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The human ribosomal RNA genes: structure and organization of the complete repeating unit

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Summary. The complete repeating unit of the human ribosomal RNA gene has been reconstructed by the cloning of approximately 27 kilobases (kb) of non-transcribed spacer. The structure of this tandemly repeated gene can now be studied in its entirety. We report the analysis of spacer DNA by molecular cloning and its organization in the genome by Southern transfer analysis. These studies reveal both length and sequence variation of the spacer. Sequence variations are distributed throughout the spacer while the length variations exist near the 5′ end of the transcript and just beyond the 3′ end. The human spacer shares extensive homology with primates but little with other mammals. Within the primates the degree of homology reflects the rapid evolutionary changes characteristic of the primate group.

Introduction

The 13 kb transcribed portion of the human ribosomal RNA gene complex alternates with a 31 kb "spacer" segment to form a 44 kb unit which is tandemly repeated (Wellauer and Dawid 1979). In this paper we report that the complete repeating unit of the ribosomal RNA gene has been reconstructed by cloning. The repeating unit consists of a 13 kb region which is transcribed as the 45S ribosomal RNA precursor and a 31 kb region whose function is not defined and is referred to as the "spacer". This 44 kb unit is tandemly repeated about 40 times on each of the five acrocentric chromosomes to form a cytogenetically distinct 1,700,000 base unit. The cytogenetically distinct area is known as the *secondary constriction,* a rather extended and thin region of the chromosome. Although the 13 kb transcribed region has been studied extensively, relatively little knowledge has been gained for the much longer region known as the "spacer". As the name implies, this DNA may serve DNA and chromosome packing requirements and not have important physiologic functions. Some regulatory functions have been found to reside in the region upstream from the promoter as has been found with the messenger RNA genes (transcribed by polymerase II). Signals for the initiation of rRNA synthesis reside in the spacer and are species specific (Morgan et al. 1984; Grummt 1981, 1982; Miesfeld and Arnheim 1982). There are probably other functions yet to be assigned to this area such as nucleolar organization, tissue specific or metabolic responsive expression of rRNA, and origins for its own DNA replication.

As is the case with the coding sequences (Gonzalez et al. 1985), the ribosomal spacer has increased in length with evolutionary time. The yeast spacer is 2.5 kb (Skryabin et al. 1984), length variants in *Xenopus laevis* are 3.4 or 5.7 kb (Morgan et al. 1983), 4.1 to 5.1 kb in *Drosophila melanogaster* (Coen et al. 1982), 28 kb in chicken (McClements et al. 1978), and 31 kb in both mouse (Kominami et al. 1981) and primates (Arnheim et al. 1980). In *Drosophila* and *Xenopus* the spacer contains tandem repeats of a 240 and a 149 bp unit respectively. The human spacer is much more heterogeneous; previous reports have shown that it contains at least one region that is tandemly duplicated, several Alu repeats, sequences that are unique to the human ribosomal repeat, and still others that are interspersed elsewhere in the genome (Erickson and Schmickel 1985; Higuchi et al. 1981; Wilson et al. 1984).

This paper reports a detailed study of the human ribosomal spacer that is contained in 27 kb of spacer DNA defined by two restriction fragments; a 12 kb EcoRI and a 15 kb BamHI fragment (Fig. 2b). This has permitted a careful examination of the total ribosomal repeating unit and provides the basis for understanding the organization of the ribosomal genes as a whole. It also allows a detailed study of human DNA variation and its relationship to primate evolution.

Materials and methods

Genomic DNA preparations

Human DNA for cloning was prepared by lysis of frozen placental tissue at 60°C with 2% sodium dodecyl sulfate (SDS), 0.1 *M* EDTA, and 0.15 *M* NaCl, pH 8.0. After phenol treatment and ethanol precipitation, the DNA was treated with ribonuclease, re-extracted with phenol, and reprecipitated as previously described (Schmickel 1973). The C fragment was enriched by digestion of 250 µg of DNA with EcoRI (BRL)

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followed by preparative agarose gel electrophoresis; fractions of the gel between 10–14 kbp were cut out, the DNA was electroeluted and adsorbed to DEAE-cellulose. The eluted fractions which hybridized with C_{EB} (isolated from λ hurib-8) (Higuchi et al. 1981) were used for cloning.

Bam HI digested genomic DNA was centrifuged to equilibrium in CsCl (1.70 g/ml) as described previously (Schmickel 1973), the heavy (G-C rich) shoulder was pooled, dialyzed against 10 m*M* Tris pH 7.4 – 1 m*M* EDTA, and precipitated. The resuspended DNA was then applied to a preparative 0.7% agarose gel and 13–18 kbp fractions were cut out and treated as above. Those fractions which hybridized to C_{EB} were used for cloning.

DNA to be used for electron microscopy was enriched by two rounds of CsCl density gradient centrifugation (specific gravity of the fractions 1.71 and 1.72 g/ml) followed by trioxsalen crosslinking by UV light. The crosslinked DNA was preparatively R-looped to 18S and 28S rRNA (Thomas et al. 1976), dialyzed against TEN (10 m*M* TRIS pH 7.4, 1 m*M* EDTA, 10 m*M* NaCl), and centrifuged (1.73 g CsCl/ml). Fractions containing ribosomal sequences were pooled.

Primate DNA was prepared as above from chimpanzee, gorilla, orangutan, and rhesus monkey spleen kindly supplied by the Yerkes Regional Primate Research Center, Atlanta, Georgia.

Vector DNA and hosts

λ EMBL-4 was grown in *E. coli* LE 392 (Sup E, Sup F, hsdR (r^-, M^+), gal^-) and λ EMBL-3 was grown in C600 (Sup E). Plasmids pBR322 (Bolivar et al. 1977), KP5 and 772 (K. Peden unpublished work) were grown in *E. coli* DH1 (rec A, hsdR (r^-, M^+), sup E). Phage DNA was prepared as described in Maniatis et al. (1982) while plasmid DNA was prepared by the method of Holmes and Quigley (1981).

Genomic cloning

λ EMBL-3 (for DC fragment) and λ EMBL-4 (for C fragment) DNA were restricted with EcoRI and Bam HI (use of second enzyme reduces the ability of the insert to religate), phenol extracted, and ethanol precipitated. Standard ligation reactions (10 µl) containing 100 µg DNA/ml and 1 unit T4 DNA ligase (BRL) were incubated at least 16 h at 12°C before packaging in vitro with extracts from *E. coli* BHB 2688 and BHB 2690 (Hohn and Murray 1971) or with the in vitro packaging kit from Promega Biotech. After packaging, the mixture was diluted in 500 µl SM (0.1 *M* NaCl, 0.1% $MgSO_4$ 0.05 *M* Tris pH 7.5, and 0.01% gelatin), titered, plated, and screened according to the method of Benton and Davis (1977) on colony/plaque screen (NEN) with nick translated ^{32}P-labeled purified C_{EB} fragment as probe.

R looping

18S and 28S rRNA were prepared from ribosomes extracted from HeLa cells essentially as described by Schmickel (1973). Genomic DNA and rRNA were mixed at 5 µg/ml each in 70% recrystallized formamide, 85 m*M* Pipes pH 7.8, and 10 m*M* EDTA and incubated at 49°C for at least 3 h according to the procedure of Thomas (1976). R-loops were spread for electron microscopy by adding 2 µl of R-loop sample to 20 µl of hyperphase solution (70% formamide).

Restriction enzyme analysis of genomic and cloned rDNA

The clones and subclones are identified by the nomenclature of Wellauer and Dawid (1979) and modified by us (Erickson et al. 1981; Erickson and Schmickel 1985). Genomic DNA was restricted with the indicated enzyme and probed with nick translated plasmid subclones from the spacer fragments plus previously isolated pB_{ES} (Wilson et al. 1982) and pD_{ES} (Erickson et al. 1981). Southern transfers (Southern 1975) were performed using GENE SCREEN^{plus} (NEN) and hybridized according to the manufacturer's instructions. Most restriction sites were verified by their presence in the appropriate subclone. Partial restriction analysis of pC_{BE} from two distinct C fragment isolates was performed by end-labeling as described by Maniatis et al. (1982).

Results

Electron microscopy of rDNA

High molecular weight total genomic DNA was separated from smaller fragments by NaCl gradient centrifugation. Since rDNA has a high G plus C content, two successive CsCl density gradients were employed to enrich it prior to a final preparative R-looping with 18S and 28S rRNA and CsCl centrifugation (see Methods). The recovered rDNA, enriched about 35-fold, was incubated with fresh rRNA under R-loop conditions and spread for electron microscopy. More than 85 molecules with R-loops were photographed and 29 molecules with at least two complete transcript regions (Fig. 1) were selected for length measurements. The mean length of 25 of the analyzed molecules was 43.85 ± 1.59 kb and that of the remaining four repeats was 49.81 ± 0.72 kb. This is in very good agreement with the observations of Wellauer and Dawid (1979) who report 22 repeats with an average size of 43.65 ± 1.27 kb and two repeats with lengths of 28.6 kb and 53.9 kb. Since the sizes of the A and B fragments (Fig. 2A) are invariant, these results suggest that the significant length variation of the rDNA repeat is localized in the nontranscribed spacer.

Isolation and analysis of spacer fragments

The 31 kb spacer has been isolated in the form of three different recombinant clones. The first 4 kb of the spacer near the 5′ end of the D fragment are contained in lambda AD_{BB}-8 and AD_{BB}-19. This region contains a length variation (Fig. 1a, b), is described in detail by Erickson and Schmickel (1985), and will not be discussed further here. The remaining 27 kb of the spacer are the subject of this report.

Fifteen separate 12 kb EcoRI C fragment recombinants were identified, plaque purified, and found to be similar by restriction analysis with EcoRI and Bam HI (see map Fig. 2C). Six of these 15 clones were subcloned into plasmid for size comparison with each other and with the clone previously isolated (Higuchi et al. 1981). The seven plasmid clones were restricted with EcoRI and Bam HI and electrophoresed through 1% agarose. The generated restriction pattern demonstrated that three of the clones had a 4.7 kb Bam-Eco fragment (C_{BE}) and four clones had a 4.5 kb Bam-Eco fragment. To investigate this variation, each of the seven clones was digested with Hind III and EcoRI and the 7 kbp (C_{HE}) and 5 kbp (C_{EH}) (Fig. 2B) fragments were isolated by electroelution and purified

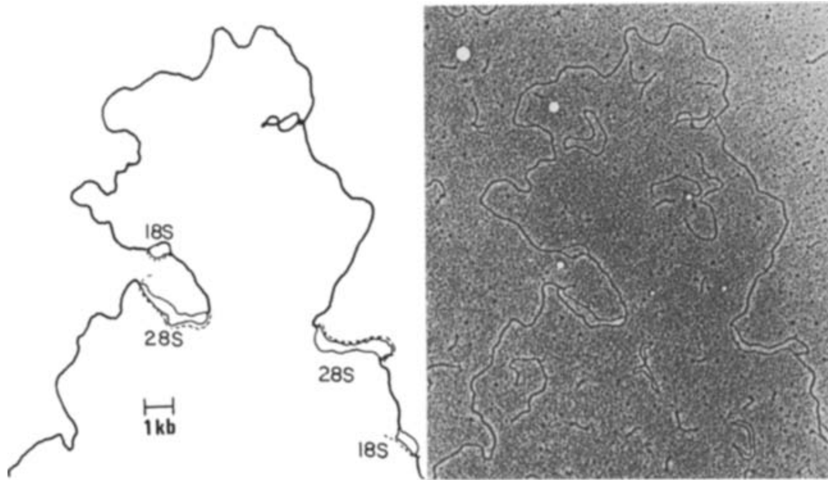

Fig. 1. Electron micrograph of human genomic DNA hybridized to two copies of 18S and 28S rRNA. The short space between 18S and 28S is the transcribed spacer (~2 kb); the long space between 28S and 18S rRNA is the nontranscribed spacer (~31 kb). SV40 and Mp7 DNA were used as internal controls for length measurements

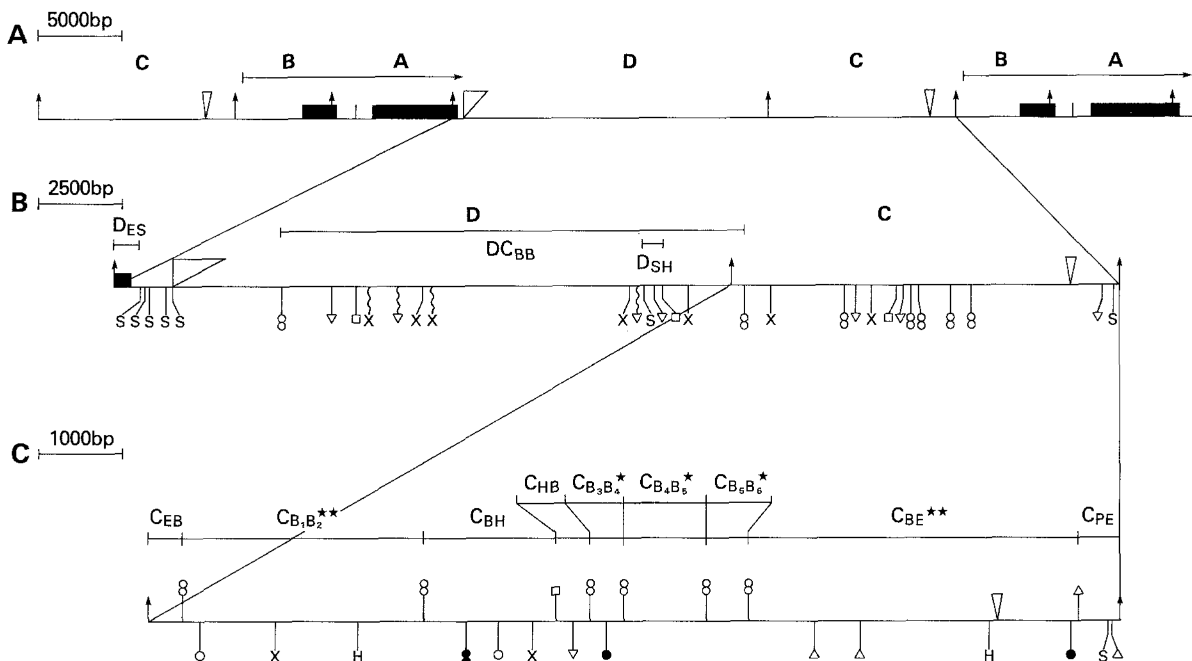

Fig. 2. A Organization of the entire ribosomal gene repeat. Letters A, B, C, and D define the four EcoRI (↑) fragments. *Long arrow* above shows the precurser 45S rRNA that yields 18S, 5.8S, and 28S rRNA *(solid rectangles)*. *Triangles* denote areas of size variations. **B** Expanded view of the 19 kb D and the 12 kb C EcoRI fragments. D_{ES}: 960 bp fragment between EcoRI and SalI sites; D_{SH}: 1050 bp fragment between SalI and HindIII sites. **C** Expanded view of the C fragment with probes and/or subclones C_{EB} (0.4 kb), C_{B1B2} (2.9 kb), C_{BH} (1.6 kb), C_{HB} (0.4 kb), C_{B3B4} (0.38 kb), C_{B4B5} (1.2 kb), C_{B5B6} (0.6 kb), C_{BE} (4.5 kb), and C_{PE} (0.5 kb). * Denotes presence of the repetitive Alu sequence, ** two separate elements within fragment. Restriction site key: EcoRI (↑); SalI (S); BamHI (8); PstI (□); HindIII (▽); Xba (X); HincII (H); BglII (●); SstII (○); SstI (●); and ApaI (Y). *Wavy line* (⌇) with restriction site marker denotes site that is variable

over DEAE-cellulose. The individual fragments were digested separately with HinfI or HpaII, end-labeled with klenow and ^{32}P-dATP or ^{32}P-dCTP, electrophoresed through 1.2% agarose or 4% acrylamide gels, and autoradiographed. Restriction patterns from each enzyme digest of C_{EH} fragments were identical for all seven clones. However, analysis of similarly restricted C_{HE} fragments showed that the three larger clones had a HinfI and HpaII fragment that was about 150 bp larger than in the smaller four clones. Partial restriction analysis of end-labeled C_{BE} fragments from two representative clones carrying different size inserts localized the length variation to 1.5 kb from the 3′ EcoRI end.

The remaining 15 kb of the ribosomal DNA gene were isolated as a BamHI fragment in λEMBL-3 as described in the Methods section. The HindIII and BamHI-HindIII fragments of this clone were subcloned into pBR322 to yield pD_{BH} (2.2 kb), pD_{HH} (9 kb), and pD_{HB} (2.3 kb). The recombinants initially obtained were used to generate a map of the BamHI fragment (Fig. 2B). Other genomic clones of this area and their comparison will be the subject of a separate detailed report (D. A. Whiteman, in preparation). Preliminary results indicate length heterogeneity of the 9 kb D_{HH} fragment in genomic DNA as detected by Southern analysis of human DNA samples. The size of this fragment varies in normal popula-

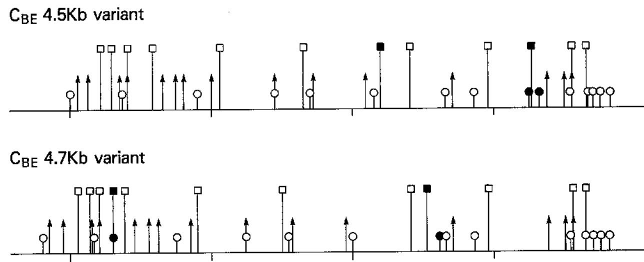

Fig. 3. Map of the two size variants of C_{BE} region; approximately 4.5 kb and 4.7 kb. HinfI (⇡), AluI (♀), and Sau3A (⍑). *Dark solid* restriction site marker denotes site not present in the other variant

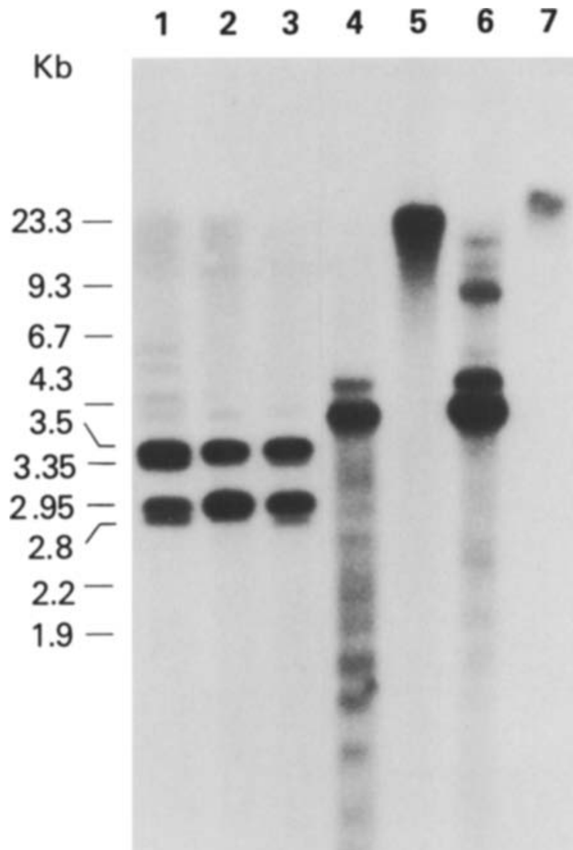

Fig. 4. Southern blot of ApaI digested genomic DNA probed with C_{PE}. *Lanes 1, 2,* and *3,* different human individuals; *lane 4,* chimpanzee; *lane 5,* gorilla; *lane 6,* orangutan; *lane 7,* rhesus monkey. *Bands* indicate size variation (2.8 and 2.95 kb) and restriction site variation (3.35 and 3.5 kb) see text and Fig. 1C

tions, but that of the other two fragments, D_{BH} and D_{HB}, does not.

Restriction map of cloned and genomic ribosomal spacer DNA

A battery of restriction enzymes was used to generate the spacer map as shown in Fig. 2B, C. The preliminary map was then used to obtain subclones (or restriction fragments) for Southern blotting experiments on genomic DNA in order to determine genomic variation within the C and D fragments. The length variation at the 3′ end of the C fragment (C_{BE}), as analyzed by partial restriction is displayed in Fig. 3. Comparison of the restriction sites for these two fragments indicates that there are multiple restriction sites that differ in the two clones, as shown in Fig. 3. These variations are apparent in genomic DNA digested with ApaI and Southern transfers probed with C_{PE}. Figure 4 demonstrates two genomic fragments of 2.95 and 2.8 kbp, indicating that the size variation exists in genomic DNA. This variation appears to be present in all DNA analyzed. The double band above (3.35 and 3.5 kb) reflects an absence of the 5′ ApaI site in about 50% of the genes. This finding further supports the observations of Wilson et al. (1984), who has previously reported sequence variation in this region of the ribosomal gene.

The restriction map of rDNA in Fig. 2A shows the basic ribosomal gene repeat with details of the spacer in Fig. 2B. This map was obtained by the data derived from cloned DNA and Southern analysis. Many enzyme digests (PstI, HindIII, Xbal) and subsequent hybridizations also revealed minor bands that are not indicated here but represent restriction site heterogeneity. Since most minor bands accounted for less than 10% of the total hybridization, an alternative interpretation could be that of partial restriction of the genomic DNA.

Comparison of rDNA spacers of humans and other primates

Areas of the rDNA transcription unit that code for the mature rRNA have been remarkably conserved throughout evolution, but both the internal and external transcribed sequences and the non-transcribed spacer DNA have diverged much more rapidly. Thus, a comparison of spacer DNA from closely related species was made to determine regions that are invariant in primates and therefore may be functionally necessary. Fragments within the human C and D fragments were used as probes for Southern blots of enzyme digested genomic DNA from primates (human, chimpanzee, gorilla, orangutan, and rhesus monkey). The results are given in Table 1.

Whereas there is extensive homology among the spacers of these primates, one can detect additional EcoRI and BamHI restriction sites in both the C and D fragments of the lower primates. Although there are more EcoRI restriction sites present for the lower primates, the total length of the D fragment is equal to that of the human. In the case of the C fragment, there are two EcoRI fragments detected by a probe from the immediate 3′ end, C_{PE}. This indicates that a portion of these ribosomal repeats have an additional EcoRI site. In fact, when probes at the 5′ end of the C fragment are used, low molecular weight bands in addition to the 12500 bp fragment are detected. Similarly the total length of fragments obtained by C_{BH} hybridization to BamHI digested DNA from chimp and gorilla is equal to the human length even though there are more BamHI sites. In the case of orangutan DNA, there is either a length or restriction site polymorphism to give the higher (2775 bp) fragment.

Since there are sequences homologous to the repetitive Alu family of human DNA present in the spacer (Higuchi et al. 1981), hybridization with C_{B1B2}, C_{B3B4}, C_{B4B5}, and C_{B5B6} gave a background smear of hybridization not only with human but also with other primate DNA. Interestingly, when these fragments are used as probes, not only was the predicted fragment visualized above the background smear, but other ribosomal spacer fragments are also visualized although at greatly reduced intensity. For instance C_{B3B4} hybridized to the predicted 380 bp BamHI fragment (Table 1) but also faintly to fragments of 2.9 kb (C_{B1B2}), 1.25 kb (C_{B4B5}), and 6.3 kb (CB_{BB}). This suggests that all of these DNA fragments contain

Table 1. Size of primate DNA restriction fragments in bp which hybridized with human rDNA probes as listed (Fig. 1b, c) in **a** EcoRI-digested genomic DNA and **b** BamHI-digested genomic DNA. Numbers in *parenthesis* denote minor or faint hybridization. The total size of the D fragment, C fragment, and C_{B2B3} fragment (sum of C_{BH} hybridizations) sequences analogous to human are tabulated. The second smaller EcoRI band detected by C_{PE} indicates an EcoRI restriction site polymorphism (see text). C_{EB} hybridization (to DC_{BB} restriction fragment) reveals additional BamHI site in lower primates

Probe	Primate DNA				
	Human	Chimpanzee	Gorilla	Orangutan	Rhesus
a EcoRI					
D_{ES}	19000	17000	14000	8500	7700
D_{SH}	19000	17000	3400	9000	13500
Total D length	19000	17000	17400	17500	21200
C_{PE}	13000	12500	12500	12500	12500
		5500	5500	7500	
Total C length	13000	12500	12500	12500	12500
b BamHI					
C_{EB}	14500	11000	8550	7500	3575
C_{B1B2}	2900	2900	2900	2900	–
C_{BH}	2150	(2150)	1000	2775	–
		1200	780	1200	
		1000	480	1000	
				535	
Total C_{B2B3} length	2150	2200	2260	2200	–
C_{B3B4}	380	1250	1250	380	380
		380			
C_{B5B6}	800	800	800	800	–

Alu sequences which cross-hybridize. These Alu sequences are only found in the higher primates and are not found in the rhesus monkey.

Discussion

The 300 or so copies of the human ribosomal gene family are tandemly repeated on five non-homologous chromosomes (13, 14, 15, 21, 22) (Henderson et al. 1972; Schmickel 1973; Evans et al. 1974). The non-transcribed portion of the ribosomal genes shows much more evolutionary divergence than the adjacent sequences which code for the mature rRNAs. Evolutionary mechanisms of sequence conservation operate differently in the spacer than in the coding region. Variation is present in particular regions of the 28S rRNA coding sequences (Gonzalez et al. 1985), but not in the entire sequence. Some variation may be explained by unequal homologous exchange, as has been shown to be operative at the 5′ end of the spacer (Erickson and Schmickel 1985). Other mechanisms such as gene conversion, transposition, slippage replication, and RNA-mediated transfers may also be operative in this gene system. Until more sequence information is obtained from discrete regions, the specific size and sequence variations await explanation.

In this paper we show that the spacer is variable both in size and nucleotide composition. Whereas there is no homology between mouse and human spacer sequences, areas within both the C and D fragments appear to be homologous to those of chimpanzee, gorilla, orangutan, and rhesus monkey. Nucleotide sequence dependent functions within the spacer may be found by detecting evolutionarily highly conserved regions within the spacer. Comparisons of genes within a single primate and between primates may help locate potential regulatory sequences in the spacer. Since there are multiple Alu repeat sequences within the spacer, the relative homology of these to each other and to the "consensus" (or primitive) Alu sequences, may allow some analysis of the stepwise formation of the spacer in the primate. Yang-Yen et al. (1985) have recently reported the sequence of 1612 nucleotides at the 3′ end of the rat spacer. They detect two regions of repetitive DNA that appear to evolve at different rates, one of which can be transcribed in vitro.

The map of the C_{BE} fragment (Fig. 3) details both size and restriction site variations at the 3′ end of the fragment. The specific mechanism which gives rise to these variations awaits comparative sequencing. In this regard, a 136 bp SalI-EcoRI fragment, C_{SE}, has been sequenced and found to be identical in six of the plasmid subclones. It is interesting that this size variation is located near the site of initiation of transcription. It raises the possibility that variable sequences in this region may play a role in the regulation of transcription. It also raises the possibility that this variation is in some way related to the two alternate processing pathways of the precursor 45S rRNA to mature products (Sameshima et al. 1981). That this region is integral to gene expression is supported by the fact that the first gene of the tandemly repeated genes on the chromosome begins just upstream of this region (R. Worton, personal communication). Previous studies have defined the DNA sequences of the ribosomal RNA transcription unit. Studies of the spacer will lead to knowledge of sequences responsible for regulation of ribosomal RNA transcription and for the organization of the nucleolus.

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