Am J Hum Genet 32:890–897, 1980

HeLa Cell Identification by Analysis of Ribosomal DNA Segment Patterns Generated by Endonuclease Restriction

R. D. SCHMICKEL,[1,2] J. R. WATERSON,[1] M. KNOLLER,[1] L. L. SZURA,[1] AND G. N. WILSON[1]

SUMMARY

Restriction endonuclease analysis of HeLa cells and cells in which origins have been questioned provides evidence in favor of a HeLa cell origin for the questioned cells. Digestion of cellular human DNA reveals a variable ribosomal DNA (rDNA) fragment that is present in up to four discrete sizes. Cell lines of known and suspected HeLa origin contain only two size variants. This pattern of variability serves to distinguish HeLa-derived cells from others. Despite repeated passage and divergence of the HeLa phenotype and karyotype, the restriction pattern is remarkably constant.

INTRODUCTION

Finding chromosome variants and isozymes characteristic of HeLa cells in cells that have presumably been derived from diverse tissues casts doubt on their purported origin [1–5]. We have examined some of the disputed cell lines by restriction nuclease analysis and show that the disputed cell lines all have the same restriction pattern for ribosomal genes as do HeLa cells. This pattern was not seen in tissue of other origins. This report demonstrates the relative stability of gene organization and the usefulness of endonuclease restriction analysis for determinations of inheritance.

Variations of DNA sequences that lead to enzyme polymorphisms have long been recognized as useful for studies of inheritance among populations. Those sequences that do not necessarily have a protein product can be studied by restriction pattern

Received October 17, 1979; revised March 6, 1980.

This study was supported by grants HD-04569 (to R. S.) from the National Institutes of Health and 5-169 (to G. N. W.) from the National Foundation, and fellowship F32 GM-06317 (to J. W.) from the National Institutes of Health.

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

[2] Department of Human Genetics, University of Michigan.

analysis. Studies of *Xenopus* have demonstrated that complex restriction patterns of transcribed and nontranscribed ribosomal genes show predictable inheritance patterns [6]. Grossman et al. [7] demonstrated the usefulness of restriction analysis to detect mouse cell contamination of a HeLa cell culture. Several studies have documented that human DNA polymorphism is demonstrable by restriction analysis. Arnheim and Krystal have discussed rDNA variation [8], and Kan and Dozy [9] have used the variant restriction pattern of sickle hemoglobin genes to predict the disease prenatally.

The dramatic chromosomal alterations in number and morphology that occur in malignant tissue and transformed cells would suggest that gene organization is continuously evolving at the chromosomal level [10]. We have examined stability of gene organization by restriction analysis in a HeLa cell line used continuously in our laboratory for 10 years.

MATERIALS AND METHODS

Cell Culture

HeLa cells and permanent cell lines were obtained from the American Type Culture Collection and grown to confluency (3×10^8 cells) in roller bottles. Eagle's minimum essential medium (Gibco, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Gibco) was used in all cases.

DNA Extraction

Trypsinized cells or surgical specimens were homogenized in 10 vol of cold buffer (0.05 M Tris-HCl, 0.025 M KCl, 0.005 M $MgCl_2$, and 0.25 M sucrose at pH 7.5) and lysed at 65°C by adjusting to 0.1% SDS, 1 M NaCl, and 1 mM EDTA. DNA was extracted by a modification of the Marmur technique as described [11] and stored at 4°C over chloroform.

Restriction Analysis

Each DNA sample (10 μg) was digested with 20 U of the appropriate restriction endonuclease (Bethesda Research Laboratories, Bethesda, Md.) for 2 hrs at 37°C in the recommended buffer. Each sample was separated by electrophoresis on a 1%, 15×30 cm agarose slab gel at 100 V for 15 hrs. The DNA was transferred to nitrocellulose by the Southern technique [12] and hybridized for 15 hrs at 65°C with 8 μg of [^{125}I]28S rRNA (10^7 cpm/μg) in 40 ml of $2 \times$ saline, sodium citrate (SSC). The [^{125}I]rRNA was prepared from HeLa cell polysomes as described [13]. Autoradiography was for 15 hrs at −70°C using Kodak X-O Mat R film and a fluorescence screen [14]. A standard curve for each gel was drawn by a least-squares fit of the logarithm of fragment molecular weight vs. the distance migrated. *Eco* RI and *Hind* III fragments of lambda DNA were used as standards. In the figures, the numbers to the left of the gels are the calculated size by comparison with lambda fragment standards. Cell name, purported tissue of origin, and American Type Culture catalog (ATCC) number are given in the figure 1 legend for each American Type Culture permanent cell line. Tissues derived from surgical specimens are indicated by tissue of origin and patient initials.

RESULTS AND DISCUSSION

Restriction patterns of rDNA were compared by transfer of the size-fractionated DNA to nitrocellulose membranes and hybridized to ribosomal RNA [12]. HeLa ribosomal DNA restriction patterns for *Eco* RI, *Hind* II, *Hind* III, *Sal* I, *Sst* I, and *Pst* I endonucleases were the same as seen in diploid tissue [15]. Within a given individual, the family of 300 repeated copies of the ribosomal genes demonstrates variability. This variation is seen in three separate restriction enzyme patterns. The first variation is that

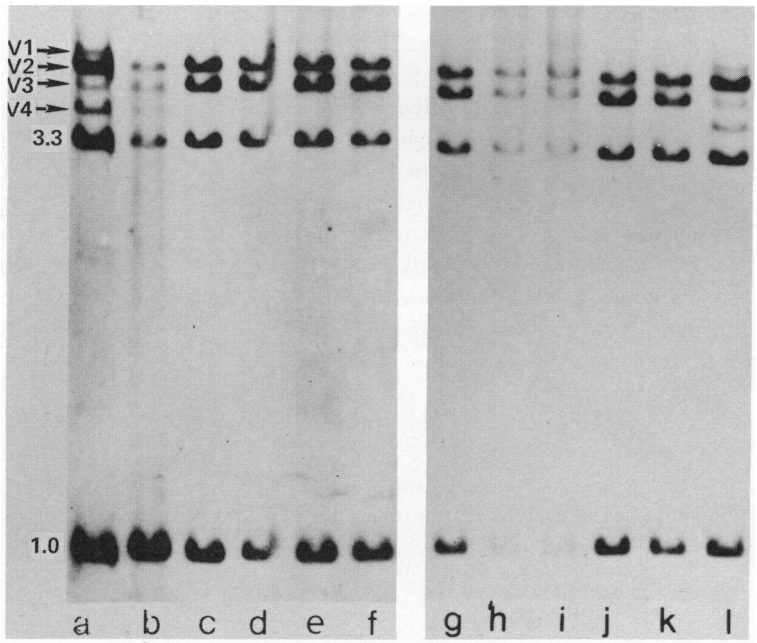

FIG. 1.—rDNA fragments in *Bam* HI digests of various cell DNAs identified by hybridization with [^{125}I]28S rRNA. DNA was restricted with *Bam* HI, transferred to nitrocellulose, hybridized with [^{125}I]rRNA, and autoradiographed. Fragments *V1*, *V2*, *V3*, and *V4* seen in *lanes a* and *l* are found in variable amounts in tissues of different origins. Note that all HeLa lines (*lanes c–k*) have approximately equal amounts of *V2* and *V3* and no detectable amounts of *V1* or *V4*. The *3.3* and *1.0 bands* are the 3.3 and 1.0 × 10^6 *Bam* HI fragments that also hybridize with [^{125}I]28S rRNA. Arrangement of these fragments within the gene can be seen in figure 2. Permanent cell culture DNA is identified by its name, tissue of purported origin, and the ATCC no. Patient tissue is identified by initials. The source of DNA: *a*, human spleen tissue, GB; *b*, ovarian teratoma tissue, GC; *c*, HeLa 229, cervix, CCL2.1; *d*, Detroit 6, marrow, CCL3; *e*, HeLa S3q, cervix, CCL2.2; *f*, HEp 2, larynx, CCL2.3; *g*, Minnesota, esophagus, CCL4; *h*, Chang, liver, CCL13; *i*, HeLa cervix, CCL2; *j*, Detroit 98, marrow, CCL18; *k*, HeLa, origin unknown; and *l*, human spleen tissue, AT.

a small number of ribosomal genes do not contain the *Eco* RI restriction site close to the beginning of rRNA transcription. The second variation is that of a *Hind* II site in the middle of the *28S* gene (see fig. 2). About one-half of the ribosomal genes contain this site. These two variations that are seen in all individuals do not serve to distinguish one individual from another. In contrast, *Bam* HI and *Bgl* II show variation between different individuals. All DNA samples digested with *Bam* HI generated a fragment with a mol. wt. of 3.3 ×10^6 and another fragment with a mol. wt. of 1.0 × 10^6, both which hybridize with 28S RNA. When [^{125}I]18S rRNA was used, a third constant fragment of mol. wt. 1.4 × 10^6 was seen. In addition to these invariant fragments, up to four fragments of which mol. wts. were 5.2, 4.8, 4.2, and 3.7 × 10^6 were detected. In figures 1 and 3, these variable fragments are labeled V1, V2, V3, and V4, respectively. The relative proportions of these varied, and for any given sample there were from two to four of these fragments present. HeLa cells showed a very distinctive pattern that had not been seen in other tissue. Because of the interest in the

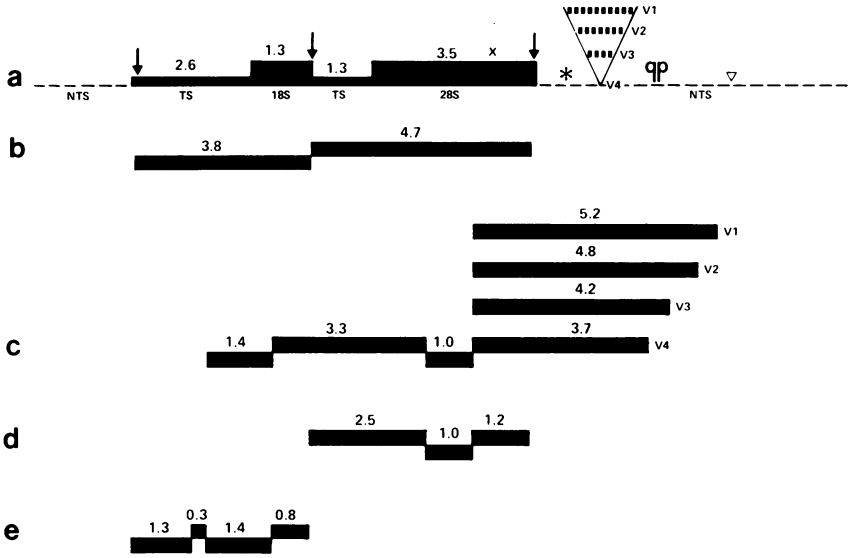

FIG. 2.—Representation of gene organization determined by restriction analysis of cellular DNA and cloned DNA. [^{125}I]rRNA was used as probe. *a*, Representation of entire gene as determined by restriction studies. Gene is composed of: — — —, nontranscribed spacer (NTS); ▬▬, transcribed spacer (TS) that is transcribed as the 45S precursor; and the *18S* and *28S* genes that are the final RNA product. Restriction sites *Hind* III (▽), *Hind* II (X), *Sal* I (*), *Eco* RI (↓), *Bam* HI (q), and *Bgl* II (p) are shown above the genes. Length of gene between the *Sal* and *Bam* sites varies in different genes within an individual. Four different length variations are detected. These are represented by the incremental lengths (▬▬▬) that would result if lengths *V1*, *V2*, or *V3* (▬▬▬) were inserted at point indicated by *V4*. *b*, The two fragment lengths of 3.8 and 4.7 × 10^6 Daltons were cloned separately when whole cell DNA was digested with *Eco* RI and inserted into the Charon 16A and λ WES vectors [14 and our unpublished data]. These clones permit exact identification of restriction sites given in *a*). *c*, Arrangement of restriction fragments detected by autoradiography in figures 1 and 3 using *Bam* HI. The 1.4 fragment is visible only in figure 3 where [^{125}I]18S rRNA is present. Fragments *V1*, *V2*, *V3*, and *V4* hybridize only with 28S RNA and show overlapping patterns when analyzed by restriction nucleases. *d*, Representation of a *Bam* HI digest of *Eco* RI cloned 28S RNA (fragment 4.7 in *b*). No length variation was seen in any of eight separately cloned fragments. *e*, Representation of *Bam* HI digests of cloned 18S RNA (fragment 3.8 of *b*). These fragments did not show any restriction site variation in 11 separately cloned segments.

classification of heteroploid cell lines, we chose to look at several HeLa cell lines and cells in which derivation was questioned. All of those cells were found to have *Bam* HI and *Bgl* II restriction patterns identical with the original HeLa cells examined (fig. 1).

To compare this pattern with other diploid cells, we obtained DNA from 15 placentas of normal births and DNA of six transformed cell lines. The six transformed cell lines were: (1) AG2804, an SV40 transformed lung, (2) GM1304, a teratoma cell, (3) ATCCG IMR-32, neuroblastoma, (4) BDDL-1, an ovarian tumor cell from our laboratory, (5) IMR90, lymphoblast, and (6) AG1947, retinoblastoma. Samples of some of these are seen in figure 3. Of these samples (lane 2), one showed the absence of the smallest variable fragment, V4. This fragment is also absent in HeLa cells. However, this sample had a different proportion of the V3 and V2 fragments. The V2 band was denser than the V3 band, whereas in HeLa, V2 and V3 are of equal density.

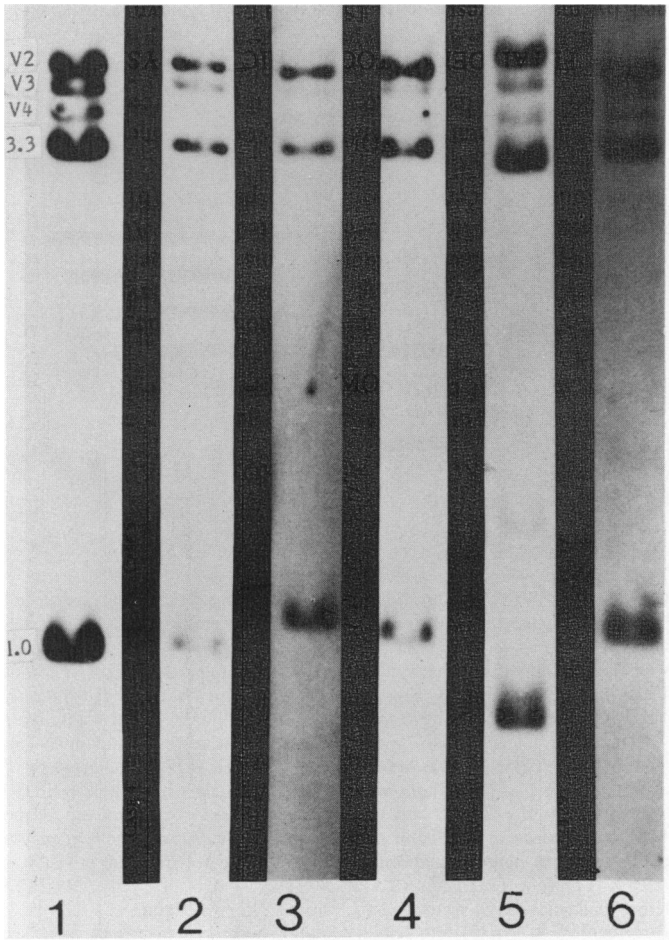

FIG. 3. —rDNA fragments in *Bam* HI digests of human placenta DNAs and transformed cell lines. Each *track* represents a separate placenta, gel, transfer, and hybridization. Analysis was repeated three times for each DNA sample with no variation in relative intensity of the bands. *Lanes 1–4*, DNA isolated from fresh placenta; *lane 5*, A62804, SV40 transformed fetal lung; *lane 6*, GM1304, teratoma cells from 27-year-old female; and *lanes 5* and *6*, cells from Mutant Cell Repository, Camden, N.J.

Simultaneous digestion of the DNA by two different restriction enzymes permitted us to order the fragments detected in the gel. Comparisons of these patterns to cloned *Eco* RI fragments of the *18S* [15] and *28S* gene (Schmickel et al., in preparation, 1980) allowed us to verify this ordering. Repeated analysis of the HeLa DNA always showed identical patterns of *Bam* HI fragments. Repeated analysis of each diploid DNA also gave consistent proportions of the variable fragments characteristic for that individual sample.

The gel patterns vary among individuals in two ways: in the total number of variable bands—any given individual has between two and four size variations, and in the relative amounts of each band. In all of the patterns, V2 is the most prominent or as

prominent as any other band. Either band V2 or V3 has been present in every pattern analysis. Based on these two features, we have constructed table 1 to show the frequency of each of these patterns. Other patterns are possible and probable, but were not seen.

Double digestion and analysis of human rDNA clones permits us to attribute the variability seen with *Bam* HI and *Bgl* II endonuclease analysis to length heterogeneity of a section of DNA to the right of the *28S* RNA gene (fig. 2). *Hind* III, *Bam* HI, and *Bgl* II restriction of DNA does not alter the four variable segments, whereas *Sal* I restriction removes all four variable segments. This indicates that the variable sites are located between the *Bam* HI/*Bgl* II and *Sal* I sites. The position of the *Sal* I and *Hind* III sites is shown in fig. 2*a*. *Eco* RI segments of the ribosomal genes have been isolated in the bacteriophage vector Charon 16A as described [15]. Digestion of cloned DNA (11 *Eco* RI human *18S* and eight *28S* fragments) with *Bam* HI disclosed no variability (fig. 2*d* and *e*). These findings also suggest that the variable region must be to the right of the *Eco* RI site near the junction of the *28S* gene and nontranscribed spacer.

The data presented in figure 4 also suggest that this variability is due to intervening segments and not to a variability of the *Bam* HI or *Bgl* II restriction sites. The HeLa pattern of variability is similar for both *Bam* HI and *Bgl* II. Furthermore, a double digest of *Bam* HI and *Bgl* II does not generate any new fragments as would be expected if these two sites were independently variable. The interpretation of electron microscope mapping by Wellauer and Dawid [16] would suggest that this variable sequence consists of repeated, discrete segments, since the reannealing of partially melted rDNA results in realignment of the strands into deletion loops. Krystal and Arnheim [8] have shown that the variation described here is limited to four segment lengths. Various tissues may have as few as two lengths, but more than four has not been seen.

Together, the number of variable lengths and the characteristic proportion of variable segments seen in HeLa cells provide an identifying characteristic of these cells that was not seen in other tissues sampled and is probably relatively uncommon. This analysis lends support to the hypothesis that several cell lines purported to represent new isolations are indeed HeLa cells.

It would be difficult to estimate how many generations have elapsed since the original HeLa isolation. The HeLa cells from this laboratory have been passed several

TABLE 1

SUMMARY OF RESTRICTION PATTERNS

Types of patterns	Examples in figures 1 and 3	Frequency in 26 non-HeLa
Four bands present V1, V2, V3, and V4	*a*, *l*	5
Three bands present V2, V3, and V4 with V2 the most prominent:		
V3 = V4	*3*, *5*, *6*	8
V3 > V4	*1*, *4*, *b*	12
Two bands present V2 and V3:		
V2 = V3	*c* thru *k*	0
V2 > V3	*2*	1

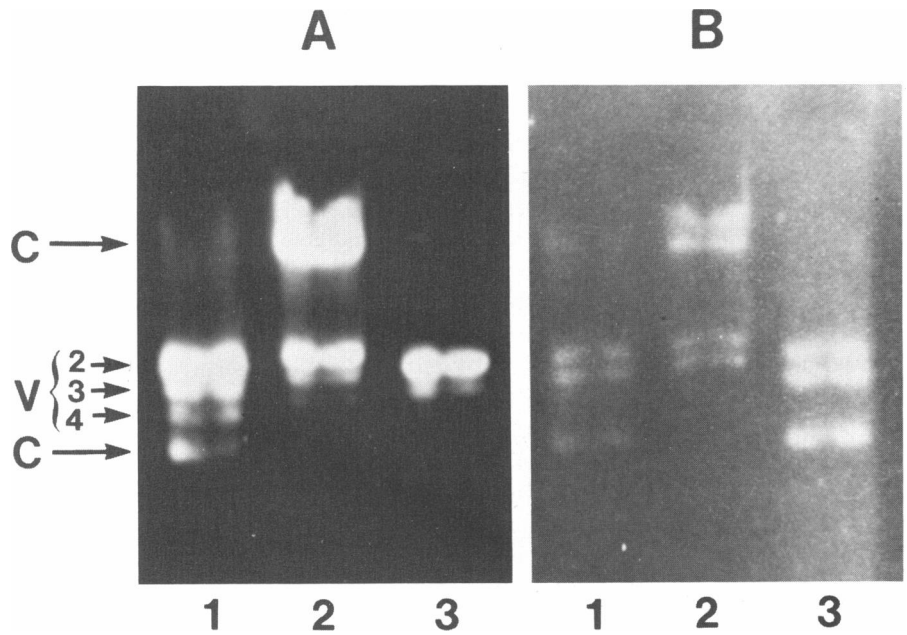

FIG. 4.—Comparison of *Bam* HI and *Bgl* II digestions for both human placental DNA (*frame A*) and HeLa DNA (*frame B*). Prints of autoradiographs of Southern transfer and [^{125}I]rRNA hybridization. Endonuclease digestions: *lane 1* is *Bam* HI, *lane 2* is *Bgl* II, and *lane 3* is digestion with both *Bam* HI and *Bgl* II. *Arrows* indicate bands; *C* bands are for the constant regions, and *V2*, *3*, and *4* are the variable bands indicated in figure 2*c*. Human placental DNA contains variable bands *2*, *3*, and *4*, whereas HeLa contains only *2* and *3*. Hybridization and autoradiography were as described in figure 1.

hundred times over the 10 years we have used them and account for at least 1,000 generations. By any estimation of total generations, the organization of the human ribosomal gene and pattern of variability have shown remarkable stability. It is possible that the pattern for HeLa endonuclease fragments was present in the original tissue from which the HeLa cells were isolated in 1952.

As more restriction fragment patterns are known, it should be possible to identify the origin of any tissue. Each cell carries its own written history and restriction analysis presents a way to interpret it.

REFERENCES

1. MILLER OJ, MILLER DA, ALLDERDICE PW, DEV VG, GREWAL MS: Quinacrine fluorescent karyotypes of human diploid and heteroploid lines. *Cytogenetics* 10:338–346, 1971
2. NELSON-REES WA, FLANDERMEYER RR, HAWTHORNE PK: Banded marker chromosomes as indicators of intraspecies cellular contamination. *Science* 184:1093–1096, 1974
3. NELSON-REES WA, FLANDERMEYER RR: HeLa cultures defined. *Science* 191:96, 1976
4. CHANG RS: HeLa marker chromosomes, Chang liver cells, and liver-specific functions. *Science* 199:567, 1978
5. NELSON-REES WA: Reply. *Science* 199:567–568, 1978
6. REEDER RH, BROWN DD, WELLAUER PK, DAWID IB: Patterns of ribosomal DNA spacer lengths are inherited. *J Mol Biol* 105:507–516, 1976

7. Grossman LI, Parker RC, Watson RW, Chandler SEW, Teplitz M: Detection of a contaminant cell culture line by restriction endonuclease cleavage patterns of mitochondrial DNA. *Nucleic Acids Res* 4:1267–1289, 1977
8. 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, 1978
9. Kan YW, Dozy AM: Antenatal diagnosis of sickle cell anemia by DNA analysis of amniotic fluid cells. *Lancet* ii: 910–912, 1978
10. Nowell PC: The clonal evolution of tumor cell populations. *Science* 194:23–28, 1976
11. Marmur J: in *Methods in Enzymology*, edited by Colowick SP, Kaplan NO, New York, Academic Press, 1963, p 726
12. Southern EM: Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 98:503–517, 1975
13. Schmickel RD: Quantitation of human ribosomal DNA: hybridization of human DNA with ribosomal RNA for quantitation and fractionation. *Pediatr Res* 7:5–12, 1973
14. Laskey RA, Mills AD: Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. *Eur J Biochem* 56:335–341, 1975
15. Wilson GN, Hollar BA, Waterson JR, Schmickel RD: Molecular analysis of cloned human 18S ribosomal DNA segments. *Proc Natl Acad Sci USA* 75:5367–5371, 1978
16. Wellauer PK, Dawid IB: Isolation and sequence organization of human ribosomal DNA. *J Mol Biol* 128:289–303, 1979.