Structure and Evolution of Human and African Ape rDNA Pseudogenes[1]

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We discuss the evolutionary significance of four aberrant 18S rDNA clones that were obtained from human, chimpanzee, and gorilla DNA libraries. We show that these clones carry representatives of a small 18S rDNA pseudogene family that arose in a common ancestor of these species. Aspects of their structure and phylogenetic distribution suggest that the 18S pseudogenes no longer interact genetically with normal ribosomal genes and therefore may not be linked to nucleolus organizer regions.

Introduction

The genes that encode 18S and 28S ribosomal RNAs (rDNA) are present in multiple tandemly arrayed copies in most eukaryotic genomes. The 18S and 28S genes of orangutan (*Pongo pygmaeus*), gorilla (*Gorilla gorilla*), chimpanzee (*Pan troglodytes*), and human (*Homo sapiens*) are found on several pairs of nonhomologous chromosomes (Henderson et al. 1972, 1974, 1976, 1979; Evans et al. 1974; Tantravahi et al. 1976). One interesting aspect of the rRNA genes in these species is that they evolve in unison, or concertedly, despite their multichromosomal distribution (Arnheim et al. 1980).

To learn more about the molecular genetic mechanisms that are responsible for this phenomenon, we isolated representatives of the functional rDNA repeats from human and chimpanzee genomic DNA libraries. In addition, we purified unusual 18S rDNA-containing recombinants from human, chimpanzee, and gorilla DNA libraries. Characterization of these aberrant clones and comparison with the normal rDNA repeats led us to ask how the unusual genes were generated and how they were evolutionarily maintained despite rDNA turnover in these species.

1. Key words: rDNA, pseudogenes, evolution.

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Material and Methods

Enzymes

Restriction endonucleases were purchased from New England Biolabs, Boehringer-Mannheim, and Bethesda Research Laboratories. We used the digestion conditions recommended by the suppliers.

Cloning Studies

We obtained the clones shown in figure 1*B–E* by screening partial human and chimpanzee *Eco*RI genomic DNA libraries (in Charon 4A) with a mouse 18S rDNA probe (Benton and Davis 1977). The human library was a generous gift from Dr. J. Slightom; the chimpanzee library was constructed using standard procedures (Blattner et al. 1977; Lawn et al. 1978). The human clones in figure 1*B* and *C* were later subcloned into the plasmid vector pBR-322 (Arnheim and Kuehn 1979). One of these subclones, a *Hin*dIII/*Eco*RI fragment from the human rDNA pseudogene (fig. 1*C*, probe 4), was used to screen a gorilla *Eco*RI genomic DNA library (kindly provided by Dr. Alan Scott). Two recombinants were isolated, one of which is shown in figure 1*F*.

Southern Transfer and Hybridization Studies

For the experiments shown in figure 2*a*, *b*, and *c* we used ^{32}P-labeled probes that were nick translated (Rigby et al. 1977) to a specific activity of at least 1.5 $\times$ 10^8 cpm/μg with ^{32}P dCTP. All hybridizations were carried out in 3 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl, 0.015 M Na citrate), 0.1% sodium dodecyl sulfate (SDS), and 0.06% each of polyvinylpyrrolidone (PVP), Ficoll, bovine serum albumin (BSA), with 15 mM ethylene diamine tetra acetic acid (EDTA) at 65 C for 16–18 h. For each experiment 2 $\times$ 10^6 cpm of probe per milliliter of hybridization buffer were used. Hybridized filters were washed three times (1 h each) at 65 C in 3 $\times$ SSC, 0.1% SDS before autoradiography.

Results and Discussion

We screened human, chimpanzee, and gorilla partial *Eco*RI genomic DNA libraries with rDNA and rDNA related probes. Five of the six clones that we isolated are shown in figure 1*B–F*. Extensive restriction enzyme mapping studies of the 18-kb human (fig. 1*B*) and the 11.5-kb chimpanzee (fig. 1*D*) clones and comparison with genomic mapping data (Arnheim et al. 1980; see fig. 1*A*) suggested that the clones were derived from functional ribosomal genes. Indeed, both were found to be capable of initiating transcription in a human in vitro assay system (Miesfeld and Arnheim 1982 and unpublished data).

In contrast, the remaining four recombinants were unusual in several respects that would support the view that they are pseudogenes. The human (fig. 1*C*), chimpanzee (fig. 1*E*), and two presumably nonallelic gorilla (one of which is shown in fig. 1*F*) "aberrant" clones had *Eco*RI fragments atypical in length (15 kb); they lacked the evolutionarily conserved mammalian 18S gene *Eco*RI site (Southern 1975; Arnheim and Southern 1977; Fuke et al. 1981); and, although they retained nontranscribed spacer and internal transcribed spacer homologies detected by restriction enzyme mapping and cross-hybridization experiments (summarized in fig. 1), they lacked 28S gene sequences. In addition, although they hybridized strongly to a normal 18S gene probe, a 250-bp fragment that contained the human

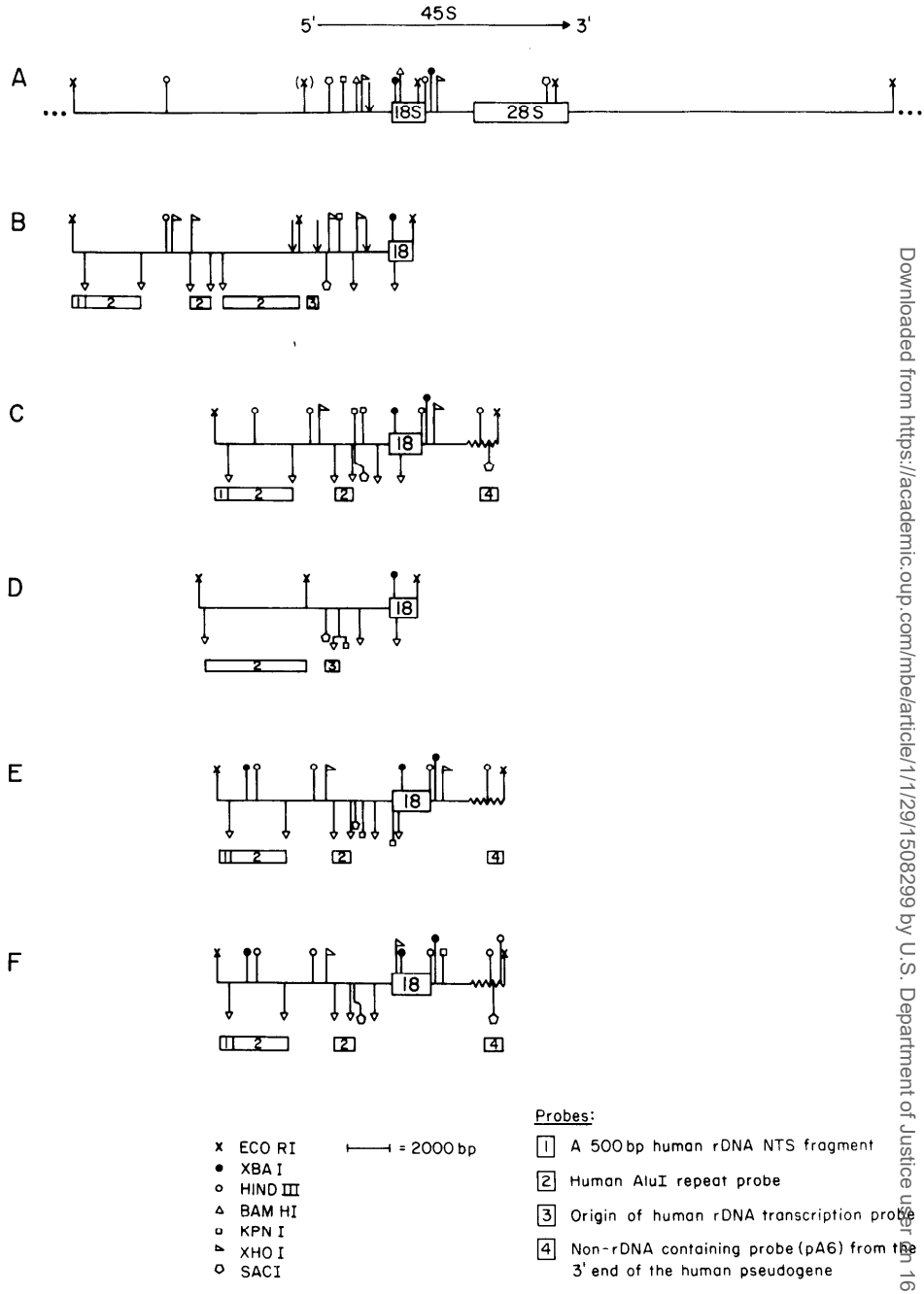

FIG. 1.—Restriction enzyme maps of human, chimpanzee, and gorilla 18S rDNA genes and pseudogenes. *A*, A human rDNA repeating unit. A polymorphic *Eco*RI site (Arnheim et al. 1977; Wellauer and Dawid 1979; Arnheim et al. 1980; Nelkin et al. 1980; Erickson et al. 1981; Higuchi et al. 1981; Micsfeld et al. 1982) is shown in parentheses. Each 45-kb repeating unit (Wellauer and Dawid 1979) is composed of nontranscribed spacer (NTS), external transcribed spacer (ETS), 18S, internal transcribed spacer (ITS), and 28S sequences. The origin and direction of transcription of the 45S precursor are shown. *B*, The human 18S rDNA clone. The probes used to define homologous regions between the normal genes and pseudogenes by cross-hybridization experiments are also shown. *C*, The human 18S pseudogene. *D*, The chimpanzee 18S rDNA clone. *E*, The chimpanzee 18S pseudogene clone. *F*, A gorilla 18S pseudogene clone. Sites above the solid lines denote complete mapping data; sites below the line signify that only partial mapping information is presented.

rDNA origin of transcription (Miesfeld and Arnheim 1982) (region 3, fig. 1*B*) reacted equally weakly with the human, chimpanzee, and gorilla 15-kb clones when compared with normal rDNA genes (human data shown in fig. 2*a*). This suggests that these rDNA variants may be deleted for regions around the origin of transcription. Preliminary in vitro transcription assays using subclones carrying the weakly hybridizing region of the human 15-kb clone as a template failed to show transcriptional initiation (Miesfeld and Arnheim, unpublished data). Other more extensive deletions of nontranscribed spacer segments were also characteristic of the pseudogenes (fig. 1).

To eliminate the possibility that the unusual human recombinant was a cloning artifact, we tested whether predicted restriction enzyme fragments derived from it could be detected in the human genome. Two human placental DNAs digested with *Sac*I and hybridized with a mouse 18S probe (fig. 2*b*, lanes *B* and *C*) gave a strong 11-kb 18S DNA-containing fragment typical of normal human rDNA repeats and a faint 6.9-kb band that corresponded in size to the 18S-containing *Sac*I piece from the aberrant human clone (lane *A*). The relative intensities of these two genomic bands also suggested that the 18S rDNA variant specific fragment might be present in low copy number in the human genome. Indeed, subsequent quantitation experiments showed that there are between four and 10 copies in each human, chimpanzee, and gorilla genome (data not shown).

Unlike normal rDNA, 28S sequences were not found 2.5 kb 3′ to the 18S region of the aberrant clones. To determine whether this 3′ end was derived from other regions of the normal rDNA repeating unit, we hybridized *Eco*RI restricted human placental DNA with a subclone from the 3′ end of the human pseudogene (the 850-bp *Hin*dIII/*Eco*RI fragment, designated pA6; fig. 2*c*, lane *D*). Because the sizes of the bands that we detected were not attributable to any of the normal rDNA *Eco*RI fragments (19 kb, 18 kb, 12 kb, 7.5 kb, 6 kb; fig. 1*A*), we concluded that pA6 contains sequences of non-rDNA origin which are themselves present in low copy number. Sequences homologous to pA6 were also found at the 3′ terminus of the chimpanzee and gorilla 18S pseudogene 15-kb fragments (fig. 1*E*, *F*). Interestingly, although each gorilla recombinant contained a large 15-kb *Eco*RI fragment that hybridized with pA6, they were flanked at the 3′ end by different, smaller *Eco*RI segments. Restriction enzyme analysis (data not shown) indicated that the 15-kb *Eco*RI fragments were identical to each other, in contrast to their 3′ flanking sequences. We inferred from this observation that the clones carried nonallelic pseudogene loci.

The presence of non-rDNA homologies at the 3′ ends of these 18S pseudogenes suggests possible ways in which these pseudogenes arose and how they were evolutionarily maintained. For example, 18S rDNA pseudogene formation might have been initiated following integration of exogenous DNA sequences into the internal transcribed spacer region of a normal rDNA repeat. Ribosomal genes, in fact, may have been the targets for the integration of mobile elements during evolution (Treco et al. 1982). In many *Drosophila* species a fraction of the ribosomal genes have insertions in the 28S gene, and repeats bearing these insertions are almost completely transcriptionally silent in vivo (i.e., pseudogenes; see Long and Dawid [1980] and Beckingham [1982]). However, it is not clear whether these insect 28S pseudogenes constitute an evolutionarily stable subpopulation of ribosomal genes within nucleolus organizing regions. It has been proposed that the insertions may move between repeats from one generation to the next through

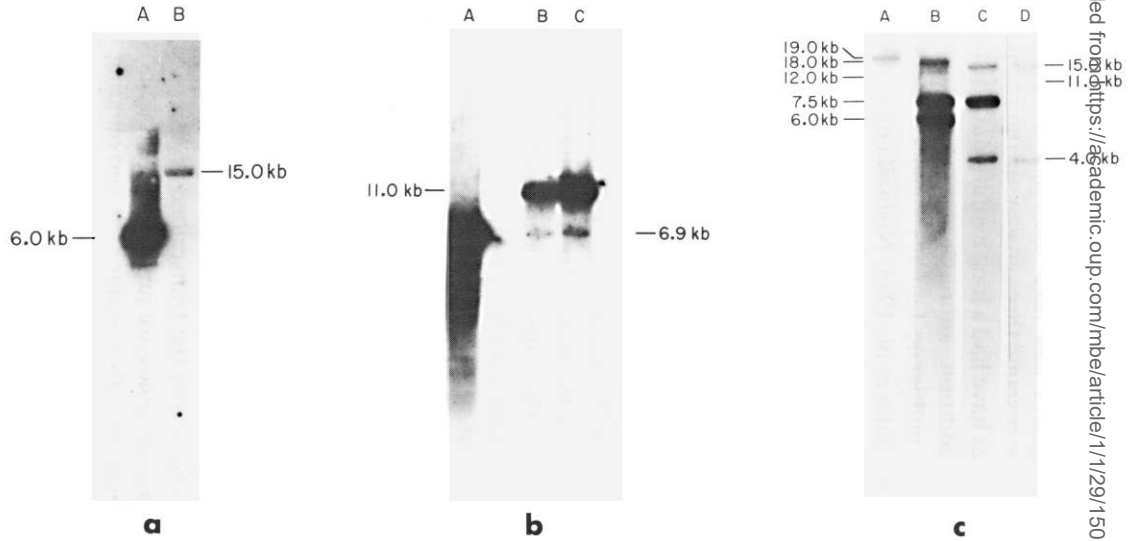

FIG. 2.—*a*, The human 15-kb clone contains only a small portion of the origin of rDNA transcription. Equimolar amounts of the normal rDNA 6-kb *Eco*RI fragment (lane *A*) and the 15-kb rDNA clone (lane *B*) were hybridized with a 250-bp probe containing the origin of human rDNA transcription in a Southern transfer experiment (fig. 1*B*, probe 3). The human 15-kb clone hybridizes at approximately 1/10 of the control level. *b*, The aberrant 15-kb human clone is represented in the human genome. *Sac*I digests of the human 15-kb clone (lane *A*) and two human placental DNA samples (lanes *B*, *C*) were hybridized with a mouse 18S DNA probe. The normal rDNA-containing bands in lanes *B* and *C* are 11.0 kb in length. The faint bands at 6.9 kb correspond in size to the *Sac*I fragment in lane *A*. *c*, The human and chimpanzee 15-kb clones contain non-rDNA sequences. *Eco*RI-digested human placental DNAs were hybridized with the following probes: Lane *A*—A small fragment including the terminal 600 bp of the human 28S gene immediately 3′ to the *Eco*RI site in the gene (see fig. 1*A*) and additional 3′ nontranscribed spacer sequences which should hybridize to the 19-kb fragment. Lane *B*—A lambda Charon 4A phage clone containing human ETS, 18S, ITS, and the 5′ portion of the 28S gene which should hybridize to the 18-kb, 6-kb, and 7.5-kb pieces. Lane *C*—pA4, a 3.4-kb *Hin*dIII subclone of the human 18S pseudogene (fig. 1*C*) adjacent to pA6. Lane *D*—The 850-bp *Hin*dIII/*Eco*RI subclone of the human 18S pseudogene (pA6) (see fig. 1*C*). The sizes of the expected *Eco*RI fragments from normal rDNA repeats are shown to the left of lane *A*; the sizes of the non-rDNA fragments we observed with probes C and D are noted to the right of lane *D*. We conclude that fragment pA4 contains both rDNA and non-rDNA sequences; fragment pA6 contains only non-rDNA.

the normal process of unequal meiotic crossing-over (Rae 1982). This would allow some inactive 28S rDNA pseudogenes to be reactivated in subsequent generations. This hypothesis is consistent with the observation that, besides the insertion itself, no obvious structural differences can be detected between the pseudogenes and normal genes even though such differences would be expected to accumulate once an active gene were permanently silenced. Even at the nucleotide sequence level no differences have accumulated within the origin of transcription region in the interrupted genes (Long et al. 1981). In contrast, the significant structural differences between the primate rDNA pseudogenes and their normal counterparts suggest that this pseudogene family cannot be rescued through genetic interactions with normal genes and that these pseudogenes have had a long evolutionary history in isolation from normal rDNA arrays. In addition, although other pseudogenes have been found in association with many multigene families (Jacq et al. 1977; Lacy et al. 1979; Bentley and Rabbits 1980; Jahn et al. 1980; Nishioka et al. 1980; Vanin et al. 1980; Denison and Weiner 1981; Selker et al. 1981; Steinmetz et al. 1981; Karin and Richards 1982; Lemischka and Sharp 1982; Martin et al. 1983), the persistence of 18S pseudogenes over the last 5 million years (i.e., since these species last had a common ancestor; Sarich and Wilson [1967]) is unexpected in view of the evolutionary behavior of the normal rDNA clusters in these three primates. The ribosomal gene repeats of man and the apes evolve concertedly (Arnheim et al. 1980); that is, within an individual the ribosomal gene units are more similar to each other than would be expected if each repeat evolved independently of all others (see Brown et al. 1972; Hood et al. 1975; Tartof 1975; Dover et al. 1982; Arnheim 1983). By examining the molecular structure of primate rDNA repeats, Arnheim et al. (1980) found that, despite a multichromosomal distribution, different species-specific restriction sites have been fixed in the clusters of even the three most recently diverged species—human, chimpanzee, and gorilla (Sarich et al. 1967). That 18S pseudogenes escaped elimination during the normal process of rDNA turnover in these three lineages suggests that the pseudogenes are genetically isolated from normal rDNA repeats. This situation would arise, for example, if pseudogenes were relegated to junctional positions between rDNA clusters and neighboring chromosomal sequences. Being at the extreme end of the array might effectively prevent genetic interaction of the rDNA pseudogene with the remaining genes. However, a recent study of yeast rDNA organization suggests that junctional segments contain rDNA sequences which have not undergone significant structural divergence in the transcribed regions (Zamb and Petes 1982). If the human and ape pseudogenes are junctional fragments, our clones are potentially useful for analyzing the DNA sequences surrounding nucleolus organizer regions.

A second situation that would lead to genetic isolation would be one in which rDNA sequences moved to a new location outside of the array (Childs et al. 1981; Denison and Weiner 1981; Jagadeeswaran et al. 1981). Possible mechanisms include the recombinational excision of looped-out normal rDNA segments during meiotic unequal alignment of the rDNA repeating units, followed by integration at a new locus. The 3′ non-rDNA sequences may define the original target site. The dispersed copy, and any descendants that arose from it by amplification, could have evolved independently of the original parental locus. Additional data will allow us to distinguish between these alternatives.

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