20, 306.
Rhodes, D. (1975), *J. Mol. Biol. 94,* 449.
Richter, D. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 707.
Richter, D., Erdmann, V. A., and Sprinzl, M. (1973), *Nature (London), New Biol. 246,* 132.
Richter, D., Erdmann, V. A., and Sprinzl, M. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3226.
Roberts, R. (1972), *Nature (London), New Biol. 237,* 44.
Robertus, J. D., Ladner, J. E., Finch, Y. T., Rhodes, D., Brown, R. S., Clark, B. F. C., and Klug, A. (1974), *Nature (London) 250,* 546.
Schmidt, J., Buchardt, B., and Reid, R. (1970), *J. Biol. Chem. 245,* 5743.
Schneider, D., Solfert, R., and von der Haar, F. (1972), *Hoppe Seyler's Z. Physiol. Chem. 353,* 1330.
Schwartz, I., Gordon, E., and Ofengand, J. (1975), *Biochemistry 14,* 2907.
Schwarz, U., Lührmann, R., and Gassen, H. G. (1974), *Biochem. Biophys. Res. Commun. 56,* 807.
Shimizu, N., Hayashi, H., Miura, K. (1970), *J. Biochem. 67,* 373.
Sigler, P. (1975), *Annu. Rev. Biophys. Bioeng. 4,* 477.
Simsek, M., Perrissant, G., and RajBhandary, U. L. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2660.
Simsek, M., RajBhandary, U. L., Boisnard, M., and Petrissant, G. (1974), *Nature (London) 247,* 518.
Sprinzl, M., Wolfrum, D.-L., and Neuhoff, V. (1975), *FEBS Lett. 50,* 54.
Svensson, J., Isaksson, K., and Hennigsson, A. (1971), *Biochim. Biophys. Acta 238,* 331.
Thiebe, R., and Zachau, H. G. (1971), *Methods Enzymol. 19,* 179.
Uziel, M., Koh, C. K., and Cohn, W. E. (1968), *Anal. Biochem. 25,* 77.
Wolfrum, D. I., Rüchel, R., Mesecke, S., and Neuhoff, V. (1974), *Hoppe Seyler's Z. Physiol. Chem. 355,* 1415.
Wong, Y. P., Reid, B. R., and Kearns, D. R. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2193.

Isolation of a Transcriptionally Active Chromosome from Chloroplasts of *Euglena gracilis*†

Richard B. Hallick,* Carol Lipper, Oliver C. Richards, and William J. Rutter

ABSTRACT: A transcriptionally active chromosome has been isolated in highly purified form from chloroplasts of *Euglena gracilis.* It contains chloroplast DNA, DNA-dependent RNA polymerase, and other proteins. Transcription occurs at low levels of endogenous DNA, and is indifferent to high levels of exogenous DNA. RNA chain elongation continues for several hours in vitro, and RNA chain initiation, determined by [γ-^{32}P]ATP incorporation, is continuous for at least 1 h in vitro. Maximal rates for RNA synthesis require only a divalent cation and the four ribonucleoside triphosphates. Apparent K_m values for adenosine triphosphate, cytidine triphosphate, guanosine triphosphate, and uridine triphosphate are 4.0, 0.6, 2.5, and 2.3 μM, respectively. As would be expected for a DNA-dependent RNA polymerase, RNA synthesis is inhibited by actinomycin D. However, rifampicin and streptolydigin, inhibitors of procaryotic RNA synthesis, and α-amanitin, an inhibitor of eucaryotic nuclear RNA polymerases II and III, do not inhibit the RNA synthesis reaction. Heparin, which is a potent inhibitor of the initiation of RNA synthesis by a nontemplate bound RNA polymerase, also does not inhibit RNA synthesis. Isolation of transcriptionally active chromosomes should prove to be a useful method to study the mechanism of selective RNA transcription of eucaryotic chromosomes.

An important approach to the study of the regulation of in vivo RNA transcription in eucaryotic cells involves attempts to reconstruct the transcription apparatus in vitro with the minimum appropriate components. This requires solubilization and purification of the RNA polymerases, relevant regulatory molecules, and the DNA template for reconstitution experiments. This approach has led to the discovery of multiple eucaryotic RNA polymerases with separate transcription functions and to the recognition of several distinct transcription systems in the cell nucleus (Roeder and Rutter, 1969; Roeder and Rutter, 1970).

In addition to the nuclear systems, independent genomes are segregated within other cellular organelles such as mitochondria and chloroplasts. DNA-dependent RNA polymerases have been described from mitochondria of *Neurospora* (Küntzel and Schäfer, 1971), *Saccharomyces* (Tsai et al., 1971; Wintersberger, 1970; Scragg, 1971), rat liver (Reid and Parsons, 1971), and *Xenopus* ovaries (Wu and David, 1972), and from chloroplasts of maize (Bottomley et al., 1971; Smith and Bogorad, 1974), wheat leaf (Polya and Jagendorf, 1971a,b) and *Euglena gracilis* (Hallick et al., 1973; Hallick and Rutter, 1973).

These enzymes have been characterized by their ability to transcribe heterologous and homologous DNAs; however,

† From the Department of Chemistry, University of Colorado, Boulder, Colorado 80309 (R.B.H. and C.L.), the Department of Biochemistry, University of Utah, College of Medicine, Salt Lake City, Utah 94112 (O.C.R.), and the Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143 (W.J.R.). *Received February 6, 1976.* This work was supported by Grants GM 21351 to R.B.H. and GM 21830 to W.J.R. from the National Institutes of Health, Grant BMS 72-02222 to W.J.R. from the National Science Foundation, and Grant PF-794 to R.B.H. from the American Cancer Society.

[1] Abbreviations used are: DEAE, diethylaminoethyl; EDTA, ethylenedinitrilotetraacetic acid; ATP, adenosine triphosphate; CTP, cytidine triphosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate.

there are as yet formidable problems associated with reconstruction of either nuclear or organelle transcription systems, making it difficult to reproduce in an in vitro system the RNA transcript produced in vivo. An alternate approach to the problem of studying RNA synthesis in purified in vitro preparations is to isolate directly complexes of endogenous RNA polymerase, putative regulatory molecules, and the genome, preferably from a single transcription system. This approach circumvents the necessity of isolation and identification of each individual component required for reconstitution. In the present work, we describe the isolation and properties of a transcriptionally active chromosome from chloroplasts of *Euglena gracilis*. A preliminary report of this work has appeared (Hallick and Rutter, 1973).

Materials and Methods

Materials. Nucleoside triphosphates were obtained from P & L Biochemicals. Prior to experiments on RNA polymerase substrate kinetics, the nucleotides were individually repurified. Samples of approximately 10 μmol were dissolved in water and applied to Whatman No. 1 paper sheets. The chromatograms were developed by descending chromatography with isobutyric acid–concentrated NH_4OH–water (66:1:33). The triphosphate spots were cut out. The paper strips were washed with ethanol and ether, and air-dried. The nucleotides were eluted with 5% concentrated NH_4OH (v/v). After removal of the NH_3 in vacuo, the samples were applied individually to 30-ml columns of Whatman DE-52 DEAE-cellulose that had been previously equilibrated with 0.01 M triethylammonium bicarbonate and eluted with a 250-ml linear 0.1–1.0 M triethylammonium bicarbonate gradient. Fractions with constant 280 nm/260 nm absorption ratios for ATP, CTP, GTP, and UTP of 0.14, 0.96, 0.67, and 0.36, respectively were pooled, evaporated to dryness in vacuo, and redissolved in water. [5,6-^{3}H]UTP (36 mCi/μmol) and [2,8-^{3}H]ATP were purchased from New England Nuclear. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappel (1964). Triton X-100 was obtained from Sigma Chemical Co.

Growth of Cells. Euglena gracilis Klebs, Strain Z Pringsheim cells were maintained axenically in a heterotrophic medium (Difco Euglena Broth). Cultures for chloroplast isolation were prepared by inoculating 16 l. of an autotrophic medium (Richards et al., 1971) to an initial cell density of 10^3 cells/ml. The cells were grown at 25 °C, with continuous illumination using a Virtis Model 43-100 fermenter, equipped with a Model 43-I light manifold and 12 Sylvania "Grolite" fluorescent lights, to a cell density of 0.9–1.3 × 10^6 cells/ml.

Isolation of Chloroplasts. Cells were harvested by centrifugation at 12 000 rpm in a Sorvall SS-34 rotor equipped with a Szent-Gyorgyi and Blum continuous flow system. Cell yield was approximately 30–70 g of cells/16 l. of culture. Washing of cells, cell lysis, and chloroplast purification were performed by the procedure of Brawerman and Eisenstadt (1964), as modified by Manning et al. (1971). Purified chloroplasts were suspended in 2 volumes of 0.37 M sucrose, 0.01 M Tris-HCl, 0.05 M EDTA, pH 7.6, and stored at −60 °C prior to use.

RNA Polymerase Assay. RNA polymerase activity was routinely determined by measuring the incorporation of [^{3}H]uridine into polynucleotides following incubation of samples with [^{3}H]UTP. Unless specified otherwise, RNA polymerase reactions contained 0.05 M Tris-HCl, pH 7.9, 0.01 M $MgCl_2$, 0.3 mM ATP, 0.3 mM CTP, 0.3 mM GTP, 0.01–0.1 mM [5,6-^{3}H]UTP (0.1–1.0 mCi/μmol), and 0.04 M $(NH_4)_2SO_4$. In addition, 40% of the reaction volume was contributed by the buffer containing the enzyme; this contributed to the assay (final concentration) 10% glycerol, 0.016 M 2-mercaptoethanol, 1.6 mM EDTA, and 0.4% Triton X-100. The mixture was incubated for 10 min at 30 °C, and then the reaction was terminated by the addition of 0.6 volume of 1% sodium dodecyl sulfate, 0.05 M sodium pyrophosphate. Aliquots were pipetted onto Whatman DE-81 filter disks. Alternatively, the reaction was stopped by pipetting an aliquot directly onto a Whatman DE-81 filter disk. The filters were subsequently washed six times for 10 min each in 5% Na_2HPO_4, twice in water, once in 95% ethanol, twice in diethyl ether, and dried. Radioactivity was estimated in a Packard Tricarb Model 3310 Scintillation Counter after solubilization of samples in a toluene based fluor containing 4 g/l. of Omnifluor (New England Nuclear), 2.5% NCS (Amersham-Searle), and 0.35% water.

Purification of a Transcriptionally Active Chromosome from Euglena Chloroplasts. In a typical preparation, 7–10 ml of chloroplast suspension was thawed, and the chloroplasts were collected by centrifugation at 3500 rpm for 15 min in a Sorvall HB-4 rotor. All subsequent operations were performed at 0–4 °C. The chloroplast pellet was resuspended in 3 volumes of a buffer containing 0.05 M Tris-HCl, pH 7.6, 0.04 M 2-mercaptoethanol, 25% glycerol, 4 mM EDTA, and 1% Triton X-100, by hand homogenization with a Teflon pestle in a Potter-Elvehjem homogenizing vessel. The resulting chloroplast lysate was centrifuged at 12 000 rpm for 30 min in a Sorvall HB-4 rotor. The dark-green supernatant was decanted, and 4 M $(NH_4)_2SO_4$ was added to a final concentration of 0.1 M. This supernatant fraction was applied to a 2.5 × 60 cm Sepharose 4B (Pharmacia) gel filtration column previously equilibrated with 0.05 M Tris-HCl, pH 7.6, 0.1 M $(NH_4)_2SO_4$, 0.04 M 2-mercaptoethanol, 25% glycerol, 4 mM EDTA, and 1% Triton X-100, and then eluted with the same buffer.

Concentration and Storage of the Transcriptionally Active Chromosomes. The Sepharose 4B column fractions containing the transcriptionally active chromosome, identified by the RNA polymerase activity, were pooled and centrifuged at 60 000 rpm for 4 h in a Spinco Type 65 rotor. The supernatant was decanted, and the pellet, containing the transcriptionally active chromosome, was resuspended in approximately 1 ml of 0.05 M Tris-HCl, pH 7.6, 0.01 M $(NH_4)_2SO_4$, 0.04 M 2-mercaptoethanol, 4 mM EDTA, 25% glycerol, 0.1% Triton X-100. Either the pooled gel filtration fractions or the concentrated transcriptionally active chromosome may be quick frozen and stored at −60 °C for at least 6 months, or at 4 °C for 24 h with no detectable loss of enzyme activity.

DNA and RNA Determinations. The determination of the buoyant density of the DNA of the transcriptionally active chromosome was obtained by centrifugation in a CsCl gradient in a Spinco Model E analytical ultracentrifuge equipped with a photoelectric scanner. *Micrococcus luteus* DNA was included in the gradient as a standard (ρ = 1.731 g/cm^3). Samples were dialyzed against 0.015 M NaCl, 0.0015 M sodium citrate prior to centrifugation. DNA concentration in the gel filtration column fractions was determined by the diphenylamine method (Burton, 1968). RNA was determined by the orcinol method (Schneider, 1957).

Glycerol Gradient Centrifugation. Rate-zonal centrifugation of the transcriptionally active chromosome was performed in 10–30% glycerol gradients. 75 μl of Sepharose 4B purified transcriptionally active chromosome was diluted with 150 μl of 0.05 M Tris-HCl, pH 7.6, 0.1 M $(NH_4)_2SO_4$, 0.04 M 2-mercaptoethanol, 4 mM EDTA, 1% Triton X-100. The re-

TABLE I: Purification of *Euglena* Chloroplast RNA Polymerase as a Transcriptionally Active Chromosome.

Purification Step	RNA Polymerase Total Act. (units)	Sp. Act.[a] (units/mg of protein)	Yield (%)
Chloroplast lysate	5.95	0.072	100
Supernatant (column sample)	7.00	0.106	118
Pooled Sepharose 4B fractions	3.31	10.1	55

[a] Protein was estimated as previously described (Rutter, 1967). RNA polymerase activity was determined as described in the text. One unit of activity is defined as 1 nmol of UMP incorporated at 30 °C in 10 min under the standard assay conditions. The UTP concentration was 50 μM. The data are reported for 2.5 ml of chloroplasts, purified as described in the text.

TABLE II: RNA and Chlorophyll Content during Purification of the Transcriptionally Active Chromosome.[a]

Purification Step	Chlorophyll (mg)	RNA (mg)
Chloroplast lysate	40.0	11.9
Supernatant (column sample)	31.2	10.3
Pooled Sepharose 4B fractions	nd	0.37
Concentrate (229 000*g* pellet)	nd	0.076

[a] Chlorophyll was determined by the method of Arnon (1949), on the preparation described in Figure 1 and Table I. The pooled Sepharose fractions and the concentrate are free of chlorophyll within the limits of the assay. RNA was determined by the orcinol method (Schneider, 1957) on a different preparation.

sulting mixture was overlayed on a 4.6-ml linear 10–30% glycerol gradient in the above buffer. After centrifugation for 3 h at 40 000 rpm in a Spinco SW 50.1 rotor, the centrifuge tube was punctured, and fractions were collected dropwise from the bottom of the tube for RNA polymerase assay.

$EcoR_I$ Restriction Endonuclease Digestion. Preparation of $EcoR_I$ restriction endonuclease and DNA digestion were by the method of Thomas and Davis (1975). Agarose-gel electrophoresis and photography of DNA fragments were by the method of Helling et al., (1974). DNA was purified from the transcriptionally active chromosome for nuclease digestion by chloroform/isoamyl alcohol extraction (24:1, v/v), followed by ethanol precipitation of the resulting aqueous phase.

Results

Purification of Chloroplast Transcriptionally Active Chromosome. The purification of the *Euglena* chloroplast chromosome from purified chloroplasts as a transcription complex is summarized in Tables I and II. When purified chloroplasts are lysed by homogenization in a buffer containing 1% Triton X-100, chloroplast RNA polymerase activity is detected in the resulting homogenate (Table I). The RNA polymerase specific activity is low in the chloroplast lysate, and there is considerable contamination by both chlorophyll and RNA (Table II). Centrifugation of the lysate at 23 500*g* for 30 min results in the pelleting of a considerable amount of

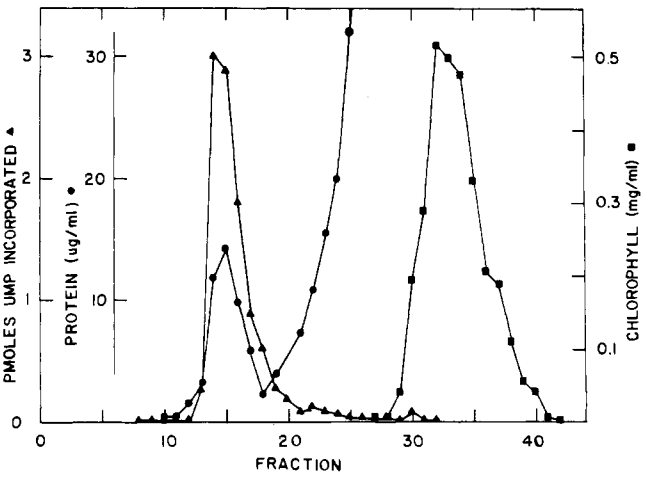

FIGURE 1: Purification of *Euglena* chloroplast transcriptionally active chromosome by gel filtration chromatography on Sepharose 4B. Gel filtration chromatography and RNA polymerase assays at 0.05 mM UTP were performed as described in the text. Fractions of 7.0 ml were collected and 20-μl aliquots were assayed in a final volume of 50 μl. The UMP incorporation represents pmol/20-μl aliquot in 10 min. No exogenous DNA was added to the reaction. The column sample was prepared from 2.5 ml of purified chloroplasts. RNA polymerase activity (▲—▲); protein (●—●); chlorophyll (■—■).

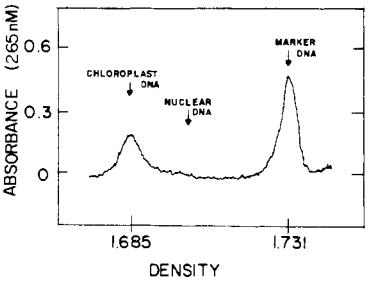

FIGURE 2: Buoyant density determination of the DNA of the *Euglena* chloroplast transcriptionally active chromosome. The DNA of the gel filtration fraction of highest specific activity was analyzed as described in the text. The DNA peak from the sample bands at ρ = 1.685 g/cm^3. Marker DNA is of the bacterium *M. luteus*, ρ = 1.731 g/cm^3.

membranous material that would interfere with subsequent purification steps. There is no appreciable increase in RNA polymerase specific activity or decrease in chlorophyll or RNA content during this step. The chloroplast RNA polymerase purification is achieved primarily during gel filtration chromatography on Sepharose 4B, the pooled Sepharose fractions showing a 140-fold increase in specific activity over the homogenate (Table I). These fractions contain no detectable chlorophyll, and less than 0.7% of the RNA of the homogenate. The chromatographic profile for the gel filtration column is shown in Figure 1. The RNA polymerase elutes in the void volume, while the chlorophyll and more than 99% of the protein are retained.

DNA of the Transcriptionally Active Chromosome. The basis for the purification of RNA polymerase during gel filtration is that the enzyme remains bound to chloroplast DNA in the presence of 1% Triton X-100 and 0.1 M $(NH_4)_2SO_4$, and, thus, elutes with the DNA in the excluded volume. We have previously shown that DNA elutes with the RNA polymerase during gel filtration chromatography (Hallick and Rutter, 1973). This endogenous DNA also serves as the template for all RNA polymerase reactions, as described below. This endogenous DNA has been characterized by sedimentation to equilibrium in neutral CsCl gradients (Figure 2). Its

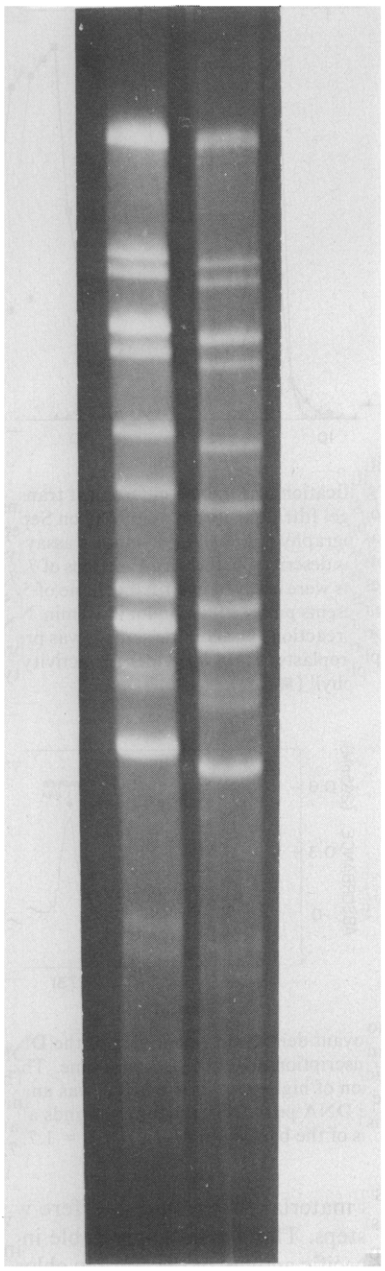

FIGURE 3: Agarose gel electrophoresis of chloroplast DNA. Purified chloroplast DNA (right frame) or DNA from chloroplast transcriptionally active chromosome (left frame) was digested with $EcoR_I$ restriction endonuclease. The resulting fragments were separated on 0.7% agarose gels.

buoyant density (ρ = 1.685 g/cm^3) is identical to that of *Euglena* chloroplast DNA (Brawerman and Eisenstadt, 1964, Ray and Hanawalt, 1964, Edelman et al., 1964). No nuclear DNA contamination (ρ = 1.707 g/cm^3) is evident (Brawerman and Eisenstadt, 1964). The endogenous DNA has also been characterized by the fragments produced upon digestion of the DNA with $EcoR_I$ restriction endonuclease (Figure 3). Following nuclease digestion, 17 fragments can be detected after electrophoresis through 0.7% agarose gels. Most of the fragments seem to be present in stoichiometric amounts, while several bands may represent two fragments of similar molecular weight, not resolved on the gel. There is no evidence for either minor bands or contamination by nuclear DNA. $EcoR_I$ digested, purified *Euglena* chloroplast DNA gives the same 17 fragments when analyzed on 0.7% agarose gels.

Sedimentation of the Transcriptionally Active Chromosome. The sedimentation properties of the purified transcriptionally active chromosome in a 10–30% glycerol gradient are illustrated in Figure 4. Sedimentation was monitored by chloroplast RNA polymerase activity, with endogenous DNA serving as the template. In vivo *Euglena* chloroplast DNA consists of homogeneous circular molecules of molecular weight 92 × 10^6 (Manning and Richards, 1972). The sedimentation profile (Figure 4) shows a heterogeneous size distribution between 20 and 50 S, with a mean size of 34 S, indicating that, although the DNA is still large, there has been some fragmentation during the isolation procedure.

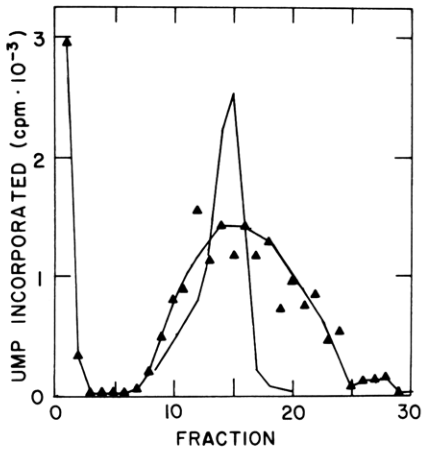

FIGURE 4: Glycerol gradient centrifugation of transcriptionally active chromosome. The sample was overlayered on a 10–30% glycerol gradient, and centrifuged at 40 000 rpm for 3 h in a Spinco SW 50.1 rotor as described in the text. Forty microliters of each fraction was incubated in an RNA polymerase reaction mixture (100 μl) containing 0.05 M Tris-HCl, pH 7.9, 0.01 M $MgCl_2$, 0.1 mM ATP, CTP and GTP, 1.0 μM [5,6-^{3}H]-UTP (36 mCi/μmol), and 0.04 M $(NH_4)_2SO_4$. After 120 min of incubation at 30 °C, aliquots of each reaction were pipetted onto Whatman DE-81 filters, and processed as described in the text. T7 phage [^{3}H]DNA was used as a sedimentation marker in this experiment. RNA polymerase activity (▲—▲); T7-DNA (—).

Properties of the RNA Synthesis Reaction. All of the RNA polymerase reactions described below utilize the endogenous chloroplast DNA of the purified chromosome as the RNA polymerase template. Addition of a large excess of denatured calf thymus DNA (100 μg/ml) or native *Euglena gracilis* chloroplast DNA (6 μg/ml) does not stimulate RNA synthesis by the transcriptionally active chromosome (Table III). Enzyme activity is maximal at the lowest ionic strength tested, 0.04 M $(NH_4)_2SO_4$, and is lowered, but not made DNA dependent, by increasing ionic strength (Table III).

There is an absolute divalent cation requirement for RNA synthesis (Figure 5). Magnesium is the preferred divalent metal ion; maximal incorporation occurs at less than 5 mM $MgCl_2$. The considerable preference for Mg^{2+} over Mn^{2+} (Mg^{2+}/Mn^{2+} activity ratio = 5.6) is noteworthy, since most isolated eukaryotic RNA polymerases utilize Mn^{2+} at least as well as Mg^{2+} (Roeder and Rutter, 1969). The solubilized RNA polymerases from chloroplast of maize seedlings (Bottomley et al., 1971) and wheat leaf (Polya and Jagendorf, 1971) also show similar preference for Mg^{2+}. However, in these cases, the Mg^{2+} concentration giving optimal activity is in the range of 10–40 mM $MgCl_2$, and exogenous DNA templates are used.

The effects of several known inhibitors of RNA synthesis are summarized in Table IV. As would be expected for a DNA-dependent reaction, RNA synthesis is inhibited by actinomycin D. Ethidium bromide is a poor inhibitor, showing only 19% inhibition at 50 μM concentration.

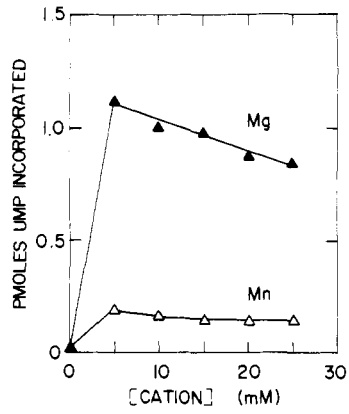

FIGURE 5: Effect of divalent metal ion concentration on RNA synthesis by *Euglena* chloroplast transcription complex. Aliquots of 20 μl were incubated in duplicate in a final volume of 50 μl as described in the text except that the concentration of divalent cation ($MgCl_2$ or $MnCl_2$) is as specified in the figure. (▲—▲) $MgCl_2$; (△—△) $MnCl_2$.

It has previously been reported that rifampicin blocks the synthesis of chloroplast rRNA and appears to promote the dissociation of chloroplast ribosomes into subunits in vivo (Brown et al., 1969). This result has led to the postulate that *Euglena* chloroplast DNA is transcribed by a "prokaryotic" RNA polymerase that is sensitive to rifampicin. However, rifampicin, even at concentrations of 100 μg/ml, has no effect on RNA synthesis by the transcriptionally active chromosome. The observed insensitivity to rifampicin is in agreement with reports that the solubilized DNA-dependent RNA polymerases of both maize seedlings (Bottomley et al., 1971) and wheat leaf (Polya and Jagendorf, 1971) are insensitive to high concentrations of rifampicin.

Streptolydigin has been reported to inhibit RNA chain elongation catalyzed by *E. coli* RNA polymerase (Cassani et al., 1971); however, no inhibition of the transcriptionally active chromosome by streptolydigin is observed. Likewise, α-amanitin, an inhibitor of RNA polymerase II (Lindell et al., 1970) and at high concentrations, RNA polymerase III (Weinmann and Roeder, 1974) of eucaryotic nuclei (Lindell et al., 1970) does not inhibit RNA synthesis, at concentrations of 100 μg/ml (Table IV).

Heparin is a potent inhibitor of initiation of RNA synthesis by *E. coli* RNA polymerase (Zillig et al., 1970). However, we find that heparin stimulates in vitro RNA synthesis in our system (Table IV). In the concentration range of 2–20 μg of heparin/ml, RNA synthesis is enhanced 1.5–1.75-fold. Apparently the in vitro initiation (see below) is due to a heparin-insensitive complex. Another recently described inhibitor of initiation of RNA synthesis is aurintricarboxylic acid (Blumenthal and Landers, 1973). This compound is also a potent inhibitor of the transcriptionally active chromosome, giving 91% inhibition at 10 μM concentration, similar to the concentration needed to inhibit QB replicase, *E. coli* RNA polymerase, and T7 RNA polymerase.

Kinetics of RNA Synthesis. The substrate kinetics for the chloroplast RNA polymerase are illustrated in Figure 6. The effect of the concentration of each of the four nucleoside triphosphates obeys classical Michaelis–Menten kinetics, even though the RNA polymerase is complexed with endogenous chloroplast DNA. From these data, apparent K_m values for each of the substrates have been determined as follows: ATP = 4.0 μM, CTP = 0.6 μM, GTP = 2.5 μM, and UTP = 2.3 μM.

TABLE III: Effect of Exogenous DNA on RNA Synthesis of Chloroplast Transcription Complex.

Experimental Conditions	Enzyme Act. (pmol of UMP incorp)	
	−DNA	+DNA
Expt 1		Denatured Calf Thymus DNA
0.04 M $(NH_4)_2SO_4$	3.54	3.40
0.08 M $(NH_4)_2SO_4$	2.29	2.14
0.12 M $(NH_4)_2SO_4$	1.17	1.01
0.16 M $(NH_4)_2SO_4$	0.60	0.56
Expt 2		Chloroplast DNA
10 min incubation	2.85	2.71
20 min incubation	3.93	4.14
80 min incubation	5.94	5.95

[a] The assay of enzyme activity was described in the text. The UTP concentration was 0.1 mM. Assays, containing 20 μl of transcription complex, and an endogenous DNA concentration of 1.2 μg/ml, were performed in duplicate. In experiment 1 (+DNA), denatured calf thymus DNA (Worthington) was added (100 μg/ml). In experiment 2 (+DNA) native *Euglena* chloroplast DNA was added (6 μg/ml).

TABLE IV: Effect of Inhibitors of RNA Synthesis on Chloroplast Transciptionally Active Chromosome.[a]

Inhibitor	Concn	% Control Act.
Actinomycin D	3.5 μg/ml	50
	50 μg/ml	15
Ethidium bromide	5 μM	86
	50 μM	81
Rifampicin	100 μg/ml	100
Streptolydigin	80 μg/ml	98
α-Amanitin	100 μg/ml	94
Heparin	2 μg/ml	175
	20 μg/ml	155
Aurintricarboxylic acid	10 μM	9

[a] The assay of RNA synthesis was described in the text. Results are expressed as percent of minus inhibitor control reactions.

The time course for both RNA synthesis and initiation of RNA chains in vitro is shown in Figure 7. RNA synthesis experiments were performed using [5,6-^{3}H]UTP to measure chain elongation and [γ-^{32}P]ATP to estimate RNA chain initiation in the same reaction. As shown in Figure 7, both elongation and initiation are continuous for 60 min in vitro. In preliminary experiment the [γ-^{32}P]RNA has been purified, and subjected to digestion by ribonuclease T1. Separation of the resulting products by electrophoresis and chromatography yielded a small number of γ-^{32}P oligonucleotides (Hallick and Lipper, unpublished observations). In other experiments not shown, we find that RNA synthesis continues for at least 4 h in vitro. From the relative incorporation of [^{3}H]UMP vs. [γ-^{32}P]ATP into RNA, we calculate that approximately one RNA chain initiates with ATP per 320–430 UMP nucleotides incorporated into RNA. Since the in vitro RNA product is 35 mol % UMP (Hallick and Lipper, in preparation), approximately one RNA chain initiates with ATP per 900–1200 nu-

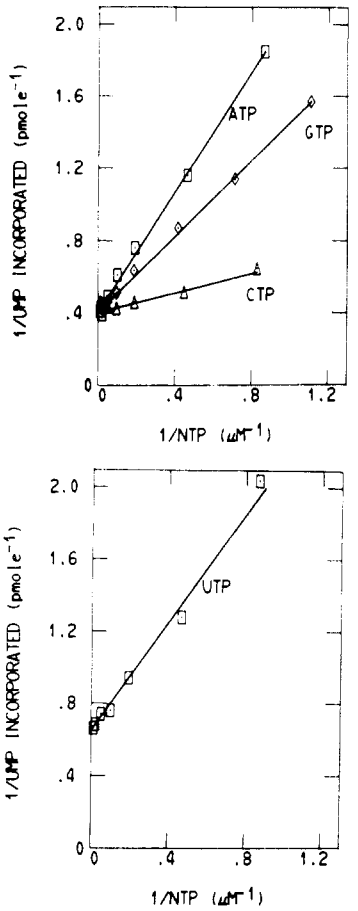

FIGURE 6: NTP substrate kinetics of RNA synthesis by chloroplast RNA polymerase. The effect of ATP, CTP, and GTP (top graph) on the rate of the RNA polymerase reaction was determined in the standard assay mixture containing 0.05 mM of the nonvaried nucleoside triphosphate including 0.05 mM [5,6-^{3}H]UTP. The concentration of one nucleoside triphosphate was varied from 1–100 μM. The effect of UTP (bottom graph) was determined in the same manner, except [2,8-^{3}H]ATP was the radioactive substrate. The results are plotted as double-reciprocal plots according to Lineweaver and Burk.

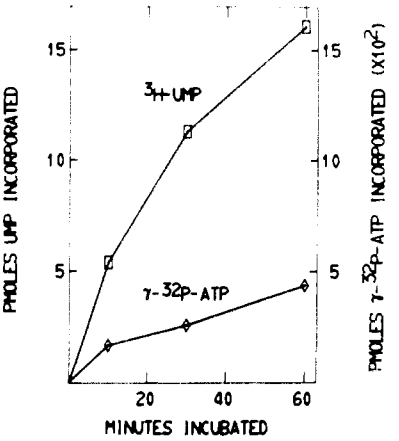

FIGURE 7: Kinetics of initiation and elongation of RNA chains by *Euglena* chloroplast transcriptionally active chromosome. RNA synthesis was determined in the standard assay mixture containing 0.05 mM CTP and GTP, 0.05 mM [5,6-^{3}H]UTP, and 1.76 μM [γ-^{32}P]ATP, specific activity of 1.8×10^5 cpm/pmol. Incorporation, shown for 20 μl of concentrated transcriptionally active chromosome in a 50-μl reaction, was 4.06×10^4 cpm of UMP and 7.92×10^3 cpm of [γ-^{32}P]ATP (60 min). Values were corrected for [^{3}H]UTP and [γ-^{32}P]ATP background radioactivities of 520 cpm and 440 cpm, respectively. ^{32}P spillover into the ^{3}H channel during liquid scintillation counting was 0.5%.

cleotides incorporated. The concentration of [γ-^{32}P]ATP (1.76 μM) used in the experiment was below the K_m for chain elongation for ATP (4.0 μM). We do not yet know the K_m for chain initiation with ATP, or whether RNA chains can initiate with the other nucleoside triphosphates. However, this experiment does demonstrate that initiation does occur in vitro, and that in vitro RNA synthesis by the transcriptionally active chromosome does not simply represent elongation of RNA molecules that had previously been initiated in vivo.

Discussion

We have described a procedure for the direct isolation and purification of a transcriptionally active chromosome from a cell organelle. By taking advantage of the fact that *Euglena* chloroplast RNA polymerase remains tightly bound to its template, a complex containing this enzyme, chloroplast DNA and other proteins has been purified in high yield from *Euglena gracilis* chloroplasts.

This new technique complements studies of transcription in vitro in many other laboratories based on solubilization of RNA polymerase from the endogenous DNA template, and it seems possible that the approach of isolating a transcription complex may be generally applicable in probing transcription in other cell organelles, such as mitochondria, and in other transcription systems with simple genomes.

The purification of the complex has several significant consequences. It provides an excellent means of purifying the chloroplast RNA polymerase. The RNA polymerase is stable, has a high specific activity, and is free of chlorophyll, a troublesome contaminant of chloroplast enzyme preparations. It should be possible now to disassociate the complex, in order to purify the RNA polymerase to homogeneity for studies on the enzyme subunit structure. Isolation of the transcriptionally active chromosome also provides a strong argument that the RNA polymerase is actually a chloroplast enzyme and does not represent a nuclear enzyme that became associated with the chloroplasts during cell fractionation. The RNA polymerase comigrates with the DNA in gel filtration and sedimentation velocity experiments. The endogenous DNA has been identified as chloroplast DNA by both its buoyant density and restriction endonuclease fragment pattern. No nuclear DNA contamination is found. The RNA polymerase transcribes the endogenous chloroplast DNA, but is indifferent to exogenous DNA templates, including chloroplast DNA. The observed indifference to exogenous DNA, and the lack of inhibition by the polyanion heparin, suggests that the chloroplast RNA polymerase may not be released from the DNA following the completion of an RNA chain. However, RNA chains are found to be initiated by [γ-^{32}P]ATP for at least 1 h in vitro. One possible interpretation of these observations is that following termination of an RNA chain the RNA polymerase is translocated along a nontranscribed region of the DNA, and subsequently reinitiates on the same DNA molecule.

Finally, isolation of the transcriptionally active chromosome has made possible the study of RNA synthesis from the chloroplast genome in a highly purified preparation. The properties of the in vitro synthesized RNA is the subject of another report (Hallick and Lipper, in preparation); however, the data are consistent with the conclusion that the RNA sequences are the same as those made in vivo. In a comparison of the extent of the chloroplast genome transcribed, using RNA-driven hybridization to denatured chloroplast[^{3}H] DNA, both the in vitro RNA product and RNA extracted from purified chloroplasts hybridize to 20–23% of the chloroplast DNA. When

the two RNA samples are mixed and hybridized to chloroplast DNA, there is no increase in the fraction of chloroplast DNA hybridized. This result, that the in vitro transcribed RNA contains the same sequences as those made in vivo, and the finding that RNA chain initiation occurs in vitro, raises the intriguing possibility that elements of transcriptive selectivity have been isolated with the transcriptionally active chromosome and that resolution and identification of the components, as well as reconstitution of the functional unit, can be achieved. Furthermore, with the recent finding that there are changes in the expression of the chloroplast genome of *Euglena gracilis* during chloroplast development (Chelm and Hallick, 1976, Rawson and Boerma, 1976), it may be possible to correlate such differences with changes in the composition of the transcriptionally active chromosome.

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References

Arnon, D. I. (1949), *Plant Physiol. 24,* 1.
Blumenthal, T., and Landers, T. A. (1973), *Biochem. Biophys. Res. Commun. 55,* 680.
Bottomley, W., Smith, H. J., and Bogorad, L. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2412.
Brawerman, G., and Eisenstadt, J. M. (1964), *Biochim. Biophys. Acta 91,* 477.
Brown, R. D., Bastia, D., and Haselkorn, R. (1969), *Lepetit Colloq., 1st, 1969 Biol. Med. 1,* 309.
Burton, K. (1968), *Methods Enzymol. 12B,* 163.
Cassani, G., Burgess, R. R., Goodman, H. M., and Gold, L. (1971), *Nature (London) New Biol. 230,* 197.
Chelm, B., and Hallick, R. B. (1976), *Biochemistry 15,* 593.
Edelman, M., Cowan, C. A., Epstein, H. T., and Schiff, J. A. (1964), *Proc. Natl. Acad. Sci. U.S.A. 52,* 1214.
Glynn, I. M., and Chappel, J. B. (1964), *Biochem. J. 90,* 147.
Hallick, R. B., Hager, G. L., and Rutter, W. J. (1973), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32,* 622.
Hallick, R. B., and Rutter, W. J. (1973), *Mol. Cytogenet, Proc. Annu. Biol. Div. Res. Conf., 26th, 1973,*227.
Helling, R. B., Goodman, H. M., Boyer, H. W. (1974), *J. Virol. 14,* 1235.
Küntzel, H., and Schäfer, K. P. (1971), *Nature (London) New Biol. 231,* 265.
Lindell, T. J., Weinberg, F., Morris, P. W., Roeder, R. G., and Rutter, W. J. (1970), *Science 170,* 447.
Manning, J. E., and Richards, O. C. (1972), *Biochemistry 11,* 2036.
Manning, J. E., Wolstenholme, D. R., Ryan, R. S., Hunter, J. A., and Richards, O. C. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1169.
Polya, G. M., and Jagendorf, A. T., (1971a), *Arch. Biochem. Biophys. 146,* 635.
Polya, G. M., and Jagendorf, A. T. (1971b), *Arch. Biochem. Biophys. 146,* 649.
Rawson, J. R., and Boerma, C. L. (1976), *Biochemistry 15,* 598.
Ray, D. S., and Hanawalt, P. C. (1964), *J. Mol. Biol. 9,* 812.
Reid, B. D., and Parsons, P. (1971), *Biochemistry 11,* 2830.
Richards, O. C., Ryan, R. S., and Manning, J. E. (1971), *Biochim. Biophys. Acta 238,* 190.
Roeder, R. G., and Rutter, W. J. (1969), *Nature (London) 224,* 234.
Roeder, R. G., and Rutter, W. J. (1970), *Proc. Natl. Acad. Sci. U.S.A. 65,* 675.
Rutter, W. J. (1967), in Methods in Developmental Biology, F. H. Wilt and N. K. Wessells, Ed., New York, N.Y., Thomas Y. Crowell, p. 671.
Schneider, W. C. (1957), *Methods Enzymol. 3,* 680.
Scragg, A. H. (1971), *Biochem. Biophys. Res. Commun. 45,* 701.
Smith, H. J., and Bogorad, L. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4839.
Thomas, M., and Davis, R. W. (1975), *J. Mol. Biol. 91,* 315.
Tsai, M., Michaelis, G., and Criddle, R. S. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 473.
Weinmann, R., and Roeder, R. G. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1790.
Wintersberger, E. (1970), *Biochem. Biophys. Res. Commun. 40,* 1179.
Wu, G., and Dawid, I. B. (1972), *Biochemistry 11,* 3589.
Zillig, W., Zechel, K., Rabussay, D., Schachner, M., Sethi, V. S., Palm, P., Heil, A., and Seifert, W. (1970), *Cold Spring Harbor Symp. Quant. Biol. 25,* 47.