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Localization of the Ribonucleotide Sites in Rat Liver Mitochondrial Deoxyribonucleic Acid

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Supercoiled rat liver mitochondrial DNA is relaxed by treatment with ribonucleases A, T_1 or H. All the supercoiled mitochondrial DNA is sensitive to ribonuclease H and ribonuclease A, but only 35% of the supercoiled population is sensitive to ribonuclease T_1. Removal of the ribonucleotides with calf thymus ribonuclease H, followed by denaturation of the mitochondrial DNA and analysis of the single-strand fragment lengths in the electron microscope, showed that the ribonucleotides were randomly located on both strands of the DNA. Endonuclease-S_1 digestion of mitochondrial DNA after removal of the ribonucleotides reveals that no unique fragments are produced and ribonucleotides are randomly distributed with respect to one another. The average number of ribonucleotide sites per molecule was estimated to be between 8 and 13. Two possible mechanisms for the origin of ribonucleotide sites are discussed.

The mtDNA of mammalian cells exists as a circular double-stranded molecule with a mol.wt. of approx. 10000000. Sedimentation-velocity studies have shown the presence of two components with $s_{20,w}$ of 38S and 27S. The 38S mtDNA (FI mtDNA) is a supercoiled circular molecule, whereas the 27S (FII mtDNA) is a relaxed circular molecule (Nass, 1969), differing only by the fact that it contains at least one phosphodiester scission in the DNA backbone (for a review see Borst, 1972).

FI mtDNA is anomalous compared with viral closed circular DNA in that it is sensitive to high pH, suffering chain scission (Piko *et al.*, 1968; Borst, 1972). The alkali-lability of FI mtDNA shows biphasic kinetics, with fast and slow components (Borst, 1972; Grossman *et al.*, 1973). The fast alkali-lability undoubtedly arises from the presence of depurinated sites in the DNA duplex, depurination occurring owing to free-radical attack on the *N*-glycosidic bond of the purine residues (Rhaese & Freese, 1968). The depurination of DNA in aqueous solutions cannot apparently be avoided even at neutral pH, although the calculated rate of depurination is slow (Lindahl & Nyberg, 1972). Reducing agents, which can react with DNA in the presence of oxygen, causing depurination and eventually chain breakage (Vinograd *et al.*, 1965; Bode, 1967), would lead to a significant increase in the depurination rate if they were present during the isolation of the DNA. Since mitochondria contain reducing agents, it is probable that a significant amount of depurination can occur during mtDNA isolation. This would account for the observed fast-alkali-labile fraction in isolated samples of mtDNA (Grossman *et al.*, 1973; Borst, 1972). Slow alkali-lability has been ascribed to unusual bases (Koch, 1973) or impurities in the DNA preparations (Borst, 1972). However, slow alkali-lability is consistent with the presence of covalently incorporated ribonucleotides. The FI mtDNA from a number of mammalian sources has been demonstrated to be susceptible to a variety of ribonucleases, including ribonuclease H, an enzyme specific for duplex DNA–RNA hybrid sequences (Grossman *et al.*, 1973; Miyaki *et al.*, 1973; Wong-Staal *et al.*, 1973; Koch, 1973; Lonsdale & Jones, 1974). The function and localization of these ribonucleotides within the mtDNA genome is therefore of interest, especially as evidence has been obtained for a direct role of RNA in the initiation and continuation of replication of DNA (Gefter, 1975). We have attempted to determine whether or not the ribonucleotides are uniquely or randomly located within the mitochondrial genome.

Abbreviations used: mtDNA, mitochondrial DNA; FI DNA, covalently closed DNA; FII DNA, relaxed circular DNA; FIII DNA, genome-size linear DNA; RNAase, ribonuclease; DNAase, deoxyribonuclease; SSC medium, 0.15 M-NaCl–0.015 M-sodium citrate, pH 7.2.

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Experimental

Materials

RNAase T_1 (grade III), agarose (type II), poly(thymidylic acid), α-amylase (type IVA) and cytochrome *c* (type VI) were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames,

Surrey, U.K. RNAase A (four-times crystallized) and DNAase I were obtained from BDH Chemicals, Poole, Dorset, U.K. RNA polymerase (EC 2.7.7.6) was obtained from Boehringer Corp. (London) Ltd., London W.5, U.K.

Methods

Preparation of FI mtDNA. Mitochondria and mtDNA were isolated by a modification of the method described by Lonsdale & Jones (1974). Excised livers were chopped and washed in 0.25M-sucrose/10mM-Tris/HCl/10mM-KCl/5mM-EDTA, pH7.4. Then 10vol. of 0.21M-mannitol/70mM-sucrose/1mM-Tris/HCl/0.1mM-EDTA, pH7.4, was added to the chopped liver and the tissue was disrupted by homogenization in a Waring Blendor at top speed for 15s. Cell lysis was performed by five strokes of a Dounce homogenizer with a close-fitting Teflon pestle. The lysate was centrifuged twice for 15min at 2°C and $700g_{max.}$ to remove whole cells and nuclei. The mitochondria were pelleted from the low-speed supernatant by centrifuging for 30min at 2°C and $5200g_{max.}$. The mitochondrial pellet was suspended in 20vol. of 0.25M-sucrose/50mM-Tris/HCl/25mM-KCl/2.5mM-$MgCl_2$, pH7.2. DNAase I was added to give a final concentration of 100μg/ml and the mitochondrial suspension was incubated at 20°C for 30min. The incubation was terminated by the addition of 0.2M-EDTA, pH7.4, to a final concentration of 10mM, followed by an equal volume of 0.21M-mannitol/70mM-sucrose/1mM-Tris/HCl/0.1mM-EDTA, pH7.4. The mitochondria were pelleted by centrifuging for 10min at 2°C and $12000g_{max.}$, resuspended and washed once.

The mitochondrial pellet was resuspended in 5vol. of 0.15M-NaCl/0.1M-EDTA, pH8.0 at 20°C, made 2% (w/v) with respect to sodium dodecyl sulphate and incubated for 5min. An equal volume of freshly redistilled phenol saturated with 75mM-NaCl/50mM-EDTA, pH8.0, was added; the phases were mixed and allowed to cool in an ice bath for 20min. The phases were then separated by centrifuging for 10min at 2°C and $2600g_{max.}$. The aqueous phase was recovered and re-extracted with phenol as described above. The phenol and EDTA were removed by dialysis for 6h against two changes of 1×SSC medium. The deproteinized lysate was layered on 50ml of 10.4–26.4% (w/v) isokinetic sucrose gradients buffered with 100mM-NaCl/10mM-Tris/HCl/2mM-EDTA, pH8.0, and centrifuged for 20h at 5°C at $61000g_{av.}$ in the 3×70ml rotor of the MSE Superspeed 65 centrifuge. FI mtDNA was recovered from the appropriate fraction by centrifuging at $180000g_{av.}$ for 16h at 5°C. The FI mtDNA was resuspended in 1×SSC medium at a concentration of 50–100μg/ml, dialysed into 10mM-NaCl/10mM-Tris/HCl/0.1mM-EDTA, pH7.9, and stored at −20°C.

Preparation of bacteriophage PM2 DNA. Bacteriophage-PM2 lysates were prepared as described by Espejo & Canelo (1968). Isolation of bacteriophage-PM2 FI DNA and FII DNA was as described by Lonsdale & Jones (1974), except that the phenol was removed from the DNA solution before sucrose gradient centrifugation by dialysis for 12h against 3litres of 1×SSC medium.

Preparation of RNAase H. Calf thymus RNAase H was prepared by the method of Stavrianopoulous & Chargaff (1973), as far as step V. The preparation of poly(dT)·[^{3}H]poly(rA) and the assay of RNAase H were as described by Stavrianopoulous & Chargaff (1973). The RNAase H unit is defined as the quantity of enzyme catalysing the conversion of 1nmol of poly(rA) into an acid-soluble form in 15min at 35°C.

The RNAase H was assayed for DNAase activity by using bacteriophage-PM2 FI DNA or *Escherichia coli* [^{14}C]DNA (specific radioactivity 1000c.p.m./μg). The assay mixture contained, in a total of 50μl: 50mM-Tris/HCl/50mM-NaCl/5mM-$MgCl_2$, pH7.9 at 37°C, 1μg of bacteriophage-PM2 FI DNA or 15μg of *Escherichia coli* [^{14}C]DNA and 18 units of RNAase H. No conversion of bacteriophage-PM2 FI DNA into FII DNA was observed after analysis by agarose-gel electrophoresis of the reaction products after incubation for 30min at 37°C. Likewise no loss of acid-precipitable radioactivity was detected after incubation of *Escherichia coli* [^{14}C]DNA with RNAase H for 4h at 37°C.

Preparation of endonuclease S_1. Endonuclease S_1 was prepared from crude α-amylase powder (from *Aspergillus oryzae*) as described by Vogt (1973), as far as chromatography on sulphopropyl(SP)-Sephadex. The assay of endonuclease S_1 was as described by Vogt (1973), 1unit of nuclease activity being defined as the amount of enzyme that renders 10μg of denatured DNA soluble in 10% (w/v) trichloroacetic acid in 10min at 45°C.

Analytical band sedimentation. Analytical-band-sedimentation experiments were performed as previously described by Lonsdale & Jones (1974) and sedimentation coefficients were calculated by the method of Bruner & Vinograd (1965). Sedimentation coefficients in alkaline CsCl experiments were calculated relative to the sedimentation velocity of single-stranded linear bacteriophage-ϕX174 DNA.

Agarose-gel electrophoresis. Agarose-gel electrophoresis was performed by the method described by Aaij & Borst (1972), on 1% agarose gels in the presence of 1μg of ethidium bromide/ml. Either rod gels (0.4cm×8cm) or slab gels (0.3cm×17cm×17cm) were used. DNA was detected by direct illumination with a short-wave u.v. lamp and photographed on Ilford FP4 film through a 4X orange filter.

Determination of thermal 'melting' curves. The thermal 'melting' curves of rat liver mtDNA and rat liver nuclear DNA are essentially identical under

similar conditions (Wolstenholme *et al.*, 1972). The thermal 'melting' profile of rat liver nuclear DNA was determined in 1×SSC/10% (w/v) formaldehyde at formamide concentrations between 0 and 40% (v/v). Above 40% (v/v) formamide the A_{260} of the formamide was sufficiently high to prevent measurement of changes in hyperchromicity. The DNA solutions, at a concentration of 10–15 μg/ml, were continuously heated in sealed cuvettes at a rate of 0.5–0.8°C/min. The temperature was measured with a calibrated thermocouple in the sample cell.

Thermal denaturation of mtDNA. For electron microscopy, a 10 μl sample containing approx. 0.1 μg of mtDNA was added to formamide (65 μl) and incubated at 40±0.5°C for 3 min. To this, 40% (w/v) formaldehyde solution (25 μl), previously equilibrated at 40°C, was added. The sample was incubated for a further 8 min at 40°C. At the end of the incubation samples were cooled rapidly in an ice/water mixture and prepared directly for electron-microscopic examination.

Before use the formaldehyde solution was treated as described by Wolstenholme *et al.* (1972).

Electron microscopy. (1) Preparation of denatured DNA. Denatured mtDNA samples in 0.1 ml of formaldehyde/formamide solutions were mixed with an equal volume of 1 M-ammonium acetate containing cytochrome *c* (0.2 mg/ml). A 40 μl sample was then allowed to flow down an inclined glass slide on to a hypophase of 0.26 M-ammonium acetate/0.5% formaldehyde.

The surface film was left for 10 min and then picked up on Formvar films supported on 200-mesh nickel grids. The grids were stained for 30 s in 95% (v/v) ethanol/10 μM-uranyl acetate/10 μM-HCl, washed in ethanol and air-dried. The grids were 'shadowed' on a rotary turntable at 2500 rev./min at an angle of 7° with 10–15 mg of Pt/Pd (4:1, w/w) evaporated at a mean distance of 10 cm from the grids. The specimens were examined and photographed with a JEOL Jem 7A electron microscope at an original magnification of 12600×. The magnification was calibrated with a diffraction-grating replica (2160 lines/mm). The electron micrographs were traced at a final magnification of 125000× and the tracings converted into digital form by using a System-II Pencil Follower (CETEC System Ltd., Glasgow, Scotland, U.K.). Lengths of molecules and frequency distributions were computed by using an ICL 1904S computer.

(2) Preparation of double-stranded DNA. Samples of mtDNA were prepared for electron microscopy by the formamide technique described by Robberson *et al.* (1972). Grids were prepared and examined as described above.

Determination of DNA. The concentration of native DNA solutions was determined by assuming that an A_{260} of 1.0 corresponds to a concentration of 50 μg/ml.

Results

Physical characterisation of rat liver mtDNA

Measurements of sedimentation velocity in neutral CsCl for FI mtDNA and FII mtDNA were made, and values for $s_{20,w}$ of 38.5±0.5S and 27.3±0.4S respectively were obtained. Electron-microscopic examination showed that the 38.5S fraction contained not less than 80% of the molecules as highly twisted circles, whereas the 27.3S fraction consists mainly of relaxed circular molecules with a few linear molecules. The physical length of FII mtDNA was determined from electron micrographs by using bacteriophage-PM2 open circular DNA as an internal standard, assuming a contour length of 3.02 μm for bacteriophage-PM2 DNA (Espejo *et al.*, 1969). The length of FII mtDNA was 5.06±0.07 μm. The linear FIII mtDNA molecules were the same length. When different preparations of rat FII mtDNA were spread under identical conditions and measured relative to electron micrographs of a diffraction grating (2160 lines/mm), values that were indistinguishable from those measured against the internal standard were consistently obtained. Thus the stability of the electron microscope and the reproducibility of spreading were sufficiently good to allow measurements without internal standards.

Alkali-lability of FI mtDNA

The results of sedimentation analysis at high pH depended strongly on the total time of exposure of the mtDNA to the alkaline conditions. When the DNA was denatured by layering a neutral solution directly on to the alkaline CsCl solution, a minor band sedimenting at about 80S was observed during the first 15 min of the experiment. The major species observed was a single band sedimenting at 20S. Prior denaturation of the mtDNA before layering on to the gradient resulted in the disappearance of the fast minor component. Preincubating the mtDNA in 0.16 M-NaOH for 3 h at 20°C, or for 1 h at 37°C, resulted in the disappearance of the 20S band, and no species sedimenting more rapidly than 8S could be observed. It is suggested that the very unstable 80S component is the collapsed supercoil (Borst, 1972), and the somewhat more stable component with an observed sedimentation coefficient of 20S (the corrected value, $s^{pH13}_{Na,w} = 25S$) represents the full-length linear single strands. Prolonged incubation in alkali results in the degradation of the full-length linear single strands to a heterogeneous population of small single-stranded fragments.

Susceptibility of mtDNA to ribonucleases A, T_1 and H

When purified FI mtDNA was incubated with RNAase A at either 40 μg/ml or 400 μg/ml in SSC medium, no appreciable effect on the sedimentation

Table 1. *Effect of RNAase A and RNAase T_1 on FI mtDNA*
Samples (1 μg) of FI mtDNA were incubated under the conditions indicated for 1 h at 37°C. The conversion into FII mtDNA was determined by analytical band sedimentation as previously described (Lonsdale & Jones, 1974).

	Conversion of FI into FII mtDNA (%)			
	RNAase A		RNAase T_1	
Buffer	40 μg/ml	400 μg/ml	0.8 μg/ml	4 μg/ml
SSC medium	0	0	15	15
50 mM-Tris/HCl/50 mM-NaCl/5 mM-$MgCl_2$, pH 7.9	9	—	15	35
50 mM-Tris/HCl/5 mM-$MgCl_2$, pH 7.9	22	100	15	35

profile was observed. Incubation in buffers of lower ionic strength showed a conversion of FI mtDNA into FII mtDNA compared with control incubations with no enzyme present (Table 1). Complete conversion of the FI mtDNA into FII mtDNA was obtained in 10 mM-Tris/HCl/5 mM-$MgCl_2$, pH 7.9, at an RNAase A concentration of 400 μg/ml.

Similar experiments with RNAase T_1 showed little dependence of the degree of conversion on the ionic strength, and the maximum amount of conversion observed was 35% (Table 1); no further conversion was produced by extending the incubation period to 2 h or by redigestion of the FI mtDNA isolated from these incubations.

The incubation of 1 μg of FI mtDNA with 2 units of RNAase H in 50 mM-NaCl/50 mM-Tris/HCl/5 mM-$MgCl_2$, pH 7.7, for 15 min at 37°C resulted in the quantitative conversion of the FI mtDNA into FII mtDNA as determined by band sedimentation, agarose-gel electrophoresis and electron microscopy.

Control experiments with bacteriophage-PM2 FI DNA showed that the sensitivity of the FI mtDNA to RNAases A, T_1, and H was not due to trace quantities of DNAase.

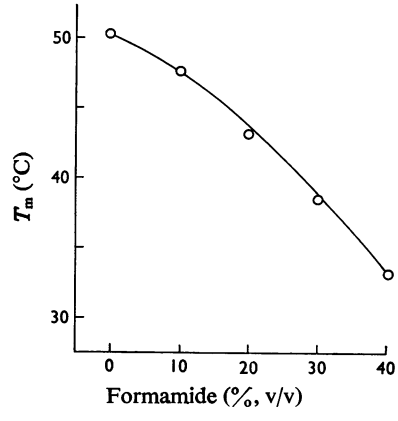
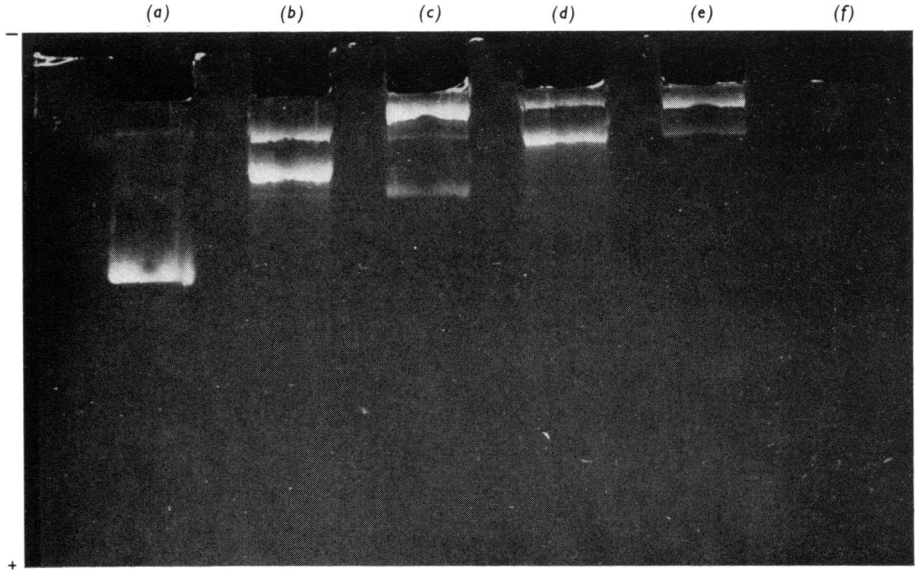


Fig. 1. *Effect of formamide on the 'melting' temperature (T_m) of rat liver nuclear DNA*
T_m was measured as described under 'Methods'. DNA was at a concentration of 10–15 μg/ml in SSC medium/10% (v/v) formaldehyde containing the indicated concentrations of formamide.

Thermal denaturation

The 'melting' temperatures of rat liver nuclear DNA and rat liver mtDNA in both SSC medium (Nass, 1969) and 50 mM-sodium phosphate/10% (w/v) formaldehyde (Wolstenholme *et al.*, 1972) are essentially the same. To minimize the risk of heat-induced strand scission during 'melting', denaturation was done in formamide/formaldehyde solutions. Rat nuclear DNA was used in place of mitochondrial DNA to study the 'melting curves' in 10% (v/v) formaldehyde at various concentrations of formamide. The 'melting' temperature of rat liver nuclear DNA in the presence of 10% (v/v) formaldehyde was determined to be 50.3°C, in agreement with the data of Wolstenholme *et al.* (1972). Formamide in increasing concentrations up to 40% (v/v) was observed to lower the 'melting' temperature of rat liver nuclear DNA (Fig. 1). At 40% (v/v) formamide, no increase in the hypochromicity was observed above 40°C. Samples of FI mtDNA containing approx. 20% of FII mtDNA were incubated in 10% formaldehyde at 0, 10, 20, 30, 40, 50 and 65% formamide at 40°C as described under 'Methods' and examined in the electron microscope. Denaturation of the FII mtDNA was observed to be essentially complete when a formamide concentration of 50% (v/v) was used. The FI mtDNA was apparently not denatured at any of the concentrations of formamide used. The resistance of FI mtDNA to denaturation has also been described by Wolstenholme *et al.* (1972). This resistance demonstrates that the method can be used with samples that are known to contain depurinated sites, which are apparently stable under these conditions.

FI mtDNA and RNAase H-digested FI mtDNA were denatured in 65% formamide/10% formaldehyde at 40°C for 10 min. The products were analysed



EXPLANATION OF PLATE 1

Effect of endonuclease S_1 on mtDNA and RNAase H-digested FI mtDNA

Samples (1 μg) of DNA were incubated in 30mM-sodium acetate/50mM-NaCl/1mM-$ZnSO_4$, pH4.6, in a total volume of 40μl for 15 min at 37°C. Samples (*b*), (*d*) and (*f*) contained 100 units of endonuclease S_1/ml; samples (*a*), (*c*) and (*e*) contained no enzyme. (*a*) and (*b*), bacteriophage PM2 FI DNA; (*c*) and (*d*) rat liver mtDNA; (*e*) and (*f*), RNAase H-digested FI mtDNA. Electrophoresis was carried out for 20h at 2V/cm on 1% agarose gels (17cm×17cm×0.3cm) in 40mM-Tris/20mM-sodium acetate/2mM-EDTA adjusted to pH7.7 at 20°, containing ethidium bromide (1 μg/ml).

Table 2. *Electron-microscopic analysis of denatured FI mtDNA and RNAase H-treated FI mtDNA*
(*a*) FI mtDNA (1 μg in 0.1 ml of 50 mM-NaCl/50 mM-Tris/HCl/5 mM-$MgCl_2$, pH 7.9) was treated with 2 units of RNAase H for 15 min at 37°C. (*b*) FI mtDNA without RNAase H digestion. The samples were denatured and examined in the electron microscope as described under 'Methods'. Sedimentation analysis of the FI mtDNA before denaturation showed it to contain about 20% of FII mtDNA and no FIII mtDNA.

	DNA (%, w/w)		
Sample	Covalently closed molecules	Single-stranded circular molecules	Single-stranded linear molecules
(*a*)	0	13.3	86.7
(*b*)	78.0	8.3	13.6

by electron microscopy, and the results are shown in Table 2. In contrast with the FI mtDNA, RNAase H-digested FI mtDNA contained no supercoiled molecules, as suggested by the band sedimentation and gel-electrophoresis analysis. Only 13.3% by weight of the molecules observed were circular. The distribution of the linear single-stranded molecules contains no unique fragment lengths, and is essentially random (Fig. 2). The mean length of the observed linear molecules was 1.37 μm. This implies the presence of approximately four ribonucleotide sites per full-length single strand, or eight ribonucleotide sites per duplex molecule.

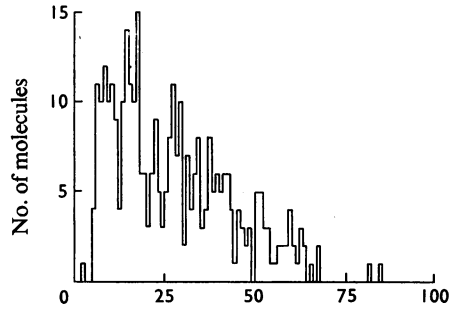


Fig. 2. *Size distribution of linear single-stranded DNA fragments after denaturation of RNAase H-digested FI mtDNA*

Endonuclease-S_1 analysis of RNAase H-digested FI mtDNA

Endonuclease S_1 from *Aspergillus oryzae* is almost completely specific for single-stranded DNA, although a very low activity towards double-stranded DNA can be detected in most preparations (Godson, 1973; Vogt, 1973). FI DNA is, however, rapidly relaxed to FII DNA by cleavage at loosely base-paired regions and, in the presence of an excess of enzyme, is converted slowly into full-length linear molecules and eventually into small fragments. Samples of bacteriophage-PM2 DNA and rat liver mtDNA containing supercoils, relaxed circles and full-length linear molecules, and RNAase H-digested mtDNA, were incubated with S_1 nuclease at 100 units/ml for 15 min at 37°C. The reaction products were analysed by electrophoresis in 1% agarose gels, and a sample of the mtDNA that had been digested with both S_1 nuclease and RNAase H was examined in the electron microscope. The endonuclease-S_1 digestion products of bacteriophage-PM2 DNA and the mtDNA consisted of full length linear molecules and relaxed circular molecules (Plate 1). The RNAase H-digested mtDNA after endonuclease-S_1 digestion appears as a heterogeneous collection of linear fragments. Similar results were obtained when endonuclease S_1 was used at 12.5, 25 and 50 units/ml. There was no evidence for discrete fragment lengths, as is obtained after digestion with restriction endonucleases (D. M. Lonsdale & I. G. Jones, unpublished work). This indicates that the ribonucleotide sites, which are converted into single-stranded gaps by RNAase H, are not uniquely located with respect to one another. Analysis of electron micrographs of the mtDNA digested with both S_1 nuclease and RNAase H revealed a range of size of linear fragments from approx. 5 μm to the lower limit of detection, approx. 0.05 μm (Fig. 3), most of the fragments being of small size. The mean length of the molecules observed was 0.38 μm, giving an estimated 13 ribonucleotide sites per molecule. The distribution observed is consistent with the ribonucleotide sites being randomly located in both strands of the mtDNA.

A computer model of random breakage of double-stranded mtDNA with a minimum fragment size of 1% of full-length molecules produces similar distributions to those observed experimentally at between 10 and 15 breaks per molecule.

Discussion

The ability of various ribonucleases to cause relaxation of rat liver FI mtDNA implies that the

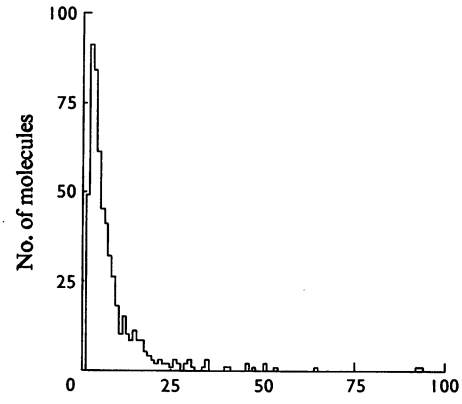


Fig. 3. *Size distribution of double-stranded DNA fragments after digestion of RNAase H-digested FI mtDNA with endonuclease S_1*

A portion of sample (*f*) of Plate 1 was prepared for electron-microscopic examination by the formamide technique of Robberson *et al.* (1972).

mtDNA contains ribonucleotides covalently linked in the phosphodiester backbone. Similar results with other mammalian mtDNA species have also been reported (Grossman *et al.*, 1973; Koch, 1973; Miyaki *et al.*, 1973; Wong-Staal *et al.*, 1973). The presence of ribonucleotides covalently linked in the phosphodiester backbone explains observations on the alkali-lability of mtDNA. Studies of the rate of nicking of FI mtDNA at high pH values have suggested that fast- and slow-reacting components are present (Borst, 1972; Grossman *et al.*, 1973; Wong-Staal *et al.*, 1973). Wong-Staal *et al.* (1973) deduced, by comparison with the rate of hydrolysis of RNA, that the fast hydrolytic phase corresponded to molecules containing 10–17 ribonucleotides, whereas the slow phase corresponded to molecules with three or four ribonucleotides. Grossman *et al.* (1973) reported 30 and 10 ribonucleotides for the fast- and slow-hydrolysed components respectively. Well over 50% of the FI mtDNA was present as the fast-alkali-labile component in these studies. For rat liver mtDNA we have found that virtually all the FI mtDNA is fast alkali-labile, in agreement with Leffler *et al.* (1970), who described the rapid fragmentation of mtDNA in alkali. This fast alkali-lability is almost certainly caused by the presence of depurinated sites, since bacteriophage-PM2 FI DNA that had been partly depurinated without strand scissions by incubation at pH5.0 exhibited fast alkali-lability: most of the untreated bacteriophage-PM2 FI DNA sediments at alkaline pH as a collapsed supercoil (Lonsdale & Jones, 1974). To obtain an estimate of the number of ribonucleotide sites without the interference of other alkali-labile sites the mtDNA was denatured under conditions that did not cause strand scissions at the depurinated sites. This was evidenced by the presence of FI mtDNA and of single-stranded circles in the denatured FI mtDNA preparation, which originally contained up to 20% of FII mtDNA. Both sedimentation data and electron-microscopy data were in good agreement as to the proportion of FI mtDNA before and after denaturation. Denaturation of rat liver mtDNA from which the ribonucleotides had been removed with RNAase H gave a random distribution of single-stranded DNA molecules, the mean length of which indicated three to four ribonucleotide sites per full-length single strand or six to eight per intact duplex. The alternative means of analysis, by using the enzyme endonuclease S_1, gave a value of 13 ribonucleotide sites per duplex molecule. This larger value for the number of ribonucleotide sites obtained by using endonuclease S_1 probably results from the low activity towards double-stranded DNA exhibited by most preparations of endonuclease S_1 (Godson, 1973). The number of ribonucleotide sites estimated from denaturation or endonuclease-S_1 cleavage of RNAase H-digested mtDNA is consistent with the number of ribonucleotide sites estimated from slow alkaline nicking kinetics of mtDNA (Grossman *et al.*, 1973; Wong-Staal *et al.*, 1973). The distribution of the fragments observed in the electron microscope indicates that the ribonucleotide sites are not uniquely located on one strand, nor are they uniquely located with respect to each other on adjacent strands. The range of fragment sizes suggests that the number of ribonucleotide sites per molecule is subject to a wide variation around the determined mean.

Substantial evidence has been presented in several systems, for example bacteriophage ϕX174, bacteriophage M13, polyoma virus and *Escherichia coli*, that an RNA polymerase synthesizes the 3′-hydroxyl primer that is required by all known DNA polymerases (Gefter, 1975). The mitochondrial RNA polymerase has been demonstrated to be sensitive to rifampicin, and the resulting inhibition of the RNA polymerase is coupled with a decrease in thymidine incorporation into mtDNA (Saccone & Gadeleta, 1973). The effect of rifampicin on mtDNA synthesis was not a secondary result of inhibition of synthesis of RNA and consequently of protein synthesis, as chloramphenicol, a direct inhibitor of mitochondrial protein synthesis, did not significantly affect the extent of thymidine incorporation. The presence of ribonucleotides in mtDNA and the inhibition of thymidine incorporation into mtDNA by rifampicin is suggestive of an RNA-priming mechanism.

The initial step in the replication of mtDNA is the formation of D-looped molecules (Kasamatsu *et al.*, 1971; Ter Schegget & Borst, 1971), the D-loop being a uniquely located triple-stranded structure containing a dissociable 7S single-stranded DNA fragment.

Analysis of the 7S DNA fragment for 5'-ribonucleotides indicated that few, if any, ribonucleotides are present (Kasamatsu *et al.*, 1973). However, as previously stated (Kasamatsu *et al.*, 1973), the proportion of D-looped molecules to molecules in the process of further replication is high, and therefore the majority of 7S DNA fragments associated with D-looped molecules have been available for further processing for some time and the primer ribonucleotide may well have been removed. However, later stages of replication appear to proceed in a similar discontinuous mode of synthesis (Koike & Wolstenholme, 1974), and the incomplete excision of the RNA primer sequences involved would result in covalently linked ribonucleotides scattered throughout the mitochondrial genome. As the estimated total number of ribonucleotides is small (Grossman *et al.*, 1973; Wong-Staal *et al.*, 1973) and of the same order as we have estimated for the number of ribonucleotide sites, it would appear that the ribonucleotides occur singly, though the existence of sites containing more than one ribonucleotide cannot be excluded. Evidence for the existence of such sites comes from the ability of *Escherichia coli* RNAase H to act on 60% of HeLa-cell mtDNA (Wong-Staal *et al.*, 1973) whereas chick-embryo RNAase H causes the complete conversion of HeLa FI mtDNA into the relaxed FII mtDNA. *Escherichia coli* RNAase H is known not to be able to function as an endonuclease at a bond linking a ribonucleotide to DNA (Berkower & Leis, 1973; Berkower *et al.*, 1973), and it seems probable that those sites susceptible to the chick-embryo enzyme but not to the *E. coli* enzyme are single ribonucleotides.

The ribonucleotides present in mtDNA, rather than being remnants of RNA primer sequences, could alternatively arise through the action of a non-stringent DNA polymerase in the presence of Mn^{2+} (Kornberg, 1974). However, Meyer & Simpson (1970) found no evidence that isolated rat liver mtDNA polymerase could incorporate ribonucleoside triphosphates even in the presence of Mn^{2+}.

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