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The Presence of Ribonucleotides in Mature Closed-Circular Mitochondrial DNA

(ribonuclease H/alkali hydrolysis of DNA)

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ABSTRACT **Mitochondrial DNA from mouse and HeLa cells is nicked by alkali and chick-embryo ribonuclease H. It has been concluded that ribonucleotides are present in the closed-circular duplex. A comparative study of the scission rates at pH 13.0 of RNA and of mitochondrial DNAs indicates that there are no more than 10 ribonucleotides in the major fraction of the mitochondrial DNAs.**

Mammalian and amphibian mitochondrial DNAs (mtDNAs) are readily obtainable at neutral pH as closed-circular duplex molecules with molecular weights of about 10 million. These DNAs are exceptional in that they suffer chain scissions at high pH (1, 2), an observation which has been routinely exploited to prepare physically separated complements from closed mtDNA without prior nicking in buoyant alkaline CsCl (3). This alkali lability has been attributed to damage incurred during DNA isolation (4) from an organelle that contains a variety of compounds that may react with DNA (5). The lability is also consistent with the presence of covalently incorporated ribonucleotides.

The quantitative conversion of closed mtDNA to nicked DNA by ribonuclease H, demonstrated here, indicates that at least a part of the alkaline lability in each molecule is due to the presence of ribonucleotides in the mature closed-circular duplex.

We have estimated the number of ribonucleotides per DNA duplex from kinetic measurements on the nicking rate of mtDNA and of RNA at pH 13.0. The mtDNA measurements indicate that two populations of molecules are present. By comparison with the rate constant for RNA hydrolysis, we can calculate that these two populations contain about 10 and 30 ribonucleotides per DNA duplex. This calculation assumes that all of the sensitive sites are ribonucleotides.

Two reports appeared (6, 7) while this manuscript was in preparation which presented evidence that ribonucleotides are contained within the covalently closed mitochondrial DNA circles from rat-ascites hepatoma cells and HeLa cells.

Mitochondrial DNA thus represents the only known mature circular DNA which contains covalently inserted ribonucleotides. Recent reports have suggested that T2, T4 (8), and T5 (9) viral DNAs contain ribonucleotides. The colicinogenic factor, col E1 DNA, in *Escherichia coli* is nicked at alkaline pH (10) and by ribonuclease H (11), but only when it is allowed to replicate in cells treated with chloramphenicol. Oligoribonucleotide primers have been found in several replicating systems (12–14), but these appear to be removed during the maturation of the DNA.

Abbreviations: mtDNA, mitochondrial DNA; EthBr, ethidium bromide; DNA-I, closed circular DNA; DNA-II, nicked circular DNA.

MATERIALS AND METHODS

Nucleic-Acid Preparations. DNA from Pseudomonas phage PM2 was labeled and isolated as described (22). MtDNA was prepared from HeLa cells and the two mouse-cell lines previously described (15). Cells were labeled with 600 μCi/liter [^{3}H]thymidine (Schwarz/Mann) until the late exponential phase. The cells were harvested and disrupted as described (15), except that HeLa cells were suspended in a hypotonic solution in which 1.5 mM $MgCl_2$ replaced EDTA. Purification of mitochondria and isolation of mtDNA was carried out essentially as described by Clayton *et al.* (16) for rabbit brains, except that the sucrose step gradient contained equal volumes of 1.5 M and 1.0 M sucrose and the DNase treatment was omitted. DNA from the lower band of ethidium bromide (EthBr)–CsCl gradients was freed of dye by dialysis against Dowex 50 (Na^+), divided into portions and stored at $-20°$.

[^{32}P]RNA was isolated from the cytoplasm of BSC-1 cells grown in low phosphate medium and labeled for 48 hr with 5 mCi/liter of $^{32}P_i$. Cells were collected and disrupted as described above for mtDNA isolation. Nuclei and mitochondria were removed by centrifugation. The supernatant was brought to 1% sodium dodecyl sulfate and precipitated at $-20°$ with 1 volume of ethanol. The precipitate was collected by centrifugation, dissolved in dodecyl sulfate buffer (17) (5 ml/liter of cell culture) and extracted twice with phenol saturated with dodecyl sulfate buffer. The phenol supernatant was extracted twice with $CHCl_3$ and again precipitated with ethanol. The precipitate was washed once with ethanol and stored frozen in 10 mM Tris·HCl (pH 7.5), 10 mM NaCl, 1 mM EDTA.

Enzymes. Alkaline phosphatase (WBAP) was purchased from Worthington Biochemical Corp. and assayed using *p*-nitrophenylphosphate (18) (Calbiochem). Chick-embryo ribonuclease H was generously provided by Dr. Walter Keller (11) and assayed with a ϕX174 DNA·RNA hybrid. The hybrid was prepared in a mixture (0.52 ml) which contained 40 mM Tris·HCl (pH 8.0), 1 mM $MnCl_2$, 5 mM $MgCl_2$, 12 mM 2-mercaptoethanol, 0.3 mM of each ribonucleotide triphosphate ([^{3}H]UTP, New England Nuclear Corp., was 125 cpm/pmol), 50 μg bovine-serum albumin, 94 nmol of ϕX174 DNA and 3.2 units of *E. coli* RNA polymerase. The DNA was a gift of Dr. Paul Johnson; the RNA polymerase preparation was previously described (19). The reaction was terminated

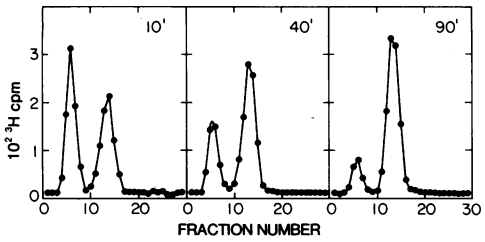

FIG. 1. CsCl–EthBr gradients of LA9 mtDNA incubated at pH 13.0. [^{3}H]mtDNA was incubated, sampled at 10, 40, and 90 min, and analyzed in CsCl–EthBr equilibrium gradients as described in *Materials and Methods*. The DNA preparation initially contained 12% nicked DNA. Several fractions were omitted from the top and bottom of each gradient for illustrative clarity. The field is directed to the left in the gradients shown in all figures.

after 2 hr at 37° by adding EDTA to 10 mM and sodium pyrophosphate to 2 mM, and the product was purified by filtration through a 0.9 × 34-cm column of porous glass beads (Sigma, G-240-50). The incorporation of UTP was equivalent to 50% net RNA synthesis. RNase H was assayed in 100 μl containing 50 mM Tris·HCl (pH 7.9), 5 mM $MgCl_2$, 10% glycerol, 25 mM NaCl, and φX174 DNA·[^{3}H]RNA hybrid equivalent to 400 pmol of RNA. Assays were incubated 30 min at 37° and acid-soluble radioactivity was determined. One unit of RNase H is defined as the amount releasing 100 pmol of UMP. For reaction with mtDNA, the incubation conditions were as described above, except that 67 mM NaCl was used and the reaction was terminated with EDTA added to 20 mM.

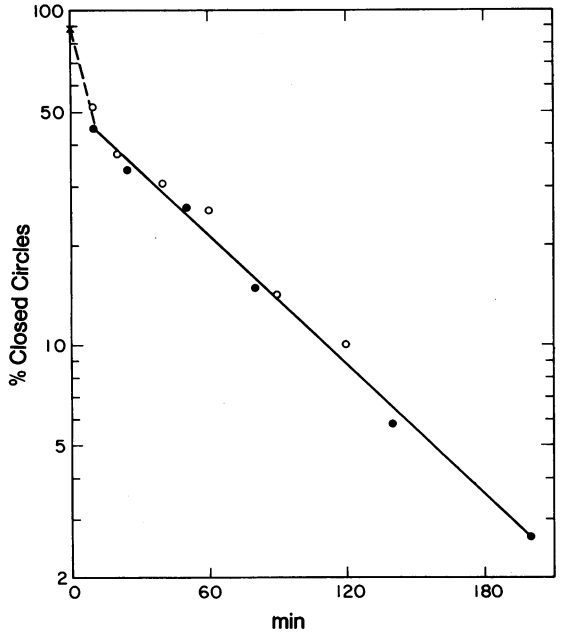

FIG. 2. Kinetics of conversion of LA9 mtDNA to nicked DNA at pH 13.0, 20°. Data were taken from gradients analogous to those shown in Fig. 1. The extrapolation to zero time is based on an analysis in a CsCl–EthBr gradient of material never exposed to high pH. The symbols represent two independent experiments. ●, incubation in the standard reaction mixture described in *Materials and Methods;* ○, incubation in the same reaction mixture, but without potassium phosphate and glycine.

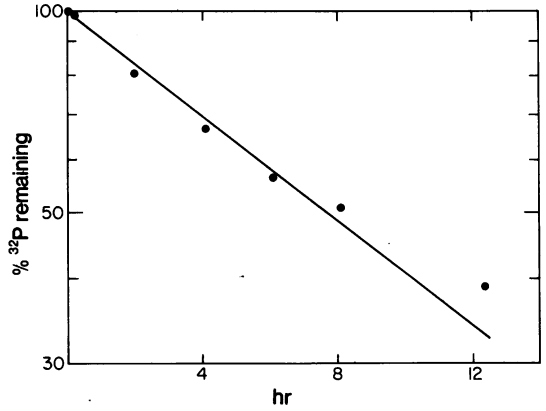

FIG. 3. Hydrolysis of [^{32}P]RNA at pH 13.0, 20°. The proportion of $^{32}P_i$ resistant to release by alkaline phosphatase is shown at various times. Details are given in *Materials and Methods*.

CsCl–EthBr Centrifugation. Gradients (about 2.5 ml) containing EthBr (300 μg/ml) and bovine-serum albumin (100–200 μg) at an initial density of 1.57 g/ml were centrifuged in the Spinco SW50.1 rotor at 36,000 rpm, 20° for at least 24 hr. Tubes were punctured; fractions were collected onto glass fiber papers (Whatman GF/A, 2.4 cm), dried, and counted in a toluene-based scintillator.

RNA Hydrolysis. RNA was incubated in 4.5 M CsCl plus 0.5 mM EDTA at 20°. pH was determined with a standard glass electrode and sleeve-type junction electrode (20) with a Beckman Research model pH meter, standardized with saturated calcium hydroxide. pH was adjusted with 2 M KOH (CO_2-free, P-H Tamm, Uppsala, obtained from Bio-Rad Laboratories). Hydrolysis proceeded in a closed Teflon container mounted in water in a thermostatted metal block on a magnetic stirrer. Argon gas was introduced whenever the sample vessel was opened for experimental manipulations. Variation in pH during the course of an experiment never exceeded ± 0.02 units.

200-μl samples were neutralized with 100 μl of Tris·HCl (0.2 M), and acidified to pH 1.3 with 100 μl of 0.25 M HCl. Samples were reneutralized after 1 hr at 37° with 100 μl of 0.3 M KOH and incubated 1 hr at 37° with 1.1 unit of alkaline phosphatase. 100 μl of a solution containing 10 mM potassium phosphate (pH 6.0) and 10 mM sodium pyrophosphate, 200 μl of bovine-serum albumin (1 mg/ml), 100 μl of 1 M HCl and 0.5 ml of a 40% (v/v) suspension of Norit A (Matheson, Coleman, and Bell) were added and mixed. The charcoal was removed by centrifugation and 100 μl of the supernatant were counted.

$^{32}P_i$ released at any time is expressed as the fraction of available cpm, determined by omitting charcoal from a similar assay mixture. The radioactivity was corrected for $^{32}P_i$ released by alkaline phosphatase in the absence of alkaline hydrolysis (2–3% of available radioactivity). Control experiments showed that the RNA preparation was rendered >98% acid soluble after overnight incubation in 0.3 M KOH, 37° and >93% of the radioactivity was releasable by alkaline phosphatase in such digests.

Scission Rate of Circular mtDNA. [^{3}H]mtDNA samples were incubated at 20° and maintained as described above for RNA, except that the CsCl solution contained 0.05 M potas-

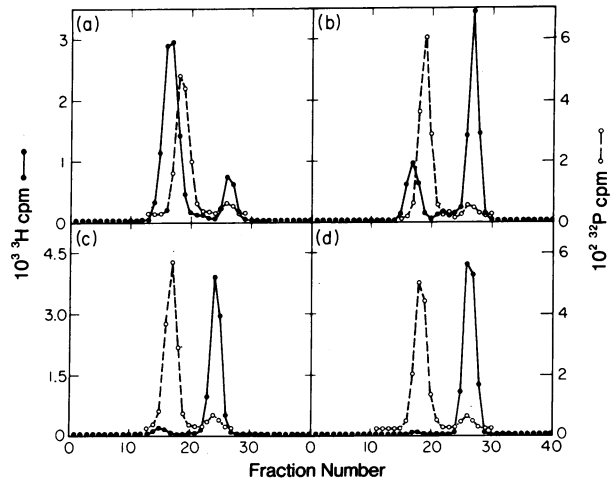

FIG. 4. RNase H treatment of LA9 mtDNA and PM2 DNA. Reactions were carried out as described in *Materials and Methods*, and analyzed by CsCl–EthBr equilibrium centrifugation. (*a*) control incubation; (*b*) 0.18 units enzyme; (*c*) 0.36 units; (*d*) 1.8 units. 0.36 μg of mtDNA and 0.67 μg of PM2 DNA were used in each experiment except in (*b*), in which 0.9 μg of PM2 DNA were used. ●——●, [^{3}H]mtDNA; O- - -O, PM2[^{32}P]DNA.

sium phosphate, 0.05 M glycine, and 9 μg of calf-thymus DNA. 1-ml samples were placed in polyallomer tubes containing 1.4 ml of CsCl (ρ = 1.55), 5 mM EDTA, 0.4 M Tris·HCl, and sufficient dye to give a final concentration of 300 μg/ml of EthBr (or 400 μg/ml of propidium diiodide). Samples were overlaid with oil and stored in the dark before centrifugation, carried out as described above. The proportion of closed-circular and nicked DNA in the samples was determined from the radioactivity in the upper and lower bands. The proportion of closed DNA in the sample was determined from samples removed from alkali at 10 sec, or never exposed to alkali.

RESULTS

Kinetics of the First Alkali-Induced Strand Scission in mtDNA at pH 13.0. Mouse and human mtDNAs were incubated at pH 13.0 as described in *Methods*, and samples neutralized periodically. The fraction of unnicked material was assayed by CsCl–EthBr density gradient centrifugation. Neutralized alkali-denatured closed-circular DNA exhibits a dye binding restriction similar to that of native closed-circular DNA and bands dense relative to single-stranded and renatured open DNA (21, 22). Examples of such gradients are shown in Fig. 1.

The first nick in mouse (LA 9) mtDNA appears to follow biphasic first-order kinetics (Fig. 2). The biphasic kinetics clearly suggest that there are at least two kinds of molecules in isolated mtDNA. The fast rate, approximately three times the slow rate, is exhibited by about 35–45% of the total DNA. The half-time for the slower rate is 47 min. A similar experiment, using HeLa cell mtDNA, and analyzed by CsCl–propidium diiodide buoyant centrifugation, provided a half-time of 46 min. Mitochondrial DNA from a mouse cell line (LD) in which the mtDNA is in the form of dimers was also examined. The half-time for nicking this dimer DNA was 24 min.

Kinetics of RNA Hydrolysis at pH 13.0. The release of ^{32}P$_i$ at pH 13.0 from cytoplasmic RNA follows first-order kinetics,

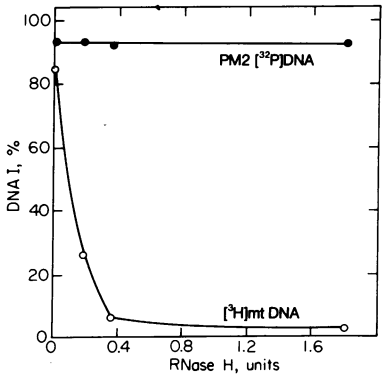

FIG. 5. Distribution of radioactivity in CsCl–EthBr gradients after RNase H treatment. The data shown in Fig. 4 were used to calculate the proportion of closed DNA at each enzyme concentration. ●, PM2 DNA; O, LA9 mtDNA.

with a half-time of 7.9 hr (Fig. 3). It should be emphasized that this half-time represents a phosphodiester-bond hydrolysis rate; thus, half the DNA molecules of any size containing one ribonucleotide will be nicked in 7.9 hr at pH 13.0. This rate for a molecule containing one ribonucleotide is 0.1 that observed for the slow rate in mtDNA and 0.03 that of the fast rate. Thus, mtDNA is nicked with a rate corresponding to 10 and 30 ribonucleotides per molecule.

Under these conditions, the nicking rates of viral and *E. coli* DNAs were interpolated for the molecular weight of mtDNA to give a value less than 1% of the slow rate observed for mtDNA (23; and Grossman, Watson, and Vinograd, unpublished results).

Ribonuclease H nicks mtDNA. The alkali sensitivity of mtDNA is compatible with, but does not prove, the existence of covalently inserted ribonucleotides. Such evidence was obtained upon exposing mtDNA to RNase H, an enzyme specific for the ribonucleotide bonds in an RNA·DNA duplex.

LA9 [^{3}H]mtDNA-I was exposed to increasing amounts of enzyme in the presence of PM2 [^{32}P]DNA-I and the reaction mixtures analyzed by CsCl–EthBr buoyant centrifugation (Fig. 4). The closed-circular forms of LA9 and PM2 DNA do not co-band in these gradients because they differ in superhelix density. Treatment with RNase H was observed to nick mtDNA increasingly, and finally quantitatively, while PM2 DNA remained completely resistant. These results are summarized in Fig. 5, in which it is shown that PM2 DNA is resistant to RNase H at a concentration at least five times greater than that required to nick LA9 mtDNA essentially completely. Similar results were obtained with HeLa mtDNA (Fig. 6) and LD dimer mtDNA. A middle band is visible when HeLa mtDNA is partially nicked (Fig. 6*b*), a consequence of the high initial catenane level in this DNA.

The chick-embryo RNase H is reported to be inhibited by salt concentrations in excess of 150 mM (11). Incubation of LA9 DNA in the standard reaction mixture modified to contain 200 mM KCl resulted in a 30% inhibition of conversion to DNA-II.

Since RNase H will not attack double-stranded RNA or DNA, the reaction product is expected to be a nicked circle, unless ribonucleotides occur within about 10 bases on complementary strands. The RNase H product of an extended digestion was analyzed on neutral sucrose gradients (Fig. 7).

The LA9 DNA sediments almost quantitatively at 28 S, the position expected for nicked circular LA9 DNA, with no evidence of sedimenting species representing full-length linear molecules or smaller degradation products.

DISCUSSION

We have shown that mouse and human mtDNAs are nicked upon exposure to alkaline pH or chick-embryo RNase H, and have concluded that these DNAs contain covalently incorporated ribonucleotides. Since all mammalian and amphibian mtDNAs so far tested are alkali sensitive (2), the presence of ribonucleotides in the DNA duplex is likely to be a general property of mtDNA.

The measured kinetics of the nicking of mtDNAs at alkaline pH were biphasic, with rates 10 and 30 times that expected for a single ribonucleotide. Approximately 55–65% of the mtDNA in our preparations nicks at the slower rate, corresponding to a maximum of 10 ribonucleotides per molecule. The number of such nucleotides may be smaller if other alkali sensitive sites are present. The complete sensitivity to RNase H implies that at least one of the alkali sensitive sites is a ribonucleotide.

The rate of nicking of mtDNA may have been slightly underestimated with our method of analysis because of the presence of catenanes in the sample. Catenanes would appear to be nicked at a slower rate than the monomers because three-fourths of the product of the first nick would be regarded as unreacted material in CsCl–EthBr gradients.

As indicated earlier, rat-ascites hepatoma cell mtDNA is sensitive to RNase A and T1 (6) and HeLa mtDNA is sensitive to RNase A, T1, and *E. coli* RNase H (7). In the first case, approximately 50% of the DNA was RNase sensitive, whereas, in the latter case, the sensitivities were reported to be 60, 49, and 17% for RNase A, H, and T1, respectively. Both HeLa and mouse mtDNA have been shown in the present study to be completely sensitive to chick-embryo RNase H. These results suggest that the chick-embryo enzyme can act endonucleolytically at a single ribonucleotide in a DNA duplex. *E. coli* RNase H will not function as an endonuclease at a bond linking a ribonucleotide to DNA (24, 25). The results with HeLa mtDNA taken together may imply that the ribonucleotides in HeLa mtDNA occur singly and as oligoribonucleotides in different molecules.

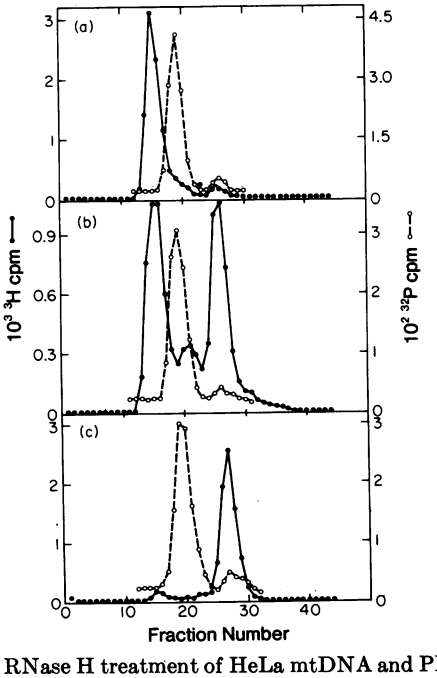

FIG. 6. RNase H treatment of HeLa mtDNA and PM2 DNA. 0.41 μg of HeLa mtDNA and 0.9 μg of PM2 DNA were treated with RNase H as described in *Materials and Methods*, and analyzed by CsCl–EthBr equilibrium centrifugation. (*a*) control incubation; (*b*) 0.22 unit of enzyme; (*c*) 0.54 unit. ●——●, [^{3}H]-mtDNA; O– – –O, PM2[^{32}P]DNA.

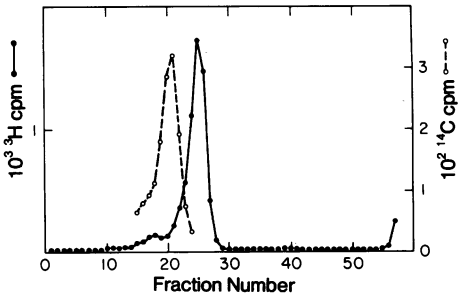

FIG. 7. Sucrose gradient analysis of LA9 mtDNA treated with RNase H. 0.9 μg of mtDNA was treated 30 min in the standard reaction mixture with 0.72 unit RNase H. 30 μl (0.3 μg of DNA) were removed and assayed to show that >95% of the material was nicked. The remainder was treated with an additional 0.35 unit for a further 30 min, mixed with EDTA (10 mM), T7[^{14}C]-DNA and sodium dodecyl sulfate (0.2%) and layered on a 5–20% sucrose gradient containing 10 mM Tris·HCl (pH 7.5), 1 mM EDTA, 0.5 M NaCl. Sedimentation in the Spinco SW50.1 rotor was at 37,000 rpm, 130 min, 20°. Fractions were collected on GF/A filters, dried, and counted. ●——●, [^{3}H]mtDNA; O– – –O, T7[^{14}C]DNA.

Wong-Staal *et al.* (7) have also reported biphasic alkaline nicking kinetics with approximately the same proportions of slow and fast degrading components as those reported here. From their own determination of the hydrolysis rate of RNA, they calculate that the slow component contains 3–4 ribonucleotides and the fast component contains 10–17. Our values, in contrast, are consistent with 10 and 30 ribonucleotides, causing a discrepancy which is currently unresolved. We should emphasize that at the current time the evidence for all but 1 to 2 of the postulated ribonucleotides per molecule can be adequately explained by the existence of other kinds of alkali sensitive sites.

The origin of ribonucleotides in mtDNA is unclear. We can envision that they are inserted during replication or repair by a relatively nonstringent DNA polymerase, or that they are remnants of RNA primers which have not been completely excised. Substantial evidence has been presented in several systems that an RNA polymerase product provides the 3′-hydroxyl primer required by all known DNA polymerases. If mitochondria contained an RNase H with the specificity of the *E. coli* enzyme, the action of this enzyme would leave a ribonucleotide at the 5′-end if excision occurred before circularization of newly synthesized strands, and at both the 3′- and 5′-ends if excision occurred after circularization. The existence and specificity of nucleolytic activities as part of mitochondrial DNA polymerases, or other mitochondrial proteins, is currently unknown.

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