PROPERTIES OF NASCENT DNA OF EHRLICH ASCITES TUMOR CELLS OBTAINED BY NITROCELLULOSE COLUMN CHROMATOGRAPHY

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SUMMARY

Ehrlich ascites tumor cells were labeled with ^{3}H-thymidine *in vitro* and their DNA was extracted by treatments with sodium dodecyl sulfate and NaCl. Bulk DNA and nascent DNA were separated by nitrocellulose column chromatography. Nascent DNA was characterized as short, single-stranded DNA by sucrose density gradient centrifugation, hydroxylapatite column chromatography and isopycnic centrifugation in cesium chloride. The properties of nascent DNA obtained by nitrocellulose column chromatography in this experiment were considerably different from those reported by other workers. The reasons for the difference are discussed and the value of this method for preparation and characterization of nascent DNA is emphasized.

Early events in the replication of DNA of phages and bacteria have been extensively studied and the short chain DNA intermediates synthesized in these microorganisms have been well characterized (1-5). Formation of short chain intermediates during replication of DNA in mammalian cells has also been detected in several laboratories, including ours (6-14). To characterize this nascent DNA further, it is essential to develop a method for its preparation in sufficiently large amount. Sucrose density gradient centrifugation and hydroxylapatite column chromatography have been employed most often for preparation and characterization of nascent DNA. However, insufficient material can be obtained by these methods. Recently, Probst *et al.* reported that the bulk of the DNA and nascent DNA of Ehrlich ascites tumor cells can be separated using nitrocellulose column

chromatography (15). We previously reported on a short chain DNA intermediate formed in Ehrlich ascites tumor cells *in vivo* (12) and the present work was performed to compare the nature of the nascent DNA obtained by nitrocellulose column chromatography with that of the material we reported previously. The nascent DNA eluted from a nitrocellulose column in the present work was confirmed to be short, single-stranded DNA. The usefulness of this method for preparation of nascent DNA is pointed out. The differences in the properties of this nascent DNA from that obtained by Probst *et al.* (15) are discussed.

MATERIALS AND METHODS

Chemicals [Methyl-^{3}H] thymidine (22.4 Ci/m mole) and [2-^{14}C] thymidine (57 mCi/m mole) were purchased from The Radiochemical Centre, Amersham. Sodium dodecyl sulfate (SDS) was obtained from Wako Ltd., Osaka and was recrystallized before use. Eagle's minimal essential medium (MEM) was a product of Nissui Co. Ltd., Tokyo. Nitrocellulose (N content 11.5-12.5 %, 1/2 sec.) was obtained from Nakarai Chemicals, Ltd., Kyoto. It was treated by the method of Boezi and Armstrong (16) and the fraction of 60-100 mesh was used for column chromatography. Hydroxylapatite was purchased from BIO RAD Lab., Calif. Cesium chloride (CsCl) was a product of E. Merck, Darmstadt. ^{14}C-Single-circular DNA of ϕX 174 was a gift from Dr. R. Okazaki, Nagoya University, Nagoya. ^{32}P-Labeled tRNA was prepared by ethanol precipitation after SDS-phenol extraction from the supernatant of rat liver homogenate.

Labeling of cells with radioactive thymidine Ehrlich ascites tumor cells were serially transplanted into the peritoneal cavity of male, ddY strain, mice. Seven days after transplantation, ascitic fluid containing tumor cells was withdrawn by syringe and mixed with 100 units of sodium heparin and MEM supplemented with 10 % calf serum. For pulse-labeling of DNA, the cell number was adjusted to 5 X 10^{7} cells/ml and 50 µCi/ml of ^{3}H-thymidine were added to the cell suspension. After incubation for 2 min at 37°C, 40 ml of ice cold phosphate buffered saline (PBS) were added and the cells were washed 3 times with PBS with centrifugation. To label the bulk of the DNA, cells were suspended in MEM supplemented with 10 % calf serum (4 X 10^{5}/ml) and 2 µCi of ^{14}C-thymidine were added to 20 ml of cell suspension. The cell suspension was incubated at 37°C in a Falcon plastic culture flask for 40 hours. Then the cells were collected and washed with cold PBS with centrifugation.

Preparation of DNA DNA was prepared by a slight modification of the method reported previously (12). Packed cells were suspended in water at a concentration of 5 X 10^{6} cells/ml and mixed with the same volume of 2 % SDS in 2 mM EDTA. The mixture was incuba-

ted for 3 hours at 37°C. Then an equal volume of 4 M NaCl was added and the mixture was centrifuged at 12,000 rpm for 20 min. Over 90 % of the acid-insoluble radioactivity was recovered in the supernatant. The supernatant was extensively dialyzed against 0.05 M Tris-HCl buffer (pH 6.75) containing 0.4 M NaCl and 1 mM EDTA (TSE buffer).

Nitrocellulose column chromatography About 50 OD_{260} units of DNA preparation were applied to a column of nitrocellulose (1.5 cm X 10 cm) and eluted successively with TSE buffer, TSE buffer containing 0.5 % SDS (SDS-TSE buffer) and 0.01 N NaOH as described by Probst *et al.*(15). The flow rate was maintained at 5 ml/hour during sample application and elution.

Characterization of nascent and bulk DNA's The fractions eluted from the nitrocellulose column with TSE and SDS-TSE buffers were dialyzed against water for sucrose density gradient and CsCl isopycnic centrifugations. For hydroxylapatite column chromatography, they were dialyzed against 0.04 M potassium phosphate buffer (pH 6.8). Centrifugation on a neutral sucrose density gradient and chromatography on a hydroxylapatite column were performed as reported previously (12). Isopycnic centrifugation on CsCl was carried out in 9 ml of 0.05 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA in a conical rotor at 45,000 rpm for 50 hours at 24°C. The initial buoyant density was adjusted to 1.68. Fractions of 0.3 ml were collected from the bottom of the tube and their refractory indices were measured. Acid-insoluble radioactivity in each fraction was counted as reported previously (12).

RESULTS

Figure 1 shows the elution profile of DNA labeled with ^{3}H-thymidine for 2 min. Almost all the material with absorbancy at 260 nm passed through the column with TSE buffer. However, acid-insoluble radioactivity was distributed equally in the eluates with TSE and with SDS-TSE buffer. About 80 % of the absorbancy and radioactivity were recovered from the column. This elution pattern was almost identical to that reported by Probst *et al.* (15). Most of the radioactivity of DNA labeled with ^{14}C-thymidine for 40 hours was eluted with TSE buffer (data not shown).

Peak fractions of ^{3}H-DNA eluted with SDS-TSE buffer and of ^{14}C-DNA eluted with TSE buffer were analysed by centrifugation on a neutral sucrose density gradient. The results are shown in Fig. 2. ^{3}H-DNA sedimented slower than ^{14}C-DNA. The S value of ^{3}H-DNA was 12-13S. Fig. 3 shows the elution patterns of ^{3}H-DNA and ^{14}C-

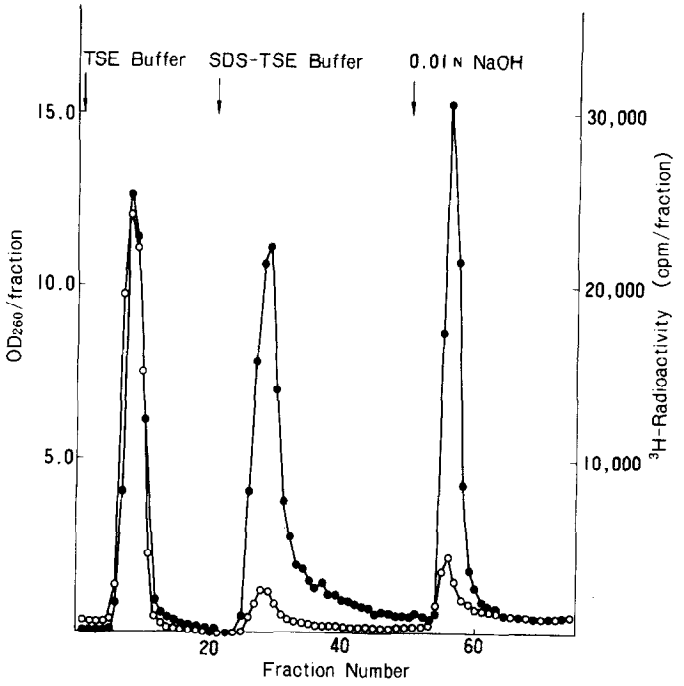

Fig. 1. Nitrocellulose column chromatography of DNA pulse-labeled with ^{3}H-thymidine for 2 min. Step-wise elution was performed successively with 0.05 M Tris-HCl buffer (pH 6.75) containing 0.4 M NaCl and 1 mM EDTA (TSE buffer) and TSE buffer containing 0.5 % SDS (SDS-TSE buffer) and 0.01 N NaOH. Absorption at 260 nm (O—O). ^{3}H-Radioactivity (●—●).

DNA on hydroxylapatite column chromatography. ^{3}H-DNA was eluted with 0.12 M potassium phosphate (pH 6.8), while ^{14}C-DNA was eluted with 0.2 M potassium phosphate (pH 6.8).

Fig. 4 shows the results of CsCl isopycnic centrifugation of ^{3}H-DNA and ^{14}C-DNA. ^{3}H-DNA showed a higher buoyant density (ρ= 1.716) than ^{14}C-labeled, bulk DNA (ρ=1.676). ^{3}H-DNA eluted from the nitrocellulose column with 0.01 N NaOH showed the same density as the bulk of the DNA.

DISCUSSION

DNA of Ehrlich ascites tumor cells was pulse-labeled with ^{3}H-thymidine for 2 min *in vitro* and chromatographed on a nitro-

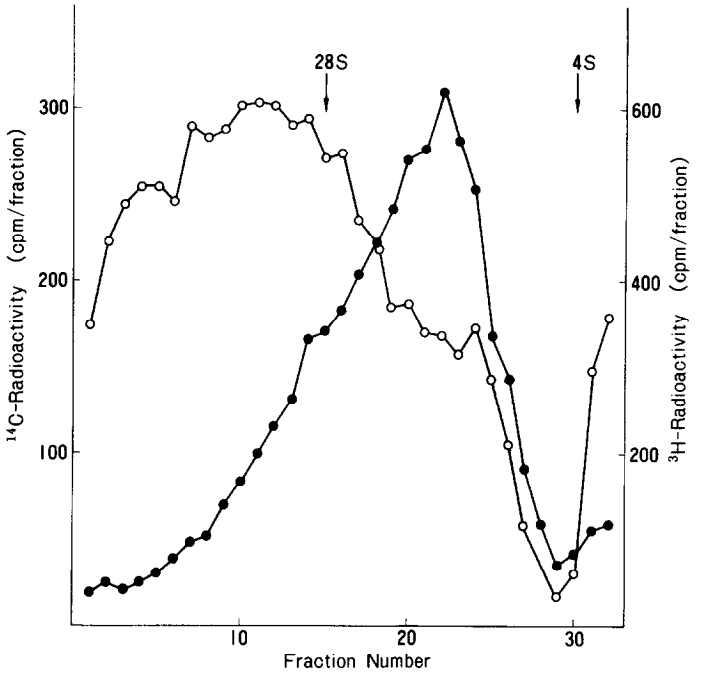

Fig. 2. Neutral sucrose density gradient centrifugation of ^{3}H-labeled, nascent DNA (●——●) and ^{14}C-labeled, bulk DNA (○——○) eluted from a nitrocellulose column. The DNA's were centrifuged separately in tubes containing 4.6 ml of 5-20 % sucrose with 1 M NaCl and 1 mM EDTA at 30,000 rpm for 6 hours at 4°C and their patterns are superimposed in the figure. The arrows indicate the positions of sedimentation of ^{14}C-single, circular φX174 DNA (28S) and ^{32}P-tRNA of rat liver (4S) used as markers.

cellulose column with buffer containing 0.5 % SDS. The resulting preparation was found by centrifugation on a neutral sucrose density gradient to be smaller than the bulk of the DNA. The S value of nascent DNA was 12-13S which is close to that of nascent DNA found in bacteria (3,5) and slightly larger than that found in some mammalian cells (14). Hydroxylapatite column chromatography showed that the DNA eluted from a nitrocellulose column with SDS was single stranded (17). In support of these findings, this nascent DNA had a higher buoyant density than the bulk of the DNA on CsCl isopycnic centrifugation. These properties of

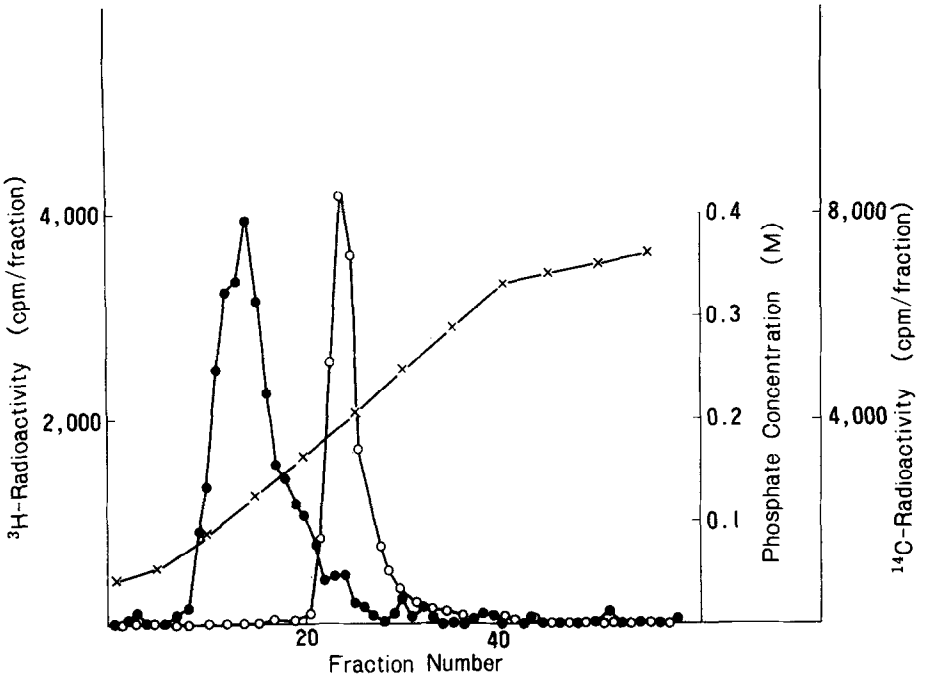

Fig. 3. Elution patterns of ^{3}H-DNA (●——●) and ^{14}C-DNA (○——○) from a hydroxylapatite column (0.5 cm X 5 cm). The two DNA's were chromatographed separately and their profiles are superimposed in the figure. Phosphate concentration (X——X).

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the nascent DNA obtained in the present work were almost identical to those reported for material from microorganisms and mammalian cells. However, they were appreciably different from the properties of the nascent DNA obtained with nitrocellulose column chromatography by Probst *et al.*(15). The latter workers reported that nascent DNA eluted from the column with SDS showed the same characteristics as the bulk of the DNA on sucrose density gradient centrifugation, isopycnic centrifugations in CsCl and $Cs_2SO_4/HgCl_2$, and hydroxylapatite column chromatography. They could only separate nascent DNA from bulk of the DNA by column chromatography on MAK. They attributed the increased affinity of nascent DNA for nitrocellulose, to its attachment to

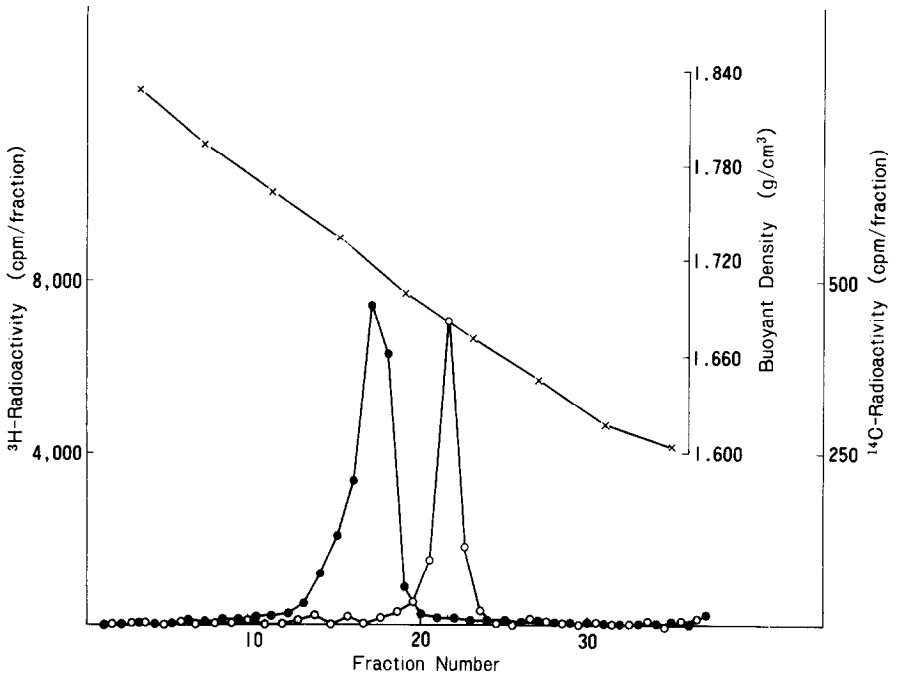

Fig. 4. Isopycnic centrifugation of ^{3}H-DNA (●——●) and ^{14}C-DNA (○——○) in CsCl. The samples were centrifuged separately in 9 ml of 0.05 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and CsCl at an initial buoyant density of 1.68 at 45,000 rpm for 50 hours at 24°C. The patterns obtained are superimposed. Buoyant density (×——×).

some cellular components. In the present work, there was no evidence of binding of nascent DNA to cellular components. The principal difference between their experiments and ours was in the procedure used for isolating DNA. Probst *et al.* collected DNA with a glass rod after treatment with an organic solvent and precipitation with ethanol. This method might not recover very short, and probably single stranded DNA. The difference in the method used for preparation of nitrocellulose powder might also contribute to the differences of properties of the DNA's obtained.

However, the present work confirmed that nitrocellulose column chromatography is a very useful method for preparing a

large amount of nascent DNA, as reported by Probst *et al.*

Recently, the participation of RNA, possibly as a primer, in DNA replication in some microorganisms was reported (18-21). Using nitrocellulose column chromatography the nascent DNA obtained from Ehrlich ascites tumor cells labeled with ^{3}H-uridine showed some radioactivity. We have some evidence that this radioactivity is due to RNA attached to nascent DNA (to be published separately).

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