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Molecular analysis of cloned human 18S ribosomal DNA segments

(human DNA/ribosomal genes/recombinant DNA/restriction endonucleases/bacteriophage λ)

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ABSTRACT **A fraction of DNA from the human fetal lung fibroblast line IMR-90, 30-fold enriched for ribosomal DNA, was cloned in the λ phage vector Charon 16A. Of 978 clones assayed by hybridization to a mixture of ^{125}I-labeled 18S and 28S ribosomal RNA, 11 recombinants containing a 3.8-megadalton segment of human 18S ribosomal DNA were identified. Restriction endonuclease analysis of these clones demonstrated variation only in orientation of the human gene segment within the phage vector. Restriction sites that we had previously detected from analysis of restriction products of unfractionated human DNA by using the Southern transfer method were also present in the cloned DNA segment. Recombinant DNA technology thus provides a valid and efficient means to define structural conservation or variation within families of human genes.**

Until recently, knowledge of human genetics has depended upon the analysis of inheritance or studies of protein expression. Recombinant DNA technology now allows direct study of specific human gene segments by their placement into defined bacterial systems. These methods provide the opportunity to study human gene structure and function at the nucleotide level and are especially useful for those DNA regions that do not code for protein. The extent to which these gene regions vary will be of great interest because they are often present in multiple copies and distributed over several chromosomes. Alterations in their individual or organizational structure may contribute to human disease.

Particularly suitable for analysis by restriction endonuclease digestion are the tandemly repetitious genes for human ribosomal RNA (rRNA), which are clustered on the telomeres of the five D and G group chromosome pairs (1–4). We have assembled (5) a preliminary restriction map for human ribosomal DNA (rDNA), which is shown in Fig. 1. A single ribosomal gene unit can be subdivided into transcribed and spacer regions on the basis of electron microscopy of rDNA transcriptional complexes in HeLa cells (6) and by analogy with the well-defined rDNA structure of *Xenopus* (7). A unit of about 17 megadaltons is estimated from the sizes of the various restriction fragments, and this agrees well with the estimate of Arnheim and Southern (8).

In order to confirm this map for human ribosomal genes and to further define variable regions both within and among individuals, we have begun isolating single ribosomal gene segments by recombination with a bacteriophage vector.

λ bacteriophage Charon 16A (Fig. 1) is an EK2 vector that was constructed by workers in Fred Blattner's laboratory and contains a bacterial lactose operon for easy detection of recombinant bacteriophage in addition to several safety features (9). We report here the isolation of 11 recombinant bacteriophage containing the smallest *Eco*RI fragment of human rDNA and a preliminary search for variation in the cloned 18S gene and adjacent spacer sequences. No variation among the cloned human gene fragments has so far been found except in their orientation within the bacteriophage vector.

METHODS

DNA Preparation. The well-characterized human fetal lung fibroblast line IMR-90 (10) was obtained from the Human Mutant Cell Repository and grown to confluency in 18 roller bottles in minimal essential medium with Earle's salts and 10% fetal calf serum (GIBCO). A modification (10) of the method of Marmur (11) was used to prepare DNA. The cells were lysed in sodium dodecyl sulfate/EDTA buffer, extracted with chloroform, digested with Pronase and RNase, and precipitated with ethanol. The preparation yielded 36 A_{260} units of DNA with a molecular mass range of 15–60 megadaltons when examined by electron microscopy.

The IMR-90 DNA was diluted to about 27 ml with CsCl (final density 1.71 g/ml) and centrifuged for 3 days at 20°C and 25,000 rpm in the SW 25.1 rotor (Beckman). Fractions (2 ml) were collected and hybridized to ^{125}I-labeled 18S rRNA to determine their content of rDNA (1). A single fraction of density 1.72 g/ml was 30-fold enriched for rDNA and was used for all recombination experiments.

Charon 16A bacteriophage was cultivated on *Escherichia coli* K802 in L broth (12) after the presence of *A* and *B* gene amber mutations and phage φ80 immunity had been confirmed (9). DNA was prepared essentially as described by Murray (13), with omission of the nuclease digestion prior to phenol extraction.

DNA Recombination. Human fetal DNA (20 μg), enriched 30-fold for rDNA by CsCl gradient centrifugation, and bacteriophage DNA (20 μg) were digested with 10 units of *Eco*RI nuclease under standard conditions (14) for 3 hr at 37°C, heated to 70°C, and dialyzed against 10 mM Tris·HCl, pH 7.5, to remove salt. Ligation reaction mixtures (100 μl) contained 2 units of DNA ligase from phage T4-infected *E. coli* (Miles), 66 mM Tris·HCl at pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.1 mM ATP, 4 μg of *Eco*RI-digested Charon 16A DNA, and 2 μg of *Eco*RI-digested human fetal DNA. All steps subsequent to the addition of ligase were performed in a P3 facility. After 24–48 hr at 4°C, 5–10 μl of the ligation reaction was mixed with calcium-shocked (15) *E. coli* K802 (2×10^8 cells in 0.2 ml) and plated on agar containing NZamine A (Humko, Norwich, NY) and the chromogenic substrate XG (5-chloro-4-bromo-3-indolyl-β-D-galactoside) as described by Blattner *et al.* (9). Larger volumes of the ligation mixture were found to inhibit the transfection assay. Although strain K802 is lactose$^+$ (9), insertion of foreign DNA into the *lac* operon of Charon 16A decreases hydrolysis of the XG sufficiently so that recombinant plaques can be distinguished from parental plaques on lawns of K802 by their pale blue color and absent halo. Recombinants were

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Abbreviations: rDNA, ribosomal DNA; *Eco*RI, *Bam*HI, *Sal* I, *Hin*dIII, *Hpa* I, and *Hae* III, restriction endonucleases from *Escherichia coli*, *Bacillus amyloliquefaciens*, *Streptomyces albus*, *Haemophilus influenzae*, *H. parainfluenzae*, and *H. aegyptius*, respectively.

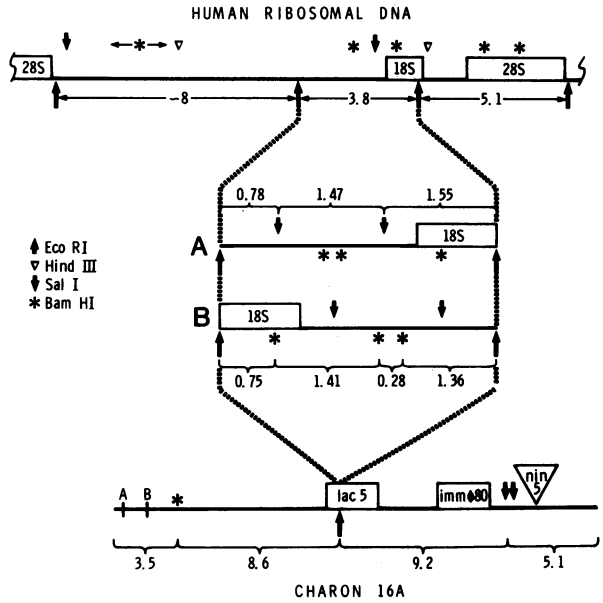

FIG. 1. Restriction maps of human ribosomal DNA, Charon 16A bacteriophage DNA, and a cloned 18S rDNA segment. At the top of the figure is shown a single repeat unit of the tandemly repeated genes for human ribosomal RNA. Restriction sites were derived from the digestion of human spleen DNA. Restriction fragments were identified by electrophoresis on 1% agarose slab gels, transfer of the size-fractionated DNA to a nitrocellulose membrane sheet, and hybridization to iodinated rRNA purified from HeLa cells as described in *Methods*. The middle of the figure shows two orientations for the cloned *Eco*RI 18S rDNA fragment, labeled A and B. Molecular masses of *Bam*HI, *Sal* I, or *Eco*RI restriction fragments of the cloned region are based upon the data in Figs. 4 and 5. The bottom of the figure shows the bacteriophage vector Charon 16A with its *A* and *B* amber mutations, bacterial lactose operon, phage $\phi 80$ immunity region, and *nin* 5 deletion. Restriction sites were positioned according to information from Blattner *et al.* (9) or estimated by analysis on agarose gels as described in *Methods*. Numbers represent fragment molecular masses in megadaltons.

picked up with a sterile capillary tube and suspended in 1 ml of 10 mM Tris·HCl, pH 7.4/100 mM NaCl/10 mM $MgCl_2$/0.05% (wt/vol) gelatin (Difco) plus a drop of chloroform.

Selection of Recombinants Containing rDNA. Lawns of *E. coli* K802 were formed by mixing 0.1 ml of an overnight culture with 3 ml of soft agar and pouring on 90-mm NZY (NZamine/yeast extract/NaCl/$MgCl_2$) agar plates (9). After the agar hardened, 5 μl of each recombinant suspension was applied, 40 to a plate. The resultant 0.5- to 1-cm diameter plaques were individually suspended in microtiter plate wells containing 100 μl of 0.1 M NaOH, incubated for 15 min at room temperature, and neutralized with 100 μl of 3 M sodium acetate, pH 5.5. The contents of each microtiter plate well were transferred to 90-mm nitrocellulose membranes (Schleicher and Schuell) by using a plastic disc (3 cm thick, 90-mm diameter) containing 50 3-mm diameter holes that served as chimneys for filtration. The disc was clamped tightly over the membrane filter, which was supported by four circles of Whatman 3 MM paper and a 90-mm Millipore filtration assembly connected to a water aspirator. The chimneys were washed before and after filtration with 2 ml of 0.5 M NaCl/0.05 M Na citrate, pH 7, and baked for 2 hr at 80°C in a vacuum oven. Hybridization was with 5 μg (5×10^7 cpm) of ^{125}I-labeled 18S and 28S rRNA prepared from HeLa cells (1) in a volume of 20 ml of 0.3 M NaCl/0.03 M Na citrate, pH 7, for 12 hr at 65°C. Autoradiography was performed for 12 hr at −70°C with Royal XOMat film (Kodak) and a fluorescent intensifier screen (16).

Growth of Recombinant Bacteriophage. Recombinant bacteriophage shown to contain ribosomal sequences by autoradiography were again tested for the presence of amber mutations by appropriate complementation tests (9). Initial growth was by lysis on a single plate and then in multiple 100-ml cultures of the weakened *E. coli* strain DP 50 *supF* (9). Phage were added at a bacterial density of 2×10^8 cells per ml and a multiplicity of 1. Titers varied from 0.5 to 3×10^{10} phage per ml, and DNA was prepared as described before (13).

DNA Restriction and Gel Electrophoresis. *Eco*RI restriction endonuclease was purified from *E. coli* RY 13 by the method of Greene *et al.* (14) involving phosphocellulose and hydroxyapatite chromatography. Restriction endonucleases *Bam*HI, *Sal* I, *Hpa* I, *Hin*dIII, and *Hae* III were obtained from Miles Laboratories. Digestions contained 0.5 μg of the appropriate phage DNA or 20 μg of human DNA and 1 unit per μg of DNA of the selected restriction endonuclease in the recommended buffer. After incubation for 1–2 hr at 37°C and 5 min at 70°C, the reactions were cooled quickly and applied to an 18-cm long × 9-cm wide vertical 1% agarose (Seakem, Rockland, ME) slab gel containing 1 μg of ethidium bromide (Sigma) per ml. Electrophoresis was for 14 hr at 11 V/cm as described by Helling *et al.* (17).

Molecular weights of the restriction fragments were estimated by comparison to a mixture of λ *Eco*RI and *Hin*dIII fragments or, for acrylamide, to *Hae* III fragments of ϕX174 DNA (Bethesda Research, Rockville, MD). A standard curve for each gel was drawn by a least squares fit of the logarithm of fragment molecular weight versus the distance migrated. In some experiments, the DNA was transferred to a nitrocellulose membrane sheet after the gel had been soaked in 0.5 M NaOH/1.5 M NaCl for 30 min and neutralized with 0.2 M Tris·HCl, pH 7.5/0.3 M NaCl/0.03 M Na citrate (18). The DNA on the sheet was then hybridized with iodinated 18S rRNA as described above and autoradiographed.

RESULTS

Digestion of human fetal DNA with the restriction endonuclease *Eco*RI yields a wide range of fragment sizes, as is shown in Fig. 2. Transfer of the *Eco*RI restriction fragments to nitrocellulose membrane sheets and hybridization to a mixture of ^{125}I-labeled 18S and 28S rRNA identifies three rDNA fragments (Fig. 2, *insert*). Separate experiments have demonstrated that the 13- and 3.8-megadalton fragments hybridize only to 18S rRNA, while the 5.1-megadalton fragment hybridizes strongly to 28S rRNA and weakly to 18S rRNA (5). The restriction map in Fig. 1 reflects these data with the provision that the 13-megadalton *Eco*RI fragment derives from a fraction of rDNA that lacks the *Eco*RI site between the 3.8- and 8-megadalton fragments.

The 5.1- and 3.8-megadalton rDNA fragments as well as 90% of the *Eco*RI fragments of the human genome were less than the 6-megadalton theoretical size limit for incorporation into the bacteriophage vector Charon 16A (9). For recombination experiments, human fetal DNA was enriched approximately 30-fold for rDNA by cesium chloride equilibrium density gradient centrifugation (1) before digestion with *Eco*RI restriction endonuclease.

Under the conditions described in *Methods*, each ligation reaction containing restricted Charon 16A and human fetal DNA yielded about 2000 viable phage, of which approximately 10% appeared to be recombinants as shown by their pale blue color when plated on *E. coli* K802 in the presence of the chromogenic β-galactoside XG (9). From several such reactions, 978 recombinants were sampled and screened for the presence of sequences complementary to 18S or 28S rRNA as shown in Fig. 3. Eleven recombinants were shown to hybridize with 18S

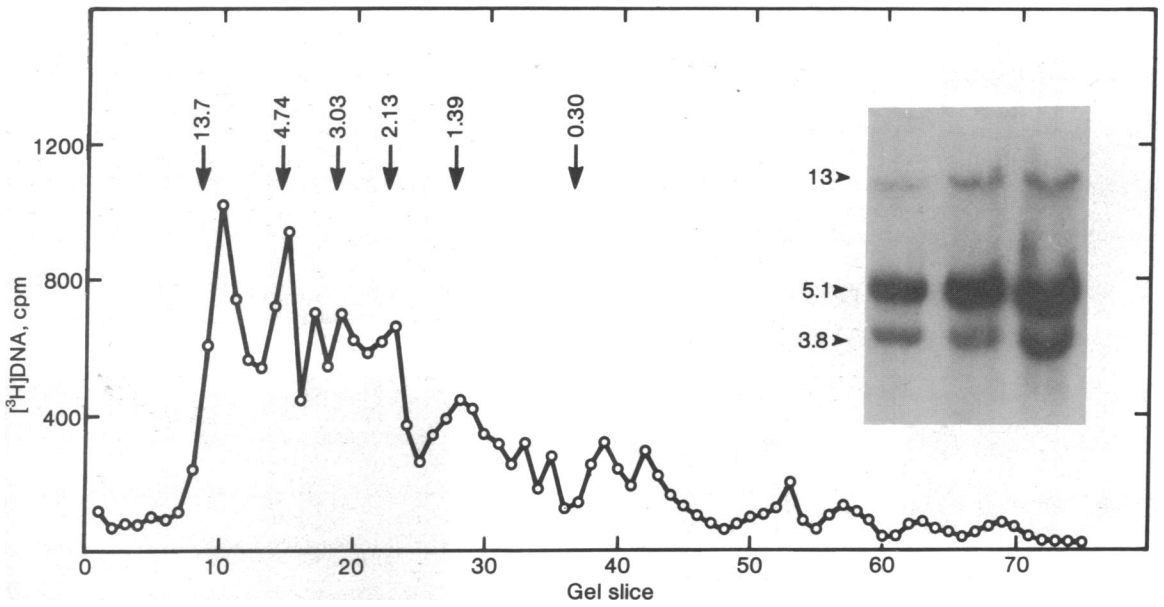

FIG. 2. Gel electrophoretic analysis of *Eco*RI-digested human fetal DNA, showing size distribution of cleavage products. IMR-90 fibroblasts were grown in Blake bottles containing minimal essential media (GIBCO) and 10% dialyzed fetal calf serum. To each of six bottles, 200 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{3}H]thymidine (20 Ci/mmol) was added at about half confluency and the cells were harvested 48 hr later. [^{3}H]-DNA was prepared (11) and 5 μg (30,000 cpm) was digested with *Eco*RI endonuclease and applied to a 1% agarose 1 × 15 cm cylindrical gel. After electrophoresis for 15 hr at 200 V, the gel was sliced and dissolved in ACS (Amersham/Searle) for scintillation spectrometry. The arrows indicate the position of certain *Eco*RI or *Hin*dIII fragments of λ DNA; molecular masses are in megadaltons. The autoradiogram (*Inset*) shows three *Eco*RI fragments of human rDNA at positions corresponding to molecular masses of 13, 5.1, and 3.8 megadaltons. The autoradiogram represents the upper portion of a 1% agarose slab gel that was loaded with *Eco*RI digestions of 10, 20, and 30 μg of human fetal DNA. Gel electrophoresis, transfer of the DNA to nitrocellulose, and hybridization to a mixture of ^{125}I-labeled 18S and 28S rRNA were as described in *Methods*.

rRNA alone, while none were isolated that hybridized to 28S rRNA.

One recombinant, Charon 16A Hr-1, was selected for the restriction analysis shown in Fig. 4. A 3.8-megadalton fragment that hybridizes to human 18S rRNA is shown to be inserted at the *Eco*RI site of Charon 16A. The inserted fragment contains at least two sites for the restriction endonuclease *Sal* I and at least three sites for *Bam*HI as shown in Fig. 4 and diagrammed in Fig. 1. No *Hpa* I or *Hin*dIII sites have been found in the cloned 18S rDNA fragments (data not shown). Charon 16A Hr-1 DNA is not digested with *Hin*dIII alone, showing that the *Hin*dIII site near the end of the 18S gene is in fact outside of the *Eco*RI site (Fig. 1).

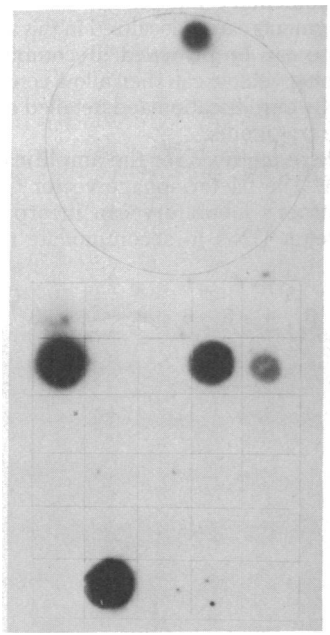

FIG. 3. Screening of recombinant bacteriophage for 18S and 28S rDNA sequences. The upper circle represents an autoradiogram of a 90-mm nitrocellulose filter containing 50 denatured plaques of recombinant bacteriophage. Note the single region of hybridization to ^{125}I-labeled 18S rRNA. Recombinants in the region of positive hybridization were retested after plaque purification, and the lower part of the figure shows the hybridization of recombinants from several different positive regions with 18S rRNA.

The similarity of 18S rDNA fragments produced from Charon 16A Hr-1 and human spleen DNA by various restriction enzymes is summarized in Table 1. The higher molecular weight estimated for the 18S rDNA fragment in human spleen DNA represents variability introduced by transfer to nitrocellulose and autoradiography, because this fragment comigrates with the cloned 18S rDNA fragment when both are applied to the same gel.

Comparison of 7 of the 11 recombinant bacteriophage containing 18S rDNA is shown in Fig. 5. *A* shows that each recombinant has a 3.8-megadalton *Eco*RI fragment. *B* shows

Table 1. Molecular masses of 18S rDNA fragments

Enzyme digestion	Human spleen DNA	Charon 16A Hr-1
*Eco*RI	4.2 ± 0.4	3.8
*Bam*HI/*Eco*RI	1.3, 0.7	1.41, 0.75
*Bam*HI/*Sal* I	0.8	0.8
Sal I/*Eco*RI	1.5	1.55
Hpa I/*Eco*RI	4.2 ± 0.4	3.8
*Hin*dIII/*Eco*RI	4.2 ± 0.4	3.8

Molecular mass values are given in megadaltons and were estimated as described in *Methods*. Human spleen rDNA fragments were identified by autoradiography, while those of the recombinant bacteriophage could be visualized directly by ethidium bromide staining.

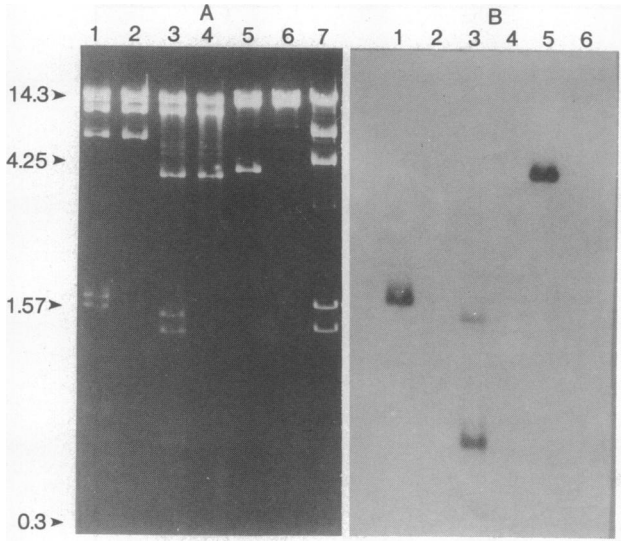

FIG. 4. Gel analysis of Charon 16A and Charon 16A Hr-1 DNA digested with restriction endonucleases. (*A*) Analysis by agarose gel electrophoresis of the digestion products of Charon 16A Hr-1 and the parent Charon 16A DNA produced by various restriction endonucleases. (*B*) Autoradiogram of the companion nitrocellulose transfer hybridized with 18S ribosomal RNA. Lanes 1, 3, and 5 show patterns of *Eco*RI/*Sal* I, *Eco*RI/*Bam*HI, and *Eco*RI digestion products of Charon 16A Hr-1 DNA, while lanes 2, 4, and 6 are controls using Charon 16A DNA. Lane 7 contains molecular weight marker fragments of λ DNA. Lane 5 of *A* shows the presence of a 3.8-megadalton fragment in an *Eco*RI digestion of Charon 16A Hr-1 DNA that is not present in the *Eco*RI digestion of Charon 16A DNA (lane 6). This fragment hybridizes strongly to 18S rRNA (lane 5, *B*). Similarly, fragments of 1.41, 1.36, and 0.75 megadaltons are present in an *Eco*RI/*Bam*HI digestion of Charon 16A Hr-1 DNA (*A*, lane 3) but absent in the corresponding digestion of Charon 16A DNA (*A*, lane 4). The 1.41- and 0.75-megadalton fragments hybridize with 18S rRNA (*B*, lane 3). Digestion with *Eco*RI/*Sal* I liberates fragments of 1.55, 1.47, and 0.78 megadaltons from Charon 16A Hr-1 DNA (*A*, lane 1) that are absent in the corresponding digestion of Charon 16A DNA (*A*, lane 2). The 1.55-megadalton fragment hybridizes to 18S rRNA (*B*, lane 1).

that *Bam*HI/*Sal* I digestions of the recombinant bacteriophage yielded identical 0.80-, 0.61-, 0.58-, and 0.28-megadalton fragments. The 0.8-megadalton fragment hybridized with 18S rRNA in each case (not shown).

A restriction map of the cloned 18S rDNA fragment can be assembled from the data in Figs. 4 and 5 and is shown in Fig. 1. Orientation of the cloned fragment within Charon 16A may place the 18S gene adjacent to the right (A) or left (B) arm of the bacteriophage DNA. The asymmetric sites for *Sal* I on the right arm and *Bam*HI on the left arm of bacteriophage DNA can be used to distinguish these possibilities. For orientation A, digestion with *Sal* I would theoretically give a 10.75-megadalton 18S rDNA fragment, while digestion with *Bam*HI/*Sal* I or *Bam*HI would give 9.95- or 15.05-megadalton 18S rDNA fragments, respectively. For orientation B, digestion of Charon 16A Hr-1 DNA with *Sal* I would give a 13.65-megadalton 18S rDNA fragment, while digestion with *Bam*HI or *Bam*HI/*Sal* I would give a 9.35-megadalton fragment that hybridizes with 18S rRNA. In Fig. 6 products of *Sal* I, *Bam*HI/*Sal* I, and *Bam*HI digests of two different recombinant bacteriophage are shown. The patterns of hybridization of 18S rRNA are those predicted for orientation B in the first case (lanes 1–3) and for orientation A in the second (lanes 4–6). Of the 11 recombinant bacteriophage, orientation A has been demonstrated in 8 cases, and orientation B in the remaining 3.

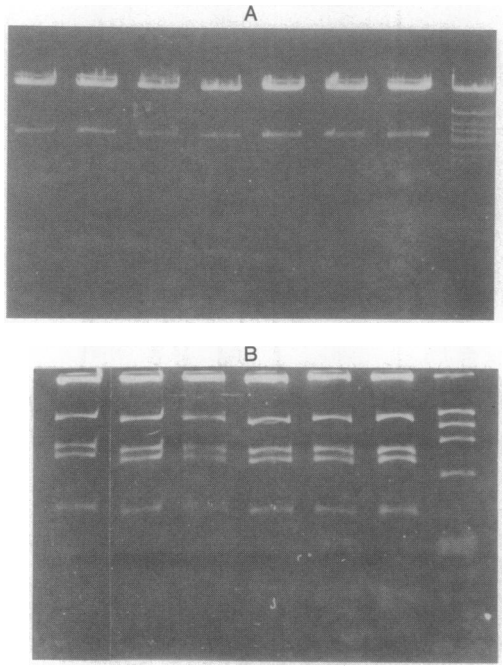

FIG. 5. Electrophoretic analysis of DNA restriction products from seven separate 18S rDNA recombinants. (*A*) Electrophoretic analysis on a 1% agarose gel of 1 μg of DNA from Charon 16A Hr-1 through Hr-7 digested with *Eco*RI restriction endonuclease. (*B*) Gel analysis on 4% polyacrylamide of six of the DNA samples digested first with *Bam*HI, then with *Sal* I restriction endonuclease. Standards were a mixture of *Hin*dIII and *Eco*RI fragments of bacteriophage λ DNA for *A* and *Hae* III fragments of φX174 DNA for *B*.

DISCUSSION

The electrophoresis of restriction endonuclease-digested DNA on agarose gels provides a unique array of human DNA fragments. If complementary nucleic acids are available, then specific gene fragments can be localized in this array and a map of gene structure can be proposed. Recombination with an appropriate cloning vehicle can then allow confirmation of the restriction map by amplification and detailed characterization of selected gene fragments.

We present here methods for the amplification of human ribosomal DNA. The bacteriophage vector Charon 16A developed in Blattner's laboratory can incorporate sufficient amounts of foreign DNA to accommodate most *Eco*RI re-

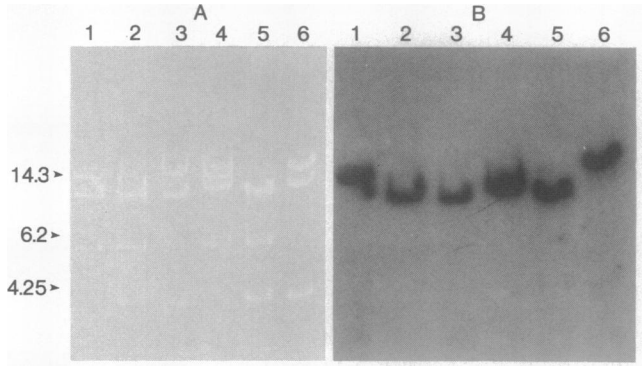

FIG. 6. Electrophoretic analysis of DNA restriction products from Charon 16A Hr-1 and Hr-2 to demonstrate different orientations of the rDNA fragment. (*A*) Digestion products of Charon 16A Hr-2 DNA (lanes 1–3) from *Sal* I, *Bam*HI/*Sal* I, and *Bam*HI, respectively; lanes 4–6 show identical digestions of Charon 16A Hr-1 DNA. (*B*) Autoradiogram of the companion nitrocellulose transfer after hybridization to 18S rRNA.

striction fragments of the human genome. The presence of a *lac* operon in this phage provides a simple indicator test to distinguish true recombinants from reconstituted parental phage. A stable repository of recombinant phage can be easily assembled and screened for the presence of rDNA or other gene sequences by hybridization and autoradiography. Our screening is very sensitive, in that very small or stippled plaques still gave positive results.

Estimates of human ribosomal gene number based upon hybridization studies range between 100 and 400 copies per diploid genome (1–4). Assuming an rDNA repeating unit of 18–20 megadaltons reported here and by Arnheim and Southern (8), 0.036–0.15% of the 5.5×10^6 megadaltons of DNA in a human diploid cell (1) would be rDNA. A further 30-fold enrichment by CsCl centrifugation would give a DNA fraction containing 1.1–4.5% rDNA or 0.21–0.86% of the 3.8 megadalton 18S rDNA fragment obtained by *Eco*RI endonuclease digestion. Of 978 recombinants isolated from this enriched human DNA, 11, or 1.1%, contained 18S rDNA sequences, and this agrees well with the predicted value. Such agreement may be spurious, however, because no correction was made for the surprising number of small *Eco*RI fragments of the human genome shown in Fig. 2, or for possible selection for fragments of a certain size range by the Charon 16A vector. Our failure to obtain recombinants containing the 5.1-megadalton *Eco*RI fragment of human rDNA may reflect such a size selection, although aberrant migration of this fragment on agarose gels or interference with phage growth should be considered.

The data in Fig. 4 and in Table 1 show that the cloned *Eco*RI fragment is identical in size and hybridization characteristics to the smaller *Eco*RI fragment in digests of human fetal fibroblast or spleen DNA. Fig. 1 shows the presence of three restriction sites in the cloned fragment that were predicted from digestion of human spleen DNA. In addition, data not shown indicate the absence of *Hpa* I and *Hin*dIII sites in the 3.8-megadalton *Eco*RI fragment both in human DNA and in Charon 16A Hr-1. The observation (Fig. 6) that the cloned 18S rDNA fragment is present in different orientations along with the fact that the 11 recombinants were obtained from five separate ligation reactions underlines the fact that they were isolated independently as single plaques and are likely to derive from different ribosomal gene units. The data in Fig. 5 shows no evidence for length heterogeneity in 11 cloned fragments of ribosomal genes despite their probable origin from rDNA regions on different chromosomes. This may reflect strict conservation of the 18S rRNA gene and adjacent promotor sequences because of their obvious functional importance and suggests the spacer region near to the 18S gene may also be conserved. Our approach should allow detection of minor variations among families of human genes and perhaps lead to an understanding of the evolutionary forces that determine variation or conservation of the genes' structure.

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