Deoxyribonucleic Acid-Dependent Ribonucleic Acid Polymerase Activity in Purified Trachoma Elementary Bodies: Effect of Sodium Chloride on Ribonucleic Acid Transcription

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Highly purified trachoma elementary bodies (T'ang strain), incubated in the presence of the four nucleoside triphosphates [Mg++, Mn++, 2-mercaptoethanol, tris(hydroxymethyl)aminomethane buffer (pH 7.5)] were found to incorporate 3H-uridine triphosphate (UTP) into ribonucleic acid (RNA) molecules. Eighty-seven per cent of the labeled molecules were sensitive to ribonuclease treatment. In vitro RNA synthesis was almost completely inhibited by actinomycin D. Rifampin was also inhibitory, but allowed some initial RNA synthesis before complete inhibition occurred. When the reaction mixture lacked Mn++, trachoma elementary bodies synthesized, for a limited period, high-molecular-weight RNA species (23 to 24S, 16 to 17S, and 10 to 11S). Addition of 0.2 M NaCl to the same reaction mixture stimulated and prolonged 3H-UTP incorporation into the same radioactive RNA species. Addition of 0.001 M Mn++ instead of NaCl also stimulated 3H-UTP incorporation but prevented the synthesis of the high-molecular-weight RNA species.

The elementary bodies are the dormant phase in the life cycle of trachoma agent, an obligate parasite of human conjunctival cells. These particles can survive outside the host cell, but initiate their growth cycle after entering the cytoplasm of host cells (4, 8, 15). Analysis of the molecular composition of purified trachoma elementary bodies (20) disclosed that these particles are cellular entities of prokaryotic nature which contain a circular deoxyribonucleic acid (DNA) genome, 660 x 10^{9} daltons molecular weight (22), ribonucleic acid (RNA) species with sedimentation coefficients of 23, 16, 5, and 4S (20; Becker, Loker, Sarov, Asher, Gutter, and Zakay-Rones, Excerpta Medica, in press), and ribosomal 50 and 30S subunits (21). Recently, it was found that the developing trachoma elementary bodies are inhibited by low concentrations of rifampin (2, 3, 5; Becker et al., Excerpta Medica, in press), a potent inhibitor of RNA polymerase in bacterial cells (10, 13, 20, 26).

The results of the present study reveal that purified trachoma elementary bodies have a DNA-dependent RNA polymerase activity which catalyzes the synthesis of trachoma RNA molecules under in vitro conditions.

MATERIALS AND METHODS

Trachoma agent. The T'ang strain of trachoma agent (TRIC/RC-PK/PK-2/OT) propagated in FL cell cultures (7, 8, 18) was used. Highly purified preparations of trachoma elementary bodies were obtained from the cytoplasm of infected FL cultures by centrifugation in 15 to 30% (w/w) sucrose gradients formed on top of a cushion of 0.4 ml of 45% (w/w) sucrose (21). The material in the cushion was removed, diluted, and rebanded in 30 to 45% (w/w) sucrose gradients (21). Elementary bodies were obtained from the single band which appears in the middle of the sucrose gradient. The yield of elementary bodies from 2 x 10^{8} infected FL cells was calculated to be about 4 x 10^{8} plaque-forming units, as determined by the plaque assay developed by Bernkopf (7). Such a purified preparation has an absorption of 1 optical density unit at 260-nm wavelength and contains 10^{6} morphological particles as determined by electron microscopy (L. Sarov, Ph.D. Thesis, Hebrew University, Jerusalem, 1969). In preliminary experiments, the material from the cushion was incubated with 100 μg of trypsin per ml at 36 C for 30 min to remove contaminating proteins and then treated...
with 100 μg of soybean inhibitor per ml before centrifugation in 30 to 45% (w/w) sucrose gradients. Since no difference in RNA polymerase activity was observed in trypsin-treated and untreated elementary bodies, the trypsinization step was omitted.

RNA polymerase assay. The complete reaction mixture for determination of the RNA polymerase activity in the purified elementary bodies contained (in micromoles) in a final volume of 0.2 ml: Mg<sup>2+</sup>, 0.8; Mn<sup>2+</sup>, 0.2; 2-mercaptoethanol, 0.2; tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride, pH 7.5, 10; adenosine triphosphate (ATP), cytidine triphosphate (CTP), and guanosine triphosphate (GTP), 0.008 each; H<sub>2</sub>uridine triphosphate (UTP), 0.003. This was found to be the optimal concentration for RNA synthesis by 10<sup>7</sup> to 2.5 x 10<sup>7</sup> elementary bodies. In different experiments, Mn<sup>2+</sup> was either omitted or substituted by NaCl (0.2 or 0.3 M) as will be specifically indicated. To 0.1 ml of purified trachoma elementary bodies (10<sup>7</sup> to 2.5 x 10<sup>7</sup>) suspended in a solution containing 0.1 M Tris-hydrochloride (pH 7.5), supplemented with 8% (w/w) sucrose, 10<sup>-4</sup> M 2-mercaptoethanol, and 2 x 10<sup>-3</sup> M Mg<sup>2+</sup>, 0.1 ml of the reaction mixture was added.

The reaction mixtures were incubated at 36 C for various time intervals. The reaction was stopped by the addition of 1 ml of ice-cold 10% (w/w) trichloroacetic acid containing 0.3% (v/v) NaH<sub>2</sub>PO<sub>4</sub>. The suspensions were kept at 4 C for 10 min and filtered through Millipore filters (0.45-μm porosity) washed with 30 ml of cold 10% trichloroacetic acid containing 0.5% Na<sub>2</sub>HPO<sub>4</sub>. The filters were dried, and the radioactivity was counted in a Packard liquid scintillation counter.

Size determination of RNA. To analyze the newly synthesized radioactive RNA samples, the reaction mixtures were chilled and treated with diethyl pyrocarbonate (bayovin; 3%, v/v), a protein-denaturating, general enzyme inhibitor and sodium dodecyl sulfate (SDS; 1%, w/v), and analyzed by centrifugation in SDS sucrose gradients.

Isotopes. H<sub>2</sub>-UTP, ammonium salt (820 mCi/mm or 2.02 Ci/mM) and protein hydrolylate-U-14C (52 mCi/matom carbon) were obtained from the Radiochemical Centre, Amersham.

Chemical, enzymes, and drugs. The unlabeled nucleoside triphosphates ATP, GTP, CTP, and UTP were purchased from Schwarz BioResearch Co., Orangeburg, N.Y. DNA (salmon sperm), sodium salt, highly polymerized (DNA, A grade), was purchased from Calbiochem, Los Angeles, Calif., and ribonuclease B, chromatographically pure, was obtained from Worthington Biochemical Corp., Freehold, N.J. Trypsin (2x crystalline, salt free) and soybean trypsin inhibitor (5x crystalline) were obtained from Nutritional Biochemical Corp., Cleveland, Ohio. Actinomycin D was purchased from Merck, Sharp, and Dohme, West Point, Pa. Rifampin and rifamide were a gift from Gruppo Lepetit, Milan, Italy. Bayovin (diethyl pyrocarbonate; reference 10) was purchased from Bayer Leverkusen, Germany.

RESULTS

RNA polymerase activity in trachoma elementary bodies. Incubation of purified trachoma elementary bodies in vitro in the presence of the four nucleoside triphosphates demonstrated the presence of RNA polymerase activity which catalyzed the incorporation of H<sub>2</sub>-UTP into RNA molecules. The RNA polymerase activity was always associated with the elementary bodies purified by the sucrose gradient technique. Further purification of the elementary bodies with trypsin did not remove the RNA polymerase activity (see above). The results presented in Table 1 demonstrate the following. (i) The in vitro RNA synthesis was dependent on the presence of divalent cations. The optimal concentration of Mg<sup>2+</sup> and Mn<sup>2+</sup>, at pH 7.5, was found to be 0.004 and 0.001 M, respectively. In the absence of either one, RNA synthesis was reduced. (ii) The addition of actinomycin D to the reaction mixture almost completely prevented RNA synthesis. (iii) Only a slight stimulation of RNA synthesis occurred when exogenous DNA was added to the reaction mixture in vitro, indicating that RNA synthesis was carried out on the DNA genomes present in the trachoma elementary bodies. (iv) Omission of the unlabeled nucleoside triphosphates from the reaction mixture resulted in a reduction of H<sub>2</sub>-UTP incorporation into trichloroacetic acid-precipitable material to about 5%. Some synthesis of poly U may have occurred, although the possibility that the trachoma elementary bodies contain a low level of nucleoside triphosphates that permitted RNA synthesis cannot be ruled out. (v) Ribonuclease treatment of the reaction product resulted in the degradation of 87% of the H-labeled molecules into trichloroacetic acid-nonprecipitable material (Table 1).

Trachoma elementary bodies were treated in various ways to obtain the highest degree of RNA polymerase activity. Treatment of trachoma elementary bodies with 10<sup>-3</sup> M 2-mercaptoethanol enhanced the RNA polymerase activity as compared to the activity in the untreated ones (Table 2). Freezing and thawing of the 2-mercaptoethanol-treated elementary bodies did not affect the RNA polymerase activity, whereas ultrasonic treatment reduced its activity.

Effect of rifampin on RNA synthesis. Since rifampin strongly inhibits the development of trachoma agent in vivo (2, 3, 5; Becker et al., Excerpta Medica, in press), the effect of the drug on the kinetics of RNA synthesis in vitro was studied. To allow penetration of rifampin into the elementary bodies, the latter was preincubated with 100 μg of rifampin for 40 min at 4 C before addition to the reaction mixture. It can be seen (Fig. 1) that the rate of H<sub>2</sub>-UTP incorporation into RNA molecules during the initial 15 min was diminished by the drug. Subsequently,
\( ^3 \text{H}-\text{UTP} \) incorporation was completely inhibited (Fig. 1). Similar results were obtained with 10 \( \mu \text{g} \) of either rifampin or rifamide per ml.

**Effect of \( \text{Mn}^{2+} \) and \( \text{NaCl} \) on the kinetics of RNA synthesis.** The kinetics of \( ^3 \text{H}-\text{UTP} \) incorporation into RNA molecules by 2-mercaptoethanol-treated trachoma elementary bodies was studied, and the results are presented in Fig. 2. Incubation of elementary bodies in a reaction mixture without \( \text{Mn}^{2+} \) (Fig. 2, "no addition") resulted in a relatively low level of \( ^3 \text{H}-\text{UTP} \) incorporation into RNA, which terminated after 15 min. Addition of 0.2 or 0.3 m \( \text{NaCl} \) to the reaction mixture (in the absence of \( \text{Mn}^{2+} \)) showed a marked stimulation of \( ^3 \text{H}-\text{UTP} \) incorporation and prolonged the synthesis of RNA. A higher rate of \( ^3 \text{H}-\text{UTP} \) incorporation was noted

<table>
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<tr>
<th>Table 1. RNA polymerase activity in 2-mercaptoethanol treated elementary bodies of trachoma agent</th>
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<tr>
<td>Additions to the complete system*</td>
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<tr>
<td>None</td>
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<tr>
<td>DNA (salmon sperm), 5 ( \mu \text{g/ml} )</td>
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<tr>
<td>Minus ATP, GTP, CTP</td>
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<tr>
<td>Minus ( \text{Mn}^{2+} )</td>
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<tr>
<td>Minus ( \text{Mg}^{2+} )</td>
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<tr>
<td>Plus actinomycin D( ^{b} ), 10 ( \mu \text{g/ml} )</td>
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<tr>
<td>Ribonuclease treatment product( ^{c} ), 40 ( \mu \text{g/ml} )</td>
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* Each reaction mixture contained 2.5 \( \times \) 10\( ^{8} \) elementary bodies (about 2.5 \( \mu \text{g} \) of protein), and the conditions are as described in Materials and Methods.

\( ^{b} \) 2-Mercaptoethanol-treated trachoma elementary bodies were preincubated with the drug for 40 min at 4 C before addition to the reaction mixture.

\( ^{c} \) At the end of the incubation period, sodium deoxycholate [final concentration 1% (w/v)] and ribonuclease, 40 \( \mu \text{g/ml} \) (pretreated at 80 C for 20 min in saline, pH 5.0) were added and further incubated for 15 min at 36 C.

**Figure 1. Effect of rifampin on the kinetics of RNA synthesis.** 2-Mercaptoethanol-treated trachoma elementary bodies (2.5 \( \times \) 10\( ^{8} \)) were pretreated with 100 \( \mu \text{g} \) of rifampin per ml for 40 min at 4 C before addition of the reaction mixture containing 0.001 M \( \text{Mn}^{2+} \). Samples were removed at different time intervals. Each point represents the average of three determinations. Untreated (○); rifampin treated (○).

<table>
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<tr>
<th>Table 2. Methods of treating trachoma elementary bodies to obtain optimal RNA polymerase activity</th>
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<tr>
<td>Reaction mixture</td>
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<td>---</td>
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<tr>
<td>Unchanged</td>
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<tr>
<td>Plus actinomycin D (10 ( \mu \text{g/ml} ))</td>
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<tr>
<td>Minus ATP, GTP, CTP</td>
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* 2-Mercaptoethanol was also omitted from the reaction mixture.

\( ^{a} \) Elementary bodies were treated with 10\( ^{-3} \) M 2-mercaptoethanol immediately before incubation at 37 C and further incubated in its presence.

\( ^{b} \) Preparations were sonically treated for 1 min at 1.5 amp in an ultrasonic disintegrator (Measuring & Scientific Equipment, Ltd.).

\( ^{c} \) 2-Mercaptoethanol-treated trachoma elementary bodies were preincubated with the drug for 40 min at 4 C before addition to the reaction mixture.

* Not done.

† Adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytidine triphosphate (CTP).
in the presence of 0.3 M NaCl. In the presence of 0.001 M Mn\(^{2+}\) in the reaction mixture (with no NaCl), a threefold stimulation of \(^3\)H-UTP incorporation was evident during the initial 15 min. This was followed by a decrease in the rate of RNA synthesis.

**Nature of RNA molecules synthesized in vitro.**

The incorporation of \(^3\)H-UTP by 2-mercaptoethanol-treated trachoma elementary bodies was allowed to proceed for 30 min, and the RNA product was analyzed in sucrose gradients. Typical radioactivity profiles of the reaction products, obtained under various conditions, are shown in Fig. 3. In the absence of Mn\(^{2+}\) (Fig. 3, "no addition"), a distinct peak of radioactive RNA molecules with a sedimentation constant ranging from 23 to 25S (average 24S) was observed. RNA molecules of about 11S were also detected. Addition of 0.2 M NaCl to the above mentioned reaction mixture resulted in a marked increase in the radioactively labeled RNA species with a sedimentation constant of 23 to 24S as well as molecules of 10 to 11S. In the presence of 0.001 M Mn\(^{2+}\), there was a greater incorporation of \(^3\)H-UTP into smaller RNA molecules with sedimentation constants ranging from 24 to 11S (Fig. 3).

**DISCUSSION**

The results of the present study demonstrated that highly purified trachoma elementary bodies (20) have a DNA-dependent RNA polymerase activity. When the elementary bodies were supplemented with the four nucleoside triphosphates under the appropriate conditions, trachoma RNA molecules were synthesized. This RNA synthesis is DNA dependent since it was completely inhibited by actinomycin D. Rifampin inhibited the in vitro RNA synthesis by the tra-
choma elementary bodies, but allowed some initial synthesis of RNA before complete inhibition occurred (Fig. 1). The results of rifampin inhibition of RNA transcription may be interpreted in the light of recent findings that rifampin inhibits the initiation of RNA synthesis by Escherichia coli polymerase (22), but has no effect on RNA polymerase molecules which had already initiated the synthesis of RNA chains. It is therefore possible that trachoma RNA synthesis was initially not completely inhibited by rifampin because the trachoma RNA polymerase molecules were bound to DNA in an initiated form, and were therefore resistant to the action of the drug. Since the initiation of new RNA chains did not occur in the presence of rifampin, further synthesis of RNA was inhibited. RNA polymerase molecules extracted from trachoma elementary bodies were template dependent and were found to be immediately and completely inhibited by low concentrations of rifampin (Sarov and Becker, Excerpta Medica, in press). The susceptibility of trachoma RNA polymerase to rifampin explains the inhibition by the drug of trachoma agent development in vivo (2, 3, 5; Becker et al., Excerpta Medica, in press).

RNA polymerase activity in trachoma elementary bodies was stimulated by NaCl at a high ionic strength (0.2 to 0.3 M), similar to the DNA-dependent RNA polymerase from E. coli (12, 23). It was recently demonstrated that at high ionic strength (0.2 M), E. coli RNA polymerase can repeatedly reinitiate the synthesis of RNA chains (14, 19). In the present system without NaCl, 3H-UTP incorporation continued for a limited time (Fig. 2). Addition of 0.2 M NaCl stimulated and prolonged the duration of the total synthesis of RNA (Fig. 2). RNA molecules with a sedimentation constant of about 24S (ranging from 25 to 23S) were synthesized in vitro by trachoma elementary bodies. These analyses do not enable us to determine whether these RNA molecules are precursors (1, 9, 24) or mature ribosomal RNA. Further analyses by acrylamide gel electrophoresis are still needed to determine the nature of the RNA molecules synthesized in vitro.

Addition of 0.001 M Mn** to the reaction mixture (in the absence of NaCl) resulted in a marked stimulation of 3H-UTP incorporation into RNA molecules (Fig. 2). Nevertheless, Mn** prevented any appreciable synthesis of 23 to 24S RNA (Fig. 3). This finding indicates that Mn** has changed the pattern of RNA transcription by the trachoma RNA polymerase in vitro and yielded a different population of RNA molecules. Intact untreated elementary bodies of trachoma agent (without 2-mercaptoethanol pretreatment) could be activated to synthesize RNA in vitro when nucleoside triphosphates were supplied (Table 2). It is assumed that after entry into the cytoplasm of the host cell, trachoma elementary bodies obtain ATP, GTP, CTP, and UTP from the host cell, and RNA synthesis by its DNA-dependent RNA polymerase is initiated. The activation of trachoma RNA synthesis in vitro by nucleoside triphosphates strengthens Moulder's hypothesis (16, 17) that chlamydial agents depend on the host cells for the supply of high energy-containing compounds such as nucleotides and other phosphorylated compounds (18, 28). The in vitro synthesis of trachoma RNA may be an initial step in the development of the conditions necessary for the cultivation of these agents in vitro.

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LITERATURE CITED