Am J Hum Genet 34:32-49, 1982

Structure and Variation of Human Ribosomal DNA: The External Transcribed Spacer and Adjacent Regions

GOLDER N. WILSON,[1] L. LYNNE SZURA,[1] CAROLINE RUSHFORD,[2] DAVID JACKSON,[2] AND JEANNE ERICKSON[3]

SUMMARY

A group of human ribosomal DNA (rDNA) recombinants that include the probable site for initiation of transcription have been examined for sequence polymorphism. A detailed restriction map of one rDNA insert was constructed using plasmid subclones and end-labeled segments. Comparison of 16 similar rDNA inserts by restriction and heteroduplex analysis demonstrated striking conservation of the external transcribed spacer and 18S gene regions, but defined a region where restriction sites for the enzymes *Sma* I, *Hpa* II, and *Hha* I become frequent or variable. This region extends for about 400–800 base pairs (bp) at the left end of the rDNA insert and is postulated to contain nontranscribed spacer sequences. The use of cloned rDNA segments as probes for the restriction analysis of genomic rDNA has demonstrated certain fixed sites in the nontranscribed spacer that do not vary significantly among different individuals or tumor cell lines. In contrast, restriction with the enzyme *Sal* I reveals several variable fragments, one of which has been found only in a retinoblastoma cell line.

INTRODUCTION

Following a general trend for concepts of biological structure, the anatomy of the eukaryotic genome becomes more dynamic as methods for dissecting protein,

Received January 25, 1981; revised July 13, 1981.

This paper was supported by grant 5-P01-HD13506 from the National Institutes of Health, and Basil O'Connor Starter Research Grant 5-169 to G. N. W.

[1] Departments of Pediatrics, University of Michigan, Ann Arbor, MI 48109.

[2] Department of Microbiology and Immunology, University of Michigan, Ann Arbor, MI 48109.

[3] Department of Human Genetics, University of Michigan, Ann Arbor, MI 48109.

gene, and chromosome morphology become more refined. In man, polymorphic loci or chromosomal variations have been extremely useful in studies of evolution and disease. The search for human variation is greatly extended by restriction endonuclease analysis of human DNA coupled with molecular cloning of selected restriction fragments. Here these complementary techniques are used to examine the variability of human ribosomal RNA genes in hopes of finding polymorphisms that will clarify the evolutionary history and functional significance of rDNA sequences.

The structure of a single repeat unit of human rDNA, based upon the electron microscopic evidence of Wellauer and Dawid [1], is shown in the upper portion of figure 1*A*. Their nomenclature for the *Eco* RI fragments of human rDNA is used in this report with the provision that sites within the A and B *Eco* RI fragments are lettered or numbered in the direction of rRNA transcription as defined in *Xenopus* [2]. The proposed structure is consistent with previous density equilibrium centrifugation [3, 4], hybridization [3, 5, 6], or restriction [7–9] analysis of human rDNA if one assumes the 44 kilobase pair (kb) unit is tandemly repeated 50–400 times per haploid genome to form clusters of G + C rich DNA. The restriction analyses referenced above did not detect portions of the C and D *Eco* RI fragments because they are far removed from regions that hybridize to rRNA. As in lower eukaryotes, nontranscribed spacer regions of rDNA alternate with transcribed regions that yield unstable nuclear (transcribed spacer) or stable cytoplasmic (5.8, 18, and 28S) rDNA segments [10].

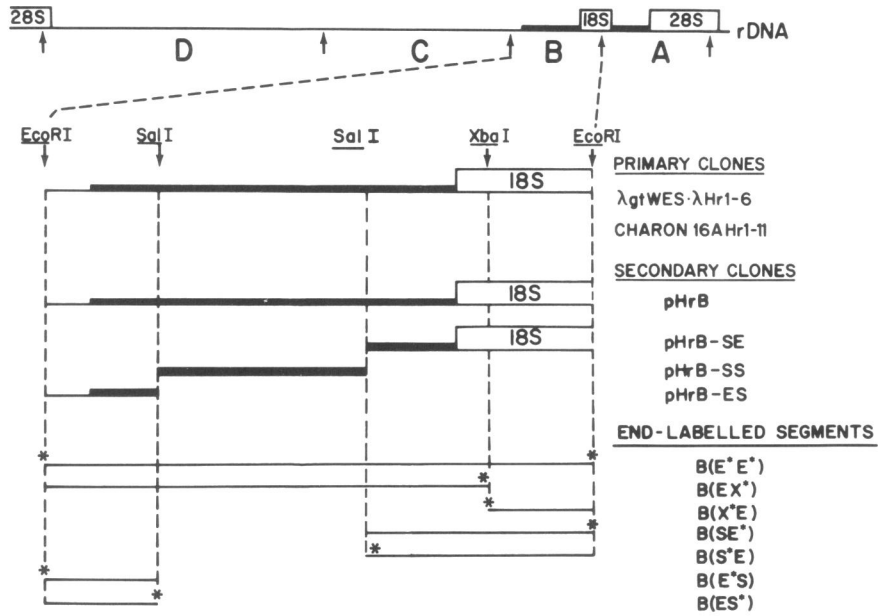
Restriction analysis employing radio-iodinated 18S or 28S rRNA demonstrated little variation in the A and B *Eco* RI fragments except for occasional absence of the *Eco* RI site at the B/C fragment border [8], and a *Hind* II polymorphism within the 28S gene [11]. The major variation demonstrated so far by restriction analysis is length heterogeneity in the nontranscribed spacer region about 2 kb from the 3′ end of the 28S gene [11, 12]. Additional evidence for length heterogeneity of the nontranscribed spacer is provided by R-loop and restriction analysis of highly enriched human rDNA [1], but the majority of repeats seem uniform in size.

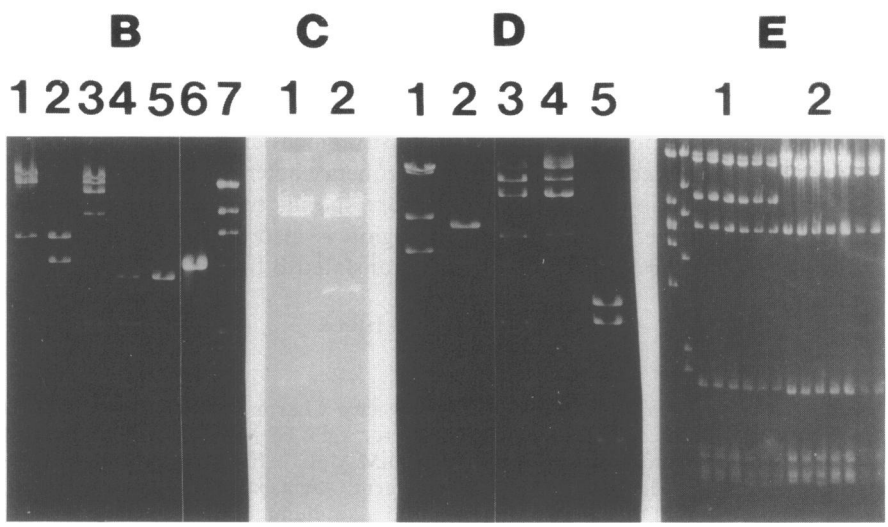
Molecular cloning of several unique *Eco* RI B fragments from the same or different individuals allows a more detailed search for variation among different rDNA repeats. This report compares unique B fragment clones by restriction or heteroduplex analysis and defines a region of sequence heterogeneity near the B/C *Eco* RI fragment junction that presumably corresponds to a region of the nontranscribed spacer. The variability of this region is also examined in genomic DNA by hybridizing Southern blots to nick-translated B fragment DNA probes.

MATERIALS AND METHODS

Cloning of rDNA Fragments

Primary cloning of *Eco* RI B fragments in the vector Charon 16A [13], from IMR-90 cellular DNA [14], has been described [8]. The vector λgtWES·λB [15] was digested with *Eco* RI and *Sst* I in 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, and 2 mM 2-mercaptoethanol in order to cleave the λB fragment. After extraction with phenol and ether, 0.5 μg of λgtWES DNA was ligated with 0.15 μg of human placental DNA that had


28S
18S
28S
rDNA
D
C
B
A
EcoRI
Sal I
Sal I
Xba I
EcoRI
18S
18S
18S
PRIMARY CLONES
λgtWES·λHr1-6
CHARON 16A Hr1-11
SECONDARY CLONES
pHrB
pHrB-SE
pHrB-SS
pHrB-ES
END-LABELLED SEGMENTS
B(E*E*)
B(EX*)
B(X*E)
B(SE*)
B(S*E)
B(E*S)
B(ES*)


B
C
D
E
1 2 3 4 5 6 7
1 2
1 2 3 4 5
1
2

been subjected to successive cesium chloride and silver cesium sulfate density gradients [4]. The ligation reaction (100 μl) contained 66 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 mM ATP, 10 mM dithiothreitol, and 1 U of T4 DNA ligase (Bethesda Research, Rockville, Md.). After ligation for 16 hrs at 4°C, the ligated DNA was incubated with shocked cells [8] prepared from the strain LE 392 [15]. Transfectants were screened with iodinated 18S rRNA isolated from HeLa cells [8] and DNA purified from positive recombinants as described by Murray [16]. Seven *Eco* RI B fragments were obtained from a pool of 650 recombinants.

Transfer of the *Eco* RI B fragment from Charon 16A HrB-1 to pBR 322 [17] was accomplished by ligating 1 μg of *Eco* RI restricted phage DNA with 0.1 μg of *Eco* RI restricted pBR322 DNA as described above. Of 100 transformants screened in the host *E. coli* K802 [13], three hybridized with 18S rRNA; DNA was prepared from one of these (pHrB) for characterization. Plasmid DNA was prepared as described by Bolivar et al. [17]. Subcloning of *Eco* RI-*Sal* I fragments from Charon 16AHrB-1 was accomplished in identical fashion using doubly-digested DNA, except that the approximately 2,000 transformants obtained from 50 μl of ligation reaction were subjected to cycloserine selection [17]. The percentage of tetracycline sensitive transformants was increased from 30% to 98% by this procedure and of five selected for characterization, two contained the insert shown in pHrB-ES (fig. 1), two contained the insert shown in pHrB-SE, and one contained an insert of about 9 kb that was probably derived from the right phage arm of Charon 16A. Subcloning of the *Sal* I rDNA segment was accomplished as described for pHrB, except that the DNA was digested with *Sal* I. Of 100 transformed colonies, seven appeared tetracycline sensitive, and two contained the segment shown as pHrB-SS.

Preparation of End-labeled DNA Segments

From 5 to 10 μg of DNA was restricted in the appropriate buffer (250 μl) and dialyzed for 6 hrs in 10 mM Tris-HCl, pH 7.5. End-labeling reactions (200 μl) contained 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 10 mM $MgCl_2$, 18 μM dGTP-dCTP-dTTP, 50 μCi of $\alpha[^{32}P]$dATP (300 Ci/mmol), and 5–10 U of reverse transcriptase (gift of J. Beard, National Institutes of Health). After 30 min at 37°C, the reaction was heated 5 min at 65°C and diluted to 500 μl of the appropriate buffer for digestion with a second restriction enzyme. End-labeled fragments were separated by applying the reaction to 5%–20% sucrose gradients in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 10 mM NaCl and centrifuging 24 hrs

FIG. 1.—Schematic structure and derived clones of human ribosomal DNA. *A* (*top*), *Upper portion* shows the four *Eco* RI restriction fragments of a single rDNA repeat as defined by Wellauer and Dawid [1]. Nontranscribed spacer (*thin lines*), transcribed spacer (*thick lines*), and gene regions are indicated. The A and B fragments can be detected by hybridization with mature 18 or 28S rRNA and have been cloned in bacteriophage vectors. Primary phage clones of the B fragment are depicted along with the plasmid clones and end-labeled segments derived from plasmid inserts. (↑), *Eco* RI site; (*), [^{32}P]-labeled 3′-end. *B*, A 1% agarose gel after electrophoresis and ethidium bromide staining (see MATERIALS AND METHODS). *Lanes 1* and *2* show the release of the 5,700-bp B fragment after *Eco* RI digestion of Charon 16AHrB-1 or pHrB DNA; *lanes 3–5*, *Eco RI-Sal* I digestions of Charon 16AHrB-1, pHrB-SE, and pHrB-ES, respectively; *lane 6*, an *Eco* RI digestion of pBR 322; and *lane 7*, standard *Hind* III fragments of phage lambda DNA (24, 9.6, 6.7, 4.3, 2.3, and 2.0 kb). *C*, Digestion of Charon 16AHrB-1 (*lane 1*) or HrB-5 (*lane 2*) with *Sst* I liberating 3.0- or 5.1-kb fragments according to the orientation of the B fragments *Sst* I site (fig. 3) relative to the *Sst* I site in the right phage arm of Charon 16A [13]. *D*, The 8.4- and 1.7-kb fragments obtained by digestion of pHrB with *Pst* I (*lane 2*) define the B fragment orientation in this plasmid (see fig. 4), while comparison of the *Eco* RI-*Kpn* I digestions of Charon 16AHrB-1 (*lane 3*), Charon 16A (*lane 4*), and pHrB (*lane 5*) defines which *Kpn* I fragments derive from rDNA. *Lane 1* shows the *Hind* III standard fragments described in *C*. *E*, Comparison of independent B fragment recombinants by digestion with *Eco RI-Sma* I. The *first two lanes* are lambda *Eco* RI or *Hind* III fragment standards, while *lanes 3–8* show digestion of λgtWES·λB fragment recombinants and *lanes 9–15* show Charon 16A recombinants.

at 40,000 rpm in the Beckman SW41 rotor. Fractions (0.5 ml) were screened by electrophoresis on 1% agarose gels and dialyzed overnight vs. 10 mM Tris-HCl, pH 7.5, before restriction. As described below, the end-labeling reaction was characterized using pBR 322 DNA to ensure the absence of internal labeling and other artifacts.

Restriction Analysis

Bacteriophage (0.5–1 μg), plasmid (0.2–0.5 μg), or human DNA (2–5 μg) was incubated with 2 U per μg of restriction enzyme (Bethesda Research) in 50 μl of the recommended buffer. After incubation for 1–2 hrs at the appropriate temperature, samples were heated 5 min at 70°C and applied to 14 × 10-cm or 14 × 30-cm 1% agarose slab gels. Electrophoresis was for 10-20 hrs at 50 V in buffer containing 40 mM Tris acetate, 20 mM Na acetate, and 1 mM EDTA at pH 8.2. Gels were stained with ethidium bromide (1 μg per ml) or transferred as described [8].

End-labeled DNA was restricted in similar fashion using 20,000–50,000 cpm (0.1–0.2 μg) per gel lane and amounts of restriction enzyme dictated by preliminary titrations (0.1–0.5 U per μg DNA for a standard incubation time of 15 min). Gels (1.5% agarose) were dried down for 5 hrs with water aspiration in the Hoefer drying apparatus at 60°C and autoradiographed for 3–48 hrs at room temperature using Kodak Royal XOMat film with enhancement screens [18].

Nicked-Translation and Hybridization Reactions

Nicked-translation reactions (100 μl) contained 50 mM Tris-HCl, pH 7.8, 50 mM $MgCl_2$, 100 mM 2-mercaptoethanol, 0.5 mg per ml bovine serum albumin, 18 μM dGTP-dCTP-dTTP, 50 μCi of $\alpha[^{32}P]$dATP (2,000–3,000 Ci/mmol), 1 μg of nicked plasmid or phage DNA, and 5 U of DNA polymerase I (Bethesda Research). DNA was nicked using 0.1 ng of DNAse I (Miles Laboratories, Elkhart, Ind.) for 10 min at 16°C in the above buffer, which is stored as a 1 mg per ml solution in 1 N HCl and diluted 1/10 in activation buffer (10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, and 1 mg/ml bovine serum albumin) for 2 hrs at 0°C before use. After polymerization for 30 min at 16°C, the reaction was diluted to 500 μl with H_2O, extracted with 1 vol of phenol saturated with 500 mM Tris-HCl, pH 7.5, and passed over a 10-ml column of Sephadex G50 in H_2O at room temperature. The excluded ^{32}P radioactivity (95% precipitable with tricholoroacetic acid) contained 2–5 × 10^7 cpm and was stored in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA at 20°C.

Hybridization of nitrocellulose membranes was performed essentially as described by Wahl et al. [19]. After preincubation with 10 ml buffer (50% formamide [Eastman, Rochester, N.Y.]; 5× SSC; 5× Denhardt's [0.1% BSA, .01% Ficoll, .01% polyvinylpyrolidine]; 1% glycine; 100 mM K phosphate; and 100 μg/ml sonicated salmon sperm DNA) in a Seal-O-Meal bag for 5 hrs at 43°C, the membrane was hybridized in the same buffer substituting 10% Dextran sulfate (Pharmacia, Piscataway, N.J.) for glycine with 1× Denhardt's solution and 10^7 cpm of nick-translated DNA (heat-denatured 5 min at 90°C before mixing with hybridization buffer). After hybridization for 8–16 hrs at 43°C, the membrane was washed for 1 hr at 60°C with 250 ml of 2× SSC-0.1% SDS, then 30 min with 250 ml of 0.1× SSC, and dried. Autoradiography was for 1–7 days as described above.

Electron Microscopy

Heteroduplexes were formed essentially as described [20] by adding 0.25 μg of each DNA to 20 μl of 50 mM NaOH-10 mM EDTA for 10 min at room temperature, then adjusting to 40 μl of 50% formamide, 100 mM Tris-HCl, pH 7.4, and annealing for 35 min at room temperature. The DNA was then diluted 25-fold into hyperphase containing 70% formamide, 100 mM Tris-HCl, pH 8.5, 10 mM EDTA, pH 8.1, and 40 μg per ml of cytochrome C. SV40 form II DNA and phage M13 mp5 DNA were included as double-stranded and single-stranded standards, respectively. Molecules were spread on a 40% formamide hypo-

phase and absorbed onto parlodion coated grids. Grids were stained for 20 seconds in 50 μM uranyl acetate, dried in isopentane, and rotary shadowed with platinum/palladium (80/20). Molecules were photographed with a Zeiss EM10-A electron microscope and measured with a Numonics graphics calculator.

RESULTS

Cloning and Preliminary Characterization of the Eco *RI B Fragment of Human rDNA*

Two lambda bacteriophage vectors were used for primary cloning of rDNA segments from enriched DNA (fig. 1). As described in [8], 11 *Eco* RI B fragment clones (Charon 16AHrB-1 through 11) were isolated independently in Charon 16A [13] from the DNA of IMR-90 fibroblasts [14]. The six B fragment clones in λgtWES·λB [15] were isolated independently from a single placental DNA sample as described in MATERIALS AND METHODS.

The B fragment insert in recombinant phage is demonstrated most clearly by *Eco* RI digestion (fig. 1*B*, lanes 1 and 2) or heteroduplex formation with parent phage (not shown). Orientation of the B fragment within recombinant phage can be shown by heteroduplex formation with other recombinant phage (figs. 2*A* and *B*) or by restriction with *Sst* I (fig. 1*C*). The difference in orientation revealed by *Sst* I digestion is most dramatic in Charon 16A clones since, in addition to the asymmetric *Sst* I site in the B fragment (fig. 3), there is an *Sst* I site on the right phage arm that is lacking in λgtWES·λB. The 18S gene region is oriented next to the left phage arm in only three of 11 Charon 16A recombinants (Charon 16AHrB-2, 5, and 7) and only one of six λgtWES·λB recombinants (λgtWES·λHrB-3).

As diagrammed in figure 1, various segments of the *Eco* RI B fragment from Charon 16AHrB-1 were subcloned in pBR 322 [17]. The plasmid pHrB contains the entire B fragment, while the subclones pHrB-ES, pHrB-SS, and pHrB-SE contain segments of the B fragment corresponding to 18S gene or transcribed spacer regions. Comigration of the *Eco* RI-*Sal* I segments in pHrB-ES and pHrB-SE with those in the parent phage Charon 16AHrB-1 is demonstrated in figure 1*B*, lanes 3–5.

Restriction Mapping of the Human rDNA Eco *RI B Fragment*

Figure 1*A* displays the various B fragment clones that are available in plasmid or bacteriophage vectors. Restriction sites may be quickly localized by comparing digests of the various clones. An example of this approach for the enzyme *Kpn* I is shown in figure 1*D*. Restriction fragments that comigrate when *Eco* RI-*Kpn* I digests of Charon 16AHrB-1 and pHrB are compared (fig. 1*D*, lanes 3 and 5) derive from B fragment rather than vector DNA (lane 4). The cleavage of pHrB-SS DNA but not pHrB-ES or pHrB-SE DNA (not shown) precisely defines the position of this restriction site. Sites for *Kpn* I, *Sst* I, *Xho* I, *Pst* I, *Xba* I, *Bam* HI, and *Bgl* I were determined by this approach, while *Hpa* I, *Bgl* II, and *Hind* III were found not to digest the B fragment (fig. 3).

Restriction enzymes that recognize many sites in the B fragment were mapped using partial digestion of end-labeled DNA [21]. Figure 1*A* shows the different

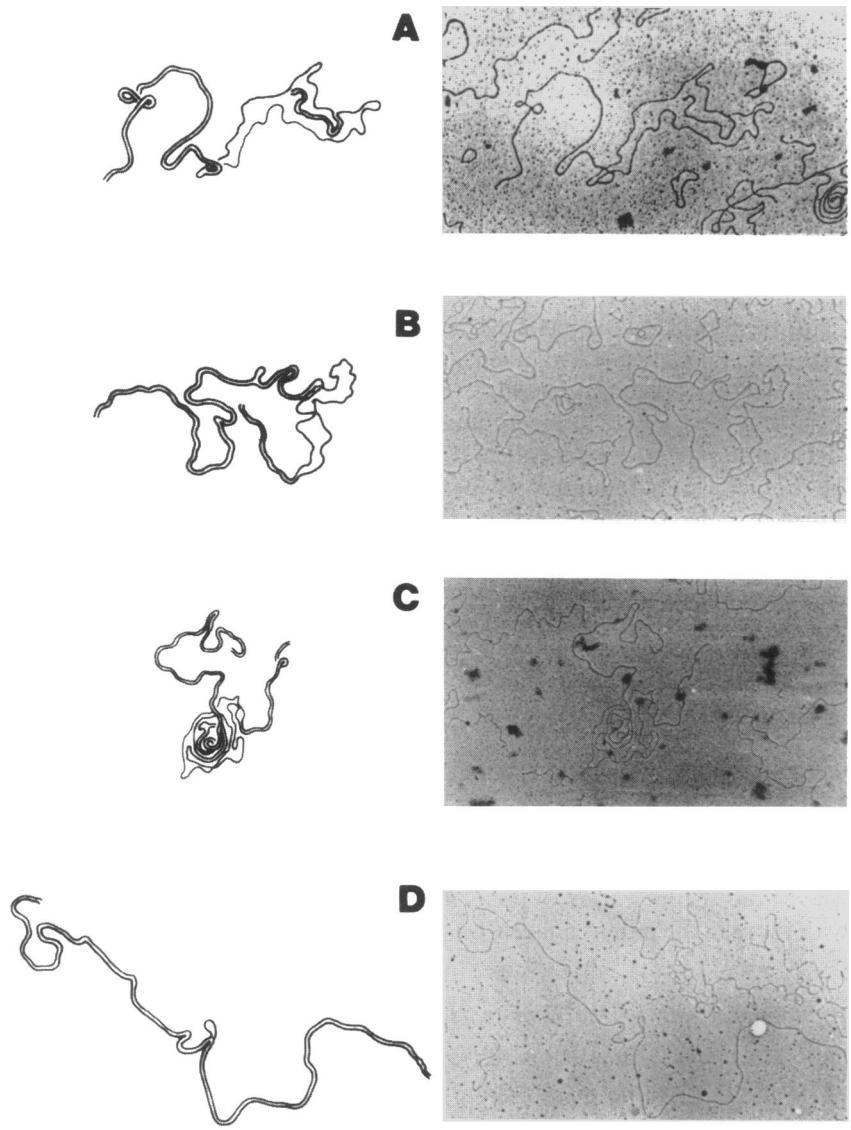


FIG. 2.—Heteroduplex analysis of cloned *Eco* RI B fragments. *A*, Heteroduplex between Charon 16AHrB-1 and λgtWES·HrB-3 showing the large substitution loop obtained when the B fragment inserts are in opposite orientation. This topology is labeled III in table 3. Magnification 45,500×. *B*, Heteroduplex between Charon 16AHrB-1 and λgtWES·HrB-1 showing the similarly oriented B fragment region between nonhomologous regions of the phage arms (structure I in table 3). Magnification 45,500×. *C*, Heteroduplex between Charon 16AHrB-2 and λgtWES·HrB-3 showing decreased length of the homologous B fragment region. Magnification 45,500×. *D*, Heteroduplex between two Charon 16AHrB-2 molecules demonstrating a deletion loop. This deletion correlates with the shortened B fragment region in *C* and indicates heterogeneity among Charon 16AHrB-2 inserts. Measurements are shown in table 3 (structure II). Magnification 35,700×.

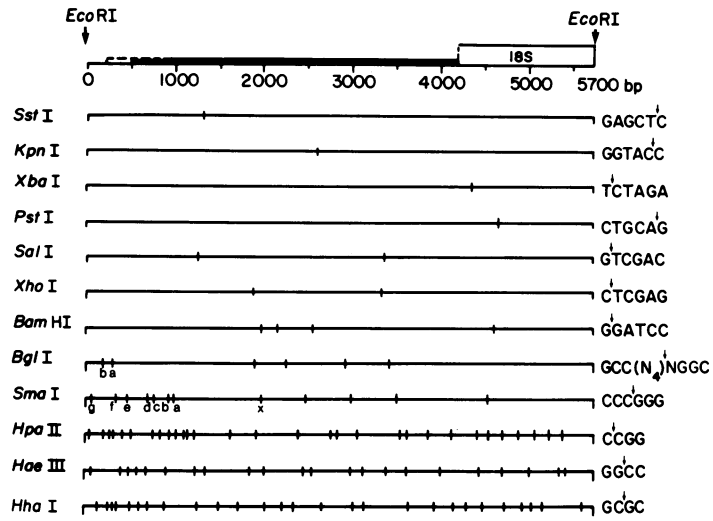


FIG. 3.—Restriction map of the human rDNA *Eco* RI B fragment. The 18S gene (*box*), external transcribed spacer (*thick line*), and nontranscribed spacer (*thin line*) regions of the *Eco* RI B fragment are displayed above restriction sites derived from the analysis of Charon 16AHrB-1 and its plasmid subclones (fig. 1). The enzymes *Bgl* II, *Hpa* I, and *Hind* III do not digest the B fragment. *Dotted lines* represent uncertainty about the position of nontranscribed/transcribed spacer and spacer/18S gene junctions. Lettered *Bgl* I and *Sma* I sites are examined for variability in figure 5.

end-labeled segments that were constructed from plasmid B fragment clones. The circular DNA was opened at a specific site and end-labeled using $\alpha[^{32}P]$dATP and reverse transcriptase. For most experiments, the two end-labeled segments were separated by cleavage with a second restriction enzyme as diagrammed in figure 4*A* for fragments B(EX*), B(X*E), and B(SE*) or B(E*S).

Before analysis of rDNA segments, control digestions were performed to assess the degree of internal labeling and the accuracy of molecular weight estimates. Partial restriction of pBR322 DNA linearized with *Eco* RI and labeled at both ends was compared with the partial restriction fragments expected from the sequence data of Sutcliffe [22]. There was general agreement between the experimental and predicted values if one assumes limited resolution of fragments that differ by less than 50 bp or fall outside a 100–4,000-bp size range.

Partial restriction of the segment B(ES*) with *Sma* I and *Hpa* II is shown in figure 4*B*. As expected, certain fragments are similar since the *Hpa* II recognition sequence is contained in *Sma* I sites. The *Hpa* II sites documented in figure 4*B* are tabulated in table 1 by converting the molecular weight of each restriction fragment to a coordinate representing the distance of that restriction site in bp from the *Eco* RI site at the B/C *Eco* RI fragment junction (fig. 1). Although the *Hpa* II digestions shown in figure 4*B* contained too much enzyme to visualize the larger restriction fragments, those sites that are demonstrated compare well with sites derived from digestion of the segment B(E*S), which is labeled at the opposite end

(table 1). Such comparisons are necessary to rule out internal labeling by reverse transcriptase. Despite such precautions, table 1 and the similar tables for *Hae* III, *Hha* I, and *Sma* I (available upon request from the authors) clearly represent a minimal estimate of restriction sites. These data are summarized in figure 3.

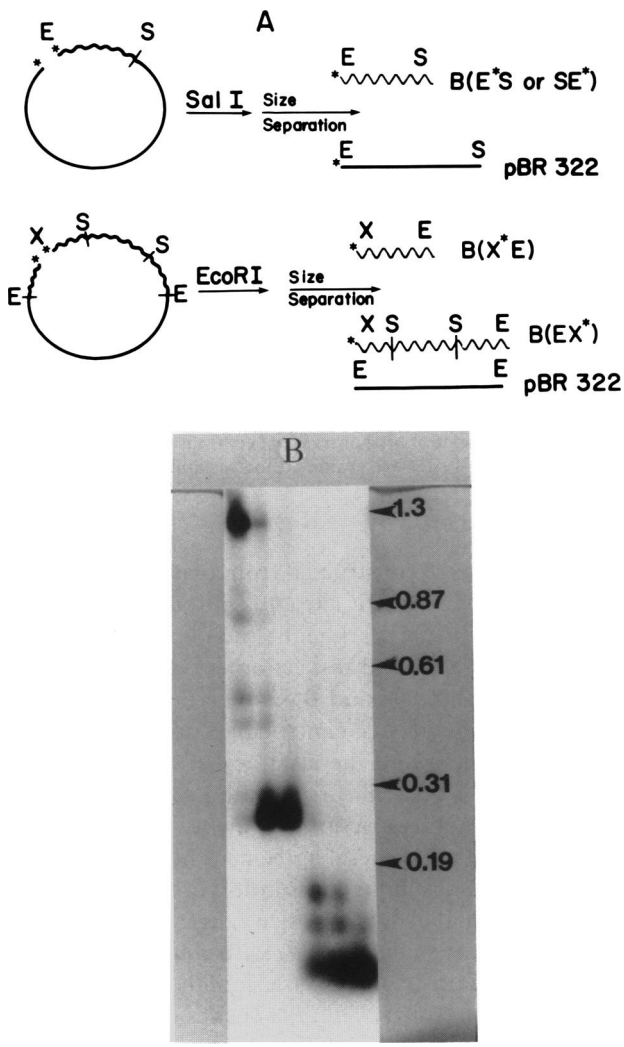


FIG. 4.—Isolation and partial restriction of end-labeled rDNA segments. *A*, The strategy for preparing end-labeled *Eco* RI-*Sal* I segments from pHrB-ES or pHrB-SE DNA (*top*) and *Eco* RI-*Xba* I segments from pHrB DNA (*bottom*) is diagrammed. *E*, *S*, and *X* represent *Eco* RI, *Sal* I, and *Xba* I sites; the plasmid (*smooth lines*) and insert (*rough lines*) DNAs are indicated schematically. *B*, Partial restriction analysis of the end-labeled segment B(ES*) with *Sma* I (*lanes 1–3*) and *Hpa* II (*lanes 4–6*). The molecular weights of certain *Hae* III fragments of ϕX 174 are indicated as standards, and the coordinates derived from molecular weights of the *Hpa* II fragments are listed under B(ES*) in table 1.

TABLE 1

PARTIAL RESTRICTION ANALYSIS OF *Eco* RI B rDNA SEGMENTS WITH *Hpa* II

END-LABELED SEGMENT										
B(E*S)			B(EX*)		B(SE*)			B(X*E)		
Exp. 1	Exp. 2	B(ES*)	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	MEAN COORDINATE
35	65									50
200	230									215
265	285									275
310	305									310
410	430									420
530	520	490	480							505
780	800	770	700							760
820	850	830	800							825
980	970	970	960							970
	1020	1020	...							1020
		1130	1090							1110
		1150	1130							1140
		1210	1230							1220
			1600							1600
			1910							1910
			2350							2350
			2770	2760						2765
			2830	...						2830
			3060	...						3060
			3560	3540	3510	...				3535
			3650	3625	...	3750				3675
			3870	3850	3820	3860				3850
			4110	4100	3940	4250				4125
					4410	4430		4450	4488	4430
					4575	4530	4550	4554	4563	4555
					4660	...	4700	4730	...	4700
					...	5000	4770	4940	4950	4890
					5150	5120	5040	5030	5000	5070
					5280	5210	5220	5240	...	5240
					5380	...	5350	5420	...	5385

NOTE: Each column represents a separate partial restriction analysis of the indicated end-labeled rDNA segment (see fig. 1). Molecular weight estimates for each fragment visualized on gel autoradiograms (i.e., fig. 4*B*) are converted to a coordinate representing distance in bp from the *Eco* RI B/C fragment junction. Coordinates derived from separate experiments are averaged to give the mean listed in the final column and diagrammed in figure 3.

Comparison of Cloned B Fragments by Restriction Analysis

Restriction analysis of independent bacteriophage B fragment recombinants with *Eco* RI-*Pst* I, *Eco* RI-*Bam* HI-*Sal* I, and *Eco* RI-*Xba* I-*Xho* I revealed no evidence of length or sequence heterogeneity among the 17 clones (not shown). A questionable difference in mobility of the *Sma* I rDNA fragment (see arrow in fig. 1*E*) extending from *Sma* I sites a to x in figure 3 was observed, but this was not confirmed by the partial restriction analysis described below. The smaller *Sma* I fragments could not be clearly visualized by ethidium bromide staining.

More detailed comparison of bacteriophage recombinants was achieved by restricting with *Eco* RI, labeling the ends, and subjecting the B(E*E*) segments to partial restriction analysis. In figure 5, B(E*E*) fragments containing the left-labeled end can be viewed separately from fragments containing the right-labeled end by utilizing *Sma* I or *Bgl* I digestions. The lettered fragments deriving from the *Sma* I cluster are clearly separated from the phage fragments (ϕ), and the many *Sma* I rDNA fragments of intermediate size that correspond closely to the sizes predicted from the map in figure 3. There was clear variation in the *Sma* I and *Bgl* I patterns for two of four recombinant phage, and the calculated fragment molecular weights are listed in table 2. Variation of the smallest fragment (g) could not be

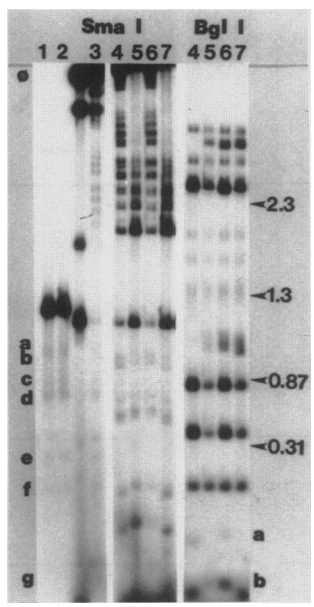


FIG. 5.—Comparative restriction of independently cloned *Eco* RI B fragments. The end-labeled segments B(E*E*) derived from λgtWES·HrB-1 (*lanes 3 and 4*), HrB-2 (*lanes 5*), HrB-4 (*lanes 6*), and HrB-6 (*lanes 7*) were restricted with *Sma* I or *Bgl* I followed by electrophoresis on a 1.5% agarose gel and autoradiography. *Sma* I digestions of the end-labeled segment B (E*S) were included for comparison (*lanes 1 and 2*). *Lanes 1–3* are taken from one gel, *lanes 4–7* from another. *Letters* identify fragments corresponding to the restriction sites for *Sma* I and *Bgl* I shown in figure 3; molecular weights calculated from the mobility of these fragments are listed in table 2.

TABLE 2

COMPARATIVE RESTRICTION ANALYSIS OF END-LABELED rDNA SEGMENTS

		B(E*S)		B(E*E*)				
ENZYME	SITE (BAND)	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
Sma I	a	980	990	990	990	1020	1000	980
	b	940	950	950	950	970	950	940
	c	770	785	780	780	820	800	760
	d	710	720	720	720	740	730	700
	e	450	455	450	465	480	470	450
	f	345	350	340	360	380	380	350
	g	60	...	...	...	...	...	...
Bgl I	a	(300)		...	310	...	325	...
	b	(200)		...	200	215	210	190

NOTE: Molecular weights in kb are listed for the restriction fragments labeled a–g (*Sma* I) or a and b (*Bgl* I) shown in the various lanes of figure 5. Values in parentheses were determined from separate experiments.

assessed because of the presence of unincorporated nucleotide even after extensive dialysis.

Comparison of Cloned B Fragments by Heteroduplex Analysis

The lambda bacteriophage vectors Charon 16A and λgtWES·λB have little homology in their central regions as indicated by the large substitution loop in heteroduplexes of these two phage DNAs (not shown). Insertion of the human rDNA *Eco* RI B fragment in the single *Eco* RI site of Charon 16A and replacement of the λB fragment of λgtWES·λB with this same insert yields a larger substitution loop in heteroduplexes between recombinant phage if the B fragments are in opposite orientation (fig. 2*A*). If the B fragments are in similar orientation, a B fragment heteroduplex is visible between two substitution loops that represent the remaining nonhomologous regions of the phage arms (fig. 2*B*). The B fragment region is thus displayed between two substitution loops and can be scrutinized for evidence of length heterogeneity between the two cloned segments. As indicated in table 3, no deletion or substitution loops were detected in heteroduplexes between recombinant phage under conditions where loops greater than an estimated 100 bp should have been visible. A minor fraction of Charon 16AHrB-2: λgtWES·λHrB-3 heteroduplexes displayed a shortened B fragment region (fig. 2*C*). This deletion amounted to 3.4 ± 0.9 kb of the Charon 16AHrB-2 B fragment as measured from heteroduplexes of Charon 16AHrB-2 with itself (fig. 2*D* and table 3). Changes in the morphology of the larger phage substitution loop indicated that the deletion involved the left portion of the B fragment.

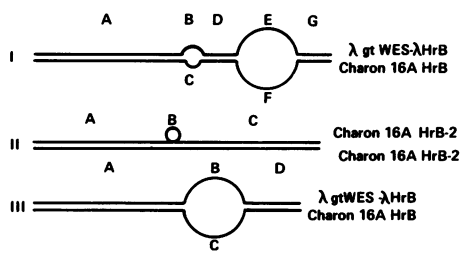
Restriction Analysis of B Fragment Regions in Genomic DNA

The examination of cloned B fragment repeats for heterogeneity was extended to adjacent regions of rDNA by hybridizing to Southern blots of restricted genomic DNA. The Southern blot in figure 6*A* shows distinct bands for *Xho* I, *Xba* I, *Kpn*

TABLE 3

LENGTH MEASUREMENTS OF HETERODUPLEXES

HETERODUPLEX TOPOLOGY	RECOMBINANT PHAGE		NO. EXAMINED	MEASUREMENTS (kb ± SD)						
	λgtWES	Charon 16A		A	B	C	D	E	F	G
I	HrB-1:	HrB-1	5							
	HrB-2:	"	13							
	HrB-4:	"	5							
	HrB-5:	"	9							
	HrB-6:	"	5							
	HrB-1:	HrB-4	6							
	"	HrB-8	5							
	HrB-3:	HrB-2	36*	18.1 ± 1.3	2.0 ± 0.4	0.3 ± 0.07	5.5 ± 0.3	7.2 ± 0.9	14.2 ± 1.9	6.1 ± 0.5
	"	HrB-5	17	18.4 ± 0.8	1.8 ± 0.2	0.3 ± 0.07	5.4 ± 0.4	6.7 ± 0.9	14.0 ± 1.7	6.0 ± 0.5
	"	HrB-7	35	18.4 ± 0.9	1.7 ± 0.3	0.3 ± 0.07	5.5 ± 0.4	7.0 ± 0.9	14.2 ± 1.9	6.2 ± 0.5
II		HrB-2	22	21.05 ± 1.7	3.4 ± 0.9	20.6 ± 2.1	...	...	...	...
III	HrB-3:	HrB-6	25	18.5 ± 1.3	13.0 ± 1.6	18.9 ± 1.4	6.4 ± 0.5	...	...	...



NOTE: Diagram I represents a heteroduplex of a λgtWES·λHrB clone with a Charon 16AHrB clone of similar orientation. Segment D is the homologous B fragment region. Diagram II represents a heteroduplex of Charon 16AHrB-2 with itself. Bottom line of diagram represents those few molecules that have B fragment deletions. The measurements do not allow certain identification of the right and left phage arms. Diagram III represents a heteroduplex of a λgtWES·λHrB clone with a Charon 16AHrB clone of opposite orientation.

* This no. includes three heteroduplexes with a shortened D segment that were not included in the measurements.

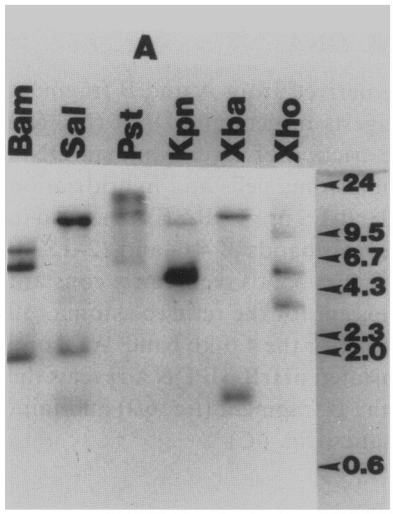
I, *Pst* I, *Bam* HI, and *Sal* I in addition to those derived from A and B fragment regions. The minor bands of 3–8 kb in *Sal* I digests of genomic DNA (fig. 6*A*) prompted further analysis with this enzyme. Restriction of several human DNA samples from spleen, placenta, HeLa, or retinoblastoma cells and hybridization with the nick-translated B fragment subclones pHrB-ES or pHrB-SE are shown in figure 6*B* and *C*. Hybridization with pHrB-ES reveals bands of 4.6- and 12–15-kb in addition to the 1.5-kb band seen in figure 6*A*. These bands seem fairly constant for DNA from different tissues and individuals except for the retinoblastoma cell line (fig. 6*B*, lane g), which substitutes a 7.2-kb band for the 4.6-kb band. A second hybridization of the identical blot with nick-translated pHrB-SE DNA reveals the 10.6-kb *Sal* I segment extending to the right of the B fragment (fig. 6*D*) and faint hybridization to segments of 7–9 kb in several lanes (fig. 6*C*).

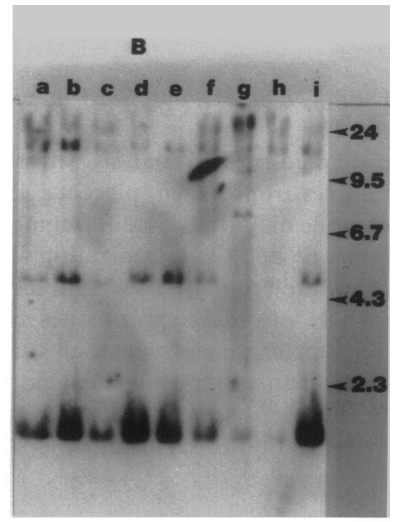
DISCUSSION

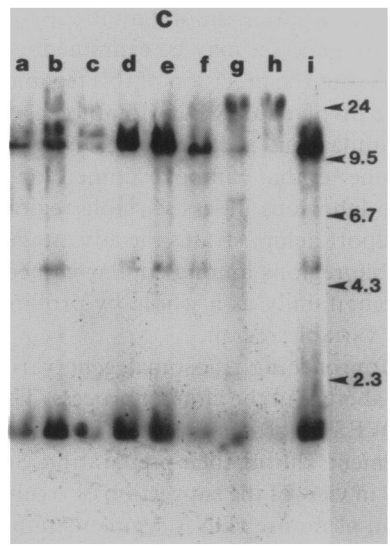
The analysis of sequence variation among multicopy genes can be approached in several ways. The entire gene set may be examined using Southern restriction analysis of genomic DNA, but the precision of this technique is limited when small restriction fragments are involved or when homologies with other gene families exist. The cloning of similar but independent DNA segments yields a clearly defined subgroup of a gene set that can be examined for variability, but sequencing of every clone is too laborious unless variable regions can be pinpointed by other techniques. This report demonstrates the advantage of a detailed restriction map for targeting variable regions among similar cloned segments, and extends this knowledge to the gene family as a whole by probing genomic DNA with a subclone containing the variable region.

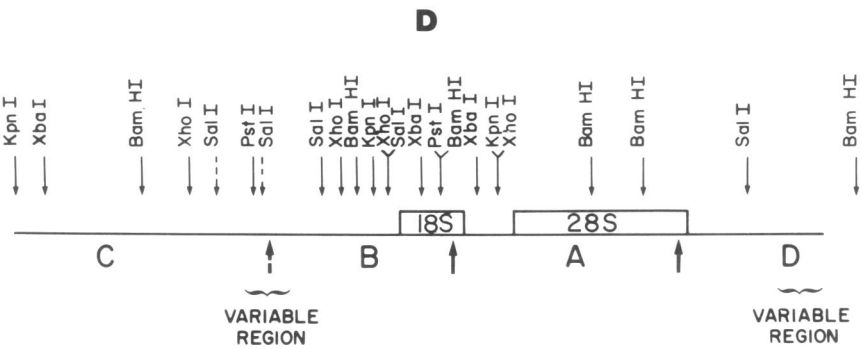
Of great concern in approaching ribosomal gene variation by the analysis of cloned segments is bias inherent in the cloning process. The deletion occurring in a small proportion of λgtWES·λHrB-2 molecules (fig. 2*D*) is the only clear example of a change in cloned segments during their preparation. Such changes must always be considered, especially in view of the suggestion of Arnheim and Kuehn [23] that growth-induced variation of mouse rDNA segments mimics the evolutionary behavior of these segments. These considerations emphasize the importance of relating features of cloned segments to their structure and organization in genomic DNA.

A detailed restriction map for the human rDNA *Eco* RI B fragment was assembled using standard or partial restriction analysis [21, 24]. Clusters of restriction sites for *Sma* I, *Hpa* II, *Hha* I, and *Hae* III were found near the 5′-end (fig. 3). No variation among B fragment clones could be detected by standard restriction or heteroduplex analysis (figs. 1*E* and 2), but partial restriction analysis suggested a 15–30-bp shift in the position of 5′-*Sma* I or *Bgl* I sites among four clones (fig. 5 and table 2). A comparable region of *Xenopus* nontranscribed spacer contains a variable 15-bp element just to the left of a *Sma* I cluster [25, 26]. Mouse rDNA also contains a *Sma* I cluster near the junction of transcribed and nontranscribed spacers [27]. A position for this junction 400–800 bp from the left *Eco* RI site of


A
Bam
Sal
Pst
Kpn
Xba
Xho
24
9.5
6.7
4.3
2.3
2.0
0.6


B
a b c d e f g h i
24
9.5
6.7
4.3
2.3


C
a b c d e f g h i
24
9.5
6.7
4.3
2.3


D
Kpn I
Xba I
Bam HI
Xho I
Sal I
Pst I
Sal I
Sal I
Xho I
Bam HI
Kpn I
Xho I
Sal I
Xba I
Pst I
Bam HI
Xba I
Kpn I
Xho I
Bam HI
Bam HI
Sal I
Bam HI
18S
28S
C
B
A
D
VARIABLE REGION
VARIABLE REGION

the human rDNA B fragment would agree with the electron microscopic estimates of Wellauer and Dawid [1].

The analysis of B fragment and adjacent regions of genomic DNA is preliminary, but demonstrates restriction sites expected from the map in figure 3 and maps of the *Eco* RI A rDNA fragment (see fig. 6*D* and [12]). New restriction sites in the C fragment region are postulated based on the fragments visualized in figure 6*A*, but homologies reported between nontranscribed spacer regions and other fragments in man and mouse [28] make these sites uncertain. Homology between regions of *Xenopus* transcribed spacer adjacent or about 1,100 bp downstream from the promoter site [29] complicates mapping of this area prior to cloning of the C fragment.

Restriction patterns of genomic rDNA with *Sal* I hybridized with pHrB (fig. 6*A*), pHrB-ES (fig. 6*B*), or pHrB-SE (fig. 6*C*) reveal many fragments in addition to those expected from the A and B *Eco* RI fragment restriction map. The *Sal* I sites indicated by the dotted arrows in fig. 6*D* represent one explanation for the extra 1.5- and 4.6-kb *Sal* I fragments specifically visualized by hybridization with pHrB-ES. This explanation requires that the *Sal* I site nearest the B fragment, like its neighboring *Eco* RI site, be absent from certain rDNA repeats and generate the 4.6-kb *Sal* I fragment. Alternatively, this 4.6-kb fragment may contain homologous non-rDNA sequences.

A restriction polymorphism involving the 4.6-kb *Sal* I segment is demonstrated in retinoblastoma cells (fig. 6*B*, lane g), where a new fragment of 7.2 kb is seen. The relation of this polymorphism to the genetic tendency for retinoblastoma caused by mutation [30] or chromosomal deletion [31] will be interesting to define. The *Bam* HI polymorphism that seemed specific for HeLa cell lines was actually a rare but normal variant that probably was present in the individual from which the cell line originated [12].

The additional *Sal* I fragments of 3–8 kb revealed by hybridization to pHrB or pHrB-SE do not fit in with the map in figure 6*D*. Similar variable fragments have been seen using A fragment probes (R. Schmickel, unpublished results, 1981). These may represent aberrant repeats, such as those defined by Wellauer and Dawid [1], or junctions of rDNA repeats with chromosomal DNA. Variation of human rDNA or related sequences thus seems considerable, but its definition awaits further cloning.

FIG. 6.—Restriction analysis of genomic rDNA using *Eco* RI B fragment clones as probe. *A*, Placental DNA was digested with the indicated restriction endonucleases, subjected to electrophoresis on a 14 × 10-cm 1% agarose gel, and blotted as described in MATERIALS AND METHODS. Autoradiography for 3 days followed hybridization with nick-translated pHrB DNA. Standards are *Hind* III and *Eco* RI fragments of lambda DNA. *B*, DNA samples (10 μg) from various sources were restricted with *Sal* I and hybridized to nick-translated pHrB-ES after electrophoresis on a 14 × 30-cm 1% agarose gel, Southern blotting, and autoradiography for 2 days. Standards are as above. *Lane a*, spleen; *lane b*, placenta; *lane c*, HeLa cell; *lane d*, spleen; *lane e*, placenta; *lane f*, spleen; *lane g*, retinoblastoma; *lanes h* and *i*, placenta. The retinoblastoma cell line (AG 1232) was obtained from the Human Mutant Cell Repository, Camden, N.J. *C*, The identical blot shown in *B* was hybridized to nick-translated pHrB-SE DNA and autoradiographed for 3 days. *D*, Composite restriction map of human rDNA.

The map in figure 6*D* is a composite derived from the restriction analysis of cloned or genomic human rDNA. Variable nontranscribed spacer regions surround a transcribed region that is highly conserved with the exception of a *Hind* II polymorphism in the 28S gene [11]. Variable regions should allow the definition of polymorphisms specific for individual acrocentric chromosomes, while clones of the constant region will provide assays for ribosomal gene expression.

ACKNOWLEDGMENT

We acknowledge helpful discussions with Roy Schmickel.

REFERENCES

1. WELLAUER P, DAWID I: Isolation and sequence organization of human ribosomal DNA. *J Mol Biol* 128:289–303, 1979
2. DAWID I, WELLAUER P: A reinvestigation of 5′→′3 polarity in 40S ribosomal RNA precursor of *Xenopus laevis*. *Cell* 8:443–448, 1976
3. SCHMICKEL R: Quantitation of human ribosomal DNA: hybridization of human DNA with ribosomal rRNA for quantitation and fractionation. *Pediatr Res* 7:5–12, 1973
4. SCHMICKEL R, KNOLLER M: Characterization and localization of the human genes for ribosomal ribonucleic acid. *Pediatr Res* 11:929–935, 1977
5. BROSS K, KRONE W: On the number of ribosomal genes in man. *Humangenetik* 14: 137–141, 1972
6. YOUNG B, HELL A, BIRNIE G: A new estimate of human ribosomal gene number. *Biochim Biophys Acta* 454:539–548, 1976
7. ARNHEIM N, SOUTHERN E: Heterogeneity of the ribosomal genes in mice and men. *Cell* 11:363–370, 1977
8. WILSON G, HOLLAR B, WATERSON J, SCHMICKEL R: Molecular analysis of cloned human 18S ribosomal DNA segments. *Proc Natl Acad Sci USA* 75:5367–5371, 1978
9. KRYSTAL M, ARNHEIM N: Length heterogeneity in a region of the human ribosomal gene spacer is not accompanied by extensive population polymorphism. *J Mol Biol* 126: 91–104, 1979
10. REEDER R: Genetics of eukaryotic ribosomes, in *Ribosomes*, edited by NOMURA M, TISSIERES A, LENGYEL P, Cold Spring Harbor Monograph Series, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1972, pp 489–518
11. ARNHEIM N, KRYSTAL M, SCHMICKEL R, WILSON G, RYDER O, ZIMMER E: Genetic exchanges among ribosomal genes on non-homologous chromosomes in man and the great apes. *Proc Natl Acad Sci USA* 77:7323–7327, 1980
12. SCHMICKEL R, WATERSON J, KNOLLER M, SZURA L, WILSON G: HeLa cell identification by analysis of ribosomal DNA segment patterns generated by endonuclease restriction. *Am J Hum Genet* 32:890–897, 1980
13. BLATTNER F, WILLIAMS B, BLECHL A, ET AL.: Charon phages: safer derivatives of bacteriophage lambda for DNA cloning. *Science* 196:161–169, 1977
14. NICHOLS W, MURPHY D, CRISTOFALO V, TOJI L, GREENE A, DWIGHT S: Characterization of a new human diploid cell strain, IMR-90. *Science* 196:60–63, 1977
15. LEDER P, TIEMEIER D, ENQUIST L: EK2 derivatives of bacteriophage lambda useful in the cloning of DNA from higher organisms: the λgtWES system. *Science* 196:1175–1177, 1977
16. MURRAY K: 5′-terminal sequences of deoxyribonucleic acid from bacteriophages λ and 424. *Biochem J* 131:569–580, 1973
17. BOLIVAR F, RODRIGUEZ R, BETLACH M, BOYER H: Construction and characterization of new cloning vehicles. I. Ampicillin resistant derivatives of the plasmid pMB9. *Gene* 2:75–93, 1977

18. LASKEY R, MILLS A: Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide. *Eur J Biochem* 56:335–341, 1975
19. WAHL G, STERN M, STARK G: Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. *Proc Natl Acad Sci USA* 76:3683–3687, 1979
20. DAVIS R, SIMON M, DAVIDSON N: Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. *Methods Enzymol* 21D: 413–428, 1971
21. SMITH H, BIRNSTIEL M: A simple method for DNA restriction site mapping. *Nucleic Acids Res* 3:2387–2398, 1976
22. SUTCLIFFE J: pBR 322 restriction map derived from the DNA sequence: accurate DNA size markers up to 4361 nucleotide pairs long. *Nucleic Acids Res* 5:2721–2728, 1978
23. ARNHEIM N, KUEHN M: The genetic behavior of a cloned mouse ribosomal DNA segment mimics mouse ribosomal gene evolution. *J Mol Biol* 134:743–765, 1979
24. WILLIAMS JG, PENMAN S: The messenger RNA sequences in growing and resting mouse fibroblasts. *Cell* 6:197–206, 1975
25. BOSELEY P, MOSS T, MACHLER M, PORTMANN R, BIRNSTIEL M: Sequence organization of the spacer DNA in a ribosomal gene unit of *Xenopus laevis*. *Cell* 17:19–31, 1979
26. BOTCHAN P, REEDER R, DAWID I: Restriction analysis of the non-transcribed spacers of *Xenopus laevis* ribosomal DNA. *Cell* 11:599–607, 1977
27. GRUMMT I, GROSS H: Structural organization of mouse rDNA: comparison of transcribed and non-transcribed regions. *Mol Gen Genet* 177:223–229, 1980
28. ARNHEIM N, SEPERACK P, BANERJI J, LANG R, MIESFELD R, MARCU KB: Mouse rDNA nontranscribed spacer sequences are found flanking immunoglobulin C_H genes and elsewhere throughout the genome. *Cell* 22:179–185, 1980
29. SOLLNER-WEBB B, REEDER R: The nucleotide sequence of the initiation and termination site for ribosomal RNA transcription in *X laevis*. *Cell* 18:485–499, 1979
30. FALLS H, NEEL J: Genetics of retinoblastoma. *Arch Ophthalmol* 46:367–389, 1951
31. KNUDSON A, MEADOWS A, NICHOLS W, HILL R: Chromosomal deletion and retinoblastoma. *N Engl J Med* 295:1120–1123, 1976