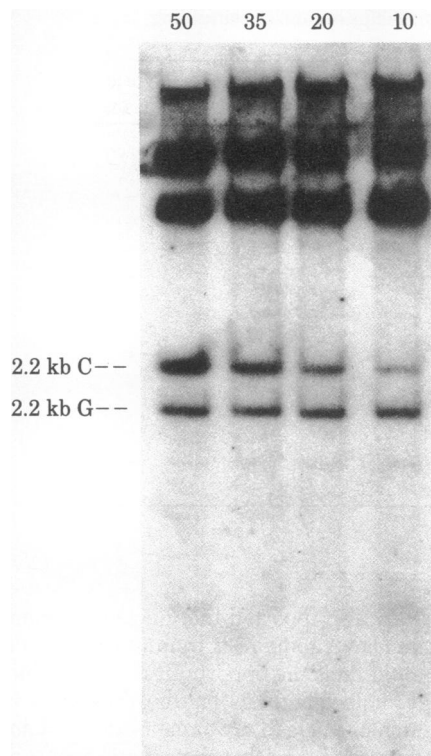


FIG. 6. Estimation of rDNA copy number in a human chromosome 21. The number at the top of each lane indicates the number of rRNA genes per diploid genome to which the added amounts of cloned rDNA fragments are equivalent. The strongly hybridizing bands above the 2.2-kb *Bam* HI cloned fragment (C) and genomic fragment (G) represent hybridization of the cloned probe with other added clone, human, or mouse rDNA fragments.

man chromosomes, only rDNA and satellite genetic material would be translocated. It is considered unlikely that trisomies involving these sequences would be physiologically disadvantageous. Therefore, the survival of chromosomes carrying rDNA crossover products would not be affected. The cytogenetic consequences of reciprocal exchange between two rDNA clusters on nonhomologous chromosomes in mice are more speculative. Such an event would result in the exchange of centromeres between the two chromosomes involved. If this led to unbalanced gametes, the products of exchange could be lost. Thus, it would seem advantageous to have rDNA sequences located terminally in homologous positions on nonhomologous chromosomes because this would lessen the chance that genetic exchanges between these sequences would yield deleterious chromosomal rearrangements. Nevertheless, in the many other mammalian species that have multiple sites in the genome, they may be located in heterochromatic short arms (apes, human, *Peromyscus*), centromeric heterochromatin (mouse, field vole)

or in telomeric regions (Chinese hamster) (see ref. 28). Within any one species, the position of these sites within each chromosome is usually the same. Further comparative studies on the mouse and human rDNA families are needed in order to provide a unified molecular explanation for the difference in their nucleolar organization pattern, genetic behavior, and chromosomal location.

The authors thank Ann Cunningham for technical assistance. This work was supported by Grants GM 2536501 (to N.A.) and GM 09966 (to F.R.). P.D. is a special fellow of the Leukemia Society of America.

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