Studies of the Inheritance of Human Ribosomal DNA Variants Detected in Two-Dimensional Separations of Genomic Restriction Fragments

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ABSTRACT

We have investigated the variation in human ribosomal DNA repeat units as revealed in two-dimensional electrophoretic separations of genomic restriction fragments that were end-labeled at *Not*I cleavage sites. The transcribed portion of the ribosomal DNA results in ~20 labeled fragments visible on each gel as multicopy spots. We have mapped these spots to the sequences responsible for their appearance on the gels, based on their migration positions and direct sequencing of spots, and describe several previously unreported sources of variation. By studying mother/father/child families we gained information on how much of the between-repeats variation is due to differences between and within repeat arrays on homologous chromosomes. Two instances in which a child exhibited more copies of a particular fragment than were present in the parents are described and hypothesized to be due to events such as multiple unequal sister-chromatid exchanges or gene conversions.

RIBOSOMAL DNA (rDNA) repeat units present several unique genetic features. In humans they are a multigene family of remarkably similar sequences of ~43 kb each that are tandemly repeated 30–60 times on each of five pairs of acrocentric chromosomes (13, 14, 15, 21, 22) (HENDERSON *et al.* 1972; WELLAUER and DAWID 1973). Although sequence differences between repeats within an individual are common, the similarities between repeats are much greater than would be expected if the repeats were evolving independently (BROWN *et al.* 1972; ARNHEIM *et al.* 1980; GONZALEZ *et al.* 1993). This finding has led to the suggestion that concerted evolution of rDNA repeats on particular chromosomes is driven largely by unequal crossing over between repeats on homologous chromosomes (SMITH 1976), while homogenization of repeat arrays across nonhomologous chromosomes must be due to occasional exchanges between them. Intrachromosomal homogenization of repeat units could also be caused by random or biased gene conversion, as well as unequal sister-chromatid exchanges (DOVER 1982). A question of some interest concerns the exact distribution of genetic variation among and between rDNA repeat units on separate chromosomes. More specifically, to what extent does a given variant found in multiple repeats tend to cluster within a chromosome, and to what extent is it disseminated across all five of the chromosomes containing rDNA units?

The relatively new technique of two-dimensional electrophoresis of enzymatically digested genomic DNA (2-D DNA) permits the visualization of up to several thousand end-labeled fragments on a single gel (HATADA *et al.* 1991). Such patterns have proven useful in genetic mapping (HAYASHIZAKI *et al.* 1994), the study of genetic variability (ASAKAWA *et al.* 1994; KUICK *et al.* 1995), and the detection of amplifications, deletions, and other genetic alterations in cancer (HIROTSUNE *et al.* 1992; NAGAI *et al.* 1994; OHSUMI *et al.* 1995). In this communication we will explore an additional application of this technique. There are ~30 multi-copy fragments visualized as large spots in these preparations. We will show that most of these are due to rDNA repeats. Further, it will be shown that many of the less common variants are predominantly inherited as if all of the variant copies are present on a single chromosome (syntenic). In two among 27 favorable opportunities to observe the transmission of multiple copies of a variant fragment from one of the parents, a child appeared to exhibit significantly more copies of the variant than were present in the parents, indicating that unequal sister-chromatid exchanges, multiple gene conversions, or other events leading to an increase in specific rDNA repeats may be fairly frequent for rDNA tandem repeats. Such mechanisms could be important in the proliferation of new rDNA repeat versions.

MATERIALS AND METHODS

Genomic DNA was obtained from Epstein-Barr virus-transformed lymphocyte cell lines established from nine nuclear families and maintained by the Radiation Effects Research Foundation, Hiroshima, Japan. The cell lines were prepared

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by exposing the mononuclear cells separated from 2.5 ml blood (~0.5 million of which are B-cells) to the virus and harvesting cells when the transformed cells numbered 500–600 million. Neither parent in any of these nine families had been exposed to the atomic bomb explosion. One family was represented by two children, whereas the other families were represented by a single child. The methods of DNA preparation and 2-D electrophoresis were as previously described (ASAKAWA *et al.* 1994, 1995). The most relevant aspects of the method are that DNA samples were digested with the restriction enzymes *Not*I (cleaves GCGGCCGC) and *Eco*RV, isotopically labeled at the *Not*I-derived 5′ protruding ends, and electrophoresed in cylindrical first-dimension agarose gels 60 cm long and 2.4 mm in diameter. A 32-cm portion of this gel containing 1–5-kb fragments was then subjected to *Hin*fI digestion and the resulting fragments electrophoresed perpendicularly in a 5.25% polyacrylamide gel (33 × 46 × 0.08 cm). The gels were then dried and autoradiographs prepared. Digital images were obtained and spots quantitated, as previously described (ASAKAWA *et al.* 1994).

*Not*I-*Hin*fI DNA fragments were cloned directly from genomic 2-D DNA patterns as follows (details in J. ASAKAWA, unpublished data). Five micrograms of genomic DNA digested with 50 units each of *Not*I and *Eco*RV was applied to the first-dimension gel, only one-fifth of which was isotopically end-labeled at the cleaved *Not*I sites by fill-in reaction with DNA polymerase. Electrophoresis was carried out as usual and autoradiography performed (without drying the gel) at −80° for 16 hr with an intensifying screen. The gel portion corresponding to each spot of interest was cut from the gel, and each gel piece was covered with 10 μl of TE solution containing 5 μg of tRNA for 10 min. Each gel piece was then embedded in 0.6% agarose. The DNA was eluted by electrophoresis onto DEAE filter paper and then eluted from the filter with 60 μl high-salt buffer (50 mM Tris/HCl, 10 mM EDTA, 1 M NaCl pH 8.0) and extracted with phenol followed by ethanol precipitation. The pellet was rinsed with 70% ethanol and air dried. The (unlabeled) fragments were ligated with DNA ligase to a Bluescript II SK+ vector whose *Pst*I cloning site was modified to generate a *Hin*fI cohesive end. The reaction mixture was used to transform Epicurian Coli XL2-Blue MRF Electroporation competent cells (Stratagene, La Jolla, CA). Colonies containing the recombinant plasmid were selected on LB-agar containing 100 μg/ml ampicillin, X-gal, and isopropylthiogalactose (IPTG). Plasmid DNA was prepared from cultures of the white colonies and digested with *Not*I and *Eco*RV (for which there is a unique site just outside the inserted fragment) to confirm the size of the inserted DNA. Sequence analysis was carried out by using a T7 primer (for *Hin*fI ends) and a T3 reverse primer (for *Not*I ends).

RESULTS

Figure 1 shows a 2-D DNA gel in which the locations of large spots (or groups of spots) deduced to be from rDNA repeats have been labeled, as have fragments from the Epstein-Barr virus (EBV) employed to immortalize the cell lines. Large spots deduced to represent EBV DNA were among the first fragments to be confidently identified due to their absence in gels prepared from nontransformed lymphocytes, and the agreement of their fragment sizes (in both dimensions) with the fragment sizes predicted from EBV sequences in DNA databases (GenBank accession V01555) was confirmed. These spots subsequently served as internal size markers, as did spots confirmed to be rDNA. These EBV and rDNA fragments of known size allowed accurate estimation of the size of (rDNA) fragments that were not directly predicted by DNA database sequences.

A restriction map that relates spots visualized on the gels with specific sequences of the rDNA repeat unit is shown in Figure 2. The known *Not*I sites in the 43-kb rDNA repeats are confined to an ~16-kb region that includes the entire 45S transcription unit. The remaining 27 kb of the repeat units is digested into a single *Not*I-*Not*I fragment that is too large to enter our standard gels. On the basis of the most complete rDNA sequence in GenBank (Genbank accession no. U13369) (GONZALEZ and SYLVESTER 1995), as well as other human rDNA sequences, there appear to be no *Eco*RV sites in rDNA repeat units, and results from pulsed-field gel electrophoresis of *Eco*RV-digested genomic DNA also indicate an absence of *Eco*RV sites in the repeats (SAKAI *et al.* 1995). Thus all of the rDNA fragments should be *Not*I-*Not*I fragments in the first-dimension gel. Indeed, gels prepared using only *Not*I before first-dimension electrophoresis exhibited identical patterns for all of the major spots identified as rDNA fragments in this report (data not shown). Additional restriction site information that was useful in confirming the identity of some spots was obtained from the patterns of larger spots in gels for which we used the restriction enzymes *Bgl*II or *Pvu*II rather than *Eco*RV, or used *Mbo*I or *Pst*I in place of *Hin*fI. Because of the absence of *Eco*RV sites, rDNA fragments appeared as pairs of large spots differing only in the second-dimension migration, and we have attempted to label the spots with names that indicate this relationship (*e.g.*, spots C1 and C2 are produced by opposite ends of a first-dimension *Not*I-*Not*I fragment). The letters used for designating spots are arbitrary except that in some cases they indicate the relationship between fragments differing only due to altered *Not*I sites, as for the related fragments labeled D1, Da1, and Db1. Variants of more common fragments caused by variability in *Hin*fI sites have simply been designated by spot numbers created during the course of matching the patterns.

In addition to those intense spots known to be due to EBV or rDNA repeats, there were several other moderately intense spots that appear to be of the same intensity in all individuals. We suggest that such spots are the result of DNA sequences other than the rDNA repeat units that occur on the acrocentric chromosomes, since such spots appear to be approximately five times larger than most surrounding spots, and sequences flanking the rDNA repeats exhibit homology between these chromosomes (WORTON *et al.* 1988).

***Not*I site variability:** In several cases it was necessary to postulate *Not*I restriction sites at positions where they have not been previously reported. In particular, the occurrence of *Not*I sites at (approximately) bases 270, 7519, 10081, and 13219 is required to explain the ob-

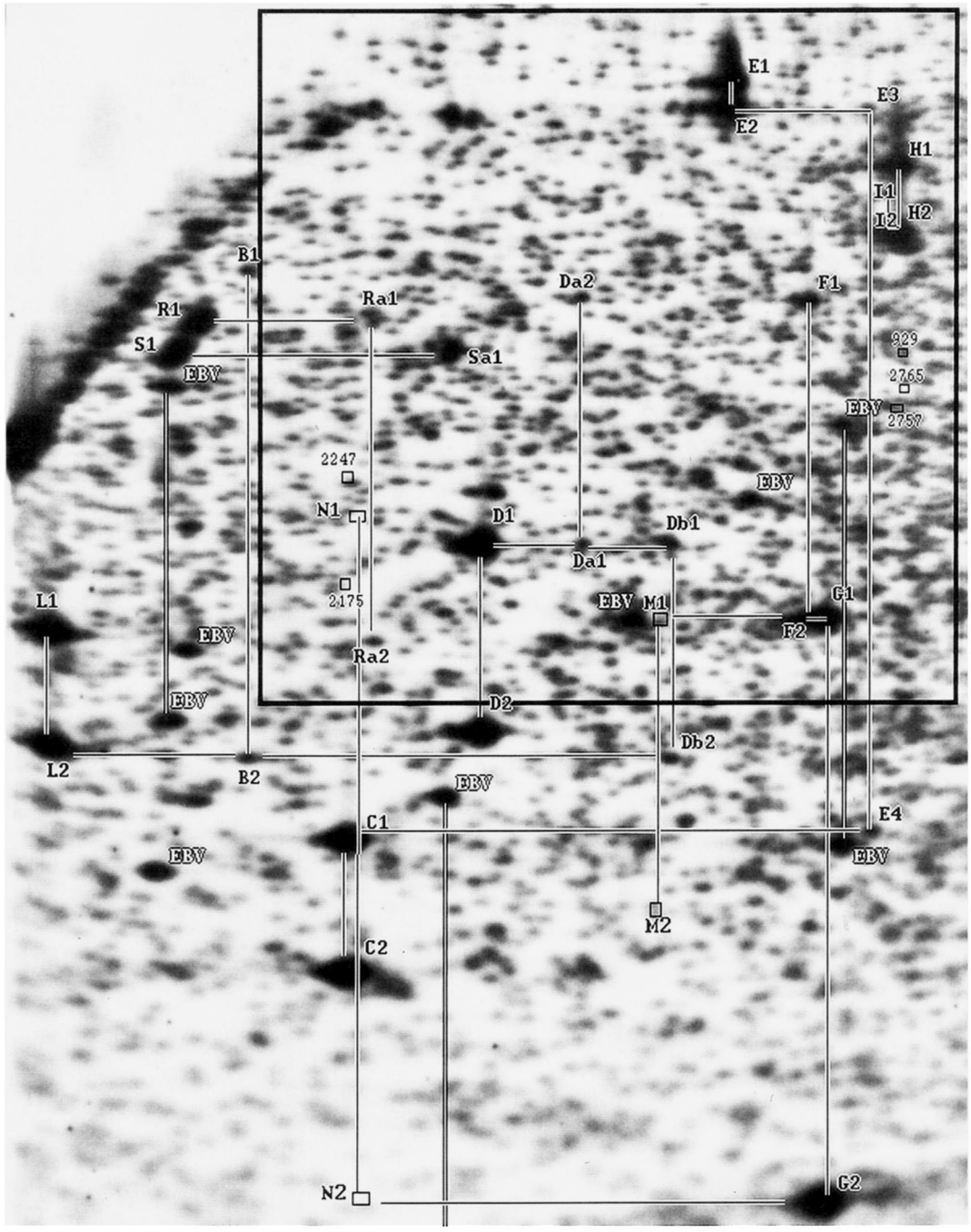

FIGURE 1.—Ribosomal DNA and EB virus fragments. This specially prepared gel, electrophoresed for a shorter time in both dimensions, shows a greater range of spot sizes than the gels routinely used, which only allow visualization of the region in the dark box. rDNA spots are labeled with the designations used in the text, for which fragment sizes and identities are given in Figure 2. EB virus-derived fragments are labeled EBV. Vertical lines join pairs of spots thought to be *Not*I-*Hin*fI fragments produced from the same first-dimension *Not*I-*Not*I fragment. Horizontal lines connect spots containing homologous partial sequences that migrated differently in the first dimension due to *Not*I site variation. The positions of rDNA spots shown in subsequent figures, but not exhibited by this particular individual, are indicated with small rectangles.

served spot patterns. (Bases are numbered according to the GenBank U13369 sequence.) Sequence analysis of fragments cloned from spots on the gels support this interpretation.

A *Not*I site in the 3′ external transcribed spacer near base 13219 appears to be present in most rDNA repeat units, as judged by the large size of the spots labeled L1 and L2 compared to the spots B1 and B2 that are predicted from published sequences. Previous sequencing at this location gave the sequence GCGCCGC (GONZALEZ *et al.* 1990). We have observed a large spot of ~400 bp that is not cleaved by *Hin*fI (though, because of its small size, this fragment migrates beyond the first-dimension region of the particular gel shown in Figure

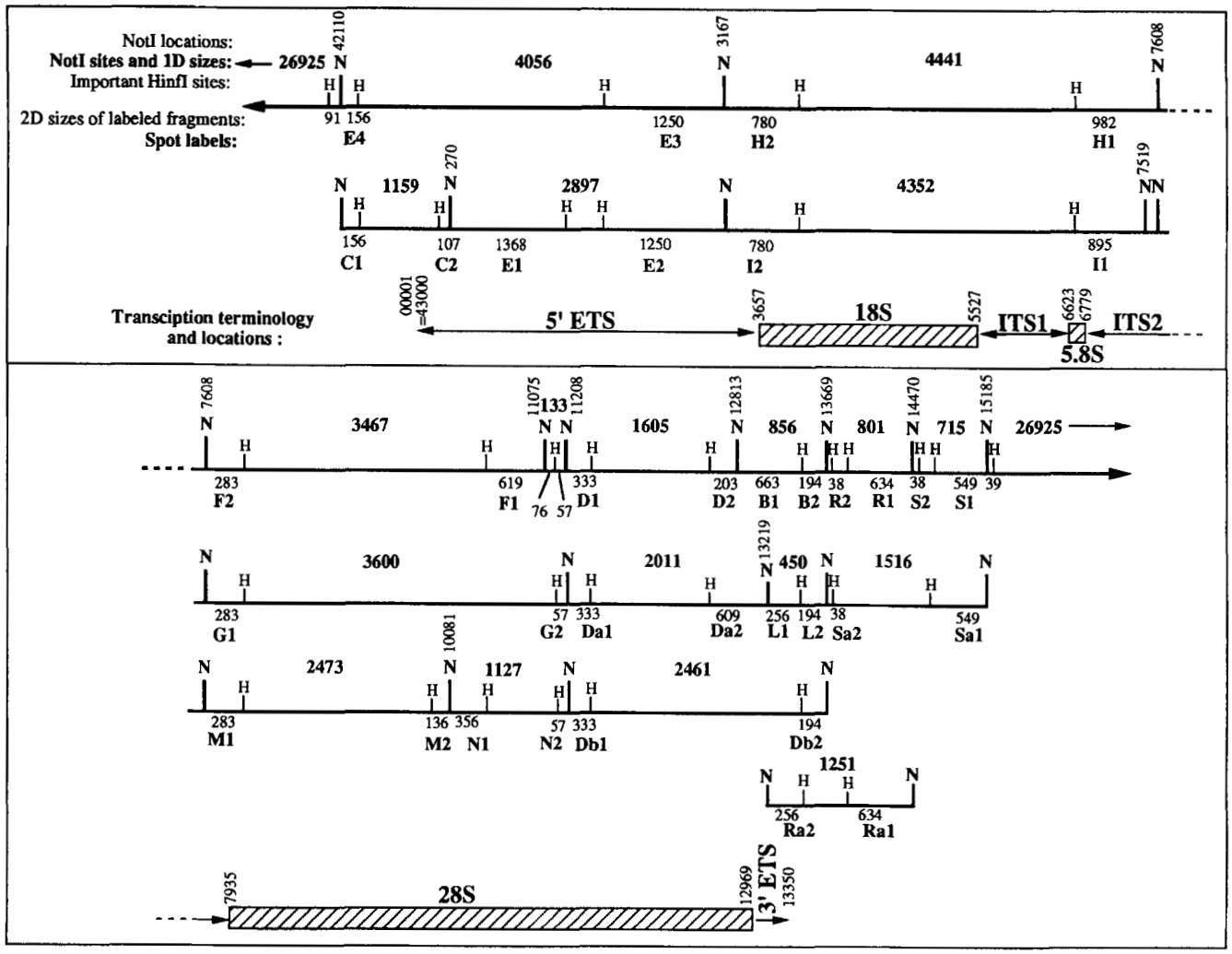

FIGURE 2.—Map of ~16 kb length of a ribosomal repeat unit. The bottom panel is the continuation of the top panel. In each panel the topmost restriction map is that predicted from GenBank sequence U13369, whereas those below it show many of the variants encountered. The bottom map in each panel shows the relative position of the 45S transcript and its components. N, *Not*I sites; H, *Hin*fI sites, though only those *Hin*fI sites creating common *Not*I-*Hin*fI fragments are shown. Locations of *Not*I sites and features of the transcription unit are written vertically and use the numbering system of U13369, from which the size estimates are calculated. Spot labels are those used in Figure 1. Several of the fragments showed length variation as discussed in the text.

1), which is consistent with the occurrence of *Not*I sites at 12813 and 13219.

The postulated *Not*I site near base 10081, which gives rise to spots M1/M2 and N1/N2 in two of the 18 parents, is in a region known to contain a variable number of GGC repeats (GONZALEZ *et al.* 1985), in which a single G to C alteration will result in a *Not*I site. The pattern of fragments for one of the parents displaying spots M1/M2 and N1/N2 is shown in Figure 3. Our explanation for these spots was partially due to the fact that patterns from individuals with any one of the spots always exhibited all four spots with sizes consistent with their being an equal number of copies of the four fragments.

Some fragments have been extracted from the gels, cloned, and sequenced to provide direct evidence for our deduced spot identity. The presence of the *Not*I site near base 270 was confirmed by partial sequencing of the *Not*I-*Hin*fI fragments cloned directly from spots E1 and E2 in the gels. This site appears to be present in almost every repeat unit for the individuals in this study, as deduced from the small size of spots E3 and E4 compared to the larger sizes for spots E1/E2 and C1/C2. The original sequence (starting at base 269 of U13369) was reported as GCGCCGCACAA (FINANCSEK *et al.* 1982), whereas sequencing of the E1 clone yielded GCGGCCGCGACAA at its *Not*I extremity (the next 28 bp being identical for the two sequences). The identity of spots in locations H1, D1, I1, and F1 has also been confirmed by direct cloning and partial sequencing. In addition, *Not*I-*Not*I fragment clones were obtained for E1/E2 and H1/H2 by screening *Not*I-*Not*I libraries using the *Not*I-*Hin*fI fragment clones from spots E1 and H1, respectively. The 2-D DNA patterns obtained from

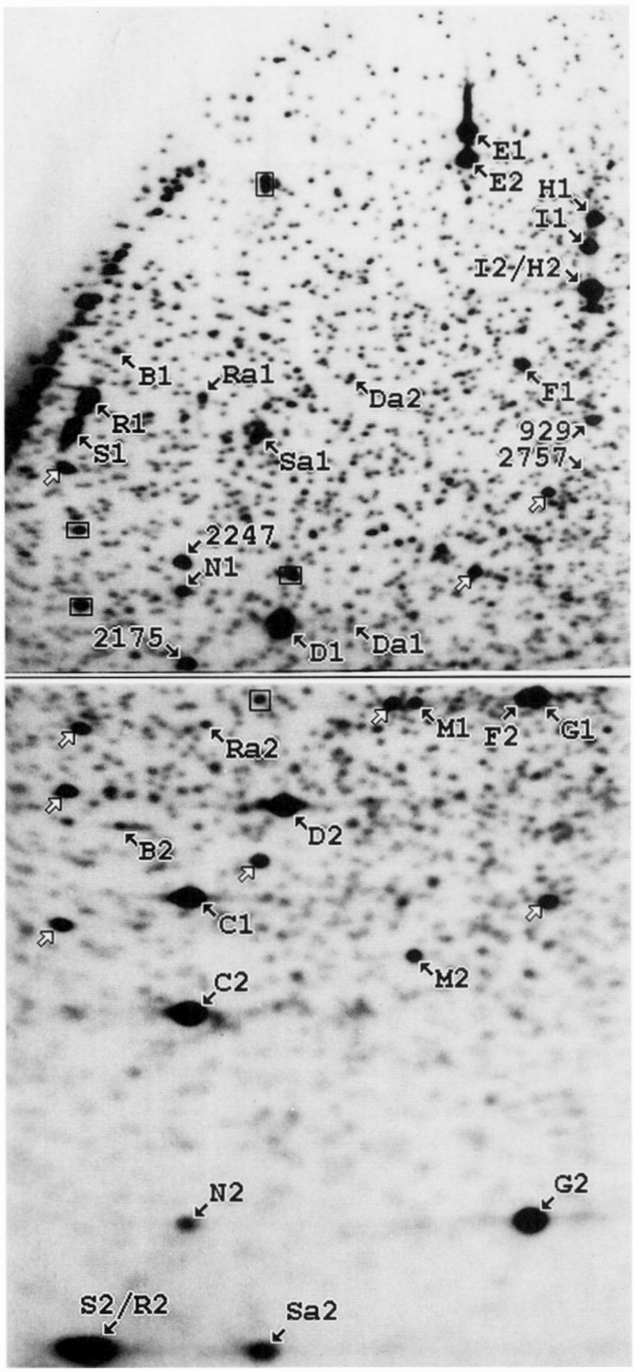

FIGURE 3.—A specially prepared pattern from an individual exhibiting several of the less common rDNA fragments. This gel was prepared using a 90-cm-long second-dimension acrylamide gel that was later cut in half and exposed to two separate pieces of film. The digital images have been joined here to recreate the total pattern. Fragment sizes are given in Figure 2. The fragments S2, R2, and Sa2 at the bottom of this pattern were too small to appear on the gel in Figure 1. EBV-derived spots are indicated with white arrows. Five black rectangles surround invariant multicopy spots that were present in all individuals and whose identity is unknown.

these *Not*I-*Not*I clones each exhibit two spots, corresponding to spots E1 and E2, and spots H1 and H2, respectively.

The *Not*I site at base 7519, which results in the I1 fragment, was present in at least some rDNA repeat units in all individuals and accounted for as many as half the repeat units in some individuals (see Figure 4, D and E). Sequence analysis of the clone obtained for I1 indicates a *Not*I site rather than the more common "GTGGCCGC" (GONZALEZ, *et al.* 1990), where the "T" is at position 7519 of U13369.

The *Not*I site at base 11075 (GONZALEZ *et al.* 1985) that gives rise to the F1 fragment appears to be present on average in approximately one-third of the repeat units for the individuals in the study despite its absence in all 35 of the distinct sequences analyzed in a previous report (LEFFERS and ANDERSEN 1993) (see Figure 4C and Figure 1).

***Hinf*I site variability:** Based on the location of spots labeled 929 and 2757 in the patterns, these spots are likely to be produced by an additional *Hin*fI site at approximately base 7062 . The presence of this *Hin*fI site shortens the common H1 fragment to a 545-bp *Not*I-*Hin*fI fragment (spot 929, seen in six families), and when present in the common I1 fragment, produces a *Not*I-*Hin*fI fragment of ~456 bp (spot 2757, seen in just two parents). We have confirmed the existence of a *Hin*fI site in approximately this location by Southern blotting in which DNA was digested with *Hin*fI and probed with our clone for the H1 spot. Spot 2765, seen in only a single parent and child thus far, is probably also a length variant of spot H1 or H2 due to a rare *Hin*fI site, leading to an ~485-bp *Not*I-*Hin*fI fragment.

A spot designated 2175 was seen in four of the parents in the study. It can be explained as being due to the absence of the common *Hin*fI site (at base 163) that creates the C2 spot when present. When this site is absent, the next *Hin*fI site 5′ of the *Not*I site at base 270 is expected to lead to a fragment of 300 bp, which is exactly the size predicted for spot 2175, based on the numerous spots of known size in this size range of the second dimension. Spot 2247, which appeared in two of the parents, is also in a position approximately above the common C1/C2 spots and thus is possibly caused by *Hin*fI variation in one of these fragments. However, its estimated second-dimension size (370 bp) is not easily explained on the basis of published DNA sequences for this region. This spot also migrates slightly further in the first dimension than spots C1, C2, and 2175 so that a more complex explanation could be needed. In favor of it being a variant of spots C1 or C2 is the fact that this spot is known to be derived from a first-dimension *Not*I-*Not*I fragment (based on first-dimension gels using only *Not*I without *Eco*RV). Yet on gels that allow visualization of second-dimension fragments as small as 35 bp (smaller than any known *Not*I-*Hin*fI fragments from rDNA), no unaccounted for extra spots are seen to migrate to the same first-dimension coordinate (see Figure 3). Thus it seems likely that the *Not*I-*Hin*fI fragment from the opposite end of the first-dimension

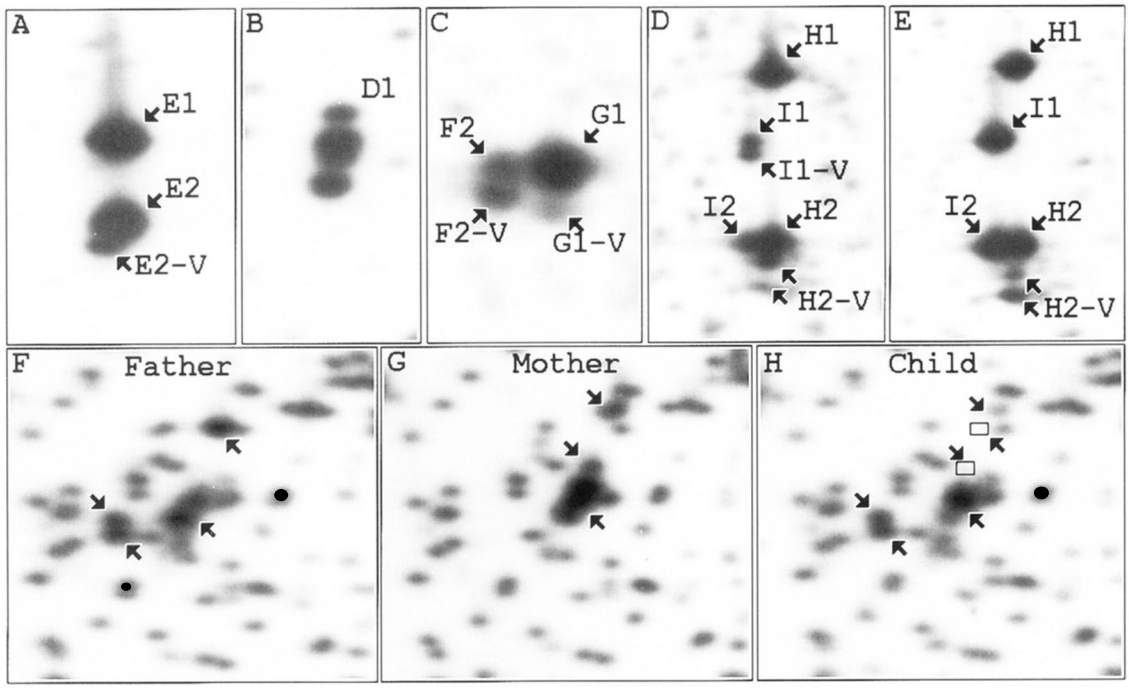

FIGURE 4.—Examples of fragment length variation. Further examples are shown in Figure 5. Some of the images are of very light autoradiograms to allow better visualization of intense spots. In the upper panels spot labels correspond with Figure 1, except that -V is appended to spot names for less common variants. (A) A length variant for the E2 fragment that presumably has a counterpart spot in the left side of the large E1 spot. (B) Typical D1 fragment length variation. (C) An individual with smaller length versions of fragments F2 and G1. (D) The single example of an apparent I1 variant. This individual also has H2 length variants. (E) Another individual with H2 length variants. Similar length variants of I2 were observed in several individuals. (F–H) A family showing just a few of the variants seen for the fragments labeled Sa1 in Figures 1, 2, and 3. Arrows point to probable Sa1 fragment versions. In some cases a single arrow points to a cluster of overlapping spots. Boxes in the child's gel occupy positions of spots absent in the child but present in one of the parents.

*Not*I-*Not*I fragment producing spot 2247 must be at a location overlapping spot C1 or C2.

Other variation: Additional minor length variation, illustrated in Figure 4, was apparent for several of the fragments. The fragment E2 showed apparent length variation in a single father and child. Several different length variants were seen for H2 (and I2), which are interpreted as due to variation in the 5′ external transcribed spacer, since length variation in this region was previously confirmed by sequencing (MADEN *et al.* 1987) and little variation has been reported for the 18S sequence (WILSON *et al.* 1978; MADEN *et al.* 1987). The multiple spots visualized at locations labeled D1, Da1, and Db1 in Figure 1 were expected on the basis of "V8" segment variability (MADEN *et al.* 1987; LEFFERS and ANDERSEN 1993). Length variants for fragments F1 (and G2) that are from the second internal transcribed spacer were common. Fragment I1 showed a well-separated variant, but we have not detected similar length variants for H1, perhaps because the greater spot intensity as well as the larger fragment size of H1 renders the equivalent length variation undetectable in these gels. Length (and restriction site) variability in the 700–800-bp repeats in the 3′ external transcribed spacer (KRYSTAL and ARNHEIM 1978; ARNHEIM *et al.* 1980; ERICKSON and SCHMICKEL 1985; LA VOLPE *et al.* 1985) created many spot variants for the fragments in this region, as expected (spots R1, S1, Ra1/Ra2, Sa1/Sa2).

Synteny: The use of data from nuclear families in this study made it possible to confirm the genetic nature of the fragment variation encountered. There were multiple instances in which children of parents with less-common variant spots inherited either none or all of the copies of the variant as illustrated in Figure 5. This finding is consistent with the hypothesis that the variant rDNA repeats were all located on the same chromosome. We now will attempt to summarize some of this evidence. We will restrict attention to spots that were cleanly separated from any common rDNA spot and for which there appeared to be at least six or more copies of a variant spot in one of the parents and no copies of this variant in the other parent. There were many additional instances (particularly for variant Sa1 and Ra1 spots; see Figure 4, F–H) of the apparent inheritance of all or none of the parent's copies, but these spots are usually small or overlapping other spots and are excluded in the following presentation.

Table 1 shows the 27 cases of potential segregating variation meeting the above criteria. We attempted quantitation of these spots and surrounding spots (on duplicate gels) in an effort to estimate precisely the copy number for each spot. However, while generally

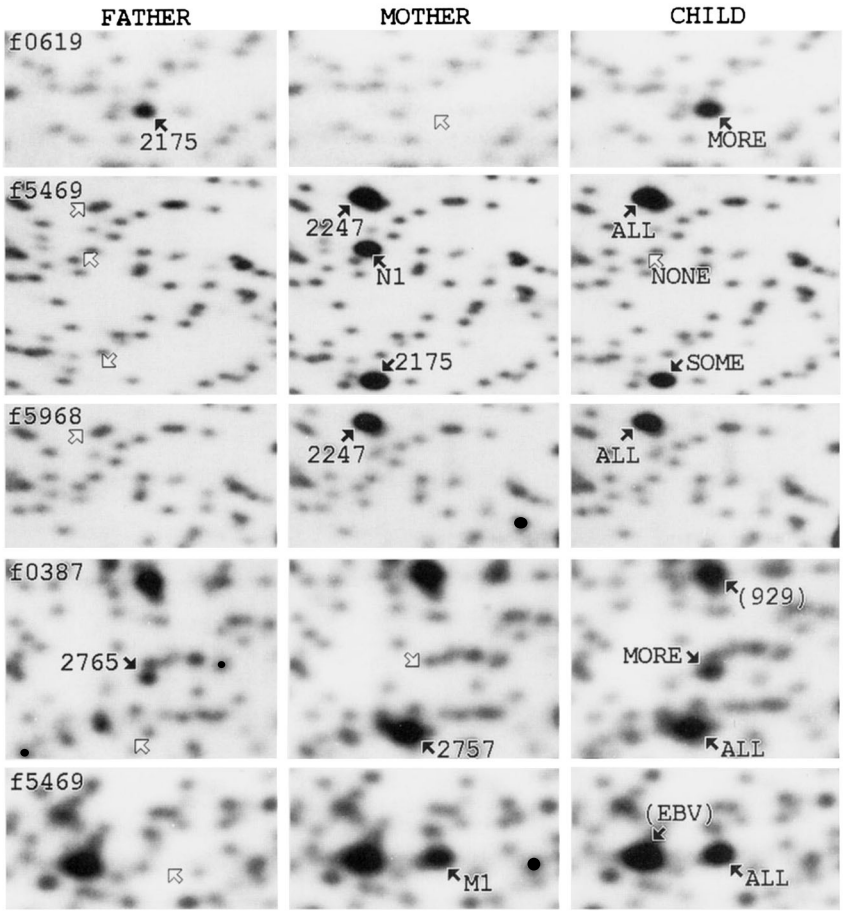

FIGURE 5.—Examples of rDNA fragment inheritance in family trios. For each trio the family number appears in the father's panel. White arrows are used to point to the location of absent spots. The assessment of the child's spot size relative to the parents is given in the child's panel. The first trio shows one of the cases in which a child appears to have more copies of the 2175 fragment than the parents. In the second trio, the child (child #2 for this family) inherited all copies of 2247, none of the copies of N1 (there is a normal spot in nearly the same position as seen in the father's gel), and some but not all of the copies of 2175. The third trio depicts another situation in which the child appears to have inherited all copies of 2247. In the fourth trio, the child appears to have inherited all copies of 2757 from the mother and to have more copies of 2765. Spot 929 is labeled but does not appear in Table 1, since both parents have copies. The last trio shows the child (child #1) inheriting all copies of M1. A fragment from the EB virus also is labeled. (EB virus-derived spots vary in size between samples and should not be used to judge the relative sizes of other spots.)

agreeing with our visual assessment regarding the number of copies inherited by the child, these estimates were not satisfactorily reproducible and exhibited indications that "saturation" of the autoradiographic film (or digitizing camera) was occurring due to the intensity of these large spots. Our visual assessment (informed by the quantitative results in the cases of moderately sized spots), as given in Table 1, is that in 11 of the 27 examples, all (or nearly all) of the variant copies present in one parent were inherited by the child, whereas in 10 cases the child inherited none of the copies. Only in four cases did the child exhibit an intermediate number of copies. In the remaining two instances the child clearly exhibited a larger spot than in the parent. If in all 27 cases the variant copies were actually distributed on two chromosomes rather than a single chromosome, an intermediate number would be expected in the child in half of the cases, and the chances of observing only four cases in which the child inherits an intermediate number of copies would be 0.00016. The chances that the variants were not syntenic in even 14 of the 27 cases is $<10\%$, and so it is likely that in most cases in Table 1 the variant copies are syntenic.

DISCUSSION

We have investigated the variation in human ribosomal DNA repeat units as revealed in 2-D electrophoretic separations of genomic restriction fragments that were end-labeled at *Not*I cleavage sites. By studying mother/father/child families we gained information on how much of the between-repeats variation is due to differences between and within repeat arrays on homologous chromosomes. The reliance on fragments that were end-labeled at *Not*I cleavage sites for 2-D analysis is intended to maximize the number of spots that result from fragments in CpG islands and that are thus likely to be in the proximity of expressed genes. Most common families of highly repetitive DNA lack *Not*I sites. Ribosomal DNA, however, because of the extremely high CG content near and within the transcribed region of the repeat units (GONZALEZ and SYLVESTER 1995), leads to many labeled fragments in the gels. The appearance of rDNA fragments on the gels affords the opportunity to study the restriction fragment length polymorphisms of rDNA in a two-dimensional setting. These fragments displayed considerable genetic variation both within and between individuals.

In this report we have described several previously unreported variations in the rDNA repeat units that occur due to restriction site polymorphisms for the enzymes employed and indicated their relative frequency in a sample of Japanese individuals, as well as the frequency of several previously reported sources of variation. The novel variation encountered is due to the efficiency of screening total genomic DNA compared

TABLE 1

Inheritance of less common variant fragments present in only one parent in a family

Spot[a]	Bases[b]	Family	Copy number[c]		Comment[d]
			Parent	Child	
2175	42969–270	0212	>20	0	None
2175		0374	>20	5	Intermediate
2175		0619	10	20	More
2175			>20	0	None (c1)
2175			>20	<20	Intermediate (c2)
2247	42110–42480 (?)	5469	>20	0	None (c1)
2247		5469	>20	>20	All (c2)
2247		5968	20	20	All
0929	7062–7608	0374	>20	2	Intermediate
0929		0619	>20	>20	All
0929		5469	20	0	None (c1)
0929		5469	20	20	All (c2)
0929		5600	15	0	None
0929		5968	>20	0	None
2757	7062–7519	0387	20	20	All
2757		5600	15	6	Intermediate
2765	7123–7608	0387	8	15	More
M1/N1	7608–7891/10081–10437	0374	20	0	None
M1/N1		5469	20	20	All (c1)
M1/N1		5469	20	0	None (c2)
I1-V	6624–7519	0693	20	0	None
F2-V	7608–7891	0212	>20	>20	All
G1-V	7608–7891	0212	20	0	None
D1-V	11208–11541	0374	>20	>20	All
D1-V		0693	20	20	All
D1-V		5600	10	10	All
D1-V		5655	10	10	All

[a] V after a spot name indicates a length variant of the named common spot (see Figure 4).
[b] Base pairs from Genbank DNA sequence U13369. See text for a discussion of each variant.
[c] Copy numbers are visual estimates.
[d] Gives the relative number of copies in the child. Family 5469 had two children marked (c1) and (c2).

to sequencing multiple individual clones and to the high resolving power of the gels employed compared to Southern blotting, by virtue of their two-dimensional nature as well as the excellent spatial resolution achieved.

Knowledge of the pattern of rDNA-derived spots is also essential for the use of these patterns for other purposes. Though most labeled rDNA fragments appear as intense spots in the same locations for different individuals, some variants are uncommon, and when present in low copy number could be confused with fragments from single copy genes. To study fragments from single-copy genes with these preparations or to scan for novel amplifications in cancer tissues, a basic understanding of the fragments that result from rDNA repeat units is needed.

Previous data on the inheritance patterns of rDNA in humans have been limited. By observing that in the case of less common restriction site variants in rDNA, the children almost always inherit either none or all of the variant copies, we can conclude that in most of these cases the variant copies are from syntenic rDNA repeat units. These results are more striking when we consider that an intermediate number of copies in the child could occur due to the copies being distributed between two homologous chromosomes in the parent, due to homologous crossing over (rarely, since the entire tandem array is likely <2000 kb on each chromosome having it) or due to other events, as in the two instances in which the child had more copies than the parents. It is perhaps not surprising that a newly arisen variant rDNA unit should be confined to a single chromosome for a considerable time until a nonhomologous chromosomal exchange spreads it to another chromosome. The apparently high copy numbers for many of the cases where synteny was likely is perhaps more surprising, suggesting high rates of homogenization among syntenic copies, since in many cases the copy number may represent nearly all the repeats on a chromosome.

There are several possible explanations for the two observations of increased spot intensities for variant fragments in the children other than copy number increases for the repeat units involved. False parentage

can be discounted on the basis of inherited alleles for polymorphic systems in the patterns of the children and their parents. The average individual displays spots from ~140 restriction fragment length polymorphisms (KUICK *et al.* 1995) in addition to those from rDNA. It is also unlikely that methylation or incomplete digestion of *Not*I sites is responsible for increased spot intensity in the children. The CpG richness of the portion of the repeats visualized on the gels make methylation unlikely. It appears that over 99% cleavage of *Not*I sites occurs, based on the near absence of at least one spot in nearly every individual for those spots that could result from incomplete digestion (*e.g.*, E3/E4, spots at Da1 and Db1). Further, except for variants due to the presence of a *Not*I site at base 10081, the consequence of incomplete cleavage at a *Not*I site for the variants used for our analysis of inheritance would be the occurrence of spots at locations on the gels that are predictable from the DNA sequence, and spots at the predicted locations have not been observed. Thus, for the increase in the copy number of the two fragments in the children to be due to methylation would require that approximately half of the variant copies be methylated in the father but that the other repeat units not be affected. Another possible explanation of increased spot intensities in children is that mutations have arisen during the course of the cell divisions needed to obtain the samples rather than in the germline, and that such a mutant clone constitutes a considerable fraction of the cells in the sample. Since there are ~380 division cycles in the male by age 28 and 21 cycles in females (VOGEL and RATHENBERG 1975) compared to <20 during expansion of the cell line, it seems unlikely that a mutation arising during cell culture is responsible for the observations, particularly since such a mutation would probably have to occur in the first few divisions to have any effect on the spot patterns. Furthermore, even if a mutant clone were positively selected in culture, it is very likely that the sample would have displayed some unusual features in the pattern of non-rDNA spots based on our experience with single-cell derived clones.

In addition to the two examples presented here, an additional instance in which a child exhibited more variant rDNA copies than the parents was described once previously (SCHMICKEL *et al.* 1985). It appears that unequal sister chromatid exchanges, multiple gene conversions, or other unspecified events would be required. In this genetic setting, these increases in copy number would appear to be best explained by unequal sister chromatid exchange in the course of one or more of the mitotic cycles of the parent. Unequal crossing over during meiosis cannot produce such an increase, though this is thought to be an important mechanism in homogenizing the rDNA units. We note that both of the mutational events resulting in copy number increases observed in this study occurred in males and that there should be a preponderance of male-derived events if this phenomenon is associated with the number of cell divisions. As noted above, a decrease in copy number for a variant due to unequal sister chromatid exchange cannot be unambiguously detected since other explanations are possible for decreases in copy number. However, decreases due to unequal sister chromatid exchange or gene conversion should be as frequent as increases, implying the phenomena is even less rare.

Whatever explanation is correct, if our observations are valid there is a mechanism whereby a variant has nearly doubled its copy number in a single generation. Such a mechanism would permit the rapid spread of a variant rDNA and appears to be relatively common. Unfortunately, it is usually difficult to determine which chromosome contains a variant rDNA repeat unit or to fix its position within the tandem repeats to more fully understand the pattern of rDNA variation. However, the approach described provides a novel means to study how rDNA variation is transmitted and enhanced, which, particularly if applied to larger pedigrees, could lead to better estimates of the frequency of various mechanisms important to the concerted evolution of rDNA repeat units.

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