Proc. Natl. Acad. Sci. USA
Vol. 77, No. 12, pp. 7323–7327, December 1980
Genetics

Molecular evidence for genetic exchanges among ribosomal genes on nonhomologous chromosomes in man and apes

(restriction maps/concerted evolution/unequal crossing-over/natural selection/chromosome abnormalities)

NORMAN ARNHEIM*, MARK KRYSTAL*, ROY SCHMICKEL†, GOLDER WILSON†, OLIVER RYDER‡, AND ELIZABETH ZIMMER§

*Department of Biochemistry and Molecular Biology Program, State University of New York, Stony Brook, New York 11790; †Department of Pediatrics, University of Michigan, Ann Arbor, Michigan 48107; ‡Research Department, San Diego Zoo, San Diego, California 92112; and §Department of Biochemistry, University of California, Berkeley, California 94720

Communicated by James V. Neel, July 21, 1980

ABSTRACT **We have found that human and ape ribosomal genes undergo concerted evolution involving genetic exchanges among nucleolus organizers on nonhomologous chromosomes. This conclusion is based upon restriction enzyme analysis of the ribosomal gene families in man and five ape species. Certain structural features were found to differ among (but not within) species even though the ribosomal genes have a multichromosomal distribution. Genetic exchanges among nucleolus organizer regions may be related to the well-known phenomenon of acrocentric chromosome associations observed in man and apes. Length variation in a region of the nontranscribed spacer was found in both chimpanzee species we examined. The nature of this length variation was found to be identical to that previously described in man. The origin of the length variation and its polymorphism within these three species might be explained by unequal alignment and unequal crossing-over among the ribosomal genes. An especially surprising finding was a nucleotide sequence polymorphism present in each individual human and ape we examined. Some ribosomal genes of each individual have a *Hin*dII site in the *28S* gene about 800 base pairs from the *Eco*RI site in this gene. The remaining *28S* genes lack this *Hin*dII site. The presence of this polymorphism within individuals of every species we examined suggests that it has been maintained by natural selection.**

Many multigene families, including the one that codes for the 18S and 28S ribosomal RNAs of higher organisms, appear to undergo concerted evolution¶ (1–6). In other words, within a single species individual members of this multigene family are far more similar to each other than would be expected if each gene had evolved independently of other family members. This fact is especially surprising for gene segments that have no known function. Such regions would be expected to accumulate mutational differences and exhibit variation within a single species. One model that can account for the concerted evolution of multigene families involves unequal crossing-over among the tandemly arranged members of the gene family (1–11).

By studying the structure of the ribosomal gene (rDNA) in man and apes, we hoped to examine the relationship between concerted evolution and the chromosomal organization of this multigene family. The ribosomal genes of man, chimpanzee, and pygmy chimpanzee are distributed among nucleolus organizers located on five pairs of chromosomes (12–17). The gorilla and orangutan have nucleolus organizers on two and eight pairs of chromosomes, respectively (15, 16, 18). The ribosomal genes of the gibbons are located on a single chromosome pair (15, 16, 19). If ribosomal genes on nonhomologous chromosomes were genetically isolated from each other then it might be expected that each nucleolus organizer region would evolve independently. Under these circumstances, we would expect to detect extensive variation in the structure of the nontranscribed spacer regions within individuals of a species. On the contrary, the greater the degree of genetic exchange between ribosomal genes on nonhomologous chromosomes, the more likely it is that the whole gene family will evolve in a concerted fashion.

In those primate species in which the ribosomal genes have a multichromosomal distribution, cytological studies have shown that at metaphase the chromosomes carrying these genes can "associate" with one another (20–23). Such associations might result from the interactions of rDNA sequences from nonhomologous chromosomes that cooperated in the formation of a single nucleolus in the previous interphase. Alternatively, these associations might be symptomatic of genetic exchanges among ribosomal genes on nonhomologous chromosomes—exchanges that would be necessary for the concerted evolution of the whole gene family by the unequal crossing-over mechanism. We interpret our structural data as supporting such genetic exchanges. Our findings also indicate that not every portion of the ribosomal gene undergoes concerted evolution. Some regions may be maintained in a polymorphic state.

MATERIALS AND METHODS

DNA Samples. Human DNA was prepared from placenta, and ape DNA was prepared from blood or liver samples obtained through the national primate centers or the San Diego Zoo (24–26). DNA from 10 humans, 20 chimpanzees (*Pan troglodytes*), 2 pygmy chimpanzees (*Pan paniscus*), 5 gorillas (*Gorilla gorilla*), 2 orangutans (*Pongo pygmaeus*), and 1 gibbon (Hylobates) were used.

Restriction Enzyme Analysis. Restriction enzymes were purchased from commercial sources. DNA was transferred from agarose gels to nitrocellulose filter paper (27).

Two types of radioactive probes were used to detect restriction fragments that carried the *18S* and *28S* genes: (*i*) mouse or human 18S or 28S RNA labeled with ^{125}I (28) or (*ii*) ^{32}P-labeled probes prepared by nick translation (29) or copy translation (30) of cloned DNA. The cloned DNAs contained portions of *18S* or *28S* genes from human or mouse (31–33). The specific activity of the probes was 2×10^8 cpm/μg of DNA.

Hybridization of the labeled probe to the nitrocellulose filter and autoradiography was carried out as described (34–36). Molecular weight markers were derived from digests of wild-type phage and a number of well-characterized deleted λ phages (37).

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Abbreviations: bp, base pair(s); kb kilobase(s).

¶ We prefer to use the term "concerted" rather than "coincidental" evolution; for a discussion see ref. 24.

RESULTS

Structural Features of Transcribed Ribosomal Gene Sequences. In all higher organisms that have been studied, each ribosomal gene copy is composed of transcribed and nontranscribed DNA segments (see Fig. 1*B*). The transcribed region in mammals is about 13 kilobases (kb) in length and is the source of the 45S precursor to the 18S and 28S RNAs (38, 39). Fig. 1*A* shows a restriction enzyme map of the transcribed region of a typical human or ape ribosomal gene; it was possible to draw a single map for all of these species because the transcribed portion was highly conserved.

Although conserved, there were some minor differences among species. For example, small additions or deletions of DNA had occurred within the *28S* gene. The amount of DNA between the two *Bam*HI sites in the *28S* gene was not identical in all species. The size of the fragment found in gibbon and orangutan digests, about 1.3 kb, was smaller by about 100 base pairs (bp) than the homologous fragment in gorilla, chimpanzees, or human digests (Fig. 2*A*). The idea that these fragment size differences arose by additions or deletions of DNA is supported by the finding that the restriction enzymes that recognize other sequences and that cut out fragments encompassing the two *Bam*HI sites are also smaller in the gibbon and orangutan.

Structural Features of Ribosomal Genes That Vary Among Species but Are Not Polymorphic Within a Species. Significant differences among human and ape ribosomal genes were found in the nontranscribed spacer region. This region varies in length among vertebrate species. In man it is about 30 kb (40). In apes the exact size is unknown, but our evidence is consistent with a length close to that found in man. For the most part, those regions of the nontranscribed spacer that varied among species were not polymorphic within each species. *Pvu* II digestion of human and ape DNAs revealed such a pattern. The *28S* gene contained one *Pvu* II site, and two fragments were detected with 28S probes after *Pvu* II digestion. The larger fragment appeared to be identical in size among all the species examined. It contained, in addition to the 5′ half of the *28S* gene, the internal transcribed spacer, the *18S* gene, the external transcribed spacer, and a small amount of nontranscribed spacer. On the other hand, the size of the smaller *Pvu* II fragment varied among the species we studied. This fragment contained the remainder of the *28S* gene and extended in the 3′ direction into the nontranscribed spacer. In human and pygmy chimpanzee digests, the fragment was 2.4 kb (Fig. 1*C*, *Pvu* IIa); in the chimpanzee and gorilla digests, it was 2.25 kb (Fig. 1*C*, *Pvu* IIb) or about 150 bp smaller. The size of the orangutan and gibbon fragments were 6.8 and 2.32 kb (Fig. 1*C*, *Pvu* IIc), respectively. We were unable to detect any variation in the size of these small *Pvu* II fragments within or among individuals of a species. The 150-bp size difference between human and chimpanzee fragments is thought to be due to the presence in every human ribosomal gene of an additional 150 bp of DNA, which must lie between the *Hin*dII and *Pvu* II sites in the nontranscribed spacer.

Another example of a structural difference that exists among species, but is not accompanied by polymorphism within or among individuals of a species, is the finding that every human ribosomal gene has an *Hpa* I and *Bgl* II restriction enzyme site adjacent to the *Bam*HI site in the nontranscribed spacer that is closest to the *28S* gene. Neither of the chimpanzee species or any of the other apes we examined had *Bgl* II or *Hpa* I sites in homologous positions (Fig. 1*C*).

Structural Features of Ribosomal Genes That Are Polymorphic Within a Species. *Length heterogeneity.* There are two *Bam*HI sites in the *28S* gene of man and apes. Therefore, three fragments containing *28S* DNA were expected after *Bam*HI digestion. One fragment (the fastest migrating band in wells 1–5 of Fig. 2) was expected to be derived from within the *28S* gene as discussed above. A second fragment contained

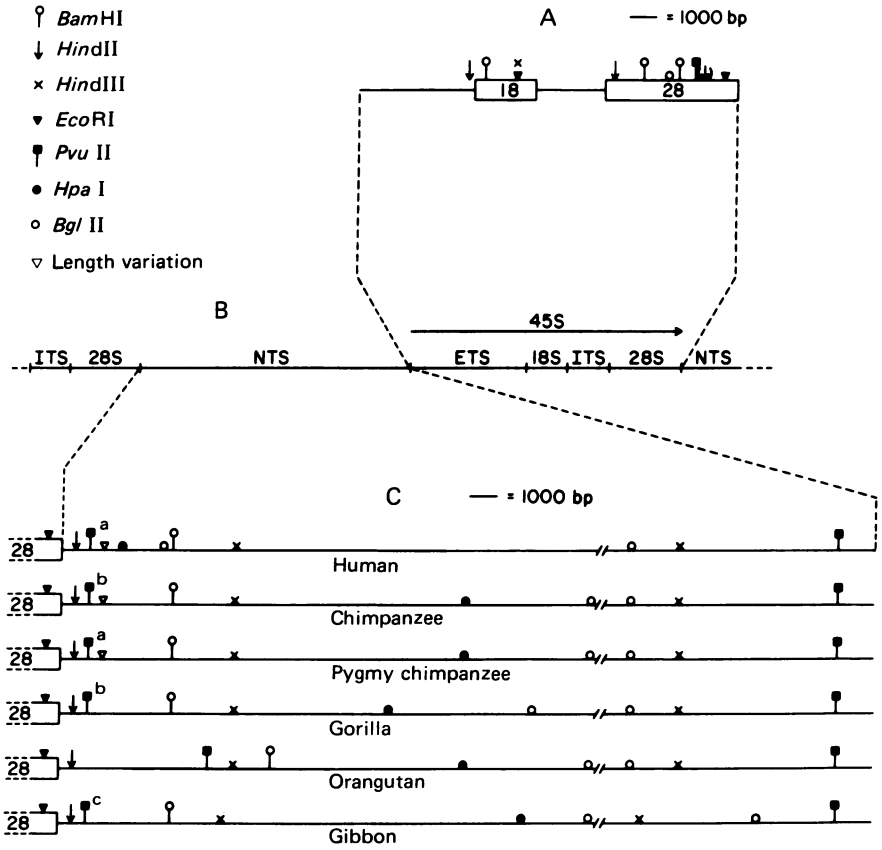

FIG. 1. Restriction enzyme maps of human and ape ribosomal genes. (*A*) Map of the transcribed region of the gene. The polymorphic *Hin*dII site is shown in parentheses. (*B*) Schematic representation of the repeating unit. In addition to the *18S* and *28S* structural genes, the internal transcribed spacer (ITS), external transcribed spacer (ETS), and nontranscribed spacer (NTS) segments are shown. The 5′→3′ orientation of the gene with respect to the origin of transcription of the 45S precursor RNA is also given. (*C*) Map of the nontranscribed spacer region. The break in the horizontal line indicates the spacer regions not mapped in our study. The human, chimpanzee, and pygmy chimpanzee rDNA show length heterogeneity in the region of the NTS between the *Pvu* II (for a discussion of Pvu II sites a, b and c, see text) and *Bam*HI sites adjacent to the end of the *28S* gene.

some *28S* DNA, the internal transcribed spacer, and a portion of the *18S* gene. The size of this fragment, 5.6 kb, was identical among the species we examined, suggesting that the length of the internal transcribed spacer has not changed significantly. The third *28S* DNA-containing *Bam*HI fragment extended in the 3′ direction from the 3′ end of the *28S* gene into the nontranscribed spacer to the position of the closest *Bam*HI site. The size of this fragment was virtually identical in the gorilla and gibbon (6.3 kb). In the orangutan it was considerably larger (9.6 kb). When human and chimpanzee rDNAs were examined, one fragment in each species was almost the same size as the fragment in gibbon and gorilla. However, several other fragments extending into the nontranscribed spacer were also detected. In man, this heterogeneity was found to result from nontranscribed spacer length variation among the ribosomal gene family within individuals (41). This length variation originates in the region of the nontranscribed spacer between the *Pvu* II site adjacent to the 3′ end of the *28S* gene and the closest *Bam*HI site in the spacer. We showed that in the chimpanzees the same region of the nontranscribed spacer also varied in length. The length heterogeneity of this region in both man and chimpanzee was discrete; only four different length classes have been found in the human population (41) and among the chimpanzee samples there were four or five major types. Some chimpanzees showed minor amounts of additional length classes (Fig. 2*B*). The size of the length classes in both man and chimpanzee were not random with respect to one another. In both species, each length class differed from the next largest or smallest by about 750 bp. Like humans, every individual chimpanzee we examined had a combination of several of the various length classes found in the population.

The size of the *Bam*HI fragment, which extends from the *28S* gene into the nontranscribed spacer in the gorilla or gibbon, was different from the homologous orangutan fragment. The absence of variation in the size of this fragment within each of these three species provides an additional example of a structural feature that varies among but not within a species.

Nucleotide sequence variation. Man and each species of ape that we examined had two types of *28S* genes. Some genes contained two *Hin*dII sites, whereas others contained only one.

When human DNA was digested with *Hin*dII, four fragments were revealed by Southern blotting experiments using 28S probe. The largest fragment also contained *18S* rDNA. Of the remaining three fragments, two were cut both by the enzymes *Pvu* II and *Bgl* II. Knowledge of the positions of the *Bgl* II and *Pvu* II sites (Fig. 1*C*) led us to conclude that these two *Hin*dII fragments contain overlapping sequences and must come from two types of *28S* genes: the larger of these two fragments comes from *28S* genes with one *Hin*dII site, and the smaller *Hin*dII fragment from genes with two sites.

The possibility exists that base modification rather than a nucleotide sequence difference was responsible for the different digestion patterns of the two types of *28S* genes. We can, however, rule out the possibility of the most common modified base in DNA of higher organisms, 5-methylcytosine (42). This modified base is found almost exclusively in the doublet CpG (43). The enzyme *Hin*dII recognizes four hexanucleotide sequences: G-T-C-G-A-C, G-T-C-A-A-C, G-T-T-G-A-C and G-T-T-A-A-C (44). The first sequence contains the CpG doublet and is also recognized by the restriction enzyme *Sal* I (45). If all of the *28S* genes contained the G-T-C-G-A-C sequence at the polymorphic site, but some of these sites were methylated and some were not, then the *Hin*dII polymorphism should be detected with the enzyme *Sal* I. On the contrary, *Sal* I did not cleave the *28S* gene. We conclude that the *Hin*dII polymorphism is the result of nucleotide sequence variation, but we cannot rule out a more exotic base modification.

Another example of nucleotide sequence polymorphism can be detected in the nontranscribed spacer with *Eco*RI. Two *Eco*RI sites exist within the transcribed regions of human rDNA (40, 46) and both were present in all of the apes we examined (Fig. 1*A*). One is 600 bp from the 3′ end of the *28S* gene (40). *Eco*RI fragments extending in the 3′ direction from this site into the nontranscribed spacer were not detected with our 28S probes. The other *Eco*RI site in the transcribed region lay in the *18S* gene. The human *Eco*RI fragment that spans these two *Eco*RI sites was virtually identical in size to those present in the other species we examined. The length variation in the *28S* gene observed by *Bam*HI analysis (discussed above) was difficult to detect in fragments almost 7.0 kb in length. Most of the *18S* gene will be on *Eco*RI fragments that extend in the 5′ direction through the external transcribed spacer and into the nontranscribed spacer. In man there were two major fragments of this type (Fig. 3, lanes 1 and 3). One came from ribosomal genes whose closest *Eco*RI site in the spacer was 6 kb from the *Eco*RI site in the *18S* gene. This site appeared to be in the nontranscribed spacer adjacent to the origin of transcription of the 45S precursor RNA. This fragment has been purified by molecular cloning (33). The second major class of ribosomal genes lacked this *Eco*RI site. The closest *Eco*RI site in the nontranscribed spacer of these genes was 18 kb (in the 5′ direction) from the *18S Eco*RI site. Studies on the ape ribosomal genes showed that only the chimpanzee, pygmy chimpanzee, and gorilla had ribosomal genes that contained the *Eco*RI site adjacent to the origin of transcription (Fig. 3, lanes 2, 4–6). Each of these three species were polymorphic and also had ribosomal genes that lacked the site adjacent to the origin of transcription, as shown by the fact that larger *18S* DNA containing *Eco*RI fragments were also detected.

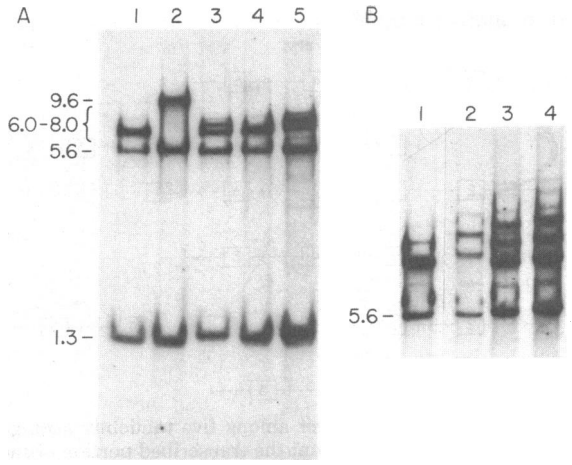

FIG. 2. *Bam*HI digestion fragments of human and ape ribosomal genes detected with a 28S probe. (*A*) Lanes: 1, gibbon; 2, orangutan; 3, chimpanzee; 4, gorilla; 5, human. The size or range in size of the fragment classes are given in kilobase pairs. (*B*) The 5.6-kb and larger fragments after more extensive electrophoresis. Lanes: 1, pygmy chimpanzee; 2, human; 3, human/chimpanzee mixture; 4, chimpanzee. Only the 5.6-kb *Bam*HI fragment is indicated. The other fragments vary in size from 6.0 to >8.0 kb in steps of ≈700–800 bp.

DISCUSSION

We found that different regions of the ribosomal gene in man and apes have different characteristic evolutionary patterns. The transcribed segments have undergone few nucleotide substitutions, although other small structural changes seem to have occurred. This is consistent with the general finding that the *18S* and *28S* RNA gene sequences are highly conserved during animal evolution (47).

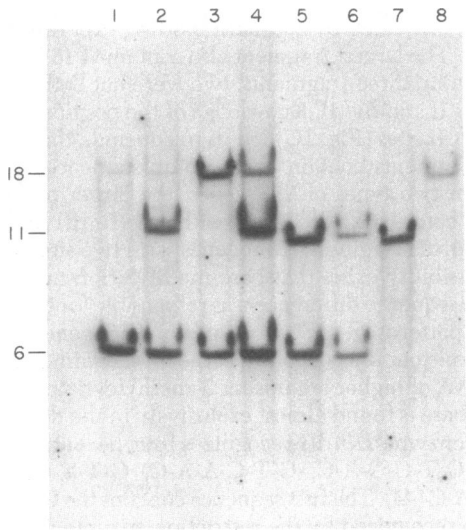

FIG. 3. *Eco*RI digests of human and ape DNA. These fragments were detected with an 18S probe. The probe carries only 75% of the *18S* gene and cannot detect the 7.0-kb *Eco*RI fragment that extends from the 3′ end of the *18S* gene through the internal transcribed spacer and almost to the end of the *28S* gene. Lanes: 1, human no. 1; 2, chimpanzee no. 1; 3, human no. 2; 4, chimpanzee no. 2; 5, pygmy chimpanzee; 6, gorilla; 7, orangutan; 8, gibbon.

Concerted Evolution. Our data also show that differences among species can exist in the structure of the nontranscribed spacer without any comparable variation existing within or among individuals of a species. This demonstrates that at least some regions of the ribosomal gene undergo concerted evolution. Consider, for example, the structure of the nontranscribed spacer in the human and the chimpanzee (Fig. 4). Each human ribosomal gene has an *Hpa* I site 2.6 kb (in the 3′ direction) away from the *Eco*RI site inside the *28S* gene. The chimpanzee lacks this site. The fact that none of the other great apes have an *Hpa* I site in this position suggests that the most recent common ancestor of man and chimpanzee also lacked this site. Therefore, the *Hpa* I site most probably originated in the human lineage after the chimpanzee–human evolutionary divergence and was eventually fixed in every ribosomal gene. The same argument applies to the absence in the chimpanzee of the *Bgl* II site that lies close to the *Hpa* I site in man. Finally, the chimpanzee and human differ in the size of the small *Pvu* II fragment that is cut out of each ribosomal gene. Regardless of what the size (or sizes) of the small *Pvu* II fragment was in their common ancestor, each species is now fixed for alternative sequences. A likely explanation for concerted evolution involves unequal crossing-over and random drift. Each unequal crossover generates a chromosome (or chromatid) that is either duplicated or deleted with respect to the number of members of the gene family (Fig. 5*A*). Computer simulations (7–11) have shown that repeated cycles of unequal crossing-over can result, eventually, in a new gene family whose members all were derived from only one or a few of the original family members

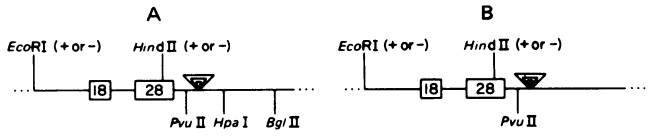

FIG. 4. Some structural features of human (*A*) and chimpanzee (*B*) ribosomal genes. Features common to both species are shown above the genes. Both species have *Eco*RI (+ or −) and *Hind*III (+ or −) nucleotide sequence polymorphism. Each species also has nontranscribed spacer length variation adjacent to the *28S* gene (▽). Species-specific structural features (shown below each gene) that show no polymorphism within individuals of these species are indicated.

as a consequence of random drift. The balance between the rate of this crossover fixation process and the rate at which new mutations occur will determine the equilibrium level of structural heterogeneity among the multigene members within a species.

Genetic Exchanges Among Ribosomal Genes on Nonhomologous Chromosomes. Our most significant finding relates the concerted evolution of the ribosomal gene family with the multichromosomal distribution of these sequences. We know that the ribosomal genes in man and chimpanzee are distributed among five pairs of chromosomes. Since the pygmy chimpanzee also has five rDNA-containing chromosomes, it is likely that the common ancestor of these species had the same multichromosomal distribution. Therefore, in spite of the fact that the ribosomal genes are found on nonhomologous chromosomes, each chromosome pair has not evolved independently. The whole gene family has undergone concerted evolution. Thus, it is likely that the mutation that gave rise to the *Hpa* I site in the human lineage arose only once and was propagated throughout the whole ribosomal gene family as a result of unequal genetic exchanges taking place between rDNA sequences on nonhomologous as well as homologous chromosomes. It is extremely unlikely that the same *Hpa* I mutation arose independently on each of the five chromosome pairs and was subsequently fixed rather than eliminated on each chromosome pair.

Our data support the idea that genetic exchanges can occur between rDNA sequences on nonhomologous chromosomes but do not indicate the frequency needed to explain concerted evolution. Smith (8) has argued that the rate need not be high in an analogy with population genetic theory, which shows that only very rare migrants are needed to maintain genetic similarity among two isolated populations of a species.

The question of rate may have some practical importance. A large body of data exists showing that the association of nonhomologous human acrocentric chromosomes is dependent upon their ribosomal gene content (21–23). The most frequent chromosome fusions (Robertsonian translocations) in man involve these same acrocentric chromosomes (48). Whether this genetic behavior is primarily caused by the association of these chromosomes in nucleolus formation or by direct genetic exchanges like those which must have occurred during human and ape evolution remains to be determined.

The question of whether genetic exchanges occur among ribosomal genes on nonhomologous chromosomes has been studied in another animal system. *Drosophila melanogaster*

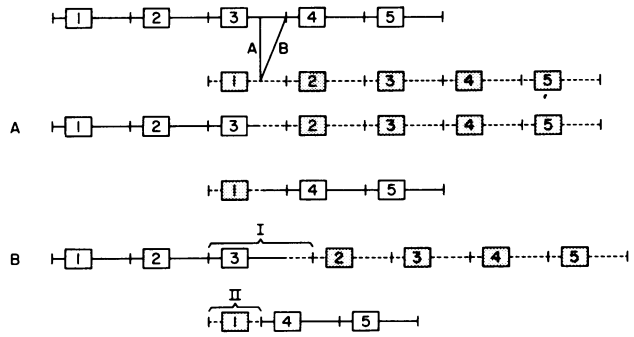

FIG. 5. Unequal crossing-over among five tandemly arranged ribosomal genes. Squares represent the transcribed portion of each of the five genes; horizontal lines represent the nontranscribed segment. Clear and dotted squares distinguish the chromosomal origin of each array. Unequal alignment and pairing of the two arrays can be followed by equal or unequal genetic exchange. The reciprocal products of equal exchange are shown in (*A*) and of unequal exchange in (*B*), in which I and II refer to the ribosomal genes that have become duplicated or deleted for nontranscribed spacer material, respectively.

rDNA exists on both the X and Y chromosomes. Some features of ribosomal gene structure are shared by genes on both of these chromosomes. Other structural traits are X- or Y-specific (49–52), which appears inconsistent with unrestricted exchange. Apparently, the *Drosophila* rDNA system differs from that of man and the apes. From our evolutionary perspective, there appear to have been no restrictions to the movement of rDNA sequences among nonhomologous chromosomes.

Ribosomal Gene Length Heterogeneity. As has been found in *Xenopus* (53, 54), *Drosophila* (55, 56), and mouse (31, 32, 46) ribosomal gene systems, a region of human, chimpanzee, and pygmy chimpanzee nontranscribed spacer DNA exhibits length heterogeneity. Although the homologous region in the other apes lack this length heterogeneity, still other segments of the nontranscribed spacer DNA undetected with our 18S and 28S probes may vary in length. However, we need to reconcile the concerted evolution of one portion of the human and chimpanzee ribosomal gene family with the persistence of the length heterogeneity in both species. This is most elegantly accomplished by the unequal crossing-over model. Fig. 5*B* shows the consequence of unequal alignment and unequal exchange between a pair of ribosomal genes. Not only do the daughter chromosomes carry either a greater or lesser number of genes than the parental chromosomes, but in addition the two genes actually involved in the crossover event are either duplicated or deleted for nontranscribed spacer DNA and differ in length from the parental genes. Such events can generate length heterogeneity in the region of the crossover and at the same time can provide the driving force for the crossover fixation process in adjacent regions. The tandem array will tend towards homogeneity for regions not involved in the actual crossover event, while at the same time remaining heterogeneous for the regions involved in such events.

Ribosomal Gene Nucleotide Sequence Polymorphisms. There is one structural feature of human and great ape ribosomal genes that does not seem easily reconcilable with a purely stochastic model of multigene evolution. In both the nontranscribed (*Eco*RI) and transcribed (*Hin*dII) region of the human, chimpanzee, pygmy chimpanzee, and gorilla ribosomal genes, a nucleotide sequence polymorphism exists in every individual examined. The nature of the polymorphism is identical among these species. The *Hin*dII polymorphism in the 28*S* gene is also found in the other apes we examined. The detection of a polymorphism *per se* is not unexpected because it is conceivable that we might catch the ribosomal gene family in the act of becoming fixed for a newly arising structural type during evolution. However, the ubiquity of the *Hin*dII polymorphism in each species tested—species which have clearly undergone fixation for alternative traits at other positions in the ribosomal gene—argues against the interpretation of our *Hin*dII data as a "transient" polymorphism. The location of the *Hin*dII polymorphism within the 28*S* gene and the *Eco*RI polymorphism immediately adjacent to the origin of transcription of the 45S precursor RNA hints at natural selection being involved in the maintenance of these polymorphisms. The molecular mechanisms responsible for this selection are unknown.

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