Ribonuclease-Sensitivity of Covalently Closed Rat Liver Mitochondrial Deoxyribonucleic Acid

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Preparations of covalently closed mitochondrial DNA of rat liver contain 10–30% of molecules that are converted into relaxed circular molecules after treatment with ribonuclease. Control experiments, with covalently closed bacteriophage PM2 DNA, indicate that ribonuclease-sensitivity cannot be induced either by depurination or by incubation with reducing agents.

The mtDNA* of mammalian cells exists as circular molecules of about 5 μ m contour length in either a covalently closed configuration (FI mtDNA) or as a relaxed molecule (FII mtDNA). Replication proceeds unidirectionally through Cairns-type intermediates (Kasamatsu & Vinograd, 1973; Cairns, 1963). Recent reports indicate that the FI mtDNA is sensitive to RNAase in both ascites AH66 (Miyaka *et al.*, 1973) and HeLa S3 (Koch, 1973) cells. Such RNAase-sensitive molecules could arise indirectly either as a function of the transformed state of the cells or perhaps as a product of a reaction with radicals which are formed in the presence of reducing agents and oxygen (Bode, 1967). It is also apparent that RNAase-sensitive molecules could constitute an intermediate in the replication of the mtDNA; such molecules have been demonstrated in Colicinogenic factor E₁ replication (Blair *et al.*, 1972). As RNAase-sensitive FI DNA has only previously been demonstrated in transformed mammalian cell lines and bacterial systems, we considered it necessary to examine the mtDNA of a normal mammalian tissue and by using a covalently closed viral DNA as a control to ensure that the RNAase-sensitive sites were not produced as artifacts. Results demonstrate that a significant proportion of the FI mtDNA of rat liver is RNAase-sensitive.

Experimental

Materials

RNAase T₁ (grade III) was obtained from Sigma Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.

Pancreatic RNAase (crystallized fourfold) and DNAase I were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

* Abbreviations: mtDNA, mitochondrial DNA; FI DNA, covalently closed DNA; FII DNA, relaxed circular DNA; RNAase, ribonuclease; DNAase, deoxyribonuclease; SSC, 0.15M-NaCl–0.015M-sodium citrate.

Methods

Mitochondria and mtDNA were isolated under conditions of low O₂ partial pressure by gassing the isolation media with O₂-free N₂ to eliminate nicking reactions which occur in the presence of reducing agents and O₂ (Bode, 1967).

Male rats (strain PVG/C) were fed *ad lib* before being killed. Livers were removed, 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 Blender at top speed for 15s. Cell lysis was achieved by five strokes of a Dounce homogenizer with 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 sedimented 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-MgCl₂, pH7.2. DNAase I was added to give a final concentration of 100 μ g/ml and the mitochondrial suspension was incubated at 0°C for 1h. The incubation was stopped 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 sedimented by centrifuging for 30min at 2°C and 5200g_{max}, resuspended and washed twice. The final mitochondrial pellet was resuspended in 5vol. of NaCl–EDTA (0.15M-NaCl–0.1M-EDTA, pH8.0) at 20°C, made 1% (w/v) with respect to sodium dodecyl sulphate and incubated for 10min. An equal volume of NaCl–EDTA-saturated phenol 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 was removed by two ether extractions; the ether was removed in a stream of O₂-free

N_2 . The deproteinized lysate was layered on 50ml 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 and 61000 $g_{av.}$ in the 3×70ml rotor of the MSE Superspeed 65 centrifuge. FI mtDNA was isolated, dialysed into SSC medium overnight and then sedimented for 16h at 5°C and 180000 $g_{av.}$. The FI mtDNA was allowed to resuspend in SSC medium to a concentration of 50–100 μ g/ml and stored at –20°C.

Preparation of covalently closed bacteriophage PM2 DNA

PM2 bacteriophage lysates were prepared as described by Espejo & Canelo (1968). The virus lysate was clarified by centrifuging for 30min at 20°C and 5200 $g_{max.}$. The virus was harvested by centrifugation for 2h at 20°C and 100000 $g_{av.}$. Virus pellets were resuspended in 1M-NaCl–20mM-Tris–HCl, pH8.1; CsCl was added to increase the density to 1.23g/ml and the suspension was centrifuged for 15h at 20°C and 90000 $g_{av.}$ in the 3×23ml rotor of the MSE Superspeed 65 centrifuge. The virus band was removed and diluted with 1M-NaCl–20mM-Tris–HCl, pH8.1, and the virus was collected by centrifuging for 2h at 20°C and 90000 $g_{av.}$. The virus pellet was resuspended in a small volume of NaCl–EDTA and made 1% (w/v) with respect to sodium dodecyl sulphate. An equal volume of NaCl–EDTA-saturated phenol was added, and the phases were mixed and cooled in an ice-bath for 20min. The phases were separated by centrifuging for 10min at 2°C and 2600 $g_{max.}$. The aqueous phase was recovered and re-extracted with phenol as described above. The phenol was removed by two ether extractions, the ether being removed in a stream of O_2 -free N_2 . The deproteinized lysate was layered on a 16ml 15.9%–26.5% (w/v) isokinetic sucrose gradient buffered with 100mM-NaCl–10mM-Tris–HCl–2mM-EDTA, pH8.0, and centrifuged for 18h at 5°C and 46000 $g_{av.}$ in the 3×23ml rotor in the MSE Superspeed 65 centrifuge. The FI DNA fraction was isolated, dialysed into SSC medium overnight and sedimented for 16h at 5°C and 180000 $g_{av.}$. The bacteriophage PM2 FI DNA was allowed to resuspend in SSC medium to a concentration of 50–100 μ g/ml and was stored at –20°C.

Purification of FI mtDNA free of transcriptional complexes

FI mtDNA (20 μ g in SSC medium) was banded in a 5.5ml-step CsCl gradient (Brunk & Leick, 1969), mean density 1.700g/ml, by centrifuging for 20h at 5°C and 130000 $g_{av.}$ in the 10×10 ml angle rotor in the MSE Superspeed 65 centrifuge. The main DNA band was isolated, dialysed against SSC medium and then sedimented for 16h at 5°C and 230000 $g_{av.}$. The DNA,

approx. 15 μ g, was allowed to resuspend in 0.2ml of SSC medium and stored at –20°C.

Preparation of RNAase-resistant FI mtDNA

FI mtDNA (20 μ g in SSC medium) was incubated with a boiled mixture of pancreatic RNAase and RNAase T_1 , at final concentrations of 50 μ g/ml and 1 μ g/ml respectively for 100min at 37°C. A 5.5ml-step CsCl gradient, mean density 1.700g/ml, was constructed with the incubation mixture and then centrifuged to equilibrium for 20h at 5°C and 130000 $g_{av.}$. The DNA was isolated and dialysed against SSC medium for a minimum of 4h. The sample was then layered on a 16ml 21.7–31.4% (w/v) isokinetic sucrose gradient buffered with 100mM-NaCl–10mM-Tris–HCl–2mM-EDTA, pH8.0, and centrifuged for 16h at 5°C and 65000 $g_{av.}$ in the 3×23ml rotor in the MSE Superspeed 65 centrifuge. The FI mtDNA fraction was isolated, dialysed against SSC medium and then sedimented for 16h at 5°C and 230000 $g_{av.}$. The FI mtDNA, approx. 10 μ g, was allowed to resuspend in 0.06–0.1ml of SSC medium.

Band sedimentation

Analytical band-sedimentation experiments were performed by the method of Vinograd *et al.* (1963). Experiments at neutral pH were done with a CsCl density of 1.397g/ml, buffered with 5mM-Tris–HCl, pH7.5, except for samples that had been incubated with 1M-sodium acetate buffer, pH5.0, in which case the CsCl buffer was 50mM-Tris–HCl, pH7.5. Experiments at alkaline pH were done with CsCl of density 1.397g/ml in 0.1M-NaOH, pH13. The data were adjusted by least-squares fit and corrected for the effects of viscosity and solvent density as described by Bruner & Vinograd (1965). Densities of 1.701 g/ml and 1.702g/ml were used for rat liver mtDNA and bacteriophage PM2 DNA respectively.

Incubations

Preparations of dithiothreitol-treated DNA. For this 0.2M-dithiothreitol in SSC medium (2 μ l) was added to 10–20 μ g of bacteriophage PM2 FI DNA in 0.2ml of SSC medium and incubated for 1h at 37°C. The dithiothreitol was removed by dialysis against SSC medium overnight at 2°C.

Depurination. Depurination was effected by adding 2 μ l of 1M-sodium acetate, pH5.0, to 1–2 μ g of DNA in 20 μ l of SSC medium and incubating for 2h at 37°C.

RNAase treatment

Mixed RNAase (1mg of pancreatic RNAase/ml and 20 μ g of RNAase T_1 /ml) was heated in a boiling-water bath for 10min before use to remove residual

DNAase activity. Solutions of 1–2 μ g of DNA in 20 μ l of SSC medium were made 50 μ g/ml and 1 μ g/ml with respect to pancreatic and T_1 RNAases respectively, and then incubated for 1 h at 37°C.

Results

Sedimentation-velocity patterns for rat liver mtDNA exhibit two components with $s_{20,w}$ 38.5 $\pm$ 0.5S and 27.3 $\pm$ 0.4S, corresponding to the covalently closed (FI) and relaxed (FII) forms respectively. Isolated FI mtDNA from rat liver when analysed by velocity sedimentation normally contained not less than 80% of the mtDNA as the 38.5S component (Fig. 1a), the remainder having $s_{20,w}$ 27.3S and probably resulting from carry-over during isolation from the sucrose gradient or by single-strand nicking during purification. Such preparations when incubated with RNAase show a 10–30% conversion of the 38.5S fraction into the 27.3S fraction (Fig. 1b). This conversion was shown not to be due to traces of DNAase or possible apurinase (Verly *et al.*, 1973) activity by incubating bacteriophage PM2 FI or depurinated FI DNA with the mixed RNAase preparation, as no detectable bacteriophage PM2 FII DNA was formed during these incubations.

The previous reports (Koch, 1973; Miyaka *et al.*, 1973) and the present results demonstrated that a significant fraction of the FI mtDNA was not converted into the FII species by RNAase digestion. The re-isolation of the FI mtDNA fraction after 60 min incubation at 37°C showed a very small conversion of FI DNA into an FII species on redigestion with RNAase. A longer incubation period of 100 min at 37°C before the re-isolation of the FI mtDNA species resulted in a FI mtDNA species that was no longer sensitive to RNAase digestion. A similar result has been obtained with mouse L-cell mtDNA labelled with [Me - 3H] thymidine (D. M. Lonsdale & I. G. Jones, unpublished work).

The possibility was considered that the observed FI into FII conversion might be due to the presence of a transcriptional complex of a FII mtDNA co-sedimenting with the FI mtDNA. Aloni & Attardi (1972) showed that such transcriptional complexes could be removed from mtDNA preparations by banding in CsCl: the presence of the RNA in association with the DNA confers a higher buoyant density on the complex. However, when FI mtDNA isolated from a sucrose gradient was banded in CsCl, only minor quantities of material of density greater than 1.701 g/ml were detected, and the DNA from the main band showed a degree of conversion from FI into FII that was similar to that of the original FI mtDNA.

The sedimentation-velocity patterns for bacteriophage PM2 DNA exhibited two components with $s_{20,w}$ 27.7S and 21.5S, corresponding to the FI and FII forms respectively. The isolated bacteriophage

PM2 FI DNA contained little or no detectable PM2 FII DNA. The treatment of bacteriophage PM2 FI DNA at acid pH (pH 5.0) or with reducing agents in the presence of O_2 was tested for the induction of RNAase-sensitivity. The depurination at pH 5.0 of bacteriophage PM2 FI DNA for 2 h at 37°C followed by incubation for a further 1 h at 37°C with or without RNAase did not cause any observable conversion of the FI into an FII species. Alkaline sedimentation patterns showed that a significant proportion (about 20%) of the bacteriophage PM2 FI DNA had been depurinated by this treatment as compared with controls. Two components with observed sedimentation coefficients of 55S and 16S were detected during runs in alkaline gradients, corresponding to the alkaline supercoils and the denatured single-stranded DNA respectively.

Fig. 1. Effect of RNAase on FI mtDNA

FI mtDNA was either (a) incubated for 1 h at 37°C or (b) incubated with RNAase for 1 h at 37°C and then sedimented through CsCl of density 1.397 g/ml buffered with 5 mM-Tris-HCl, pH 7.5, at 25000 rev./min. Photographs were taken after 5500s.

The incubation of bacteriophage PM2 FI DNA with 2mM-dithiothreitol at 37°C followed by dialysis at 4°C against SSC medium to remove the dithiothreitol resulted in about a 40% conversion of FI into the bacteriophage PM2 FII DNA. No further conversion was observed after incubation at 37°C for 1h. Incubation of the dithiothreitol-treated bacteriophage PM2 FI DNA with RNAase did not produce PM2 FII DNA.

The alkaline sedimentation-velocity patterns of rat liver FI mtDNA preparations normally contained little or no alkaline supercoils, indicating that most of the FI molecules contained at least one discontinuity which was either a depurinated site or a RNAase-sensitive site.

Discussion

Koch (1973) and Miyaka *et al.* (1973) have demonstrated the presence of RNAase-sensitive sites in ascites AH66 and HeLa S3 FI mtDNA molecules respectively. Our results demonstrate that FI mtDNA molecules isolated from normal rat livers also possess one or more RNAase-sensitive sites. The actual proportion of the RNAase-sensitive molecules in both rat liver mtDNA and mouse L-cell mtDNA (D. M. Lonsdale & I. G. Jones, unpublished work) is between 10 and 30% of the FI mtDNA population that could be isolated. The suggestion that the sensitivity could be due to an unusual base (Koch, 1973) seems unlikely, as RNAase-insensitive FI mtDNA molecules can be isolated and it would be expected that any unusual bases would be a general feature of, rather than a restricted distribution among, the mtDNA population. A restricted distribution of RNAase-sensitive molecules would be expected if intermediates in mtDNA transcription were involved; however, results indicate that this is unlikely. Similarly the induction of RNAase-sensitive sites might be possible during the isolation of the mtDNA. Attempts to induce such sites in bacteriophage PM2 DNA by depurination or by incubation with a reducing agent in the presence of O₂ (Bode, 1967; Rhaese & Freese, 1968) were unsuccessful. It is therefore apparent that the RNAase-sensitive FI mtDNA molecules present in mtDNA from transformed and normal cells constitute a normal phenomenon. The model proposed for the replication of

Colicinogenic factor E₁ plasmid (Blair *et al.*, 1972), in which the RNAase-sensitive FI molecules constitute a terminal intermediate in its replication, could equally well be applied to the replication of mtDNA. Terminal intermediates with RNA discontinuities have also been demonstrated in the replication of bacteriophages ϕ X174 and M13 in systems *in vitro* (Westergaard *et al.*, 1973). With the exception of ϕ X174 (Schekman *et al.*, 1972), RNA-mediated DNA replication is inhibited by rifampicin (Schekman *et al.*, 1972; Clewell *et al.*, 1972). A report by Saccone & Gadeleta (1973) indicates that mtDNA replication in rat liver is sensitive to rifampicin. It therefore appears on the available evidence that an RNA-mediated DNA-replication system exists within the mitochondrion of mammalian cells.

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