

Structure and variation of human ribosomal DNA: molecular analysis of cloned fragments

(Recombinant DNA; moderately repetitive DNA: DNA polymorphism; rRNA processing; R-loops)

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SUMMARY

Eco-RI-A fragments of the human ribosomal RNA gene family from two types of tissue and three individuals were cloned in λ vectors and compared by restriction enzyme digestion and electron microscopy. The *Eco*RI fragment A contains (i) 0.2 kb of the 3' end of the 18S rDNA, (ii) 2.5 kb of internal transcribed spacer and the 5.8S rDNA, and (iii) 4.6 kb of the 28S rDNA gene. All of the six cloned rDNA fragments isolated are identical by these analyses. Moreover, all contain a *Hinc*II site that is absent in about 50% of the rDNA identified by genomic blotting. Polymorphism in the nontranscribed spacer rDNA was studied on genomic blots of *Bam*HI-digested DNA, using the 3' end of the 28S rDNA as a probe. The boundaries between the 18S rDNA, internal transcribed spacer, 28S rDNA, and external nontranscribed spacer were determined by R-loop analysis, further defining the organization of the ribosomal RNA precursor.

INTRODUCTION

The DNA sequences for eukaryotic ribosomal RNA (rRNA) constitute a moderately repetitive family of genes which vary in structure and organization among species (for review see Long and Dawid, 1980). In humans, the 5S ribosomal DNA (rDNA) is found on chromosome 1 (Steffensen and Duffey, 1974), while the 45S precursor for the 28S, 18S and

5.8S rRNAs is transcribed from DNA arranged as tandemly repeating units (Wellauer and Dawid, 1973) in the nucleolar organizer regions of the five acrocentric chromosome pairs, Nos. 13, 14, 15, 21, 22 (Henderson et al., 1972). There are approx. 160 of these repeat units per haploid genome (Schmickel, 1973). This family of genes is of special interest since it contains both highly conserved transcribed sequences and variable nontranscribed spacer regions (Krystal and Arnheim, 1978; Schmickel et al., 1980). Structural studies of a polymorphism seen in the length of nontranscribed spacer rDNA for both humans and apes suggest that there may be genetic exchanges between rDNA sequences found on non-homologous chromosomes (Arnheim et al., 1980a). An analysis of independently cloned rDNA fragments

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Abbreviations: bp, base pairs; dNTPs, deoxynucleoside triphosphates; DTT, dithiothreitol; EtBr, ethidium bromide; kb, kilobase pairs; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl, 0.015 M Na⁺ citrate, pH 7.6.

reveals the extent to which homology is maintained among gene copies (Wilson et al., 1978; 1981). Moreover, the repetitive nature of ribosomal genes allows for a comparison of cloned fragments between individuals and also within the same individual. Examination of the gene and flanking spacer DNA permits identification of the genetic regions involved in the processing of 45S rRNA (see Perry, 1976, for a review of rRNA processing). Thus, rDNA is useful in the study of interchromosomal recombination, evolutionary variation and conservation, and rRNA transcription and processing.

Here we report the detailed structure of cloned portions of human rDNA in which we have localized the 3' end of the 18S rDNA, the 5.8S rDNA, and all of the 28S rDNA. In addition, we compare the rDNA *EcoRI*-A fragments from six independently isolated clones, representing DNA from two tissues and three individuals. Cloning of the human rDNA *EcoRI* fragment B, which contains the promoter region and the 5' portion of the 18S rDNA, has been previously reported by our laboratory (Wilson et al., 1978). We now have clones representing the complete length of the transcribed rDNA.

MATERIALS AND METHODS

(a) Human DNA

Human DNA was prepared from fetal human liver or from frozen placental tissue as previously described (Schmickel, 1973). DNA was enriched approx. 100-fold for ribosomal sequences by density gradient centrifugation (Schmickel and Knoller, 1977; Wilson et al., 1978).

(b) Phage λ vector DNA

λ Charon 16A was grown in *E. coli* K802 (Blattner et al., 1977), and λ gtWES \cdot λ B' (Leder et al., 1977) in *E. coli* LE392. Phage DNA was prepared as described by Tiemeier et al. (1977).

(c) Ligation and transfection of recombinant λ DNA

Both λ vector DNAs were digested with *EcoRI* for the insertion of *EcoRI*-digested human DNA.

λ gtWES \cdot λ B' DNA was also digested with *SstI*, which cuts twice in the middle *EcoRI* fragment and reduces re-ligation of parental phage DNA. 0.5 μ g of digested lambda DNA was mixed with 0.15 μ g of digested human DNA in a total volume of 50 μ l containing 66 mM Tris \cdot HCl pH 7.4, 10 mM MgCl₂, 10 mM DTT, 0.1 M ATP, and 0.5 units of T4 DNA ligase. After incubation at 4°C for 16 h, 1 μ l of the ligated DNA was mixed with 0.2 ml of calcium-shocked *E. coli* cells which were then held at 42°C for 2 min, chilled on ice, and plated on NZY agar as previously described (Wilson et al., 1978).

(d) Hybridizations and autoradiography

DNA bound to nitrocellulose filters (Southern, 1975) was hybridized to ¹²⁵I-labeled 28S rRNA (Wilson et al., 1978) or to ³²P-labeled cloned rDNA, nick translated to 10⁸ cpm/ μ g (Rigby et al., 1977). Filters hybridized to DNA probes were first prehybridized for 4 h at 65°C in 3 \times SSC, 0.1% SDS, 25 μ g/ml salmon sperm DNA and 0.02% each polyvinyl-pyrrolidone, Ficoll-400, and bovine serum albumin (Denhardt, 1966). Hybridization was in the same solution, with 10⁵ cpm/ml of [³²P]rDNA, at 65°C for 12–24 h. After rinsing in 2 \times SSC, 0.5% SDS, filters were dried and exposed with XR5 Royal Xomat film (Kodak) at –70°C for 12–72 h in the presence of a fluorescent intensifier screen.

(e) Subcloning of rDNA in plasmid vectors

The 7.3-kb *EcoRI* fragment A was subcloned in the plasmid pBR322 (Bolivar et al., 1977) by digestion of DNAs with *EcoRI*, with *BamHI*, or with *EcoRI* + *BamHI*, followed by ligation and transfection (Chisholm et al., 1980) into *E. coli* DHI (*recA1* strain kindly provided by D. Hanahan). *EcoRI*-digested plasmid DNA was treated with calf intestine alkaline phosphatase before ligation to reduce formation of nonrecombinant molecules (Ulrich et al., 1977). Ampicillin-resistant colonies (screened for tetracycline sensitivity when DNA was restricted with *BamHI* prior to ligation) were checked for inserts by digestion of plasmid DNA prepared from 0.5 ml cultures (Birnboim and Doly, 1979) followed by agarose gel electrophoresis.

(f) Partial-digestion restriction mapping

Three subclones of rDNA in pBR322 were used for partial-digestion mapping (Smith and Birnstiel, 1976) of the *EcoRI* fragment A. 5 to 10 μg of DNA from the *BamHI-EcoRI* clones (pA_{EB} , pA_{BE} , Fig. 1) was cut at the *BamHI* or *EcoRI* site, end-labeled with the appropriate [α - ^{32}P]dNTPs using reverse transcriptase (Wilson et al., 1981) or DNA polymerase I Klenow fragment (Klenow and Henningsen, 1970), and then cut with *EcoRI* or *BamHI* to free the rDNA fragment from the pBR322 DNA. Fragments were loaded into a single well of a 0.8% low melting temperature agarose gel on a 2" \times 3" glass microscope slide, and electrophoresed in 50 mM Tris \cdot borate, pH 8.3, 1 mM EDTA at 100 V for 30 min. DNA bands were identified by UV fluorescence after EtBr staining, wells were cut in front of the fragments desired, and DNA was electrophoresed into the wells at 300 V for ten 30-s pulses. After each pulse, buffer was removed from a well and fresh buffer added. Eluted fractions were monitored for ^{32}P by Cerenkov counting, pooled, and further purified by DEAE-cellulose column chromatography. After ethanol precipitation, fragments were resuspended and digested with various dilutions of enzyme (0.001 to 1.0 units/ μg of DNA) for 1 h at 37°C. Digested and partially digested DNA was electrophoresed in 1.5% agarose or 5% polyacrylamide. ^{32}P end-labeled DNA standards (*Sau3A* and *HinFI* fragments of pBR322, *HaeIII* fragments of ϕX174) were run on each gel. Gels were dried onto Whatman paper and autoradiographed as described above.

pA_{BB} DNA was cut with *BamHI*, end-labeled as described and recut with *BglII*, yielding two labeled rDNA fragments (see Fig. 1). Conversely, the DNA was cut first with *BglII*, and then with *BamHI* after end-labeling.

(g) Electron microscopy

R-loops were formed as described by Thomas et al. (1976). DNA and rRNA at 5 $\mu\text{g}/\text{ml}$ each were incubated in 70% formamide, 85 mM Pipes (piperazine-*N,N'*-bis(2-ethanesulfonic acid)) pH 7.8, 10 mM EDTA at 49°C for 16 h. Heteroduplex formation was performed using Westmoreland et al. (1969) formamide procedure as described by Davis et al. (1971). Samples were diluted 10-fold into 70% formamide,

100 mM Tris \cdot HCl, pH 8.5, 10 mM EDTA, and 40 $\mu\text{g}/\text{ml}$ cytochrome *C*. DNA was spread on a hypophase of 40% formamide. SV40(II) and M13 DNA were included in each sample as double- and single-stranded DNA standards, respectively.

(h) Enzymes, chemicals and supplies

Restriction enzymes were purchased from Bethesda Research Labs (BRL), New England Biolabs, or Boehringer. T4 DNA ligase and DNA polymerase I were purchased from BRL. DNA polymerase I Klenow fragment and calf intestine alkaline phosphatase were purchased from Boehringer. DNase I (RNase-free, bovine pancreas) was purchased from Sigma Chemical Co. [α - ^{32}P]-dNTPs (400 Ci/mmol) were purchased from Amersham or New England Nuclear. Chemicals were purchased as follows: agarose and low melting temperature agarose, SeaKem or BRL; polyacrylamide, BRL; CsCl, Kawecki Berylco Industries; NZY amine "A", Humko-Sheffield; XG (5-bromo-4-chloro-3-indoyl- β -D-galactoside), Sigma Chemical Co.; nitrocellulose membranes, Schleicher and Schuell; bacteriological medium, Difco; DEAE-cellulose (DE52), Whatman; electron microscopy supplies, Pelco.

RESULTS

(a) Identification of recombinants containing rDNA

Recombinant Charon 16A phage, identified by their colorless phenotype when plated on medium containing the chromogenic XG substrate (Blattner et al., 1977), were picked with sterile capillary tubes and stored in 0.2 ml PSB (0.1 M NaCl, 10 mM Tris \cdot HCl pH 7.5, 10 mM MgCl_2 , 0.05% w/v gelatin) in a 96-well microtiter dish. The frequency of colorless recombinants varied from 2–6% of all plaques. 576 Charon 16A recombinants were picked for screening. Recombinant λWES phage could not be identified phenotypically, but ligations constructed in parallel without human DNA indicated that the efficiency of ligation events restoring the parental $\lambda\text{gtWES} \cdot \lambda\text{B}'$ (or λB) phage was small when phage DNA was digested with *EcoRI* and *Sst* I. We estimated that 97%

of the plaques obtained after transfection were recombinants. 650 of these plaques were also picked and stored in microtiter dishes. A wooden block with 96 straight pins was used to transfer phage from the 96-well microtiter dishes onto lawns of *E. coli*. Incubations at 37°C overnight produced lysed areas of 2–5 mm in diameter. λ WES recombinant phage DNA from lysed areas could be lifted directly onto nitrocellulose filters as described by Benton and Davis (1977). However, Charon 16A DNA did not transfer well using this method, so individual lysed areas were scraped, suspended in 100 μ l of 0.1 N NaOH, neutralized with 100 μ l of 3 M sodium acetate pH 5.5, and filtered through nitrocellulose paper under suction, using an epoxy block with 96 holes for the individual lysates. Thirteen Charon 16A clones containing 28S rDNA sequences were identified by their hybridization to 28S [¹²⁵I]rRNA. Only two of these were stable enough to allow large scale growth, and both of these contained deletions in the vector. Only one clone contained the full-length 7.3-kb *Eco*RI rDNA fragment A. Both were analysed in detail by electron microscopy (manuscript in preparation).

Five λ WES ribosomal recombinants were identified by their hybridization to ³²P-nick-translated plasmid DNA containing the rDNA *Eco*RI fragment A subcloned from the stable Charon 16A recombinant. All five contained the 7.3-kb *Eco*RI fragment A. DNA prepared from one fetal liver was the source for three of the *Eco*RI-A fragments cloned in the λ WES vector. A single placenta was the source of DNA for the Charon 16A clones and one of the λ WES clones. The final λ WES clone was derived from a second placental DNA preparation.

(b) Physical map of rDNA clones

The orientation of the rDNA inserts in both λ vectors was determined by digestion with *Bam*HI, which cuts asymmetrically in the λ arms. These orientations were confirmed by heteroduplex formation between DNA from the Charon 16A full-length clone and each of the five λ WES clones. The differences in the two λ vectors allowed for recognition of heteroduplex formation and marked the left and right λ arms as well as the ends of the cloned human rDNA. The Charon 16A clone and four of the five λ WES clones contain the *Eco*RI-A fragment in a 3' to 5' orientation with respect to the left and right λ arms.

A detailed restriction map of the cloned 7.3-kb *Eco*RI rDNA fragment A was prepared (Fig. 1). Restriction sites for enzymes cutting once or twice within the rDNA were identified by digestion of the recombinant λ DNA, agarose gel electrophoresis, and Southern transfer. These sites were confirmed by digestion of the rDNA subcloned in pBR322. Enzymes that do not cut the cloned rDNA fragment are listed in the legend to Fig. 1. Restriction sites for *Sma*I and *Alu*I, which each cut many times in the *Eco*RI fragment A, were mapped by a modification of the Smith and Birnstiel (1976) method using the three subclones of the A fragment in pBR322: pA_{EB}, pA_{BB}, pA_{BE}. Sites were determined by labeling one end of the fragment and confirmed by labeling the other end (see MATERIALS AND METHODS). This allowed for more precise measurement of all partial digestion products.

(c) Comparison of rDNA clones from different sources

Six λ clones containing the 7.3-kb *Eco*RI rDNA fragment A from two tissues of three individuals were compared by restriction digestion and heteroduplex analysis. All cloned fragments produced identical restriction enzyme patterns on agarose and acrylamide gels, even when digested with *Alu*I and *Sma*I. Heteroduplexes of the Charon 16A clone against the four λ WES clones in the same orientation showed no visible areas of non-homology within the 7.3-kb *Eco*RI fragment.

(d) Identification of gene and transcribed spacer region

The 7.3-kb *Eco*RI rDNA fragment A contains the 3' end of the 18S rDNA gene and most of the 28S rDNA. R-looping was used to locate the boundaries between gene and transcribed spacer DNA (Fig. 2). R-loop analysis of 28S human rRNA with the rDNA *Eco*RI fragment A cloned in a plasmid vector reveals 2.7 kb of DNA duplex within the cloned fragment A, a 4.6-kb R-loop, and a 3' rRNA tail. The single-stranded rRNA tail was difficult to measure but appeared to be about 500 bp long. A cloned 5.8-kb rDNA *Bam*HI fragment extending from 1.6-kb 5' to the *Eco*RI site, through the end of the 28S rRNA gene, and out into the non-transcribed spacer DNA

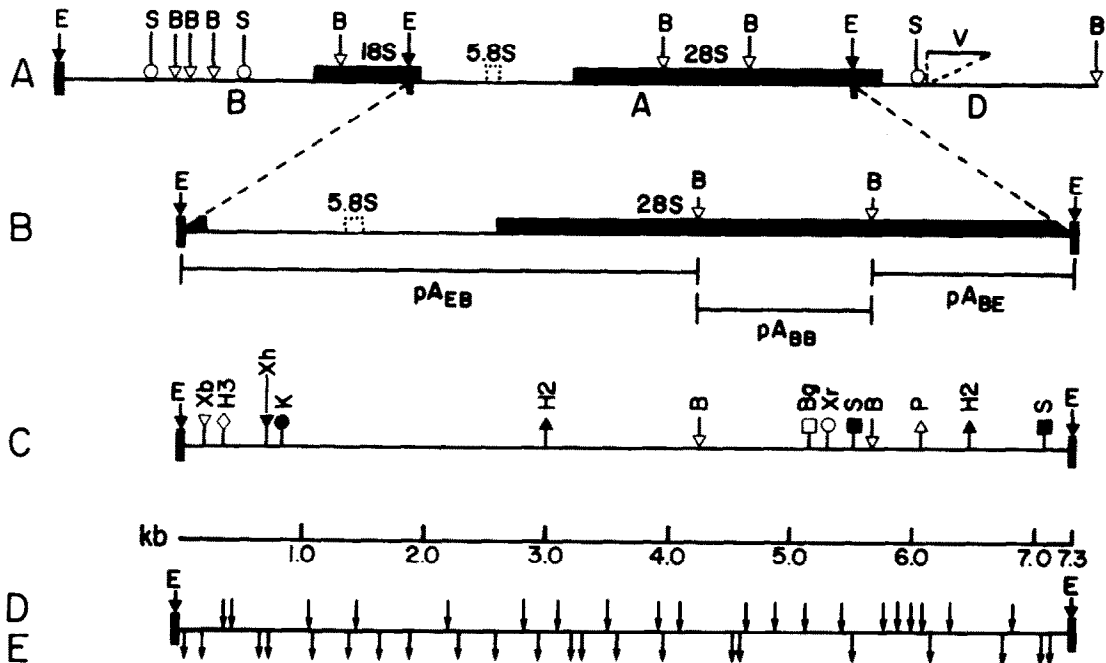


Fig. 1. Restriction map of cloned human rDNA fragments. (A) *EcoRI* fragments B (Wilson et al., 1978), A, and part of D (for fragment notation see Wellauer and Dawid, 1979), showing *EcoRI* (\downarrow), *BamHI* (\downarrow), and *Sall* (\diamond) restriction sites. Black horizontal bars \blacksquare give the position of sequences coding for the 18S and 28S rRNAs. The tentative position of the 5.8S rRNA is shown by a dashed box. A variable insert region in the nontranscribed spacer of fragment D is indicated by V. (B) Enlargement of the 7.3-kb *EcoRI* fragment A, originally cloned in λ vectors and subcloned in pBR322 as illustrated. Notation for plasmid subclones (e.g., pA_{EB}) gives the *EcoRI* fragment involved, while subscripts indicate restriction sites used for insertion into pBR322. (C) Restriction sites in the 7.3-kb *EcoRI* fragment A. Numbers are the fragment size in kb from the 5' *EcoRI* site: \downarrow , *EcoRI*, 0, 7.3; Υ , *XbaI*, 0.20; ϕ , *HindIII*, 0.35; ∇ , *XhoI*, 0.72; \uparrow , *KpnI*, 0.78; \uparrow , *HindI*, 3.0, 6.5; \downarrow , *BamHI*, 4.3, 5.7; \square , *BglII*, 5.2; ∇ , *XorII*, 5.3; \boxplus , *SsrI*, 5.5, 7.1; $\hat{\phi}$, *PvuII*, 6.1. Enzymes which do not cut in this fragment are *BclI*, *ClaI*, *HpaI*, *PstI*, *Sall*. (D) Position of *SmaI* sites in 7.3-kb *EcoRI* fragment A determined by end labeling and partial digestion mapping (see Material and Methods). From the 5' *EcoRI* site the position of *SmaI* sites in kb is: 0.38, 0.40, 1.07, 1.47, 2.18, 2.81, 3.10, 3.52, 3.92, 4.10, 4.65, 4.88, 5.13 (not indicated by an arrow), 5.14, 5.42, 5.77, 5.89, 5.99, 6.08, 6.32, 6.83. (E) Position of *AluI* sites in 7.3-kb *EcoRI* fragment A, determined as in 1D. Numbers represent size of fragments, in kb, from 5' *EcoRI* site: 0.05, 0.20, 0.68, 0.71, 1.10, 1.40, 1.65, 1.90, 2.30, 2.61, 2.95, 3.21, 3.31, 3.58, 3.96, 4.52, 4.55, 5.12 (not indicated by an arrow), 5.52, 6.14, 6.74, 7.06, 7.11.

(manuscript in preparation), was R-looped to confirm the 3' endpoint of the 28S rDNA. The R-loop measured was 2.1 kb, indicating that the 3' end of the gene lies about 500 bp beyond the *EcoRI* site. The 3' end of the 18S rDNA was positioned by R-loop analysis of the 5.7-kb *EcoRI* fragment B (Wilson et al., 1978) and of a 5.3-kb *BamHI* fragment cloned in pBR322 that extends from the *BamHI* site in the 18S rDNA to the 5' most *BamHI* site in the 28S rDNA (clone designated pA_{BB}). Sizes of all R-loops obtained are indicated in Fig. 2C. The presence of the 5.8S rDNA in our cloned fragment A was confirmed by identification of the 5.8S gene by DNA sequencing. The position of the 5.8S rDNA has been tentatively assigned (Fig. 1) by comparison of detailed

restriction sites found in the internal transcribed spacer with sites predicted from the known HeLa 5.8S rRNA sequence (Khan and Maden, 1977).

(e) Comparison of cloned rDNA fragments with genomic rDNA

Restriction-enzyme digestion of human genomic DNA, followed by agarose gel electrophoresis, transfer of DNA to nitrocellulose paper, and hybridization with the cloned rDNA fragments has shown considerable conservation of the restriction patterns within the transcribed rDNA sequences. One major exception is the *HincII* site toward the 3' end of the *EcoRI* fragment A. Genomic blotting of *HincII*-

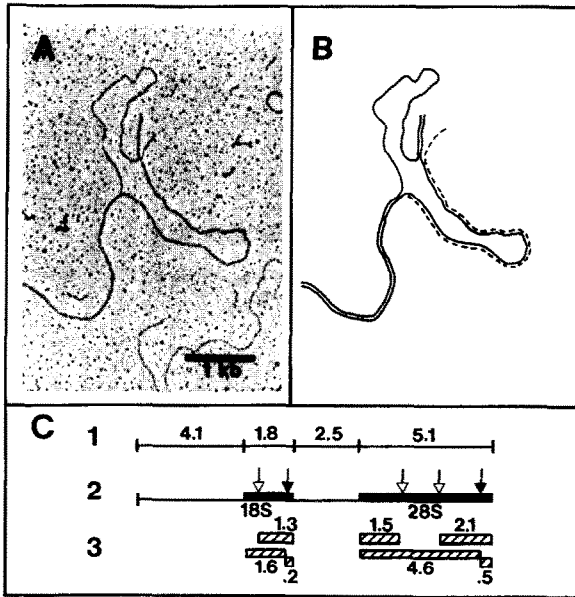


Fig. 2. R-looping of cloned human rDNA with 18S and 28S human rRNA. (A) Electron micrograph of R-loop between recombinant plasmid pA (pBR322 containing the 7.3-kb *EcoRI* rDNA fragment A and human 29S rRNA. pA DNA was cut at the *SalI* site before R-looping. (B) Tracing of R-looped molecule in 2A. Dashed line indicates 28S rRNA. Single solid line represents the single rDNA strand displaced by the rRNA. Solid double lines are double-stranded DNA (both vector and *EcoRI* fragment A sequences not found in the mature 28S rRNA). The short, unpaired rRNA corresponds to the 3' end of the 28S rDNA not contained in the *EcoRI* fragment A. (C) Sizes of gene and spacer regions identified by R-looping. (1) Sizes of gene and spacer regions (obtained from R-loops in C3): 4.1 kb, external 3' spacer; 1.8 kb, 18S rDNA; 2.5 kb, internal transcribed spacer; 5.1 kb, 28S rDNA. (2) Location of 18S and 28S rDNA coding sequences (black bars), and restriction sites used for subcloning rDNA in pBR322 prior to R-looping. ∇ *Bam*HI, \blacktriangledown *Eco*RI. (3) Size of R-loops (kilobase pairs) and rRNA tails (kilobases) measured on R-looped molecules as follows:

| Subclone | 18S rRNA | | 28S rRNA | |
|--------------------------------|----------|-----------|----------|-----------|
| | R-loop | rRNA tail | R-loop | rRNA tail |
| pBA _{BB} ^b | 1.3 | a | 1.5 | a |
| pB | 1.6 | 0.2 | | |
| pA | | | 4.6 | 0.5 |
| pAD _{BB} ^c | | | 2.1 | a |

^a Not measured.

^b pBA_{BB}: pBR322 containing 5.3-kb *Bam*HI fragment from *Bam*HI site in 18S gene to 5' most *Bam*HI site in 28S gene (see C2).

^c pAD_{BB}: pBR322 containing 5.8-kb *Bam*HI fragment extending from 3' most *Bam*HI site in the *Eco*RI fragment A to 5' most *Bam*HI site in the *Eco*RI fragment D.

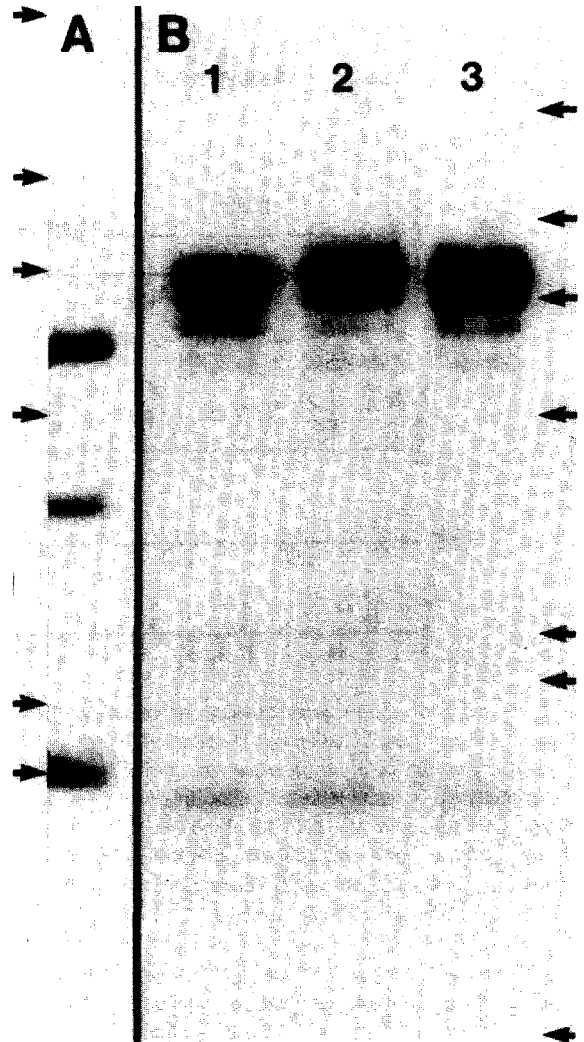


Fig. 3. Agarose gel electrophoresis of restriction endonuclease-digested human DNA transferred to nitrocellulose paper (Southern, 1975) and hybridized to ³²P-nick-translated cloned human rDNA (pA_{BB}). 5 μ g of human DNA prepared from white blood cells was digested with 5 units of restriction endonuclease for 1 h at 37°C, heated at 65°C for 5 min, and electrophoresed through a 1% agarose gel in 40 mM Tris · HCl, 1 mM Na-acetate, 1 mM EDTA, pH 8.2. Position of λ *Hind*III fragments used as size markers are indicated by arrows as follows, from top to bottom: 23.72, 9.46, 6.67, 4.45, 2.25, 1.96, 0.59 kb. (A) *Hinc*II-digested human DNA. Hybridizing bands are seen at 1.8, 3.5, and 5.3 kb (see Fig. 1C for positions in fragment A). (B) *Bam*HI-digested human DNA from parents and offspring. Hybridizing bands are seen in all lanes at 7.6, 6.7, 5.8, 4.9, and 1.2 kb. Lane 1, Mother. Lane 2, Father. Lane 3, offspring. 8.5 and 16.0-kb bands are present in father and child but not in mother.

digested human DNA and hybridization to 28S [¹²⁵I]rRNA or ³²P-nick-translated *Eco*RI fragment A reveals a heterogeneity in this site (Fig. 3A). From

the intensity of the bands, it appears that in about 50% of the rDNA this *HincII* site is absent, producing an additional DNA fragment that is 5.3 kb (see Fig. 1). All six cloned *EcoRI* fragments contain this *HincII* site.

Genomic blotting of *BamHI*-digested human DNA and hybridization with the ^{32}P -labeled subclone pA_{BE} reveals a polymorphism in the nontranscribed spacer region. Six bands are seen regularly in most individuals (Fig. 3B). Five of these fragments vary in length by about 900 bp and range from about 5 to 9 kb in length. This same type of heterogeneity is also seen when the DNA is digested with *BglII* (Schmickel et al., 1980). In addition, certain families exhibit other variant *BamHI* fragments (Fig. 3B, lanes 2–3). No variability is observed when genomic DNA is digested with both *EcoRI* and *BamHI* prior to hybridization with ^{32}P -labeled pA_{BE} DNA, and the size of the *BamHI-EcoRI* genomic fragment corresponds to that cloned in pA_{BE}.

DISCUSSION

The cloning of human rDNA sequences isolated from different tissues and individuals has allowed the first direct and detailed comparative analysis of this family of genes. We initially chose to clone the *EcoRI* rDNA fragments in Charon 16A. The 5.7-kb *EcoRI* fragment B had been cloned in this same vector with no difficulty (Wilson et al., 1978), but the 7.3-kb *EcoRI* fragment A was unstable in Charon 16A. From 13 clones identified by hybridization to 28S [^{125}I]rRNA, we were able to isolate only one that contained a stable, full-length *EcoRI* fragment A. We cannot rule out the possibility that the instability of this fragment A in Charon 16A is related to specific rDNA sequences, but since the stable, full-length clone contains deletions in the vector, and since one other stable clone contains deletions in both vector and insert, we suspect that the size of the 7.3-kb fragment cloned in Charon 16A is responsible for the observed instability. All of the five λ WES recombinants containing the 7.3-kb rDNA *EcoRI*-A fragment are stable, and none of the phage have any observable deletions. This finding is consistent with the supposition that the instability in the lambda Charon vector was due to rDNA fragment size and

was not a property of the rDNA sequence.

The six λ clones containing rDNA *EcoRI* fragment A were derived from three individuals and two types of tissue as previously indicated. Detailed restriction mapping of these clones and heteroduplex analysis reveal no differences between any of the cloned fragments, either in the genes or in the internal transcribed spacer regions. At this level of resolution, then, there is a conservation of sequence in the transcribed rDNA. In humans, the rDNA repeats are located on the five acrocentric chromosomes Nos. 13, 14, 15, 21, and 22, and while our procedure does not allow identification of the original chromosomal location of the fragments isolated by cloning, probability would dictate that our isolated genes are derived from more than one chromosome. The fact that we see no differences in clones isolated from different individuals and tissues indicates a remarkable mechanism for conservation of these rDNA sequences within and between individuals. One exception to this conservation within transcribed rDNA is a *HincII* site variability. And, while about 50% of genomic DNA appears to be missing the 3'-most *HincII* site in the 28S rRNA (Fig. 3A), all six of the cloned fragments contain this site. Although the small sample size may be responsible for this discrepancy, modification of the genomic DNA is an alternative explanation. Methylation of the cytosine of a CpG doublet in eukaryotic DNA is a widespread phenomenon (Grippio et al., 1968) and has been suggested as a means of inactivating transcription of rDNA sequences (Tantravahi et al., 1981). Since the *HincII* recognition site is GTPyPuAC, insertion of CpG for the PyPu sequence results in a *SalI* recognition site (GTCGAC). However, none of our cloned fragments are cut by *SalI*. It is possible that another type of eukaryotic DNA modification is responsible for the variation seen in genomic DNA but not in the cloned fragments.

Genomic blotting of *BamHI* or *BglII*-digested DNA and hybridization to ^{32}P -labeled pA_{BE} reveals an interesting polymorphism in the rDNA (Fig. 3B). Since this is not seen with the *BamHI-EcoRI* digestion and since our cloned fragments are identical, we know this variability lies beyond the 3' *EcoRI* site in the 28S rDNA. The polymorphism can be explained by a DNA fragment of discretely variable length inserted between the *EcoRI* site and the *BamHI* site in the nontranscribed spacer rDNA. This possibility is

depicted in Fig. 1 and is consistent with restriction digestion and heteroduplex analysis of *Bam*HI fragments recently cloned in Charon 27, which extend past the end of the 28S rDNA gene and into the non-transcribed spacer region (J.M. Erickson and R.D. Schmickel, manuscript in preparation). Similar heterogeneity in the nontranscribed spacer rDNA has been observed in R-looped preparations of genomic human DNA (Wellauer and Dawid, 1979). Thus, in contrast to the strong conservation of sequence within the transcribed rDNA, we see a marked but apparently regular heterogeneity in the length of nontranscribed spacer rDNA. Although this variation has been previously described, the construction of a plasmid subclone containing the 3' end of the *Eco*RI fragment A has permitted us to examine more closely the polymorphism of the spacer region adjacent to the 3' end of the 28S gene. In addition to the four bands already noted (Schmickel et al., 1980; Arnheim et al., 1980a), hybridization of *Bam*HI-digested DNA with ³²P-labeled pA_{BE} DNA (Fig. 3B) reveals a 4.9-kb band. This new band is about 900 bp smaller than the next larger band, and seems to be part of the series of fragments that differ from each other by the same increment. One other *Bam*HI band detected in all blots, using the ³²P-labeled pA_{BE} DNA probe, is 1.2 kb and is present in all tissues examined (spleen, placenta, leukocytes). This may represent an end unit in a tandem series, or the presence of rDNA sequences located elsewhere in the genome (Arnheim et al., 1980b; Childs et al., 1981). In certain individuals, other *Bam*HI restriction fragments are seen, and are often transmitted to offspring (Fig. 3B). These fragments may arise through infrequent events but, once generated, may be rather stable in the germ line. This phenomenon has been observed in genomic blots from several mother-father-offspring sets (unpublished results).

R-looping and restriction mapping of our cloned rDNA fragments have allowed the positioning of the mature 18S and 28S rDNA sequences within the ribosomal repeat, as well as the tentative location of the 5.8S human rDNA. These assignments have been quite useful, and various cloned fragments are being studied in relation to human rRNA processing (Dranginis et al., 1980). Nucleotide sequencing of cloned rDNA fragments is in progress and should help clarify (i) sequences pertinent to control of

rDNA transcription and processing, (ii) the extent of homology between cloned rDNA fragments within and between individuals and tissues, (iii) the basis for the *Hinc*II variation in genomic rDNA, and (iv) the molecular basis for the regular heterogeneity observed in nontranscribed spacer rDNA.

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Since the submission of this work for publication, we have improved the resolution of small DNA fragments on a 4–8% gradient polyacrylamide gel and

find that digestion of one of the cloned *EcoRI* A fragments with *SmaI* produces a band that is 3 bp larger than the comparable *SmaI* band found in the other five cloned A fragments.