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Human mitochondrial DNA: Analysis of 7S DNA from the origin of replication

(gel electrophoresis/restriction endonucleases/electron microscopy/hybridization/multiple components)

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ABSTRACT **Heat-treated samples of human mitochondrial DNA (mtDNA) exhibited a set of three low molecular weight DNA bands in addition to the major mtDNA band when electrophoresed in polyacrylamide gels. These DNA components were seen only after heat treatment or after relaxation of the mtDNA with a restriction endonuclease. The three components were single stranded and had sizes of 550, 585, and 620 nucleotides, close to the size (600 nucleotides) estimated from contour length measurements for the 7S DNA from the D loop of human mtDNA. Hybridization of the components with restriction endonuclease fragments of known position in the mtDNA confirmed this identification. Digestion of each 7S DNA component with the restriction endonuclease *Hae* III produced three fragments, two of which were identical in size among the components and the third of which varied. This third fragment, shown to be from the 5′ end of each component, differed in length by ~35 nucleotides among the components. These results suggest that human 7S mtDNA synthesis is terminated at a distinct position and that it is either initiated at one of three possible sites in the same mtDNA or that the mtDNA population consists of three subpopulations, each differing from the others by the presence or absence of a nucleotide sequence immediately adjacent to the origin of replication.**

The human mitochondrial genome is a closed circular duplex DNA molecule of 16,500 ± 300 base pairs that is replicated asymmetrically and unidirectionally from a fixed point. Some animal mitochondrial DNAs (mtDNAs), including human mtDNA, possess a characteristic structure at the origin of replication, the D loop, that can be seen by electron microscopy (1). D loop formation occurs via the displacement of one of the strands (the H strand) of mtDNA in the region of the origin of replication by the synthesis of a short complementary piece of DNA, referred to as 7S DNA, the synthesis of which is terminated by an unknown mechanism. As shown by 7S DNA turnover (2, 3), extension of the 7S DNA and synthesis of DNA complementary to the displaced strand both occur more slowly than D loop formation. This gives rise to a subpopulation of the mtDNA that consists of molecules having D loops of a uniform size. These molecules co-band with closed circular DNA that does not contain a D loop in a CsCl/ethidium bromide buoyant density gradient. Molecules in later stages of replication band at positions intermediate between the closed circular (lower band) and open circular plus linear (upper band) DNA (4). This allows the simultaneous purification and separation of D loop mtDNA from other replicating forms.

In this report we describe the dissociation of the 7S DNA from human mtDNA preparations and the analysis of this DNA by restriction endonuclease digestion and gel electrophoresis. The implications of the results on our concept of the structure of the origin of replication of human mtDNA and the mechanism of D loop formation are discussed.

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MATERIALS AND METHODS

DNAs. mtDNAs were prepared as described (5, 6). The sources of mtDNA were: human, HeLa (S3) cells and placentas; green monkey (*Cercopithecus aethiops*), BSC-1 cells; talapoin monkey (*Miopithecus talapoin*), primary cells; woolly monkey (*Lagothrix cana*), primary cells; and mouse (*Mus musculus*), LA9 cells. The cell culture conditions used have been described (5, 6). Bacteriophage PM2 DNA, prepared by the method of Espejo *et al.* (7), was a gift from Bob Watson. Bacteriophage M13 DNA digested with the restriction endonuclease *Hae* III was a gift from Peter Seeburg.

Electron Microscopy of DNA. DNA in 50% formamide was spread by the procedure of Davis *et al.* (8). Estimates of the D loop frequency were obtained by scoring 200–300 molecules per sample. Contour length measurements were performed as described (6).

Gel Electrophoresis of DNA. The electrophoresis of DNA in polyacrylamide slab gels (9) and in agarose slab gels (10) has been described. Gels were either photographed after staining with ethidium bromide or, for ^{32}P-labeled samples, dried on Whatman 3MM paper and autoradiographed with Kodak NS-2T x-ray film. The sizes of DNA fragments in the gels were estimated by use of PM2 duplex DNA digested with restriction endonuclease *Hin*dIII (11, 12) and M13 single-stranded DNA digested with restriction endonuclease *Hae* III (13) as size standards.

Preparation of 7S DNA. Kasamatsu *et al.* (1) have described the small, noncovalently bound DNA found in the D loop of mouse mtDNA as 7S DNA, according to its sedimentation coefficient. We have designated the corresponding DNA from mtDNAs of different species as 7S DNA, but no correspondence with the actual sedimentation coefficient is implied. Unless otherwise noted, 7S DNA was dissociated from D loop mtDNA by heating at 80° in 50 mM NaCl/10 mM Tris, pH 7.8/1 mM EDTA for 3–5 min. The 7S DNA was separated from the mtDNA by electrophoresis through a polyacrylamide (0.2% bis) slab gel at 100 V for 3–4 hr (room temperature). After electrophoresis of human mtDNA, three 7S bands were seen, designated A, B, and C in order of decreasing size. For some experiments the individual bands were cut out of the gel and the DNA was extracted as described (14).

Enzymes and Enzymatic Reactions. Ribonuclease-free bacteriophage T4 polynucleotide kinase, prepared by the

Abbreviations: mtDNA, mitochondrial DNA; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate.

method of Panet *et al.* (15), was a gift of Herb Heyneker. [γ-^{32}P]ATP was prepared and used with polynucleotide kinase to label the 5′ termini of DNA molecules as described (14). Terminal transferase was a gift of R. L. Ratliff. The reaction conditions are given in the following section. The restriction endonucleases *Hin*cII, *Kpn* I, *Hae* III, and *Hha* I were purchased from New England Biolabs (Beverly, MA). These enzymes and their respective reaction conditions are described in ref. 16.

Preparation of Labeled 7S DNA with High Specific Activity. A 7S DNA of high specific activity was prepared by synthesizing poly([^{32}P]dT) tails on the 3′ termini of the 7S DNA with terminal transferase. A 30-μl mixture containing ~1 ng each of the 7S DNA components A, B, and C in 0.2 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (Hepes), pH 7.5/400 μg of bovine serum albumin per ml/0.5 mM 2-mercaptoethanol/1 mM $CoCl_2$ was chilled to 0°, then added to a tube containing 0.1 mCi of dried [α-^{32}P]dTTP (Amersham). Terminal transferase (1 μl, 250 units) was added and the mixture placed at 37°. The reaction was monitored at 5-min intervals and stopped after 20 min by the addition of 1 μl of 0.33M EDTA (pH 8.5) and 1 μl (10 μg) of carrier tRNA. The total incorporation of ^{32}P into acid-insoluble material was 3×10^6 cpm, or ~1×10^9 cpm/μg of DNA. This level of activity is routinely obtained and the contribution of spontaneously initiated synthesis of poly(dT) is small (~10%) if the reaction is stopped after ~20 min (H. J. Kung, personal communication).

An equal volume of 0.5 M NaCl/0.2% sodium dodecyl sulfate/10 mM Tris (pH 7.8)/1 mM EDTA was added to the reaction mixture. The labeled DNA probe was separated from unincorporated [α-^{32}P]dTTP on a 0.6 × 15-cm Sephadex G50 column, using the same buffer for the elution. The fractions containing the DNA probe were pooled and extracted once with phenol/chloroform. The aqueous phase was made 0.3 M in sodium acetate (pH 5.8) and precipitated with ethanol. After centrifugation and removal of the ethanol the DNA probe was resuspended in 100 μl of 10 mM Tris (pH 7.8)/1 mM EDTA. Based on a total incorporation of 3×10^6 cpm, the recovery at this stage was 30% (9×10^5 cpm).

Hybridization of 7S DNA to mtDNA Restriction Fragments. Restriction endonuclease digests of HeLa mtDNA with *Kpn* I, *Hin*cII, and *Hha* I were electrophoresed in a 2% agarose slab gel for 2.5 hr at 100 V. *Hin*dIII fragments of PM2 DNA, labeled at their 5′ ends with ^{32}P, were included as size standards. The gel was stained and photographed; then the DNA in the gel was transferred to a nitrocellulose filter and immobilized (17).

The high specific activity 7S DNA was diluted to a volume of 4 ml with hybridization buffer [50 mM Hepes (pH 7.5)/3 mM EDTA/500 μg of yeast carrier RNA per ml/]/5% sodium dodecyl sulfate/0.45 M NaCl/45 mM Na citrate (pH 7.5)/50% formamide]. The nitrocellulose filter was placed on Saran Wrap, saturated with the hybridization mixture, sealed in Saran Wrap and aluminum foil, and placed at 37° for 60 hr. The filter was washed for 60 min in 500 ml of 0.3 M NaCl/30 mM Na citrate at pH 7.5, at 25°, and for 18 hr in 500 ml of 0.9 M NaCl/90 mM Na citrate at pH 7.5, with four buffer changes, after which the filter was air dried and then autoradiographed with Kodak NS-2T x-ray film.

5′-End Analysis. DNA labeled at the 5′ ends with ^{32}P was digested to 5′-mononucleotides by incubation at 37° for 3 hr with 200 μg each of DNase I and venom phosphodiesterase (Worthington) per ml in 100 mM Tris (pH 8.5)/50 mM $MgCl_2$. The 5′-dNMPs were separated by high-voltage paper electrophoresis in 5% acetic acid/0.5% pyridine/10 mM EDTA at pH 3.5.

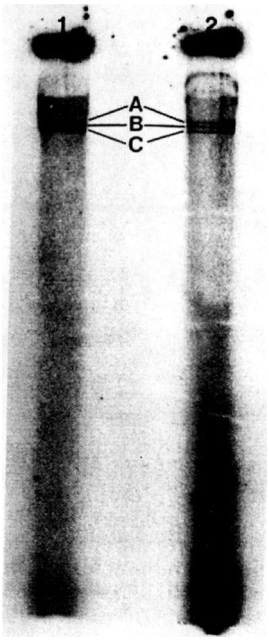

FIG. 1. Polyacrylamide gel electrophoresis of 7S DNA dissociated from human placenta mtDNA by introducing double-stranded scissions (lane 1) and by heating (lane 2). The 7S DNA migrates as three components, designated A, B, and C in order of decreasing size. The double-stranded scissions were produced by the restriction endonuclease *Kpn* I, which does not cleave human mtDNA within the D loop (unpublished data).

RESULTS

Frequency and Size of D Loops in Human mtDNA. Electron microscopy was used to estimate the frequency of D loops in molecules isolated from lower band preparations of human mtDNAs. The D loop frequency in five different preparations of HeLa cell mtDNA averaged 8% (range: 5–12%), in agreement with the value, 7%, published by Kasamatsu *et al.* (18). Much higher frequencies (53 and 57%) were observed in mtDNA preparations from two human placentas.

The average contour length of the duplex branch of the D loop, expressed as a fraction of the total contour length of the mtDNA molecule, was 0.036 ± 0.004 SD (54 HeLa mtDNA molecules) or 595 ± 65 nucleotides, based on an estimated genome size of 16,500 base pairs. For 12 placenta mtDNA molecules the value was 0.037 ± 0.006, a value not significantly different from the value for the HeLa D loops.

Dissociation of 7S DNA from mtDNA. 7S DNA may be dissociated from mtDNA by heating (18) and by the introduction of single- or double-strand scissions (2, 6). Human placenta mitochondrial 7S DNA was prepared by each of these methods and compared by polyacrylamide gel electrophoresis after 5′-end labeling with ^{32}P. In both cases the 7S DNA migrated as three distinct bands of equal intensity, designated A, B, and C (Fig. 1). The darkening of the negative in the lower third of lane 2 is presumably due to mitochondrial RNA species complexed with the mtDNA until their release by the heat treatment. Their absence in lane 1 (except at the extreme bottom) is probably due to a small amount of RNase activity present in the *Kpn* I enzyme preparation.

Sizes of Human 7S DNA Components. The A, B, and C components of human placenta mitochondrial 7S DNA were separated by polyacrylamide gel electrophoresis. The region of the gel containing each component was excised and the DNA was eluted from it. A portion of each component was re-electrophoresed along with an aliquot from the original mixture.

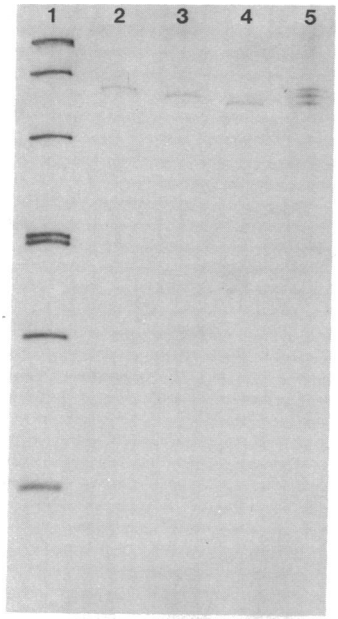

FIG. 2. Polyacrylamide gel electrophoresis of the individual A, B, and C components of 7S DNA isolated from human placenta mtDNA by preparative polyacrylamide gel electrophoresis. Lanes 1–5 contain *Hin*dIII-digested PM2 DNA, 7S-A, 7S-B, 7S-C, and the unseparated 7S DNA components, respectively.

Each component ran according to its original mobility (Fig. 2) and each was ~95% free of contamination by the other two components. The mobilities of the components were also unchanged by annealing at 24° below the T_m for 14 hr and by incubation at high pH (data not shown). These results are consistent with the interpretation that the differences in the electrophoretic mobilities of the components are due only to size differences.

The sizes of the components were estimated by polyacrylamide gel electrophoresis, with the *Hae* III fragments of the single-stranded DNA from bacteriophage M13 as size standards (13). The sizes obtained for the A, B, and C components were 680, 645, and 615 nucleotides, respectively. These size estimates are within experimental error but slightly larger than those for the size of the D loop of human mtDNA estimated by contour length measurements. We regard the sizes from contour length measurements as the more reliable, since the sizes of the *Hae* III, M13 fragments are not known with precision. However, the size differences among the components, 30–35 bases, are less sensitive to error than the absolute sizes, and we consider these estimates to be reliable.

Multiple 7S Components in Other mtDNAs. The occurrence of multiple 7S components in mtDNAs is not an isolated phenomenon, but appears to be ubiquitous, at least among mammalian species. The electrophoretic mobilities of 7S DNA components from three different human placenta mtDNA preparations, two HeLa mtDNA preparations, and mtDNA preparations from four other mammalian species were compared. Of the two HeLa mtDNA preparations used, one came from the lower band of a CsCl/ethidium bromide buoyant density gradient and the other from the portion of the gradient between the lower and upper band positions.

The human samples (Fig. 3, lanes 1–4) all exhibited identically migrating A, B, and C bands. The two HeLa samples (Fig. 3, lanes 3 and 4) showed differences in the relative intensities of the bands, the band corresponding to the C component being less intense than the other two bands. The HeLa sample from

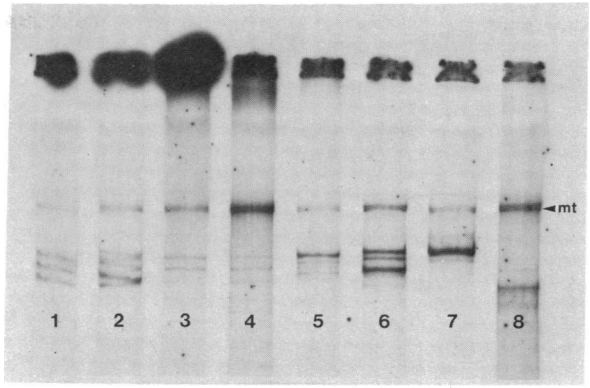

FIG. 3. Polyacrylamide gel electrophoresis of heated mtDNAs from two human placenta samples (lanes 1 and 2), HeLa cells (lanes 3, lower band, and 4, intermediate; see text), green monkey (lane 5), talapoin monkey (lane 6), woolly monkey (lane 7), and mouse (LA9) cells (lane 8). The slanted bands in lanes 1 and 2 are due to uneven drying of the gel prior to autoradiography. The heavy band (labeled mt) that appears in all lanes is due to a fast migrating portion of full-length linear duplex mtDNA (see text).

the intermediate region of the gradient (Fig. 3, lane 4) showed an additional fast component not seen in the other samples. The band indicated by the arrow in Fig. 3, common to all of the samples, has been shown to correspond to full-length linear duplex mtDNA, most of which does not enter the gel (W. Brown and F. DeNoto, unpublished results).

mtDNA samples from green monkey (lane 5), talapoin monkey (lane 6), woolly monkey (lane 7), and mouse (LA9) cells (lane 8) all exhibited multiple 7S components (Fig. 3). These differed in both number and position from the human 7S components, but their estimated sizes fell within a narrow range (450–700 bases).

Hybridization of 7S DNA to Restriction Endonuclease Fragments of mtDNA. To establish that the 7S DNA came exclusively from the D loop of the mtDNA, we hybridized 7S DNA to restriction endonuclease fragments of mtDNA. Digests of HeLa mtDNA with the restriction endonucleases *Kpn* I, *Hin*cII, and *Hha* I were electrophoresed in a 2% agarose gel (Fig. 4*a*). The DNA was denatured and transferred onto a nitrocellulose filter as described (17). A high-specific-activity 7S DNA was prepared and hybridized with the filter-bound DNA as described in *Materials and Methods*. After hybridization, the filter was autoradiographed. The result (Fig. 4*b*) shows that the 7S DNA hybridized to the smaller *Kpn* I fragment and to the largest fragments in each of the *Hin*cII and *Hha* I digests. From restriction endonuclease cleavage mapping studies (unpublished data), we know that the D loop of human mtDNA occurs on these same three fragments and, furthermore, that the only portion of the mtDNA common to the three fragments is from 0.956 to 0.040 genome units. Since the origin of replication is at 0 and the D loop extends to 0.036 genome units, this is good evidence that the 7S DNA corresponds to the single-stranded DNA segment present in the D loop. The 7S DNA used in the hybridization contained all three components; thus this result also indicates that the components originate from the same portion of the genome. Sequence data (unpublished results) confirm this conclusion.

Examination of Fig. 4*b* also indicates that a significant amount of the 7S DNA hybridized with the band containing the larger *Kpn* I fragment. This could have been due, in part, to the presence in this band of incompletely digested linear mtDNA molecules. However, it is more likely that the observed hybridization was with the smaller *Kpn* I fragments exclusively,

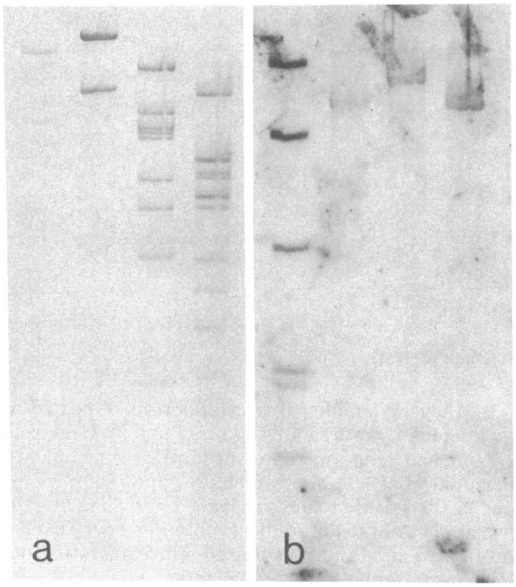

FIG. 4. (*a*) Agarose gel electrophoresis of *Kpn* I, *Hin*cII, and *Hha* I digests of HeLa mtDNA. (*b*) Hybridization of a ^{32}P-labeled 7S DNA probe to the DNA fragments shown in *a*. The four lanes shown in both *a* and *b* are (from left to right) *Hin*dIII-digested PM2 DNA and *Kpn* I, *Hin*cII, and *Hha* I digests of HeLa mtDNA. The *Hin*dIII-digested PM2 DNA fragments were labeled at the 5′ ends before electrophoresis.

and that some of these were "trapped" in the larger fragment band of the gel. The "trapping" of smaller DNA fragments in bands containing larger DNA fragments is, in our experience, a routine feature of DNA electrophoresis in both agarose and polyacrylamide gels (see also Figs. 5 and 6).

Differences among 7S DNA Components. The mitochondrial 7S DNA components were labeled at their 5′ ends and digested with the restriction endonucleases *Hae* III and *Hha* I, both of which cleave single-stranded DNA (19, 20). The results (Fig. 5) indicate that *Hha* I does not cleave any component, that *Hae* III cleaves all three, and that at least some of the

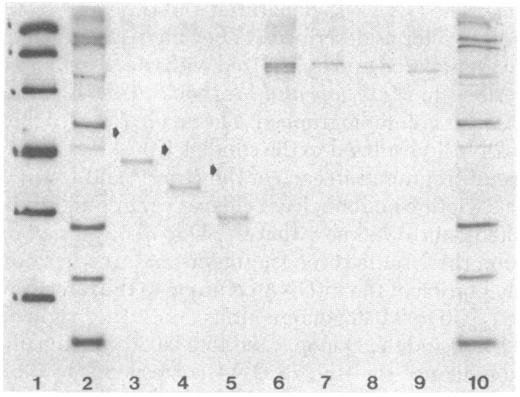

FIG. 5. Polyacrylamide gel electrophoresis of 5′-end-labeled components of human placenta 7S mtDNA digested with *Hae* III and *Hha* I restriction endonucleases. The lanes contain: 1, *Hin*dIII-digested PM2 DNA; 2 and 10, *Hae* III-digested M13 DNA; 3, 4, and 5, *Hae* III-digested 7S components A, B, and C, respectively; 6, undigested (control) heated mtDNA; 7, 8, and 9, *Hha* I-digested 7S components A, B, and C, respectively. In each of lanes 3, 4, and 5 there is one faint band (arrows) that migrates more slowly than the main band. This faint band represents an incompletely digested *Hae* III fragment with a size ~50 nucleotides greater than the main band, in each case, and indicates that there is a *Hae* III fragment of ~50 nucleotides immediately adjacent to the 5′-terminal *Hae* III fragment.

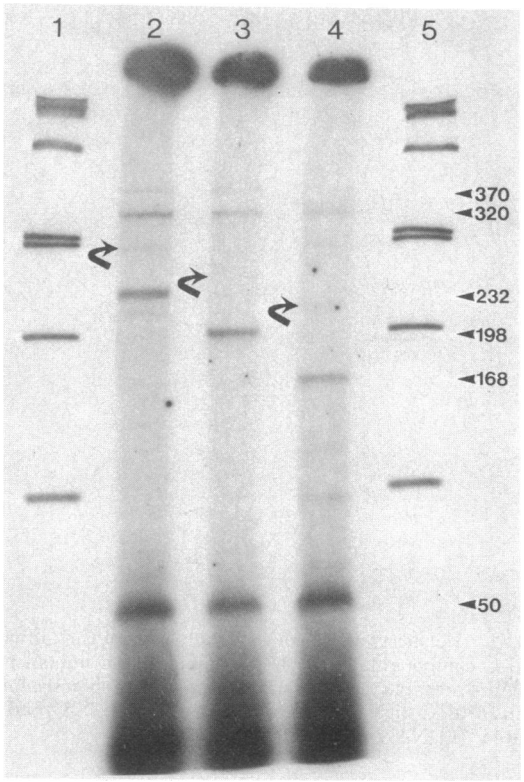

FIG. 6. Polyacrylamide gel electrophoresis of components of human placenta 7S mtDNA digested with *Hae* III restriction endonuclease and then 5′-end labeled. The lanes contain: 1 and 5, *Hin*dIII-digested PM2 DNA; 2, 3, and 4, *Hae* III-digested 7S DNA components A, B, and C, respectively. Single-stranded fragment sizes (in nucleotides) are shown at the right. The faint bands indicated by the curved arrows and the faint bands at a size of 370 nucleotides are incomplete *Hae* III digestion products.

variation in the electrophoretic mobilities of the components is due to a difference in size at or near the 5′ ends. The estimated sizes of the 5′-terminal *Hae* III fragments of the A, B, and C components are 232, 198, and 168 bases, respectively.

To determine if the size differences resided exclusively at or near the 5′ ends, we digested the three components with *Hae* III and then labeled them at their 5′ ends. Each component was cleaved into three pieces by *Hae* III (Fig. 6). The largest (~320 bases) and smallest (~50 bases) fragments did not vary in size among the three digests. The incompletely digested fragments, present in low amounts and noted in Figs. 5 and 6, revealed that the smallest *Hae* III fragment is the internal piece, since it is linked to both the intermediate-sized fragment, shown to be the 5′ end, and the largest fragment, which must correspond to the 3′ end. Thus, the size variation among the 7S DNA components resides entirely within the first 170–230 bases of the 5′ termini (Fig. 7).

With three exceptions, the faint bands seen in Figs. 5 and 6

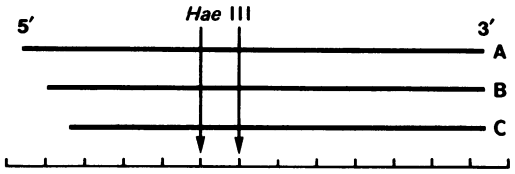

FIG. 7. Locations of the *Hae* III cleavage sites in the 7S DNA components. The horizontal scale is graduated in units of 50 nucleotides. The vertical arrows show the positions of the *Hae* III cleavage sites.

Table 1. 5′-End analysis of mitochondrial 7S DNA components

DNA	% of total ^{32}P, cpm					Total ^{32}P, cpm
	Origin	dCMP	dAMP	dGMP	TMP	
7S-A	2.3	6.9	17.1	24.5	49.2	3226
7S-B	2.3	4.8	46.4	32.3	14.3	2495
7S-C	10.6	25.7	6.0	25.4	32.4	2083
*Hin*dIII, PM2	0.4	0.4	99.3	0.0	0.0	2297

may be explained by incomplete digestion and by "trapping." One exception is the band in lane 2 of Fig. 6, at a position equivalent to ~215 nucleotides, which also occurs in lane 3 of Fig. 5. The other exceptions are the two bands seen in lane 4 of Fig. 6, between the main bands at 168 and 50 nucleotides. These faint bands are noted but not understood.

5′-Terminal Nucleotides of 7S DNA Components Are Not Unique. The 7S DNA components were labeled at their 5′ ends with ^{32}P and digested to 5′-mononucleotides with pancreatic DNase and venom phosphodiesterase, and the mononucleotides were separated by high-voltage electrophoresis. The results of one set of determinations are given in Table 1. Although the 5′-end analysis of the *Hin*dIII-digested PM2 DNA control gave the expected result (*Hin*dIII cleavage produces exclusively 5′-dA fragments), the 7S components showed no unique 5′ end for any component. Determinations on two other 7S DNA samples obtained from different placentas showed similar 5′ heterogeneity, but yielded percent base compositions different from each other and from those in Table 1. Whether this latter difference is biologically significant or, e.g., an artifact of preparation, is unknown. Heterogeneity at the 5′ end of the 7S DNA from mouse (LA9) mtDNA has also been observed (18). Because of this it has not been possible to obtain the base sequences at the 5′ ends of the human 7S DNA components.

DISCUSSION

The results indicate that the 7S mtDNA corresponds to the small, noncovalently bound DNA fragment found in the D loop of human mtDNA by the criteria of size and specificity of hybridization. The lack of bands that migrate more slowly than the A component of the 7S DNA in the HeLa sample from the intermediate region of the CsCl/dye gradient (lane 4, Fig. 3) suggests that the stepwise synthesis of mtDNA is confined to the formation of the 7S species. The origin of the fast migrating band observed in this same sample has not been investigated.

On the basis of the data from electron microscopy and hybridization to restriction endonuclease fragments of mtDNA we have assumed that the three human 7S DNA components represent the same mtDNA sequence, except at the 5′ ends where the components differ in size.

We have recently determined the sequence of the first 33 bases from the 5′ end of the smallest *Hae* III fragment of the 7S DNA (unpublished data). This was done by pooling the A, B, and C components before *Hae* III digestion; hence the sequence is derived from the smallest *Hae* III fragment common to each of the components. A sequence could not have been obtained if this *Hae* III fragment differed among the three components. From these data we conclude that the three 7S DNA components are probably identical in sequence, except for a region at or near the 5′ ends, and also that the 7S DNA is complementary to one and not to both of the human mtDNA strands, a fact already known for mouse 7S mtDNA (1).

The exact nature of the size variation at the 5′ end of the human 7S mtDNA is unknown. The most intriguing possibility is that the size variation is produced by 7S DNA synthesis that is initiated at any one of three sites in the mtDNA, located 30–35 nucleotides apart. It is also possible that the three 7S DNA components are produced on three classes of mtDNA molecules, each class differing from the other two by an added or deleted sequence of 30–35 nucleotides near the origin of replication. A third possibility is that the initiation point of human mtDNA synthesis is not specified by a sequence, but occurs instead as a consequence of some other regional feature of the mtDNA or in relation to another macromolecular species that is associated with the mtDNA. In this case the size variation among the 7S components could be related to normal variation in superhelix density, and heterogeneity in the 5′-terminal nucleotide would be expected. This latter heterogeneity could also be related to heterogeneity at the 3′ end of a hypothetical primer.

The lack of size variation at the 3′ end of the human 7S DNA does not support the hypothesis (18) that the termination of 7S DNA synthesis is due to superhelix contraints. It is possible that a specific sequence terminates the synthesis of 7S DNA in the human mitochondrial genome.

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