RIBONUCLEOTIDES IN CLOSED CIRCULAR MITOCHONDRIAL DNA FROM HELA CELLS*

F. Wong-Staal, J. Mendelsohn and M. Goulian

Department of Medicine
University of California, San Diego
La Jolla, California 92037

Received May 14, 1973

SUMMARY

Closed circular mitochondrial DNA from HeLa cells is sensitive to both alkali and ribonucleases. The kinetics of ring opening in alkali suggest at least two classes of molecules. One class undergoes rapid breakdown, ultimately to fragments smaller than unit length, in contrast to the second class, which is more resistant to alkaline cleavage and is converted in large part to unit length single strands. Ribonucleases A, T_1 and H relax the supercoiled molecules, indicating that the alkali susceptibility is due to the presence of ribonucleotides in the DNA. By comparison with the rate of hydrolysis of RNA, the alkali-resistant class of mitochondrial DNA molecules is estimated to contain approximately 3 ribonucleotides and the alkali-sensitive class 10-18.

INTRODUCTION

Observations that animal mitochondrial DNA (MtDNA) undergoes chain scission in alkali have not been well understood and have been ascribed to an artifact of the method of preparation (1). A reevaluation of this phenomenon was stimulated by recent evidence for the participation of RNA in DNA synthesis (2-6). We present here results of experiments to detect the presence of ribonucleotides in mitochondrial DNA from HeLa cells by determination of its sensitivity to ribonucleases. Based on the presumption that ribonucleotides are present in mitochondrial DNA, the kinetics of bond scission in alkali are used to estimate the number of residues per molecule.

MATERIALS AND METHODS

Enzymes

Ribonuclease A (beef pancreas), ribonuclease T_1 and bacterial alkaline

* This research was supported by U. S. Public Health Service Grants CA 11705, CA 11971 and AM 05702 and American Cancer Society Grant NP-102A.

phosphatase were purchased from Worthington Biochemical Corp. Pronase CB was purchased from Calbiochem. E. coli ribonuclease H was a gift from Drs. M. G. Rosenfeld and G. N. Gill.

Nucleic Acids

Mitochondrial DNA. HeLa cells were grown in suspension in modified Eagle's medium supplemented with 10% fetal calf serum. To label mitochondrial and nuclear DNA, (methyl ^{3}H)-thymidine (final concentration 8.3×10^{-8} M, 0.5 μCi/ml) was added to cells in logarithmic phase (4×10^{5}/ml), and growth was continued for 16 hours. Cells were then harvested and homogenized as described by Smith and Vinograd (7), and the extract was centrifuged through a step gradient containing 1.0, 1.5 and 2.0 M sucrose at 25,000 rpm for one hour in the SW 40 rotor (7). The mitochondrial band at the 1.0 and 1.5 M interface was removed and lysed in hypotonic buffer (0.01 M NaCl, 0.001 M EDTA, 0.01 M Tris-HCl pH 7.8) and 1% SDS. The lysate was incubated for two hours with Pronase (500 μg/ml), which had been preincubated for 30 min at 37°, and extracted three times with redistilled phenol saturated with 0.1 M Tris-HCl pH 8.8, keeping the interface until the last extraction. After removal of phenol with ether and N_2, the closed circular MtDNA was isolated by banding in CsCl in the presence of ethidium bromide (8). Ethidium bromide was removed by isopropanol (9), and the DNA was dialysed against 0.01 M Tris-HCl pH 7.8, 0.002 M EDTA.

^{32}P-RNA. E. coli B was labeled with ^{32}P-orthophosphate and the RNA extracted as described by Hurlbert and Furlong (10).

^{14}C-fd RF I. ^{14}C-labeled covalently closed (double circular) replicative form of coliphage fd DNA (fd RF I) was prepared by the procedure of Schekman, et al (11).

^{3}H-polyoma DNA. ^{3}H-labeled covalently closed polyoma DNA was donated by Dr. T. Friedmann.

RESULTS AND DISCUSSION

Alkali and ribonuclease susceptibility of supercoiled MtDNA.

In accordance with previous observations, supercoiled MtDNA undergoes rapid breakdown in alkali. The rate of chain scission increases with temperature and pH. By one hour of incubation at 40° in 0.2 M NaOH, almost all of the supercoils have undergone at least one cleavage event (Fig. 1).

To investigate whether this alkali lability is due to the presence of ribonucleotides, MtDNA was treated with different ribonucleases (Figs. 2 and 3). About 60%, 49% and 17% of the molecules are relaxed by RNase A, H and T_1, respectively. Under parallel conditions, covalently closed circles of viral DNA are completely conserved (Figs. 2 and 3). Increasing the ribonuclease concentration or time of incubation does not lead to greater conversion of closed circular MtDNA to open circles (data not shown). A

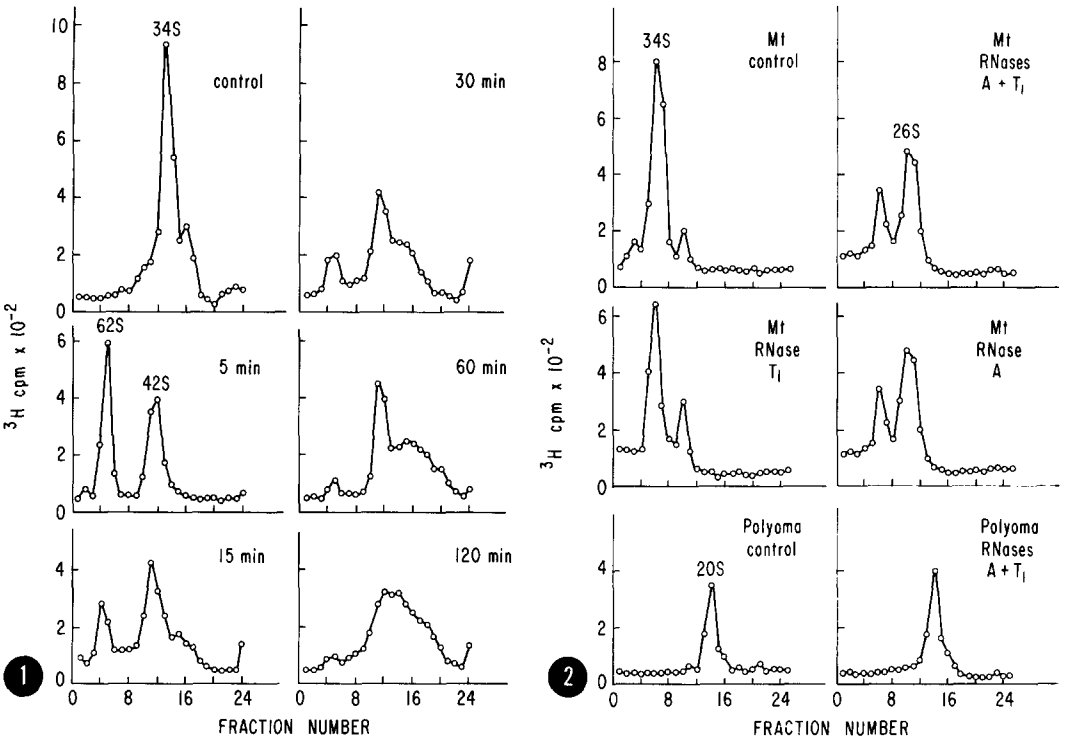


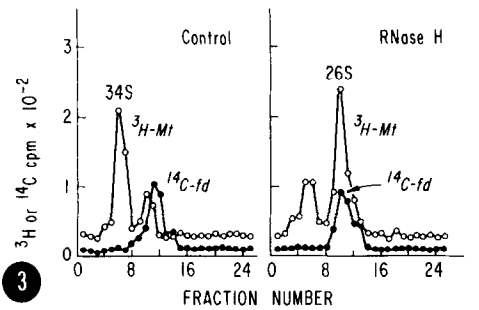
Fig. 1. Effect of alkali on covalently closed MtDNA.

MtDNA was incubated in 0.2 M NaOH at 40° for the times indicated, neutralized with KH_2PO_4, and centrifuged in 4 ml neutral 5-20% sucrose gradients (1 M NaCl, 0.005 M EDTA, 0.05 M Tris-HCl pH 7.6), for 60 min at 56,000 rpm, 20°, in the Spinco SW 56 rotor. Fractions were collected from the bottom of the tube directly onto paper discs, washed and counted. Unit length single strands, circular and linear, that result from a single cleavage event sediment at 42 S, whereas those that were not cleaved remain irreversibly denatured (16) and sediment at 62 S. The intact supercoiled control MtDNA (not treated with alkali) sediments at 34 S.

Fig. 2. Effect of ribonucleases A and T_1 on covalently closed MtDNA.

Reaction mixtures (0.1 ml) containing 0.2 µg of MtDNA or 0.1 µg polyoma DNA in 0.02 M NaCl, 0.01 M Tris pH 7.6, 0.002 M EDTA, and 0.08 mg RNase A and/or 60 units RNase T_1 were incubated for 30 min at 37°. (Both RNase A and RNase T_1 were heated for 15 min at 80° before use to destroy contaminating DNase activities.) Controls were treated in the same way but without enzyme. All mixtures were incubated for an additional 10 min with Pronase (30 µg) to prevent pelleting of the DNA (15), and then centrifuged in neutral sucrose gradients (see Fig. 1) for 110 min at 56,000 rpm, 20°, in the Spinco SW 56 rotor. Nicking converts supercoiled MtDNA to relaxed circles, which sediment at 26 S.

combination of both ribonucleases A and T_1 still affects only 60% of the molecules (Fig. 2), suggesting that molecules with guanylate also contain a pyrimidine ribonucleotide. In addition to possible base effects, there may be structural barriers to ribonuclease action by neighboring deoxyribonucleotides,



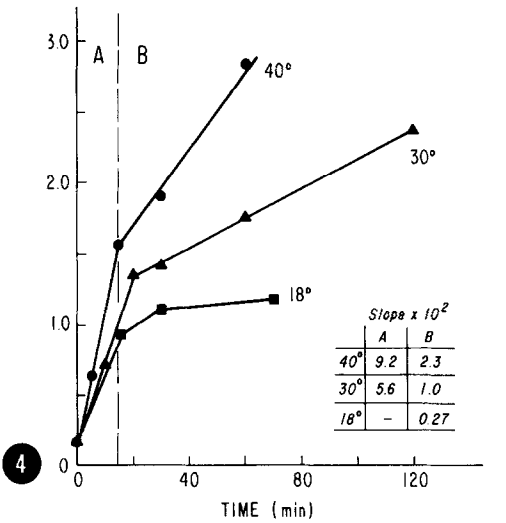


Fig. 3. Effect of ribonuclease H on covalently closed MtDNA. Reaction mixture (0.05 ml)(7) containing 0.02 M Tris-HCl pH 8.0, 0.01 M $MgCl_2$, 0.006 M dithiothreitol, 2 µg albumin, ^{3}H-MtDNA, ^{14}C-fd RF I and E. coli RNase H was incubated for 20 min at 37°; control was the same but without enzyme. Both were treated with Pronase and centrifuged in neutral sucrose gradients as described in Fig. 2.

Fig. 4. Kinetics of disappearance of supercoiled species of MtDNA. MtDNA was treated with alkali at 18, 30 and 40° and analyzed in neutral sucrose gradients following the procedure in Fig. 1. The natural logarithm of the fraction of intact (but denatured, 62 S) molecules remaining, $P_{(o)}$, was plotted on the ordinate with time of incubation on the abscissa. The Poisson formula gives $P_{(n)} = e^{-m} m^n/n!$ where $P_{(n)}$ is the probability that a given molecule undergoes n cleavage events, and m is the mean number of cleavage events per molecule. Consequently, $P_{(o)} = e^{-m}$, or $-\ln P_{(o)} = m$. Therefore, the slopes of the curve represent the average number of cleavage events/molecule/min.

which prevent a cleavage event at some sites containing ribonucleotide(s). For example, it has been reported that RNase H does not cleave phosphodiester bonds that covalently link ribonucleotides to DNA (12).

Recent reports of Miyaki, et al (13) and Grossman, et al (14) also describe sensitivity of MtDNA to alkali and ribonucleases and propose on this basis that ribonucleotides are present.

Kinetics of alkali induced breakdown of supercoiled MtDNA and estimation of number of ribonucleotides per molecule.

When MtDNA is incubated with alkali and the rate of disappearance of the 62 S peak (irreversibly denatured supercoils) is measured, at least two populations can be distinguished (Fig. 4). About 70% of the molecules

TABLE I

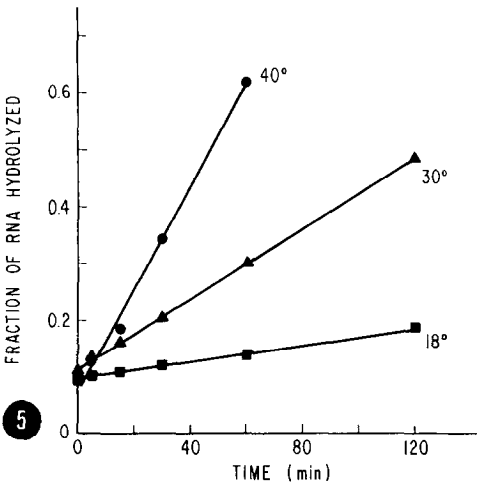
Estimation of number of ribonucleotide residues per molecule of mitochondrial DNA

Temperature of incubation	Fraction of ribonucleotides hydrolyzed/ min x 10^2*	Average no. of bonds cleaved/molecule MtDNA/min x 10^2**		Average no. of ribonucleotides/ molecule MtDNA***	
		A	B	A	B
18°	0.07	-	0.27	-	3.75
30°	0.32	5.60	1.00	17.5	3.08
40°	0.90	9.20	2.80	10.22	3.11

* From Fig. 5
** From Fig. 4
*** Obtained by dividing figures in third column of table by figure in second column

undergo rapid alteration (class A), and the remaining 30% comprise a more resistant class (class B). Although the straight line plots suggest that all of the molecules within a class open at the same rate, some variation could be missed because of the relatively few experimental points.

If the presence of ribonucleotides in MtDNA is the basis for alkali lability (as indicated by the ribonuclease sensitivity), the rate of opening supercoils should be proportional to the number of ribonucleotides per molecule. The latter can be estimated by comparison with the rate of hydrolysis of RNA under the same conditions. ^{32}P-labeled RNA was treated with alkali as described for MtDNA and the kinetics of hydrolysis determined at the same three temperatures used for MtDNA (Fig. 5). Assuming ribonucleotides covalently linked to deoxyribonucleotides are hydrolyzed at the same rate, the fraction of ribonucleotides hydrolyzed in a given time should be the same for RNA and MtDNA. Table I summarizes these results and lists the estimated number of ribonucleotides per molecule of MtDNA derived



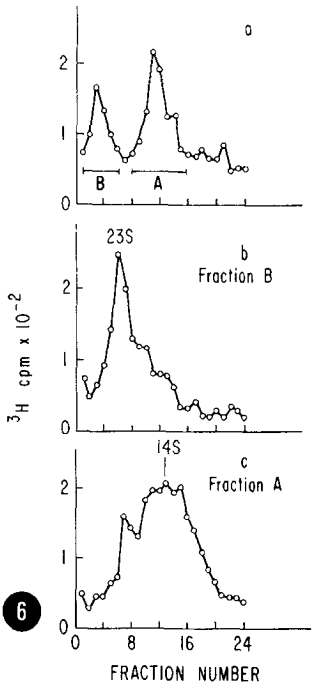


Fig. 5. Rate of hydrolysis of RNA in alkali.
Reaction mixtures containing 0.25 µg ^{32}P-labeled RNA in 50 µl 0.2 M NaOH were incubated at the specified temperatures and the reaction terminated by neutralization with HCl. The terminal phosphates exposed by the hydrolytic events were released by bacterial alkaline phosphatase (10 µg) and the values, assayed by charcoal non-adsorbability, are plotted on the ordinate against time of incubation in alkali.

Fig. 6. Size distribution of the two populations of MtDNA after extensive alkali treatment.
a. MtDNA was incubated in 0.2 M NaOH for 10 min at 40° and then centrifuged on neutral sucrose gradients as in Fig. 1. Fractions were pooled as indicated into fractions A and B, dialyzed, and concentrated by rotary evaporation under reduced pressure.
b. Fraction B was incubated in 0.4 M NaOH at 40° for 150 min and centrifuged through a 4 ml alkaline 5-20% sucrose gradient (0.3 M NaOH, 0.7 NaCl, 0.001 M EDTA) for 180 min at 56,000 rpm, 20°, in the Spinco SW 56 rotor.
c. Fraction A was treated as described in b.

from the rate measurements. Class A contains 10-18 ribonucleotides per molecule and class B approximately 3. In a preliminary report, Grossman, et al (14) conclude that there are approximately 10 ribonucleotides per molecule, but no reference is made to heterogeneity in ribonucleotide content.

Distribution of ribonucleotides in the two classes of molecules.

Brief exposure to alkali preferentially nicks class A molecules so that

primarily class B molecules remain closed. When the fractions corresponding to the closed double circles and single strands after such a treatment are reisolated and then extensively hydrolyzed in alkali, the size distribution of the limit digest products in the two classes can be determined (Fig. 6). Both fractions contain some molecules predominating in the other class, as would be expected from the procedure used to separate the two classes.

At least half of fraction B DNA is converted to single strands sedimenting at 23 S, the expected value for unit length linear DNA (Fig. 6b). Fraction A molecules are converted to heterogeneous fragments, mostly smaller than unit length and distributed around a mean of 14 S, corresponding to approximately 1 x 10^6 daltons (15) (compared to 10 x 10^6 for the intact molecule). The smallest fragments in this latter mixture are approximately 5-7 S, and prolonged incubation in alkali does not further degrade these fragments. These results suggest a correspondence between number of ribonucleotides per MtDNA molecule and number of alkali-sensitive sites, and they provide further evidence for heterogeneity in the population of MtDNA molecules.

From the amount and estimated molecular weight for each fraction in the sucrose gradient (Fig. 6c), the average size of fragments in fraction A is calculated to be 1/7 of the single strand length, which implies an average of 14 alkali-sensitive sites per double strand molecule. Each alkali-sensitive site may contain more than one ribonucleotide; the data of Leis, *et al* (12) indicate that the 49% of molecules that are susceptible to *E. coli* RNase H contain at least two adjacent ribonucleotides.

If the figure of 3 ribonucleotides is correct for class B molecules (Table I), some additional inferences may be made about the distribution of the 3 residues. Since a component of single strand circles is not prominent in the sucrose gradient (Fig. 6b), it is unlikely that molecules with all 3 ribonucleotide residues on one strand are predominant; however, the limited resolution in the type of fractionation used here does not preclude some

molecules of that type. Molecules with one residue in one strand and two on the other strand either as singles or a pair are both consistent with the results.

Possible significance.

There is now accumulating evidence that nascent DNA contains RNA probably functioning as primer, which is removed soon after replication (2-6). If ribonucleotides in mature MtDNA result from the same mechanism, their stability represents a difference from the systems thus far described. Closed circular MtDNA isolated from cells that are labeled with ^{3}H-thymidine for 16 hours and chased with cold thymidine for 60 hours shows the same degree of alkali sensitivity, indicating that the ribonucleotides are not removed at a later time. In addition, although great variability in alkali sensitivity of MtDNA has been described (1), with the procedure described here the ratio of class A and class B molecules, as well as the rate of breakdown of class B molecules, were within 10% of each other for four separate preparations.

The present studies do not exclude an alternative mechanism for insertion of ribonucleotides, e.g., a high misincorporation rate by MtDNA polymerases. Further studies will be needed to clarify this as well as the possible role of limitations in error recognition and removal mechanisms for MtDNA, and even the remote possibility of a function for the ribonucleotides in mature MtDNA. The apparent high proportion of ribonucleotides present as singles seems surprising if caused by a partial retention of primer fragments. On the other hand, the probable existence of significant numbers of clusters argues against a misincorporation mechanism.

REFERENCES

1. Borst, P., (1972) Ann. Rev. Biochem. 41, 333.
2. Sugino, A., Hirose, S. and Okazaki, R., (1972) Proc. Nat. Acad. Sci. USA 69, 1863.
3. Wickner, W., Brutlag, D., Schekman, R. and Kornberg, A., (1972) Proc. Nat. Acad. Sci. USA 69, 965.
4. Schekman, R., Wickner, W., Westergaard, O., Brutlag, D., Geider, K., Bertsch, L.L. and Kornberg, A., (1972) Proc. Nat. Acad. Sci. USA 69, 2691.
5. Sugino, A. and Okazaki, R., (1973) Proc. Nat. Acad. Sci. USA 70, 88.

6. Blair, D.G., Sherratt, D.J., Clewell, D.R. and Helinski, D.R., (1972) Proc. Nat. Acad. Sci. USA 69, 2518.
7. Smith, C.A. and Vinograd, J., (1972) J. Mol. Biol. 69, 163.
8. Radloff, R., Bauer, W. and Vinograd, J., (1967) Proc. Nat. Acad. Sci. USA 57, 1514.
9. Sebring, E.D., Kelley, T.J.,Jr., Thoren, M.M. and Salzman, N.P., (1971) J. Virol. 8, 478.
10. Hurlbert, R.B. and Furlong, N.B., (1967) in Methods in Enzymology, Vol. XII, eds. Grossman, L. and Moldave, K., Academic Press, New York, p. 193.
11. Schekman, R.W., Iwaya, M., Bromstrup, K. and Denhardt, D.T., (1971) J. Mol. Biol. 57, 177.
12. Leis, J.P., Berkower, I. and Hurwitz, J., (1973) Proc. Nat. Acad. Sci. USA 70, 466.
13. Miyaki, M., Koide, K. and Ono, T., (1973) Biochem. Biophys. Res. Comm. 50, 252.
14. Grossman, L.I., Watson, R. and Vinograd, J., (1973) Fed. Proc. 32, 529 Abs.
15. Studier, F., (1965) J. Mol. Biol. 11, 373.
16. Pouwels, P.H., Rotterdam, J.V. and Cohen, J.A., (1969) J. Mol. Biol. 40, 379.