RNA BOUND TO NASCENT DNA IN EHRLICH ASCITES TUMOR CELLS

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SUMMARY

Ehrlich ascites tumor cells were labeled with ^{3}H-uridine in vitro for a short period and the nascent DNA fraction was isolated using nitrocellulose column chromatography. After heat-denaturation, the nascent DNA fraction was analyzed by isopycnic centrifugation in cesium sulfate. Its ^{3}H-radioactivity separated into two peaks, one with a buoyant density of 1.47 which was slightly heavier than single-stranded DNA, and the other with the same density as free RNA (ρ=1.66). The radioactivity of lighter fraction was converted to an acid-soluble form by treatments with RNase and alkali. Moreover, this radioactive material showed the same characteristics as nascent DNA on centrifugation in a neutral sucrose density gradient and hydroxylapatite column chromatography. These findings suggest that newly synthesized short RNA chain is covalently bound to nascent DNA and serves as a primer for DNA replication in these cells.

Recently, it was reported that RNA participates in DNA replication, possibly as a primer in some bacteria (1,2), phages (3,4) and plasmids (5,6) and in vitro systems (7,8). An RNA primer for DNA synthesis was also found in the RNA dependent DNA polymerase reaction in RNA tumor viruses (9-11). It is well known that in DNA replication, short chain DNA intermediates are synthesized and joined together to form long chains in microorganisms (12-14) and also mammalian cells (15-18). We also reported on DNA intermediate of this type in Ehrlich ascites tumor cells (19,20). Binding of RNA to such a short chain DNA intermediate was reported in E. coli (2). To see whether RNA is involved in DNA replication in mammalian cells, the interaction of RNA and nascent DNA of Ehrlich ascites tumor cells was analyzed. The results

described in this paper suggest that RNA serves as a primer for DNA synthesis in these cells.

MATERIALS AND METHODS

Chemicals [5-^{3}H]-Uridine (21 Ci/mmole) was purchased from the Radiochemical Centre, Amersham. Cesium sulfate (Cs_2SO_4) was obtained from E. Merck, Darmstadt. Pancreatic RNase and RNase T_1 were products of Worthington Biochemical Corp., Freehold. Other chemicals used were reported previously (19,20).

Labeling of Ehrlich ascites tumor cells with ^{3}H-uridine A suspension of 5 x 10^7 cells/ml of Eagle's minimal essential medium was prepared as reported previously (20) and incubated with 50 μCi/ml of ^{3}H-uridine for 10 min at 37°C. The reaction was stopped by adding about 40 ml of ice-cold phosphate buffered saline and the cells were washed 3 times with centrifugation.

Preparation of the nascent DNA fraction DNA was extracted as reported previously (20). Nascent DNA was isolated by chromatography on a nitrocellulose column as originally reported by Probst et al. (21) and confirmed by us (20). The DNA sample was applied to a 1.5 cm x 10 cm column of nitrocellulose and eluted stepwise with 0.05 M Tris-HCl buffer (pH 6.75) containing 0.4 M NaCl and 1 mM EDTA (TSE buffer) and then with the same buffer containing 0.5 % sodium dodecyl sulfate (SDS-TSE buffer). The fraction of eluate with SDS-TSE buffer containing the peak of nascent DNA with ^{3}H-radioactivity were pooled and subjected to further analyses.

Cs_2SO_4 isopycnic centrifugation of the nascent DNA fraction The nascent DNA fraction was dialyzed against water, heated at 100°C for 5 min in 0.01 M NaCl and then subjected to a tube containing 10 ml of 0.05 M Tris-HCl buffer (pH 7.4), 1 mM EDTA and Cs_2SO_4. The initial buoyant density was adjusted to 1.52. The tube was centrifuged in a conical rotor at 45,000 rpm for 50 hours at 24°C. Then, fractions of 25 drops were collected from the bottom of the tube and their refractory indices and acid-insoluble radioactivities were measured as described previously (19,20). The density markers used were the heat-denatured bulk of DNA labeled with ^{14}C-thymidine (20) and ^{3}H-uridine-labeled RNA eluted from the nitrocellulose column with TSE-buffer.

Treatments of RNA-DNA complex with RNases and alkali The fractions containing peak B of radioactivity obtained by Cs_2SO_4 isopycnic centrifugation (Fig. 2) were combined, dialyzed against water and digested with RNases in 0.5 ml of solution containing 0.05 M Tris-HCl buffer (pH 7.4), 100 μg/ml of pancreatic RNase and 100 μg/ml of RNase T_1. The mixture was incubated at 37°C for 30 min and remaining acid-insoluble radioactivity was measured. Alkali treatment was performed in 0.3 N NaOH at 100°C for 5 min and acid-insoluble radioactivity was assayed. As controls, free RNA eluted from the nitrocellulose column with TSE-buffer and the bulk of DNA labeled with ^{14}C-thymidine after heat-denaturation were also treated with RNases and alkali.

Sucrose density gradient centrifugation and hydroxylapatite column chromatography of RNA-DNA complex Peak B in Fig. 2 was dialyzed against water or 0.04 M potassium phosphate buffer (pH 6.8) and subjected to centrifugation in a neutral sucrose density gradient or hydroxylapatite column chromatography, respectively as reported previously (19,20).

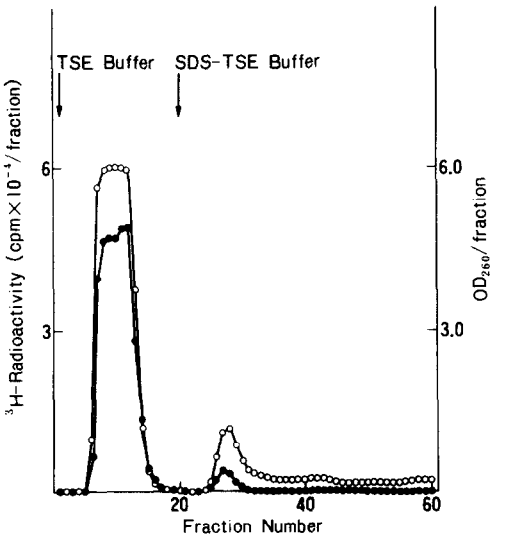

Fig. 1. Nitrocellulose column chromatography of DNA fraction extracted from cells labeled with ^{3}H-uridine for 10 min. ^{3}H-Radioactivity (●——●) and absorbancy at 260 nm (○——○).

RESULTS

Fig. 1 shows the elution pattern of DNA fraction extracted from cells labeled with ^{3}H-uridine for 10 min. The elution pattern of absorbancy at 260 nm was identical to that reported previously (20). Most of the radioactivity passed through the column with TSE-buffer. This radioactivity is that of the bulk of RNA (22), which was confirmed by Cs_2SO_4 isopycnic centrifugation and RNase and alkali treatments as described below. A small but definite amount of radioactivity was eluted in the position of nascent DNA with SDS-TSE buffer.

Fig. 2 shows the pattern of banding of ^{3}H-radioactivity eluted from the nitrocellulose column with SDS-TSE buffer in Fig. 1 after heat-denaturation and that of the heat-denatured bulk of DNA labeled with ^{14}C-thymidine. The ^{3}H-radioactivity of nascent DNA fraction bands with buoyant densities of 1.66 and 1.47. The radioactive peak with a density of 1.66 (A) seems to be free RNA because it has the same density as that of RNA eluted from the nitrocellulose column with TSE-buffer. The other peak (B) is

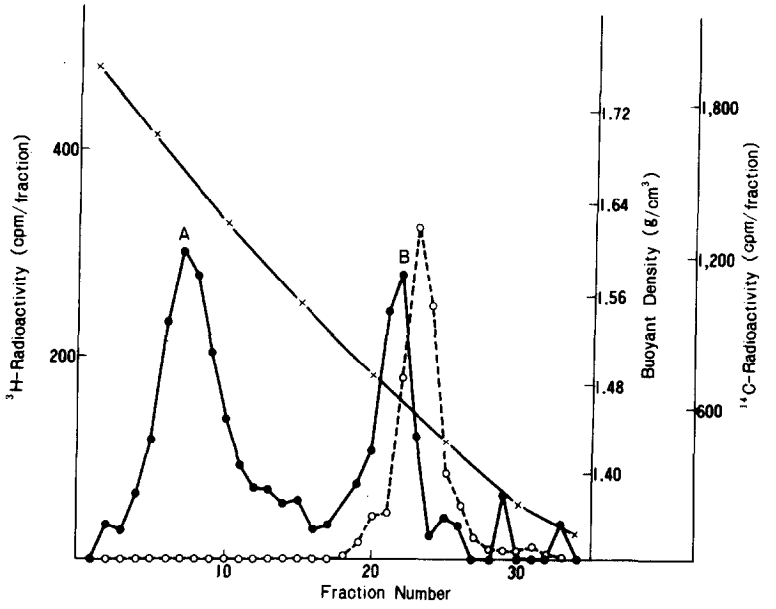

Fig. 2. Isopycnic centrifugation in Cs_2SO_4 of the heat-denatured nascent DNA fraction labeled with ^{3}H-uridine (●——●). The bulk of DNA labeled with ^{14}C-thymidine after heat-denaturation was also analyzed and superimposed in the figure (O----O). Buoyant density (X——X).

slightly more dense than heat-denatured DNA which has a buoyant density of 1.45. Peak B is tentatively referred to RNA-DNA complex.

Treatment of this RNA-DNA complex with RNases converted 57 % of the radioactivity to an acid-soluble form, while treatment with alkali converted 76 % to an acid-soluble form (Table 1). These treatments converted almost all the radioactivity of the bulk of RNA eluted from the nitrocellulose column with TSE-buffer to an acid-soluble form, but had no significant effect on heat-denatured DNA.

Fig. 3 shows the pattern of RNA-DNA complex in a neutral sucrose density gradient. The position of the radioactivity peak was almost identical with that of nascent DNA labeled with ^{3}H-thymidine (20). On hydroxylapatite column chromatography also, the ^{3}H-radioactivity of the RNA-DNA complex eluted with the same concentration of phosphate buffer (0.12 M) as nascent DNA (Fig. 4) (19,20).

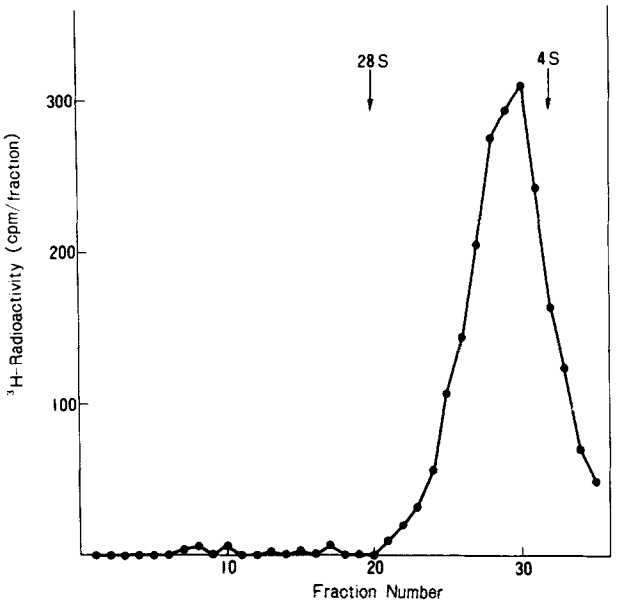

Fig. 3. Neutral sucrose density gradient centrifugation of peak B in Fig. 2. Arrows indicate the positions of sedimentation of single, circular ϕX174 DNA (28S) and tRNA of the rat liver (4S) used as markers.

DISCUSSION

In this work, when Ehrlich ascites tumor cells were incubated with ^{3}H-uridine, the nascent DNA fraction was found to take up some radioactivity. On Cs_2SO_4 isopycnic centrifugation after heat-denaturation, this labeled nascent DNA had a slightly greater buoyant density than the heat-denatured bulk of DNA from the same cells. The radioactive material was sensitive to RNase and alkali treatments but behaved as short, single-stranded nascent DNA. These findings suggest that RNA labeled with ^{3}H-uridine is bound to nascent DNA, not through hydrogen bonds but covalently. The relative insensitiveness to RNase and alkali treatments of RNA strand in the RNA-DNA complex compared to that of the free bulk of RNA might be due to structural differences between the RNA-DNA complex and free RNA. Resistance of RNA in DNA strand to RNase T_1 was also reported in the colicinogenic factor E_1 plasmid (6).

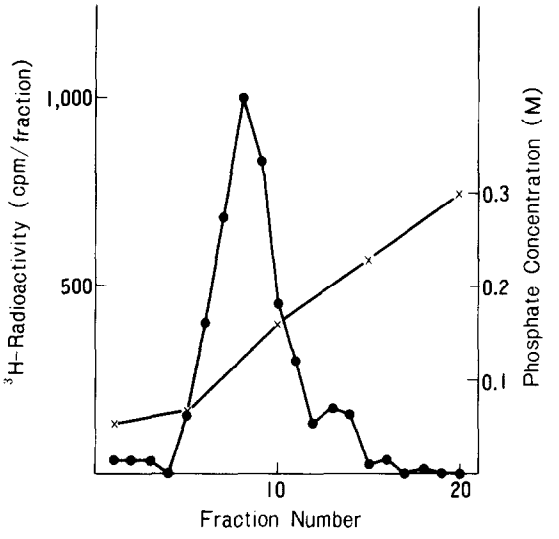

Fig. 4. Hydroxylapatite column chromatography of peak B in Fig. 2. Phosphate concentration (X—X).

Table 1

Susceptibility of RNA-DNA complex, free RNA and DNA to RNase and alkali treatments

	Conversion to acid-soluble form (%)	
	RNase	Alkali
RNA-DNA complex	56.8	75.6
RNA	99.2	94.9
DNA	5.9	3.8

The exact length of RNA bound to nascent DNA was not estimated. However, the finding that the buoyant density of this RNA-DNA complex was only slightly more

than that of the heat-denatured bulk of DNA suggests that the RNA chain length is rather short. Keller reported that an RNA-DNA complex synthesized in vitro using mammalian cell DNA polymerase had a buoyant density very close to 1.46 in Cs_2SO_4 isopycnic centrifugation and that RNA bound to DNA had a chain of 20-50 nucleotide units (7). Sugino et al. also reported that nascent DNA-RNA complex showed a slightly heavier buoyant density than denatured DNA and estimated the chain length of RNA bound to nascent DNA to be 50-100 nucleotide units in E. coli (2). Our results seemed to be in agreement with these reports. Moreover, the fact that RNA bound to nascent DNA was synthesized within a relatively short period suggests that this RNA synthesis occurs concomitantly with that of nascent DNA. Actually, incorporation of radioactivity into nascent DNA fraction was also observed in cells labeled with ^3H-uridine for 2 min (data not shown).

The present work shows that newly synthesized, short chain RNA is bound to nascent DNA in Ehrlich ascites tumor cells, possibly by a covalent linkage, and might serve as a primer for DNA synthesis as was reported in microorganisms. The exact mode of binding of RNA and DNA and the side of nascent DNA to which RNA is attached require elucidation.

ACKNOWLEDGEMENTS

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REFERENCES

1. Bazzicalupo, P. and Valentini, G. P. T., Proc. Nat. Acad. Sci. USA, 69, 298 (1972).
2. Sugino, A., Hirose, S. and Okazaki, R., Proc. Nat. Acad. Sci. USA, 69, 1863 (1972).
3. Lark, K. G., J. Mol. Biol., 64, 47 (1972).
4. Wickner, W., Brutlag, D., Schekman, R. and Kornberg, A., Proc. Nat. Acad. Sci. USA, 69, 965 (1972).
5. Kline, B. C., Biochem. Biophys. Res. Commun., 46, 2019 (1972).
6. Blair, D. G., Sherratt, D. J., Clewell, D. B. and Helinski, D. R., Proc. Nat. Acad. Sci. USA, in press.

7. Keller, W., Proc. Nat. Acad. Sci. USA, 69, 1560 (1972).
8. Karkas, J. D., Proc. Nat. Acad. Sci. USA, 69, 2288 (1972).
9. Verma, I. M., Meuth, N. L., Bromfeld, E., Manly, K. F. and Baltimore, D., Nature New Biology, 233, 131 (1971).
10. Flügel, R. M. and Wells, R. D., Virology, 48, 394 (1972).
11. Taylor, J. M., Faras, A. J., Varmus, H. E., Levinson, W. E. and Bishop, J. M., Biochemistry, 11, 2343 (1972).
12. Oishi, M., Proc. Nat. Acad. Sci. USA, 60, 1000 (1968).
13. Okazaki, T. and Okazaki, R., Proc. Nat. Acad. Sci. USA, 64, 1242 (1969).
14. Sugino, A. and Okazaki, R., J. Mol. Biol., 64, 61 (1972).
15. Tsukada, K., Moriyama, T., Lynch, W. E. and Lieberman, I., Nature, 220, 162 (1968).
16. Painter, R. B. and Schaefer, A., Nature, 221, 1215 (1969).
17. Schandl, E. K. and Taylor, J. H., Biochem. Biophys. Res. Commun., 34, 291 (1969).
18. Schandl, E. K., Cancer Res., 32, 726 (1972).
19. Sato, S., Tanaka, M. and Sugimura, T., Biochim. Biophys. Acta, 209, 43 (1970).
20. Sato, S., Ariake, S., Saito, M. and Sugimura, T., Biochem. Biophys. Res. Commun., in press.
21. Probst, H., Ullrich, A. and Krauss, G., Biochim. Biophys. Acta, 254, 15 (1971).
22. Boeji, J. A. and Armstrong, R. L., in "Methods in Enzymology" XII, Part A, p. 684, Academic Press, New York and London (1967).