RNase and Alkali Sensitivity of Closed Circular Mitochondrial DNA of Rat Ascites Hepatoma Cells

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SUMMARY: A preparation of the closed circular DNA duplex was obtained from whole rat ascites hepatoma cells, AH66, by lysis of cells with SDS and purification by CsCl-dye buoyant-density centrifugation. RNase A converted the closed circular mitochondrial DNA to open circular molecules. The closed circular DNA was also sensitive to alkali. The conversion to the open form was shown from the results of centrifugal analyses on neutral and alkaline sucrose density gradients and CsCl-ethidium bromide. These results indicate the presence of at least one RNA region in closed circular double stranded mitochondrial DNA.

INTRODUCTION: Previously we obtained evidence by sucrose gradient centrifugation analysis of bulk DNA that the RNA chain might be integrated into chromosomal DNA of ascites hepatoma cells, AH66 (1). The sedimentation properties of mitochondrial DNA from various mammals have been established. We examined circular DNA of AH66 cells to see whether the RNA chain was integrated in the mitochondrial DNA duplex. The results in this paper indicate the presence of a linkage in closed circular DNA which is susceptible to RNase and to alkali.

METHODS:

Preparation of closed circular DNA --- Three days after inoculation of ascites hepatoma, AH66 into rats, 100 μCi of ^{3}H-thymidine (25 Ci/mmole, Radiochemical Centre) was injected intraperitoneally. Ascites fluid was withdrawn 18 hr later and the cells were harvested, washed and suspended in physiological saline at a concentration of 2×10^{7} cells/3 ml. The cells were treated with 3 ml of a solution containing 1.2% sodium dodecyl sulfate, 20 mM Tris-HCl and 20 mM EDTA (pH 8.0) for 30 min at room temperature, as described by Hirt (2) and 1.5 ml of 5 M NaCl was added to the resulting lysate. The mixture was stood at 4° for 18 hr and then centrifuged at 17,000xg for 45 min. The supernatant was dialysed against 10 mM Tris-HCl-10 mM EDTA (pH 8.0) for 18 hr at 4° and concentrated to 6 ml using Ficoll (Pharmacia Fine Chemicals).

Then the closed circular DNA was separated from nicked circular DNA by the method of Radloff et al. (3) as follows: The solution was mixed with 5.71 g CsCl and 750 μg ethidium bromide (Boots Pure Drugs), and centrifuged at 43,000 rpm for 44 hr at 20° in a Beckman 50Ti rotor. Material with a density of 1.61 was collected and treated three times with isoamyl alcohol. Then it was dialysed against 200 volumes of 10 mM Tris-HCl-10 mM EDTA (pH 8.0) with three changes of the buffer, and concentrated to 0.3 ml using Ficoll. The resulting DNA preparation was submitted to centrifugal analysis and examined by electron microscopy.

Sucrose gradient centrifugation --- The closed circular DNA (0.05-0.2 μg) was treated with pancreatic RNase A (Boehringer Co.), RNase T_1 (Sankyo Co.), Pronase P (Kaken Kagaku Co.) or pancreatic DNase I (Worthington Co.). The DNA samples were overlayed on 4.5 ml of a gradient of 5-20% sucrose containing 0.1 M NaCl, 10 mM Tris-HCl and 1 mM EDTA (pH 8.0) and centrifuged at 38,000 rpm for 2.5 hr in a Hitachi RPS40 rotor. Fractions were removed from the bottom of the tube and spotted on paper discs (Whatman 3-MM) which were washed with cold 5% CCl_3COOH and ethanol, and dried. Then radioactivity was measured in toluene scintillator fluid. When the DNA solution was layered on an alkaline 5-20% sucrose gradient containing 0.3 N NaOH and 0.7 M NaCl (4), the tube was centrifuged at 38,000 rpm for 1.5 hr at 4° and then fractions were treated as described above. A mixture of 18S and 28S-RNA of rat liver was used as an external marker during sedimentation analysis.

CsCl buoyant density analysis --- The DNA solution (0.2 ml) was mixed with 2.8 ml of 10 mM Tris-HCl-10 mM EDTA (pH 8.0) and 2.86 g CsCl and 400 μg ethidium bromide, and centrifuged at 38,000 rpm for 44 hr in a Hitachi RPS 40 rotor at 20°. When no dye was added to the CsCl solution, the initial density was adjusted to 1.707 and the solution was centrifuged at 38,000 rpm for 44 hr in a Hitachi RPS 40T rotor.

RESULTS: An electron micrograph of the dense band (ρ = 1.61) in the CsCl-dye gradient showed twisted circular molecules and some open circular ones which were about 5 μ long. The circular DNA and nuclear DNA had densities of 1.702 and 1.705, respectively in CsCl and these values were similar to those reported for rat liver mitochondrial DNA and nuclear DNA (5). In sucrose density gradient centrifugation of the dense peak from the CsCl-dye gradient, the major component sedimented at 35S in neutral solution (Fig. 1a) and at 68S in alkaline solution (Fig. 2a). These values were consistent with the sedimentation coefficients of closed circular mitochondrial DNA (6-10).

When closed circular DNA was incubated with pancreatic RNase

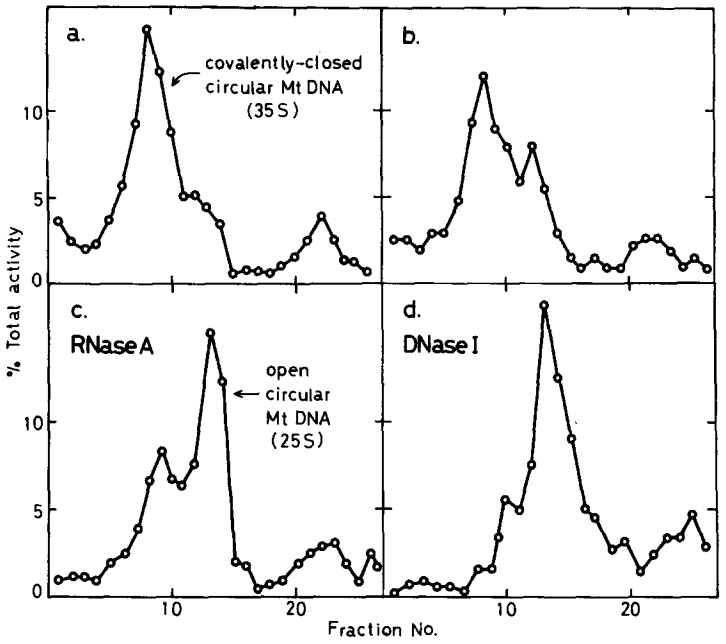

Fig. 1. Centrifugation of mitochondrial DNA on a neutral sucrose gradient. Closed circular mitochondrial DNA (a) was incubated in medium containing 10 mM Tris-HCl and 5 mM EDTA (pH 7.5) without (b) or with (c) RNase A (25 μg/ml) at 37° for 2 hr. After addition of SDS (0.4%), the reaction mixture was placed on a neutral sucrose gradient and centrifuged. (d): Closed circular mitochondrial DNA was incubated with DNase I (2 mμg/ml) in buffer containing 10 mM Tris-HCl, 5 mM EDTA, 18 mM $MgCl_2$ and 15 mM NaCl (pH 8.0) at 20° for 20 min. After addition of EDTA (50 mM) and SDS (0.4%), the reaction mixture was layered on the gradient and centrifuged.

A (25 μg/ml) at 37° for 2 hr, the amount of 35S DNA decreased and a peak appeared at 25S, which corresponded to that of open circular DNA (6-9) (Fig. 1c). RNase T_1, at the same concentration was less effective than RNase A for this conversion. Pronase P (50 μg/ml) did not affect the 35S peak. Digestion with a minimum amount of pancreatic DNase I converted the 35S molecules to 25S molecules (Fig. 1d). The effect of RNase was not due to contaminating DNase because the RNase preparations were heated at 100° for 20 min before use to inactivate DNase. Moreover, the possibility that traces of DNase might survive after this treatment was dis-

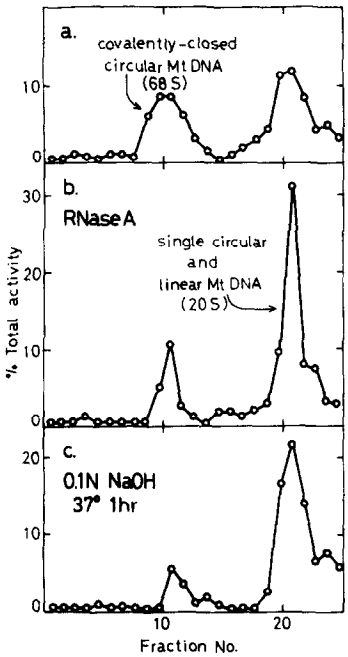

Fig. 2.

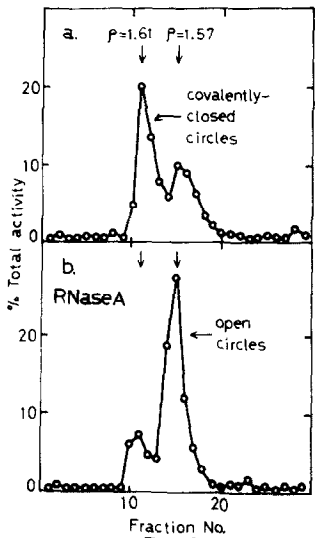

Fig. 3.

Fig. 2. Centrifugation of mitochondrial DNA on an alkaline sucrose gradient. Closed circular mitochondrial DNA was incubated without (a) or with (b) RNase A under the same conditions as for Fig. 1 and then layered on an alkaline sucrose gradient and centrifuged. (c): Closed circular mitochondrial DNA was treated with 0.1 N NaOH at 37° for 1 hr before centrifugation.

Fig. 3. Buoyant density centrifugation of mitochondrial DNA on CsCl-ethidium bromide. Closed circular mitochondrial DNA was incubated without (a) or with (b) RNase A under the same conditions as for Fig. 1 and then centrifuged in a CsCl-dye gradient as described in the Methods.

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proved by tests on the effect of incubating RNase with purified linear λ-phage DNA for 18 hr or with AH 66F-DNA for 290 hr at room temperature (1). Centrifugation of the closed circular DNA in alkaline sucrose gave a peak at 68S (denatured closed circular duplex molecules) and at 20S (denatured single stranded circular molecules or single stranded linear molecules) and the latter seemed to be formed even during centrifugation (Fig. 2a). Treatment of the closed circular molecules with RNase A decreased the amount of the 68S peak and increased that of the 20S peak

(Fig. 2b). When closed circular DNA was incubated in 0.1 N NaOH at 37° for 1 hr before centrifugation, the size of the 68S peak was decreased and that of the 20S peak was greatly increased (Fig. 2c) as in the case on treatment with RNase. The 68S molecules disappeared on prolonged incubation in alkali, and the 20S peak could not be resolved into two components even on longer centrifugation. On a CsCl-dye gradient closed circular DNA gave a major peak at a density of 1.61 (Fig. 3a), while DNA which had been incubated with RNase gave a major band at the light density of 1.57, corresponding to open circular or linear molecules (Fig. 3b).

DISCUSSION: The present results indicate the conversion of closed circular mitochondrial DNA to open circular molecules by breakage in the RNA region which is integrated into the DNA strand and is sensitive to both RNase and alkali, as shown in Fig. 4. The lability of RNA in alkali can account for the sensitivity of the 68S peak to alkali observed by other workers (7, 8, 10). Incubation of 35S DNA alone also resulted in formation of a small amount of 25S DNA (Fig. 1b). Storage at 4° for 1 month also resulted in conversion of about 50% of 35S DNA to 25S DNA and the amount of 35S DNA remaining decreased on incubation with RNase. Degradation of form I during storage has been reported (7, 9, 10), but it is uncertain whether the linkage broken was the same as that which is sensitive to RNase and alkali.

Blair et al. found that closed circular colicinogenic factor E_1 DNA prepared from *E. coli* cells grown in the presence of chloramphenicol was sensitive to RNase and alkali (12). Recently, it has been found that RNA primer is involved in DNA synthesis (13-16). It is postulated that transcription of primer RNA takes place during replication, DNA polymerase couples with RNA polymerase, and the primer RNA is removed by RNase H (17) after

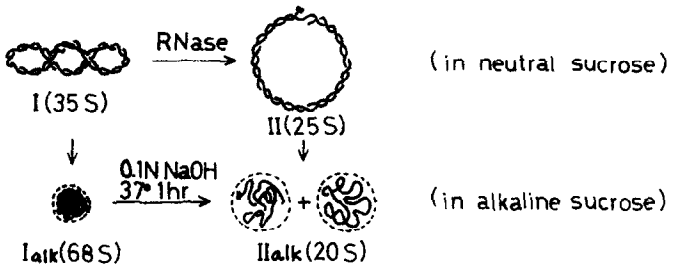

Fig. 4. Shematic presentation of the conversion of mitochondrial DNA, adapted from the paper of Vinograd et al. on polyoma DNA (18). The rod represents the RNA region.

initiation. If DNA duplex contains RNA or the RNA remains attached to it, it is possible that nicking the RNA chain by some enzyme, such as RNase H, leads to initiation of replication at a specific position on the closed circular molecule. It remains to be investigated: at what step this RNA chain is synthesized during the process of replication, and whether RNA is present in mitochondrial DNA from normal liver.

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