DNA Products in *In Vitro* DNA Synthesis Using Bacteriophage φX174 Single-stranded DNA

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We described product analysis of DNA synthesized in chloroplast lysate from liverwort *Marchantia polymorpha* L. cell suspension cultures. Characteristics of *in vitro* DNA synthesis by chloroplast lysate using bacteriophage φX174 single-stranded DNA were very similar to those in the case of double-stranded calf thymus DNA reported previously. Autoradiographic analysis clearly showed the incorporation of radioactive \[^{32}P\]-dCTP into DNA molecules associated with bacteriophage φX174 single-stranded template DNA, indicating conversion of bacteriophage φX174 single-stranded DNA to double-stranded DNA (RF III, double-stranded linear molecule). Experiments on the fate of \[^{32}P\]-labeled single-stranded DNA also showed a clear conversion of the single-stranded DNA to double-stranded DNA. Furthermore, patterns of sucrose density gradient centrifugations (neutral and alkaline) showed the production of two major components in *in vitro* DNA synthesis by chloroplast lysate. This also indicated conversion of bacteriophage φX174 single-stranded DNA to double-stranded DNA (RF III form). Our results suggest that the mechanism of chloroplast DNA replication could be the mode of strand-displacement DNA synthesis as seen in animal mitochondrial DNA synthesis.

In a previous paper we described an *in vitro* DNA synthesis system in chloroplast lysate from liverwort *Marchantia polymorpha* L. cell suspension cultures, and found that heat-denatured calf thymus DNA as well as bacteriophage φX174 single-stranded DNA exhibited markedly high template activity. Little information has been reported on *in vitro* chloroplast DNA synthesis. Recently Zimmermann and Weissbach reported the characterization of *in vitro* DNA synthesis by chloroplasts isolated from maize. However, analysis of the products synthesized in *in vitro* DNA synthesis has not been performed extensively. Here, we have described product analysis in *in vitro* DNA synthesis by a liverwort chloroplast lysate using bacteriophage φX174 single-stranded DNA as a template in order to elucidate the mechanism of DNA replication in plant chloroplasts.

**MATERIALS AND METHODS**

**Chemicals.** Deoxyribonucleoside triphosphates and ribonucleoside triphosphates were obtained from Boehringer Mannheim. \([\text{Methyl-}^{3}H\text{-dTTP (80 Ci/mmol)}\) and \([\alpha-^{32}P\text{-dCTP (3000 Ci/mmol)}\) were purchased from New England Nuclear Corp. Dideoxythymidine triphosphate (ddTTP) was supplied by P-L Biochemicals, and N-ethylmaleimide (NEM) and arabinofuranosyl cytosine triphosphate (araCTP) were from Nakarai Chemicals Ltd. Aphidicolin was a product of Wako Pure Chemicals Industries Ltd.

**Preparation of chloroplast lysate.** Chloroplasts were isolated from *Marchantia polymorpha* L. cell suspension cultures by the method of Ohyama. Chloroplast lysate was extracted from purified chloroplasts as described in the previous paper, and used as an enzyme source for *in vitro* DNA synthesis.

**Preparation of bacteriophage φX174 single-stranded DNA and labeling.** Bacteriophage φX174 single-stranded DNA and \[^{32}P\]-labeled DNA were prepared by the procedure of Jazwinski *et al.*

**Assay conditions for *in vitro* DNA synthesis.** A standard
reaction mixture (100 µl) contained 50 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, 10 mM KCl, 250 µg/ml bovine serum albumin, 100 µM each of dATP, dCTP and dGTP, 3 µM [³H]-dTTP (2 µCi), 1.5 mM ATP, 1 µg of bacteriophage φX174 single-stranded DNA, and 90 µg protein of chloroplast lysate. After 30 min incubation at 30°C, reactions were terminated by the addition of 10% cold trichloroacetic acid (TCA). Acid insoluble materials were collected on a Whatman GF/C glass filter and the radioactivity was counted in a Beckman scintillation counter.

Autoradiographic analysis of the DNA products synthesized in chloroplast lysate. Experiments were performed with the following, (1) non-labeled template DNA and radioactive [α-³²P]-dCTP, and (2) with radioactive [³²P]-labeled template DNA and non-labeled deoxyribonucleoside triphosphates. Aliquots were withdrawn at the indicated times and deproteinized by the addition of an equal volume of phenol. DNA was precipitated by the addition of ethanol. DNA samples were analysed on 1.4% agarose gels in TAE buffer (40 mM Tris-acetate, pH 8.1, 5 mM sodium acetate, 1 mM EDTA). After electrophoresis (3.3 V/cm, 4 hr), gels were dried on a Whatman 3 MM filter paper and exposed to X-ray film for 24 hr at -70°C.

Sedimentation analysis of the DNA products synthesized in the chloroplast lysate. Experiments on in vitro DNA synthesis were performed in a standard reaction mixture. Reactions were terminated by the addition of EDTA and Sarkosyl to final concentrations of 24 mM and 2%, respectively, followed by incubation at 42°C for 10 min to completely stop the reaction. Samples were applied to 5 to 20% linear sucrose gradients, either neutral (50 mM Tris-HCl, pH 7.4, 1 M NaCl, 3 mM EDTA) or alkaline (0.3 M NaOH, 3 mM EDTA, 0.5 M NaCl, 0.1% Sarkosyl) gradients. Neutral gradients were centrifuged at 37,000 rpm for 4.5 hr at 2°C in a Hitachi RPS40T-2 rotor. Alkaline gradients were centrifuged at 37,000 rpm for 7 hr in the same rotor. After fractionation, acid insoluble radioactivity of each fraction was counted with a liquid scintillation counter in a toluene-based scintillation fluid.

RESULTS

Characteristics of in vitro DNA synthesis by chloroplast lysate

Chloroplast lysate prepared from Marchantia polymorpha cultured cells exhibited incorporation of [³H]-dTTP into acid insoluble materials when bacteriophage φX174 single-stranded DNA was exogenously added as a template. As shown in Fig. 1, bacteriophage φX174 single-stranded DNA had a higher template activity than the double-stranded DNA. The incorporation directed by the single-stranded DNA increased gradually for the first 10 min, and then markedly increased for the next 50 min of incubation. The incorporation by the single-stranded DNA always exceeded by approximately 5 times that directed by the double-stranded DNA (RF I, covalently closed circular DNA of bacteriophage φX174) (Fig. 1A). The optimum concentration of single-stranded template

![Fig. 1. Kinetics of the Incorporation of [³H]-dTTP in Chloroplast Lysate Using Bacteriophage φX174 Single-stranded and Double-stranded DNA.](image)

(A): One µg of single-stranded DNA (○—○) or double-stranded DNA (RF I form) (●—●) was incubated in a reaction mixture.

(B): Various amounts of DNA (○—○, single-stranded DNA; ●—●, double-stranded DNA) were incubated in reaction mixtures.
TABLE I. REQUIREMENTS FOR IN VITRO DNA SYNTHESIS IN CHLOROPLAST LYSATE USING BACTERIOPHAGE φX174 SINGLE-STRANDED DNA AS A TEMPLATE

The incorporation was measured in a standard reaction mixture as described in MATERIALS AND METHODS. The value of 100% corresponds to incorporation of 2.9 pmol of dTMP into acid insoluble materials.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>dTMP Incorporated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100</td>
</tr>
<tr>
<td>−MgCl₂</td>
<td>1.1</td>
</tr>
<tr>
<td>−KCl</td>
<td>90.3</td>
</tr>
<tr>
<td>−ATP</td>
<td>91.0</td>
</tr>
<tr>
<td>−dCTP, dGTP, dATP</td>
<td>6.6</td>
</tr>
<tr>
<td>−DNA</td>
<td>1.0</td>
</tr>
<tr>
<td>+UTP, CTP, GTP</td>
<td>114.0</td>
</tr>
</tbody>
</table>

TABLE II. EFFECT OF VARIOUS INHIBITORS ON IN VITRO DNA SYNTHESIS IN CHLOROPLAST LYSATE USING BACTERIOPHAGE φX174 SINGLE-STRANDED DNA

The assay conditions were the same as described in Table I except for addition of the indicated amounts of inhibitors.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>dTMP Incorporated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>N-Ethylmaleimide, 10 mM</td>
<td>1.5</td>
</tr>
<tr>
<td>ddTTP, 100 μM</td>
<td>12.7</td>
</tr>
<tr>
<td>EtBr, 20 μM</td>
<td>1.3</td>
</tr>
<tr>
<td>araCTP, 100 μM</td>
<td>122</td>
</tr>
<tr>
<td>Aphidicolin, 50 μg/ml</td>
<td>102</td>
</tr>
<tr>
<td>α-Amanitin, 100 μg/ml</td>
<td>80.3</td>
</tr>
<tr>
<td>Rifampicin, 100 μg/ml</td>
<td>104</td>
</tr>
</tbody>
</table>

DNA was 1 μg in the standard reaction mixture. On the other hand, the double-stranded DNA did not significantly enhance the incorporation on increasing the amount of the DNA (Fig. 1B).

The incorporation was definitely dependent on magnesium ions (optimum concentration of 5 mM), deoxyribonucleoside triphosphates and bacteriophage φX174 single-stranded DNA added exogenously. The addition of ribonucleoside triphosphates caused no significant stimulation of the incorporation (Table I). Among the various inhibitors of DNA synthesis tested, N-ethylmaleimide (NEM), ethidium bromide (EtBr) and dideoxythymidine triphosphate (ddTTP) markedly inhibited the incorporation in in vitro DNA synthesis. Experiments on in vitro DNA synthesis were performed at 30°C with 1 μg bacteriophage φX174 single-stranded DNA and 2 μCi [α-32P]-dCTP. Aliquots were withdrawn at the times indicated. DNA samples were separated on a 1% agarose gel and subjected to autoradiography as described in MATERIALS AND METHODS. Marker DNAs, bacteriophage φX174 single-stranded DNA (SS) and double-stranded DNA (RF I, covalently closed circular form; RF II, open circular form; RF III, linear form) were run on the same gel.

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Figure 4. Sedimentation Analysis of DNA Products Synthesized in Chloroplast Lysate.

DNA samples were layered on 5 to 20% (A) neutral or (B) alkaline sucrose density gradients. Arrows indicate marker DNA positions of bacteriophage φX174 single-stranded DNA (SS for the circular molecule; 1 for the linear molecule) and double-stranded DNA (RF II).

synthesis. However, aphidicolin, a specific inhibitor of DNA polymerase α did not affect the incorporation at all. The addition of α-amanitin and rifampicin, inhibitors of RNA polymerase, to a reaction mixture had no inhibitory effect on the in vitro DNA synthesis (Table II).

Product analysis of the in vitro DNA synthesis

To analyse DNA products, DNA synthesized by chloroplast lysate in a reaction mixture containing [α-32P]-dCTP was separated by agarose gel electrophoresis, and autoradiography was performed (Fig. 2). In the 10 min incubation, the radioactivity was first detected as a major band corresponding to an RF III form (double-stranded linear DNA of bacteriophage φX174) (Fig. 2, lane 2). Further incubation produced broad bands between the positions of the single-stranded DNA and RF III DNA (Fig. 2, lanes 3 and 4). In order to confirm that the single-stranded template DNA (SS) was converted to double-stranded DNA (RF III form), radioactive [32P]-labeled bacteriophage φX174 single-stranded DNA was used as a template. Radioactivity was detected as a distinct band at the position of RF III DNA (Fig. 3, lanes 2, 3 and 4).

DNA products synthesized were further analysed by sedimentation centrifugations through neutral and alkaline sucrose density gradients. On neutral sucrose gradient centrifugation, the newly synthesized DNA labeled with [3H]-dTTP was distributed over two maj-
or components (Peaks a and b) (Fig. 4A). The faster sedimenting component (Peak a) corresponded to RF II-like DNA (double-stranded open circular form) and the slower sedimenting component (Peak b) to the RF III form of DNA. In a short incubation (less than 10 min). Peak a was found at the position of single-stranded DNA of bacteriophage ϕX174. On alkaline sucrose density gradients, the major DNA product was clearly detected at the position of full length single-stranded linear DNA molecules together with shorter DNA fragments (Fig. 4B). The former DNA fragments were from Peak b in neutral sucrose density gradients, the later from Peak a.

DISCUSSION

In a previous study we developed an in vitro DNA synthesis system in chloroplast lysate and found that denatured calf thymus DNA or bacteriophage ϕX174 single-stranded DNA possessed higher template activity than double-stranded DNA.1) In this paper we have described product analysis of DNA synthesized in chloroplast lysate using bacteriophage ϕX174 single-stranded DNA as a template in order to determine the mechanism of DNA synthesis in the chloroplast lysate.

Results of characterization of the incorporation of [3H]-dTTP into acid insoluble materials by bacteriophage ϕX174 single-stranded DNA were very similar to those of the incorporation by double-stranded calf thymus DNA reported previously.1) However, kinetic studies of the incorporation indicated that two kinds of products (different incorporation rates in the first 10 min and the next 50 min incubation) were synthesized in in vitro DNA synthesis by chloroplast lysate with bacteriophage ϕX174 single-stranded DNA as a template.

On neutral sucrose density gradient centrifugation, Peak b (the slow sedimenting component appearing after 10 min incubation) appeared first as a major band (Fig. 4A). This component corresponded to a band at the position of RF III (double-stranded linear molecule of bacteriophage ϕX174) in agarose gel electrophoretic analysis patterns. This was confirmed by the experiment of the in vitro DNA synthesis system using [32P]-labeled single-stranded ϕX174 DNA as a template. These results showed rapid and distinct formation of the RF III form of DNA at the beginning of the incubation (10 min) (Fig. 3, lane 2). On alkaline sucrose density gradient centrifugation, a product (Peak b) synthesized also corresponded to the complete linear single-stranded DNA of bacteriophage ϕX174 (Fig. 4B).

Components in Peak a (the fast sedimenting component) on neutral sucrose density centrifugation after longer incubation were intermediate components between single-stranded template DNA and RF II (double-stranded open circular DNA of bacteriophage ϕX174 replicative form) because a smaller size of DNA fragments than that of single-stranded linear DNA molecules appeared on alkaline sucrose density gradient centrifugation (Fig. 4B). These components were also found to correspond to smear bands between the positions of bacteriophage ϕX174 single-stranded DNA and RF III (double-stranded linear DNA) (Fig. 2, lanes 3 and 4).

These results indicate that the chloroplast lysate was able to convert bacteriophage ϕX174 single-stranded DNA to double-stranded DNA (RF III form). This suggests that, as reported in animal mitochondrial DNA synthesis,7) chloroplast DNA synthesis can initiate the DNA replication by means of a strand-displacement type of DNA synthesis.

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REFERENCES

4) W. Zimmermann and A. Weissbach, Biochemistry,
21, 3334 (1982).

