Volume 15 Number 2 1987 Nucleic Acids Research

Length mutations in human mitochondrial DNA: direct sequencing of enzymatically amplified DNA

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Received October 2, 1986; Revised and Accepted December 17, 1986

ABSTRACT

A specific segment of mitochondrial DNA from 18 people was examined by two methods of direct DNA sequencing. This segment includes a small noncoding region (V) shown before by restriction analysis to exhibit length polymorphism. All 11 of the human mtDNAs previously reported to have a deletion in this region proved to lack one of the two adjacent copies of a 9-base-pair sequence normally present in human mtDNAs. Phylogenetic analysis suggests that this deletion occurred only once during the evolution of modern types of human mtDNA and that it will be a valuable anthropological marker for peoples of East Asian origin. The one human mtDNA reported to have an addition in region V differs from the wild type by two mutations in the first copy of the 9-base-pair sequence: one transition and an addition of four cytosines, thereby producing a run of 11 cytosines. One of the direct DNA sequencing methods uses a single oligonucleotide primer to facilitate dideoxy sequencing from purified mtDNA templates. The second, more successful, method first amplifies this mtDNA segment enzymatically with two flanking primers (the "polymerase chain reaction") and then uses a third primer for DNA sequencing. This latter method, which works on the DNA extracted from small amounts of blood as well as on purified mtDNA, is shown to be a rapid means of defining sequence variants without purifying and cloning the same DNA segment from many individuals.

INTRODUCTION

In a survey of 176 human individuals, 146 types of mitochondrial DNA (mtDNA) were found by restriction analysis (1, 2, 3). A comparison of fragment sizes shows that 12 of these types seem to carry length mutations in region V, which contains a small noncoding region lying between the genes for cytochrome oxidase II and $tRNA^{Lys}$. In 11 cases, a deletion of about 7 base pairs was inferred, while the twelfth case appeared to involve an addition of about 7 bp (4, 5). These length mutations are of interest from the standpoint of mutagenic mechanisms and may provide markers for studying the evolutionary history of human populations.

We have explored two direct ways of determining the sequences of such

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polymorphic regions without first cloning them in bacteria. One approach was to sequence purified mtDNA directly with a single primer (6). Another approach was to take advantage of the polymerase chain reaction (PCR), which employs two primers to amplify enzymatically a specific region of DNA in vitro (7, 8). Here we demonstrate that with a third primer one can sequence the amplified product.

MATERIALS AND METHODS

Human mtDNA

Highly purified mtDNA from 16 individuals was prepared by R. Cann and M. Stoneking (1-5, 9). In regard to the length of region V, as judged by restriction fragment analysis, 11 of these mtDNAs [types 46, 67, 70, 77, 78, 79, 80, 81, 82, 122, and 127 in Fig. 1A of Stoneking et al. (3)] bear a deletion, one (type 101) bears an addition, and four are apparently normal in length (types 1, 47, 100, and 102).

Human DNA from Blood

Mitochondrial DNA was also obtained from platelets and white blood cells using a modified version of the extraction procedure described by Giles et al. (10). Ten ml of fresh, anticoagulated blood (collected in heparin-coated tubes) was placed in 15-ml Corex tubes and spun for 15 min. at 180 x g. The platelet-rich plasma was collected in the supernate, while the white-cell layer was removed with a Pasteur pipet. The white cells were resuspended in four volumes of a solution containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA, and disrupted with 3 strokes of a Dounce homogenizer; then the preparation was spun in 15-ml Corex tubes at 4° C for 5 min. at 500 x g. The supernate was respun at 4° C for 5 min. at 500 x g, and the mitochondria were recovered in a final spin of this supernate for 20 min. at 20° C and 12,000 x g. Platelets, most of which are enucleate but have mitochondria (11), were pelleted by further centrifugation of the original supernate for 20 min. at 1000 x g. Both the platelet and the white-cell mitochondrial pellets were resuspended in 0.7 ml of 10 mM Tris-HCl (pH 8), 1 mM EDTA, to which was added 10 µl of 25% SDS. The solutions were incubated for 10 min. at 37° C, followed by an addition of 15 µl of 5 M NaCl. The lysates were extracted three times with an equal volume of Tris-buffered phenol (pH 7) plus chloroform (1:1) and then once with an equal volume of water-saturated ether. The aqueous phase was placed into an uncapped microcentrifuge tube at 65° C for 10 min. to evaporate much of the dissolved ether and then lyophilized while being centrifuged in a Savant "Speed-Vac". The dried material that remained was resuspended in 100 µl of water, to which was added 250 µl of ethanol. Nucleic acid precipitates were collected by centrifugation at 12,000 x g

for 30 min. These pellets were washed with 70% ethanol, dried under vacuum and resuspended in 10 mM Tris-HCl (pH 8) plus 1 mM EDTA.

Primers

Three 20-base oligonucleotide primers were made on a Biosearch DNA synthesizer. They are complementary to segments of human mtDNA and have the following sequences, which are numbered according to Ref. 12 :

Primer A 8316 - 5' ATGCTAAGTTAGCTTTACAG 3' - 8297
Primer B 8196 - 5' ACAGTTTCATGCCCATCGTC 3' - 8215
Primer C 8225 - 5' ATTCCCCTAAAAATCTTTGA 3' - 8244

Single Primer Method

Purified mtDNA was linearized by digestion with SacI (from New England Biolabs) according to the manufacturer's recommendations. DNA was purified away from the restriction enzyme on Dupont "NENsorb" columns according to the manufacturer's instructions. 0.01 pmole (100 ng) of cut mtDNA was mixed with 0.05-0.10 pmole of ^{32}P-labeled primer C (see above). The total volume of the mtDNA template and primer mixture, which included 70 mM Tris-HCl (pH 7.6), 50 mM NaCl, 5 mM ß-mercaptoethanol, 5 mM $MgCl_2$ and 0.1mM EDTA (13), was 10 µl. This oligonucleotide was labelled at the 5' end to a specific activity of about 500 Ci/mmole primer by the method described in Maniatis et al. (14). The template-primer mixture was placed in a boiling water bath for 10 min, centrifuged at 12,000 x g for 10 sec and placed in a temperature block at 50° C. Into each of a set of four 1.5-ml Eppendorf microcentrifuge tubes, 2 µl of the appropriate "dddNTP" mixture was placed: dddG — 2 mM dideoxy GTP, 6 µM deoxy GTP, and 60 µM each dATP, dTTP, dCTP; dddA — 3.6 mM ddATP, 6 µM dATP, and 60 µM dGTP, dTTP, dCTP; dddT — 1.6 mM ddTTP, 6 µM dTTP, and 60 µM dGTP, dATP, dCTP; dddC — 1.6 mM ddCTP, 6 µM dCTP, and 60 µM dGTP, dATP, dTTP. All dddNTP mixes contained 70 mM Tris-HCl (pH 7.6), 50 mM NaCl, 5 mM ß-mercaptoethanol and 0.1 mM EDTA (13). Five units of DNA polymerase I Klenow fragment (Pharmacia) were then added to the template-primer mixture and 2.2 µl of this mixture were then added to each of the four tubes. The reactions were left at 50° C for 50 min. One µl of chase solution (containing 1 mM of each dNTP) was then added to each tube, and the mixture was left at the reaction temperature for an additional 15 min. The reactions were dried down and resuspended in 3 µl of water. Three µl of a formamide-dye mixture (formamide with 0.27% bromphenol blue, 0.27% xylene cyanol and 10 mM in EDTA) were added to each tube. The tubes were heated to 90° C for 3 min, after which the samples were loaded on a 9% polyacrylamide sequencing gel (15). Gels

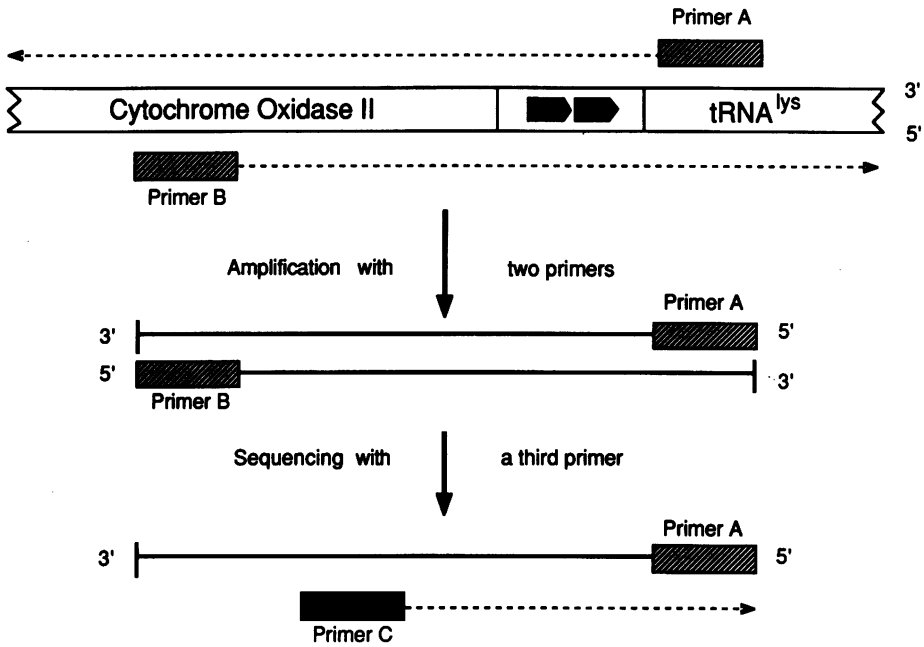

Fig. 1. Triple primer method for sequencing region V of human mtDNA. Two synthetic oligomers of 20 bp in length (primers A and B) match invariant sequences flanking region V, which contains two adjacent copies of a 9-bp sequence (shaded double arrows). In the first cycle of the polymerase chain reaction (PCR; Refs. 7 and 8) the template mtDNA is denatured and these oligonucleotides, in the presence of deoxynucleotides and DNA polymerase I, prime the synthesis of new DNA in the directions shown. In the next cycle, the newly made strands are separated from the template strands and can themselves hybridize to more primer DNA and serve as templates for further DNA synthesis. Repetition of denaturation and synthesis for many cycles leads to the exponential increase in copy number of the DNA segment bounded by primers A and B. Primer C, labeled with ^{32}P, is then used to sequence the amplified DNA fragment directly.

were dried on Whatman filter paper and exposed to X-ray film overnight at -70° C using an intensifying screen.

Triple Primer Method

As outlined in Fig. 1, this method involves first the amplification of a specific segment of human mtDNA by means of the polymerase chain reaction as described by Saiki et al. (7) and then the sequencing of the amplification product. The polymerase chain reaction was carried out with 10 µl of each primer (10 µM each of primers A and B), 15 µl of each dNTP (10 mM), 10 µl of DNA polymerase I buffer [300 mM Tris-acetate (pH 7.9), 600 mM Na-acetate, 100 mM Mg-acetate, 2.5 mM dithiothreitol], and from 1 to 100 ng of DNA (consisting of

highly or partially purified mtDNA, which need not be linearized) in a total volume of 100 µl. The mixture was heated for 10 min at 95° C to separate the strands, spun for 10 sec in an Eppendorf model 5414 microcentrifuge to bring down condensate and cool the sample, and then cooled for an additional 2 min at room temperature. Two µl of DNA polymerase I Klenow fragment (0.5 units/µl) were added and the reaction was allowed to proceed for 2 min at room temperature. The reaction mixtures were then heated again for 2 min at 95° C and the cycle just described was repeated a total of 20 times. Amplification was verified by the electrophoresis of 1/10 of the reaction volume in a 4% "NuSieve" (FMC BioProducts) agarose gel (Fig. 2). A known amount (1 µg) of ØX174 DNA cleaved with a restriction endonuclease such as HhaI was included in the gel so that the amount of amplification and the size of the product could be estimated, after staining the DNA with ethidium bromide, by comparing the ultraviolet light induced fluorescence of the amplified DNA band to that of the known amounts of DNA in the size marker bands.

Before sequencing the amplified DNA, excess deoxynucleotides were removed by centrifuge-driven dialysis in an Amicon "Centricon 30" micro-concentrator. Roughly 0.2-0.25 pmole of the amplified target sequence was then mixed with 2-2.5 pmole of ^{32}P-labeled primer C in 10 µl of 70mM Tris-HCl (pH 7.6), 50 mM NaCl, 5 mM ß-mercaptoethanol, 5 mM $MgCl_2$ and 0.1 mM EDTA (13). The reaction was then treated as in the sequencing protocol given above for the single primer method except that the DNA polymerase I Klenow fragment was added not to the template-primer mixture but to the individual dideoxy-terminated reaction mixtures. Residual dNTP's from the amplification step could otherwise sustain DNA synthesis before the addition of dideoxy chain terminators.

RESULTS

Single Primer Method

Five mtDNAs appearing from restriction analysis (4, 5, 9) to contain a deletion in region V were sequenced by the single primer method. All of these mtDNAs (types 46, 67, 77, 79, and 127 in Table 1) were alike in having a 9-bp deletion of **CCCCCTCTA** in comparison to the published (12) base sequence for one human individual, which contains two tandem copies of these 9 bp, as did two of our other mtDNAs (types 1 and 47 in Table 1) when also sequenced by this method. [The observed deletion may alternatively be viewed as resulting from loss of one tandem copy of **ACCCCCTCT** (Table 1)]. The location of this deletion, encompassing the nucleotides between either 8272-8280 or 8281-8289 bp [as

Table 1. Sequences of Length Polymorphisms in Region V of Human MtDNA

Length of Region V[a]	Type of mt DNA[b]	Sequence
Normal	1, 47, 100, 102, 119, L-1	CACCCCCTCTACCCCCTCTAGAG
Short	46, 67, 70, 77, 78, 79, 80, 81, 82, 122, 127, R-1	CACCCCCTCTAGAG
Long	101	CACCCCCCCCCCCTACCCCCTCTAGAG

[a] As reported in Refs. 4 and 5 or, for samples L-1 and R-1, in this work.

[b] As designated in Ref. 3 or, for L-1 and R-1, in this work. Type 47 is from the HeLa cell line and type 119 is the mtDNA whose complete sequence has been published (12).

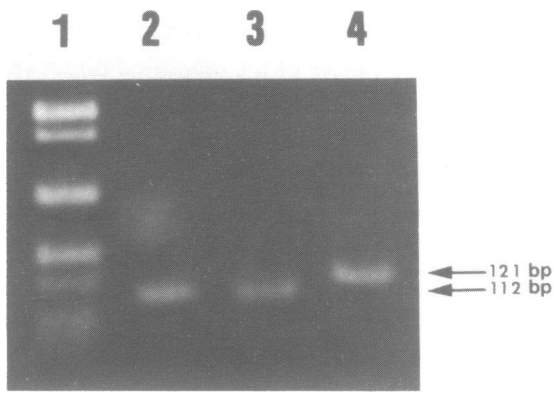

Fig. 2. Three examples of enzymatic amplification of a specific segment (region V) of human mtDNA. The templates were in one case (lane 2) highly purified human mtDNA and in two cases (lanes 3 and 4) total DNA from the blood of two individuals. Lane 1 contained a size marker (ØX174 DNA cut with HhaI). Electrophoresis was carried out in a 4% agarose gel. The DNA was stained with ethidium bromide and visualized by its fluorescence under ultraviolet illumination. Lane 2 has the amplification product from mtDNA type 127, which has one copy of the 9-bp sequence (Table 1). The 112-bp fragment in lane 3 (sample R-1) also contains a deletion of one of the 9-bp repeats, and is seen to have the same electrophoretic mobility as the type 127 fragment. The 121-bp fragment in lane 4 (sample L-1) corresponds in mobility to that seen in most mtDNAs and was found by sequencing to contain both copies of the 9-bp repeat (as diagrammed in Fig. 1). The individuals represented by samples R-1 and L-1 are of East Asian and European origin, respectively.

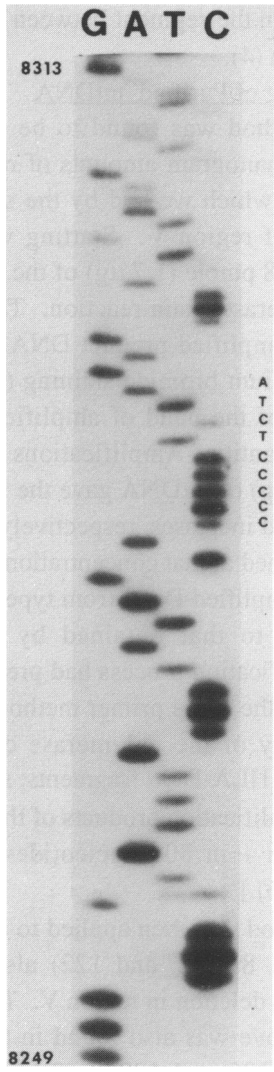

Fig. 3. Sequence of region V by the triple primer method. Shown is an autoradiograph of a 9% polyacrylamide gel containing the sequence of region V from human mtDNA type 82, which contains one copy of the sequence 5' CCCCCTCTA 3' (Table 1). The template for dideoxy sequencing was made using the polymerase chain reaction as in Fig.1. The 9-bp segment is found as a tandem repeat of two copies in most human mtDNA's (9). Except for the 9-bp deletion, the sequence of mtDNA 82 in this region (shown here from bp 8249 to 8313 as numbered in Ref. 12) matches the published sequence (12). The Cs at 8297, 8305, and 8309 and the A at 8312 are clearly visible on the original autoradiograph. The bands in the T and C lanes accompanying or slightly above the G at position 8255 seem to be sequencing artifacts, since they appear sporadically when this mtDNA region is sequenced. Furthermore, an insertion between 8255 and 8256 would lead to abolition of the termination signal for cytochrome oxidase II.

numbered in (12)], was within the segment between 8250 and 8303 bp mapped as region V by Cann and Wilson (4).

Amplification and Sequencing of Purified mtDNA

The triple primer method was found to be effective in determining the sequence of region V from nanogram amounts of mtDNA. This procedure was first tested on type 127, for which we had by the single primer method already obtained a clear sequence of region V. Starting with 0.01 pmole (100 ng) of mtDNA we obtained about 28 pmole (1.7 μg) of the desired 112-bp DNA product after 20 cycles of the polymerase chain reaction. This is a 2800-fold increase as assayed by first subjecting amplified product DNA and marker DNA to agarose gel electrophoresis and ethidium bromide staining (as shown in Fig. 2) and then comparing the fluoresence of the band of amplified DNA to that of a band of marker DNA of a known quantity. Amplifications that began with 0.002 pmole (20 ng) or 0.0001 pmole (1 ng) of mtDNA gave the same final amount of product (14,000-fold and 280,000-fold increases, respectively), which suggests that a limit to the amplification was reached at that concentration of product DNA (0.2 μM).

The sequence of the amplified DNA from type 127 mtDNA was determined and found to be identical to that obtained by the single primer method, demonstrating that the amplification process had preserved sequence fidelity. An example of sequencing with the triple primer method appears in Fig. 3. [A more detailed study of the fidelity of the polymerase chain reaction was based on amplification of ß-globin and HLA-DQα fragments; sequence analysis of multiple, independently generated, amplification products of the same fragment indicated an error frequency of less than 1 in 800 nucleotides for 20 to 27 cycles of the polymerase chain reaction (16).]

The triple primer method was then applied to eight additional mtDNAs, six of which (types 70, 78, 80, 81, 82, and 122) also appeared from restriction mapping (4, 5, 9) to contain a deletion in region V. The 9-bp deletion identified in the five types mentioned above was also found in these six mtDNAs (Table 1). Two other mtDNAs (types 100 and 102 in Table 1) were found to have the standard two copies of this 9-bp sequence. The reason for sequencing the latter two mtDNAs is their close genealogical relationship to type 101 (see below).

Mitochondrial DNA type 101 (Table 1) was identified (4) as having an addition within region V. Attempts to sequence this DNA directly by both the single and triple primer methods failed to provide a clear sequence for the polymorphic region. This DNA was amplified enzymatically and then cloned into an M13mp18 vector (16, 17). The dideoxy method (13) gave the sequence shown

in Table 1, which is four bases longer than the sequence present in the common types of mtDNA.

Amplification of Unpurified mtDNA

As described in Materials and Methods, amplification and sequencing of region V were also done using partially purified mtDNA extracted from blood components. DNA recovered from both the platelet and white cell fractions from 10 ml of human blood yielded 1.3 μg of amplified DNA (lanes 3 and 4, Fig. 2) without the need for either the time-consuming purification of mtDNA by ultracentrifugation through CsCl gradients or conventional, bacterially-mediated cloning. Since 1/100 of this amount of product is adequate for sequencing, we would predict that such sequence determinations could be done on blood samples of only a few drops. Fig. 2 shows amplification products from a highly purified mtDNA (lane 2) as well as from the two blood samples (lanes 3 and 4). Each of the three samples shows a single product. Further, since type 127 (lane 2) has the 9-bp deletion and an apparent size of 112 bp, we inferred that the blood sample in lane 3 (from individual R-1) also has the deletion, whereas the blood sample in lane 4 (from individual L-1) exhibits a product whose length is standard (121 bp) and thus does not have the deletion. These inferences were confirmed by sequencing the amplification products (Table 1).

DISCUSSION

Deletions

Cann and Wilson (4) pointed out the presence of a direct repeat of a 9-bp sequence within region V and speculated that it was a likely cause of length mutations generated by slipped mispairing during replication (18), a mechanism also invoked for explaining length mutations in other noncoding regions of mtDNA (19-21). Our work demonstrates that the deletions in region V are, in fact, due to the loss of one of the two copies of this 9-bp sequence.

The tree (Fig. 4) relating 147 types of mtDNA [*i.e.*, the 146 types mapped by R. Cann and M. Stoneking (2, 3) plus the mtDNA of known sequence (12)] shows this deletion occurring only once during the descent of these types from a common ancestor. The 11 types sharing this deletion are grouped within the shaded region of Fig. 4, and the lineage on which it occurred is labeled "**D**". Horai and Matsunaga (22) found in a study of 62 types of mtDNA from Japan that 11 of them had the deletion described by Cann and Wilson (4). Those 11 Japanese types are also united in their phylogenetic tree by several point mutations (22), which suggests again that this deletion has occurred only once in the evolutionary descent

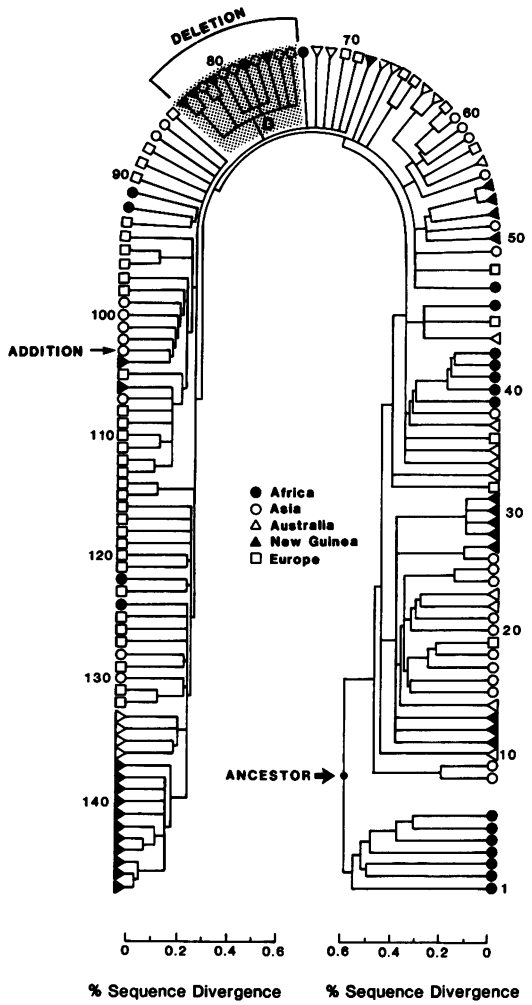

Fig. 4. Revised phylogenetic tree for 147 types of human mtDNA. All types possessing the deletion in region V (Table 1) are grouped into one clade, shown as numbers 74-84 within the shaded region. Because this tree differs slightly in branching order from that based on point mutations alone (3), the mtDNA types originally numbered 1, 47, 100, 101, 102, and 119 in Table 1 appear here as numbers 1, 46, 102, 103, 104, and 121, respectively. The mtDNA types, branching order, and placement of nodes with respect to accumulated sequence divergence were determined by mapping an average of 370 restriction sites in each of 177 individuals (2, 3). This tree is a rearrangement of the one presented by Stoneking et al. (3), which did not include the region V deletion in the parsimony analysis, and it shows all individuals containing this mutation as coming from the lineage marked "D". The tree shown previously (3) would require that the deletion event occurred four times; this tree, which is based on a single occurrence of the deletion, requires only three additional point mutational events and thus provides an equally parsimonious explanation of the data.

of modern mtDNA types from a common ancestor. Consistent with this suggestion, Hauswirth and Clayton (21) found no evidence for length polymorphism in region V in cultures of human cells.

One would expect a mutation that has occurred only once to be a valuable anthropological marker. Accordingly, we note that apart from one Black American all of the people known to bear this mutation stem from East Asia or New Guinea [Cann and Wilson (4) reported in error that this deletion was also found in an Australian]. Moreover, the order of branching within the cluster defined by the deletion is consistent with the idea that migrants from East Asia contributed to the founding of populations in New Guinea but not to the founding of the aboriginal Australian population.

The exceptional Black American mtDNA could be one result of introgression between the Black American and American Indian populations in areas where slavery existed. If so, the founders of the aboriginal American Indian populations probably included Asians bearing the deletion in region V. The triple primer method will facilitate testing of this hypothesis.

Addition

The addition detected by Cann and Wilson (4) is not due, as they proposed, to three copies of this 9-bp repeat. Instead, it is most easily explained by two mutational events, a T to C transition followed by a slipped mispairing insertion of four more cytosines in this region:

```
5'   CCCCCTCTACCCCCTCTA    3'
          ↓
5'   CCCCCCCTACCCCCTCTA    3'
          ↓
5'  CCCCCCCCCCCTACCCCCTCTA  3'
```

The resulting run of 11 Cs in the region may have caused the difficulty encountered in sequencing through that region (23). The fact that sequence analysis of region V from the nearest relatives of type 101, namely types 100 and 102, shows that neither has the T to C change or the addition of four cytosines implies that both events occurred relatively recently on the lineage leading to type 101. [Because of the restructuring of the tree brought about by our present work, the types previously numbered 100, 101, and 102 are now numbered 102, 103, and 104 (Fig. 4).]

Oligonucleotide Probes

We want to use methods based on the polymerase chain reaction to examine not only the nature of mutations in other regions showing length polymorphisms (4, 21), but also base substitutions seen as point-mutational "hot spots" clustered in the major noncoding region of human mtDNA (24). Our hope is to learn from this more about mutational mechanisms in mtDNA as well as to generate markers for human population studies. One approach would be to synthesize a panel of oligonucleotide probes spanning each region of length mutation. The presence of a length mutation would greatly diminish the ability of these probes to form stable hybrids, making possible a plus/minus screening procedure for their presence (25, 26). There are at least 10 such sites of length mutation in human mtDNA (4, 22), and the sum total of their presence or absence in any individual's mtDNA may constitute a simple means of distinguishing mtDNA types.

Direct Sequencing

By the triple primer method, the analysis of multiple samples of DNA for sequence variation in the same region is made more convenient than by previous methods. The standard method, which involves the production and screening of multiple libraries of M13 clones, requires about two or three weeks for determination of the sequence of a particular region in six mtDNAs. By contrast, this same sequence information can be obtained with the triple primer method in about three days. The ability to start with small amounts of DNA from blood is also a convenience when compared with the purification of the amounts of mtDNA needed to construct M13 libraries.

Addendum

The triple primer sequencing method has since been tried and works with human mtDNA regions other than region V (L. A. Wrischnik, R. G. Higuchi and P. Bluford, unpublished results); however, some 20-base third primers resulted in sequencing gels with high background bands that obscured the reading of the sequence. When a 14-base truncated version of one of these 20-base third primers was used as a primer, the problem disappeared.

ACKNOWLEDGEMENTS

We thank Rebecca Cann for mtDNA samples; Kary Mullis, Thomas White and Peter Bluford for helpful discussions; Cory Levenson for oligonucleotide synthesis; and Ellen Prager for helpful discussion and editorial assistance. This research was supported in part by grants from the National Science Foundation, the National Institutes of Health, and the Genetics Society of America.

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