THE STRUCTURE OF *TETRAHYMENA PYRIFORMIS* MITOCHONDRIAL DNA

The alkali stability of the DNA

R. W. GOLDBACH

Section for Medical Enzymology and Molecular Biology, Laboratory of Biochemistry, University of Amsterdam, Eerste Constantijn Huygensstraat 20, Amsterdam, The Netherlands

Received 19 May 1977

1. Introduction

Alkali lability appears to be a common feature of animal mtDNAs [1–4]. At least part of the lability is due to the presence of (oligo)ribonucleotides in this DNA [5–11]. In contrast, previous experiments done in this laboratory with the mtDNA of the protozoa *Tetrahymena pyriformis* showed that this DNA is rather stable at high pH [12,13]. In this paper we present a quantitative analysis of this stability. The results suggest that mature *T. pyriformis* mtDNA does not contain ribonucleotides.

2. Methods and materials

2.1. *DNA preparations*

Phage T7 DNA was prepared as reported by Yamamoto et al. [14]. ^{3}H-Labelled DNA (17 000 cpm/μg) was prepared from cultures supplemented with [^{3}H]thymidine (1 μCi/ml, 20 Ci/mmol) to the culture medium. Rat-liver mtDNA was isolated as described by Borst et al. [15] for chicken-liver mtDNA with some modifications as described in [16] (method II). Closed circular (component I) molecules were further purified by CsCl–EthBr equilibrium centrifugation. EthBr was removed with isopropanol [17]. mtDNA from *T. pyriformis* strain ST (obtained from Dr Y. Suyama, Philadelphia, Pa., USA) was labelled and isolated as described previously [13], except that ribonuclease treatment was omitted.

2.2. *Treatment with alkali*

DNAs were incubated, at pH 13.0, in 50 mM glycine, 50 mM Na_2HPO_4, 5 mM EDTA at 20°C. The pH was adjusted with 2 M NaOH and determined with a Copenhagen pH meter, model 28, equipped with a type GK 2301 C electrode. Samples were neutralized as described in het legends to the figures.

2.3. *CsCl–EthBr centrifugation*

Neutral denatured rat-liver mtDNA was analysed as described by Grossman et al. [7] in CsCl gradients (initial density 1.58 g/ml) containing EthBr (300 μg/ml). Gradients were run in a Spinco SW60 rotor at 45 000 rev/min and 20°C for 20 h. After the run, tubes were illuminated at 350 nm with a Universal ultraviolet lamp, type TL-900 (Camag) and photographed through a Kodak Wratten filter No. 16, with a Nikon F camera equipped with Agfa copex pan rapid film. Suitable negatives were scanned with a Gilford 2400 spectrophotometer scanner, equipped with a linear transport accessory.

2.4. *Analytical sucrose gradient centrifugation*

^{3}H-Labelled or ^{32}P-labelled DNAs were sedimented through 3.5 ml linear sucrose gradients (5–21% in 1 M NaCl, 10 mM Tris–HCl, 1 mM EDTA (pH 8.0)) in a Spinco SW60 rotor at 58 000 rev/min and 20°C for 30 min. After the run, gradients were siphoned, drops were collected on Whatman 3 MM filters and dried at 80°C. Radioactivity was counted in a toluene-based scintillation fluid.

Abbreviation: EthBr, ethidium bromide

2.5. *Radioactive compounds*

[^{32}P]Orthophosphate, carrier free and [^{3}H]thymidine were purchased from The Radiochemical Centre, Amersham, England.

3. Results

T. pyriformis mtDNA was incubated, at pH 13.0, as described in Materials and methods. As bacteriophage DNAs appear to be stable at this pH [11,18] phage T7 DNA was used as alkali-stable control in the assays. Figure 1 shows the sedimentation in sucrose gradients of the alkali-treated DNAs after neutralization. In spite of the presence of some smaller fragments in the starting material (particularly phage T7 DNA), it is clear from the distribution of the radioactivity in the gradients that both phage T7 DNA and *T. pyriformis* mtDNA are not significantly broken down after 3 h, at pH 13.0.Using phage T7 DNA as standard ($s^0_{20,w}$ = 84.2 S [19]) we calculate from the gradients an average $s^0_{20,w}$ of about 92 S (range 90–94 S) for neutral denatured *T. pyriformis* mtDNA. This corresponds to a molecular weight of 15.2 X 10^6 for the single-stranded DNA [19],which is in good agreement with het molecular weight found for the double-stranded mtDNA [20].

A quantitative evaluation of this experiment is given in table 1. Less than 5% of the mtDNA is fragmented at pH 13.0 within 3 h (not corrected for the 'fragmentation' of phage T7 DNA, see fig.2).

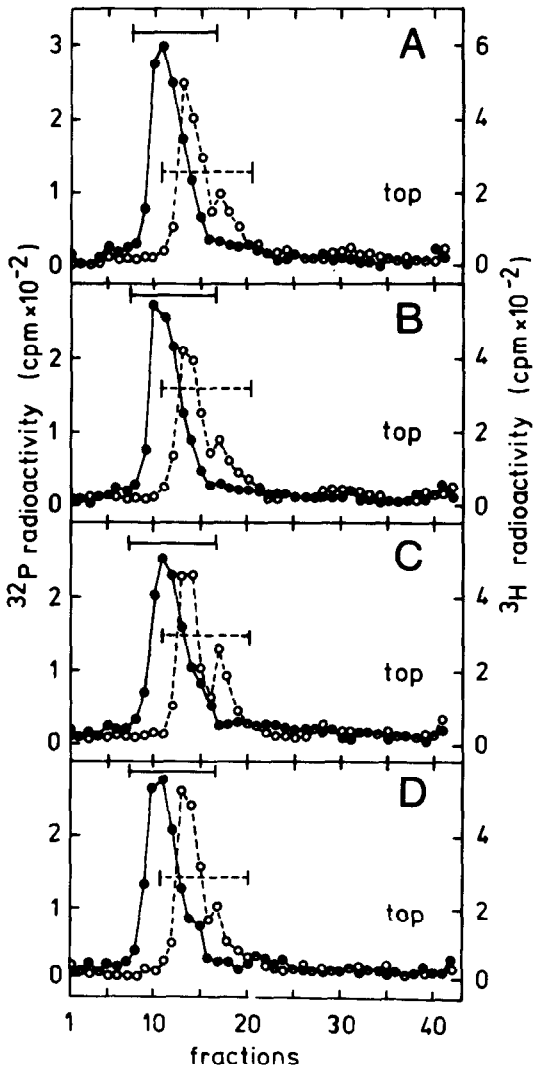

Fig.1. Sucrose velocity sedimentation of alkali-treated phage T7 DNA and *T. pyriformis* mtDNA. ^{3}H-Labelled phage T7 DNA and ^{32}P-labelled *T. pyriformis* mtDNA were incubated, at pH 13.0, and 125 μl aliquots (i.e., 0.15 μg) were neutralized by addition of an equal volume of 1 M Tris–HCl, 5 mM EDTA (pH 7.0). The samples were analysed on linear (5–21%) neutral sucrose gradients as described in Methods and materials. (o- - -o) Phage T7 DNA; (•——•) ^{32}P-labelled *T. pyriformis* mtDNA; (|- - -|) phage T7 DNA molecules considered to be intact; (|——|) *T. pyriformis* mtDNA molecules considered to be intact. (A) *t* = 0 min (sample directly neutralized after denaturation); (B) *t* = 30 min; (C) *t* = 60 min; (D) *t* = 180 min.

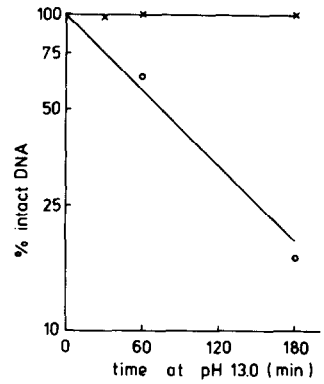

Fig.2. Effect of incubation, at pH 13.0, on rat-liver mtDNA and *T. pyriformis* mtDNA. The data are from tables 1 and 2. The fragmentation of *T. pyriformis* mtDNA is not corrected for fragmentation of phage T7 DNA. (o——o) Rat-liver mtDNA; (X——X) *T. pyriformis* mtDNA.

Table 1
Effect of incubation, at pH 13.0, on phage T7 DNA and *T. pyriformis* mtDNA

Time (min)	Longer material (%)	Intact (%)	Fragments (%)	Fragmentation[a] A	B	(A + B)/2
Phage T7 DNA						
0	7.3	73.9	18.8	0	0	0
30	6.3	73.8	19.4	0.6	0.1	0.4
60	7.4	70.3	22.3	3.5	3.6	3.6
180	7.1	73.7	19.2	0.4	0.2	0.3
T. pyriformis mtDNA						
0	5.4	75.5	19.1	0	0	0
30	5.6	72.4	22.0	2.9	2.1	2.5
60	6.3	74.0	19.7	0.6	1.5	1.1
180	6.4	74.3	19.3	0.2	1.2	0.7

[a]Corrected for t = 0 min

The data are from fig.1. Fragmentation has been calculated in two ways: based on the increase of smaller fragments (method A) and based on the decrease of intact material (method B). The averages of the results of both methods (A + B)/2 are used in fig.2.

This fragmentation rate is much slower than the rate of hydrolysis of phosphodiester bonds in RNA at pH 13.0 (half-time 7.9 h [7]). Similar results were obtained in three additional experiments.

As a control we have incubated rat-liver mtDNA, at pH 13.0, under the same conditions as *T. pyriformis* mtDNA. Nicking was analysed by CsCl–EthBr gradient centrifugation, in which denatured closed circular DNA shows a restricted EthBr uptake and bands at a higher density than the nicked circular equivalent [21]. The results are shown in fig.3 and table 2. It is clear that, at pH 13.0, most of the closed circular rat-liver mtDNA is converted into open circles within 3 h. Half-maximal conversion takes about 75 min (fig.2). Using a half-time of 7.9 h for the hydrolysis of a phosphodiester bond in

Table 2
Nicking of rat-liver mtDNA, at pH 13.0

Time (min)	Intact DNA (%)	Nicked DNA (%)	DNA broken in alkali (%)
0	75	25	0
60	47	53	37
180	13	87	83

The numbers are calculated from the gradients of fig.3.

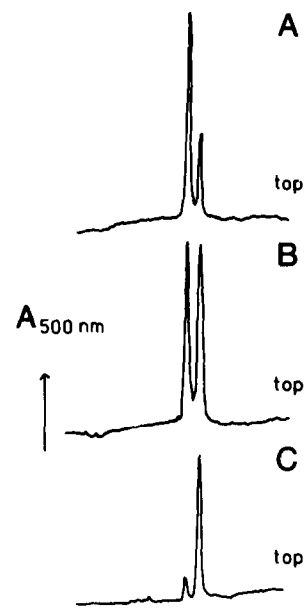

Fig.3. CsCl–EthBr equilibrium gradients with alkali-treated rat-liver mtDNA. 20 μg quantities of rat-liver mtDNA (in 500–μl volumes) were incubated, at pH 13.0. The 500 μl portions were reneutralized by transferring to SW60 centrifuge tubes with 1.5 ml CsCl solution (rho = 2.10 g/ml, 400 μg EthBr/ml, in 0.5 M Tris–HCl, 5 mM EDTA, pH 7.0. Gradients were run and analysed as described in Methods and materials. (A) t = 0 min (sample directly neutralized after denaturation); (B) t = 60 min; (C) t = 180 min.

RNA, at pH 13.0 [7] we calculate that six (oligo)ribonucleotides are present per molecule.

4. Discussion

Our results show that *Tetrahymena* mtDNA does not contain randomly-inserted ribonucleotides. The presence of (oligo)ribonucleotides is, therefore, not a feature common to all mtDNAs, as suggested by Grossman et al. [7].

In *T. pyriformis* mtDNA replication starts in the middle of the molecule [12]. The presence of a ribonucleotide sequence at this point would lead to breakage of at least 50% of the DNA strands, which is not the case. Hence, our results also exclude that *Tetrahymena* mtDNA retains a RNA primer at the origin of replication, a possibility raised by Miyaki et al. [5] for animal mtDNAs. We cannot exclude the possibility, however, that short primer RNA pieces are present at the 5'-ends of the linear *T. pyriformis* mtDNA. The sensitivity of this DNA to *Escherichia coli* exonuclease III [22] does not exclude this possibility, since this enzyme is able to attack the DNA strand in DNA–RNA hybrids [23,24].

Miyaki et al. [5] have calculated that in rat-ascites hepatoma cells mtDNA contains about 3–4 (oligo)ribonucleotides per molecule. Lonsdale and Jones [10], on the other hand, found ribonuclease-sensitive sites only in 10–30% of rat-liver mtDNA molecules. Our results show that almost all closed circular rat-liver mtDNA molecules are nicked, at pH 13.0, within 3 h, suggesting that all molecules contain at least one (oligo)ribonucleotide. We cannot exclude, however, that depurinated sites in the DNA contribute to its lability in alkali.

Acknowledgements

I thank Professor P. Borst for his advice and support and Mrs Francesca Fase-Fowler for generous gifts of phage T7 DNA and rat-liver mtDNA. This work was supported in part by a grant to P. Borst from The Netherlands Foundation for Chemical Research (SON) with financial aid from The Netherlands Organization for the Advancement of Pure Research (ZWO).

References

[1] Dawid, I. B. and Wolstenholme, D. R. (1967) J. Mol. Biol. 28, 233–245.
[2] Pikó, L., Blair, D. G., Tyler, A. and Vinograd, J. (1968) Proc. Natl. Acad. Sci. USA 59, 838–845.
[3] Nass, M. M. K. (1969) J. Mol. Biol. 42, 529–545.
[4] Borst, P. (1972) Ann. Rev. Biochem. 41, 333–376.
[5] Miyaki, M., Koide, K. and Ono, T. (1973) Biochem. Biophys. Res. Commun. 50, 252–258.
[6] Wong-Staal, F., Mendelsohn, J. and Goulian, M. (1973) Biochem. Biophys. Res. Commun. 53, 140–148.
[7] Grossman, L. I., Watson, R. and Vinograd, J. (1973) Proc. Natl. Acad. Sci. USA 70, 3339–3343.
[8] Koch, J. (1973) Eur. J. Biochem. 33, 98–103.
[9] Porcher, H. H. and Koch, J. (1973) Eur. J. Biochem. 40, 329–336.
[10] Lonsdale, D. M. and Jones, I. G. (1974) Biochem. J. 141, 155–158.
[11] Brown, W. M., Watson, R. M., Vinograd, J., Tait, K. M., Boyer, H. W. and Goodman, H. M. (1976) Cell 7, 517–530.
[12] Upholt, W. B. and Borst, P. (1974) J. Cell. Biol. 61, 383–397.
[13] Arnberg, A. C., Goldbach, R. W., Van Bruggen, E. F. J. and Borst, P. (1977) Biochim. Biophys. Acta 477, 51–69.
[14] Yamamoto, K. R., Alberts, B. M., Benzinger, R., Lawhorne, L. and Treiber, G. (1970) Virology 40, 734–744.
[15] Borst, P., Ruttenberg, G. J. C. M. and Kroon, A. M. (1967) Biochim. Biophys. Acta 149, 140–155.
[16] Smit, E. M. (1972) Ph. D. Thesis, Eindhoven.
[17] Sebring, E. D., Kelly, T. J., jr., Thoren, M. M. and Salzman, N. P. (1971) J. Virol. 8, 478–490.
[18] Hill, W. E. and Fangman, W. L. (1973) Biochemistry 12, 1772–1774.
[19] Studier, F. W. (1965) J. Mol. Biol. 11, 373–390.
[20] Goldbach, R. W., Arnberg, A. C., Van Bruggen, E. F. J., Défize, J. and Borst, P. (1977) Biochim. Biophys. Acta 477, 37–50.
[21] Grossman, L. I., Watson, R. and Vinograd, J. (1974) J. Mol. Biol. 86, 271–283.
[22] Arnberg, A. C., Van Bruggen, E. F. J., Borst, P., Clegg, R. A., Schutgens, R. B. H., Weijers, P. J. and Goldbach, R. W. (1975) Biochim. Biophys. Acta 383, 359–369.
[23] Richardson, C. C., Lehman, I. R. and Kornberg, A. (1964) J. Biol. Chem. 239, 251–258.
[24] Keller, W. and Crouch, R. (1972) Proc. Natl. Acad. Sci. USA 69, 3360–3364.