Godfrey, J. E., Eickbush, T. H., & Moudrianakis, E. N. (1980) *Biochemistry 19*, 1339-1346.
Gordon, V. C., Knobler, C. M., Olins, D. E., & Schumaker, V. N. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 660-663.
Gordon, V. C., Schumaker, V. N., Olins, D. E., Knobler, C. M., & Horowitz, J. (1979) *Nucleic Acids Res. 6*, 3845-3858.
Harrington, R. E. (1981) *Biopolymers 20*, 719-752.
Isenberg, I. (1979) *Annu. Rev. Biochem. 48*, 159-191.
Jorcano, J. L., & Ruiz-Carrillo, A. (1979) *Biochemistry 18*, 768-774.
Klevan, L., Dattagupta, N., Hogan, M., & Crothers, D. M. (1978) *Biochemistry 17*, 4533-4540.
Klug, A., Rhodes, D., Smith, J., Finch, J. T., & Thomas, J. O. (1980) *Nature (London) 287*, 509-516.
Kornberg, R. D., & Thomas, J. O. (1974) *Science (Washington, D.C.) 184*, 865-868.
Lewis, P. N., & Chiu, S. S. (1980) *Eur. J. Biochem. 109*, 369-376.
Libertini, L. J., & Small, E. W. (1980) *Nucleic Acids Res. 8*, 3517-3534.
Lutter, L. C. (1978) *J. Mol. Biol. 124*, 391-420.
Manning, G. S. (1969) *J. Chem. Phys. 51*, 924-933.
Martinson, H. G., True, R. J., & Burch, J. B. E. (1979) *Biochemistry 18*, 1082-1089.
McGhee, J. D., & Felsenfeld, G. (1980) *Annu. Rev. Biochem. 49*, 1115-1156.
McGhee, J. D., Rau, D. C., Charney, E., & Felsenfeld, G. (1980) *Cell (Cambridge, Mass.) 22*, 87-96.
Mirzabekov, A. D. (1980) *Q. Rev. Biophys. 13*, 255-295.
Mirzabekov, A. D., Shick, V. V., Belyavsky, A. V., & Bavykin, S. G. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4184-4188.
Montenay-Garestier, T. (1976) in *Excited States of Biological Molecules* (Birks, J. B., Ed.) pp 207-216, Wiley, New York.
Record, M. T., Jr., Lohman, T. M., & DeHaseth, P. (1976) *J. Mol. Biol. 107*, 145-158.
Ruiz-Carrillo, A., Jorcano, J. L., Eder, G., & Lurz, R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3284-3288.
Simon, R. H., & Felsenfeld, G. (1979) *Nucleic Acids Res. 6*, 689-696.
Spiker, S., & Isenberg, I. (1977) *Biochemistry 16*, 1819-1826.
Suau, P., Bradbury, E. M., & Baldwin, J. P. (1979) *Eur. J. Biochem. 97*, 593-602.
Thoma, F., Koller, T., & Klug, A. (1979) *J. Cell Biol. 83*, 403-427.
Thomas, G. J., Jr., Prescott, B., & Olins, D. E. (1977) *Science (Washington, D.C.) 197*, 385-388.
Thomas, J. O., & Kornberg, R. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2626-2630.
Weber, G. (1960a) *Biochem. J. 75*, 335-344.
Weber, G. (1960b) *Biochem. J. 75*, 345-352.
Wilhelm, M. L., & Wilhelm, F. X. (1980) *Biochemistry 19*, 4327-4331.
Windgender, E., Maass, K., & Bode, J. (1981) *Int. J. Biol. Macromol. 3*, 114-120.
Wu, H.-M., Dattagupta, N., Hogan, M., & Crothers, D. M. (1979) *Biochemistry 18*, 3960-3965.
Zama, M., Bryan, P. N., Harrington, R. E., Olins, A. L., & Olins, D. E. (1977) *Cold Spring Harbor Symp. Quant. Biol. 42*, 31-41.
Zama, M., Olins, D. E., Prescott, B., & Thomas, G. J. (1978) *Nucleic Acids Res. 5*, 3881-3897.

Deoxyribonucleic Acid Synthesis in Isolated Chloroplasts and Chloroplast Extracts of Maize†

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ABSTRACT: Isolated chloroplasts are capable of synthesizing chloroplast DNA in the presence of Mg^{2+} and deoxynucleoside triphosphates. The in vitro reaction proceeds for at least 60 min and is inhibited by KCl and *N*-ethylmaleimide. Stretches of several hundred nucleotides in length are synthesized within an hour. Little or no inhibition is shown by aphidicolin (an inhibitor of eukaryotic DNA polymerase α), dideoxythymidine triphosphate (an inhibitor of eukaryotic DNA polymerases β and γ), nalidixic acid, or rifampicin. Ethidium bromide is a moderate inhibitor of DNA synthesis in the isolated chloroplast. Soluble extracts of chloroplasts will copy exogenously added recombinant plasmid circular DNA containing fragments of chloroplast DNA, and this reaction is strongly inhibited by ethidium bromide. Copying of the plasmid DNA takes place on the relaxed circular or linear forms of the DNA, but no specific initiation sites on the chloroplasts' DNA fragments of the recombinant plasmids have been detected. Our data are consistent with a repair mechanism operating in vitro but may also represent incomplete replicative DNA synthesis.

Eukaryotic cells are characterized by compartmentalization of those cellular components which contain their own genetic apparatus. In animal cells, the nuclear compartment has a genomic organization and DNA replication machinery which is apparently quite different from that in the other cellular compartment, the mitochondria (Kasamatsu et al., 1974). Higher plant cells contain three, rather than two, cellular compartments (Olson, 1981) since they contain another autonomous organelle, the plastid, of which the chloroplast is the best known example (Schnepf, 1980). In each case, the various cell compartments cooperate and communicate with each other in unknown ways to achieve the proper symbiosis.

The genome of the eukaryotic organelles is considered prokaryotic in nature since both mitochondrial and chloroplast DNAs exist as naked double stranded supercoiled circular DNA molecules (Bedbrook & Kolodner, 1979). This view is tempered by the observation that *Chlamydomonas* chloroplast DNA and yeast mitochondrial DNA contain intervening sequences (Rochaix & Malnoe, 1978; Borst & Grivell, 1981).

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0006-2960/82/0421-3334$01.25/0

The replication of mitochondrial DNA in the animal cell is quite different in its mode and enzymatic requirements from that of nuclear DNA in that the mitochondrial DNA is replicated in an asymmetric manner by a strand displacement mechanism (Robberson et al., 1972). Chloroplast DNA (ct DNA) with a size of 100–200 kilobase pairs (kbp) is much larger than the mitochondrial DNA (15–20 kbp) from animal cells but not too different in size from plant mitochondrial DNA which is also in the 100–200-kbp range (Lewin, 1980). The available evidence indicates that in some cases ct DNA replication may involve intermediate structures similar to the D-loop forms of mitochondrial DNA, suggesting a strand displacement mechanism for replication (Kolodner & Tewari, 1975a). However, the existence of Cairn's type replicating DNA structures and rolling circular forms of chloroplast DNA has also been reported (Kolodner & Tewari, 1975b). At the enzymatic level, reports of a unique DNA polymerase in chloroplasts have now been reported (Sala et al., 1980a; Keller & Ho, 1981).

In order to investigate the synthesis of chloroplast DNA, we have now established in vitro DNA synthesis both in intact maize chloroplasts and in a soluble extract derived from these chloroplasts. These systems and their initial characterization are the subject of this paper.

Materials and Methods

Hybrid sweet corn (125 jubilee) seeds were ordered from J. Harris Co. Inc., Rochester, NY. Deoxyribonuclease I, pyruvate kinase, phosphoenolpyruvate, dithioerythritol (DTE), ribo- and deoxyribonucleoside triphosphates, and phenylmethanesulfonyl fluoride were purchased from Sigma, and *p*-toluenesulfonyl fluoride was from Aldrich. Proteinase K and cesium chloride were obtained from Merck; Brij 58 was from Atlas Chemical Industries, Wilmington, DE. Agarose (Seakem) was ordered from Marine Colloids Division, FMC Corp., Rockland, ME. Ribonuclease A was purchased from Worthington, the restriction endonucleases were from Bethesda Research Laboratories, and calf intestinal alkaline phosphatase was from Boehringer Mannheim. The [^{32}P]orthophosphate and α-^{32}P-labeled deoxyribonucleoside triphosphates were ordered from Amersham, and tritiated deoxyribonucleoside triphosphates were from New England Nuclear. Dideoxythymidine triphosphate (dideoxy-TTP) was obtained from P-L Biochemicals. Aphidicolin was a generous gift of the National Cancer Institute, Bethesda, MD. The bacteria strain containing the plasmid pZmc100 was the kind gift of L. Bogorad.

Isolation of Chloroplasts. All steps were performed at 4 °C unless otherwise stated. Solutions were sterilized by filtration, and glassware was autoclaved. A 10-g sample of 6-day-old corn seedlings, grown at 27 °C in the light, was washed with a solution containing 0.1% sodium hypochlorite and 0.05% Brij 58 at 0 °C and rinsed 3 times with cold distilled water. After the plants were minced, they were homogenized in a Waring blender in 30 mL of ice-cold buffer A [330 mM sorbitol, 30 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8, 10 mM NaCl, 10 mM $MgCl_2$, and 2 mM DTE] with two 3–4-s bursts at low setting. The homogenate was filtered through eight layers of sterile cheesecloth, and chloroplasts were pelleted by centrifugation for 1 min at 400*g* in a Sorvall HB-4 rotor at 4 °C. The crude chloroplast pellet was resuspended in 1 mL of buffer A, diluted to 30 mL with the same buffer, and recentrifuged. The chloroplast pellet was resuspended in 1 mL of ice-cold buffer A and immediately used for in vitro DNA synthesis.

Isolation of ^{32}P-Labeled Corn Chloroplast DNA. Six-day-old corn seedlings were washed once in tap water and 3 times with distilled water to remove soil and then kept for 3 h in distilled water and rinsed again 3 times with distilled water. Twenty-five washed plants were labeled with ^{32}P by incubating them for 24 h in 50 mL of distilled H_2O containing 5 mCi of carrier-free [^{32}P]orthophosphate. At the end of incubation, the plants were rinsed, the root system was cut off, and chloroplast DNA was isolated according to Kolodner & Tewari (1975c) with the following modifications.

Chloroplasts were isolated by differential centrifugation (omitting the initial low-speed centrifugation step), treated with DNAse I (50 μg/mL) for 45 min at 0 °C to remove contaminating nuclear DNA, and washed free of DNase I with 300 mM sucrose, 50 mM Tris-HCl, pH 8, and 20 mM ethylenediaminetetraacetic acid (EDTA) (buffer B). The chloroplast pellet was resuspended in 0.8 mL of 50 mM Tris-HCl, pH 8, and 20 mM EDTA (buffer C). After the addition of 0.1 mL of 2 mg/mL proteinase K in buffer C and 0.05 mL of 10% sodium dodecyl sulfate, the chloroplast lysate was incubated overnight at 37 °C. The lysate was extracted with an equal volume of phenol/chloroform (1:1) saturated with 10 mM Tris-HCl, pH 7.9, and 1 mM EDTA. The phases were separated by centrifugation at 10000*g* for 15 min at 4 °C in a Sorvall HB-4 rotor, and the interphase was reextracted with 1 mL of 20 mM Tris-HCl, pH 8, and 1 mM EDTA. DNA was precipitated from the combined aqueous phases, after addition of 5 μg of pBR322 DNA as carrier, with 0.1 volume of 3 M sodium acetate, pH 4.8, and 2 volumes of ethanol and stored at −20 °C overnight. The precipitate was collected by centrifugation at 16000*g* for 30 min, dissolved in 200 μL of 20 mM Tris-HCl, pH 8, and 1 mM EDTA, and stored at −70 °C.

Assay of DNA Synthesis in Isolated Chloroplasts. A 50-μL sample of a freshly prepared chloroplast suspension was added to 50 μL of a 2-fold concentrated assay mix, to yield the following concentrations of components: 115 mM sorbitol, 45 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 5 mM NaCl, 1 mM DTE, 20 mM phosphoenolpyruvate, 12 units/mL pyruvate kinase, 2 mM ATP, 0.2 mM each of CTP, GTP, and UTP, 50 μM each of dATP, dCTP, and dGTP, and 7.8 μM [^{3}H]-TTP (specific radioactivity 12.8 Ci/mmol). Incubations were carried out, unless otherwise stated, at 37 °C in the dark. The final protein concentrations in the incubations are specified in the figure legends. After the indicated incubation times, a 90-μL portion of the reaction mix was spotted onto GF/C glass-fiber filters (Whatman), which were washed once with 5% trichloroacetic acid containing 1% sodium pyrophosphate, 3 times with 5% trichloroacetic acid, and once each with ethanol and ether. Filters were air-dried and immersed in liquid scintillation cocktail (LSC) (Amersham), and radioactivity was counted in a liquid scintillation counter.

Protein Determination. The protein content of chloroplast extracts was determined according to Lowry et al. (1951). For the determination of the protein content of chloroplast suspensions, a 100-μL aliquot was extracted twice with 2 mL of acetone/H_2O (4:1), and the resulting precipitate was dissolved in 0.5 mL of 0.1 N NaOH by heating to 65 °C for 10 min. After removal of undissolved material by centrifugation at 10000*g* for 10 min, an aliquot was used for protein determination.

Preparation of Chloroplast Extracts. The chloroplast pellet isolated from 30 g of plant material, as previously described, was resuspended in 0.6 mL of buffer A (resulting volume 0.8 mL) and diluted with 0.8 mL of extraction buffer containing 30 mM Tris-HCl, pH 8, 700 mM NaCl, 2 mM DTE, and 20% glycerol to which phenylmethanesulfonyl fluoride and *p*-

toluenesulfonyl fluoride were added from stock solutions in 2-propanol, to a final concentration of 50 μg/mL (resulting concentration of 2-propanol, 1% v/v). After incubation for 15 min with occasional mixing on ice, we centrifuged the chloroplast lysate for 15 min at 20000*g* in a Sorvall HB-4 rotor. The light green supernatant was desalted by loading 0.7 mL of extract on a 5-mL Sephadex G50 fine resin (Pharmacia) in a 5-mL syringe and centrifuging at 3300*g* for 10 min at 4 °C. The Sephadex resin had been previously equilibrated with buffer containing 30 mM Tris-HCl, pH 8, 2 mM DTE, 20% glycerol, 50 μg/mL phenylmethanesulfonyl fluoride, 50 μg/mL *p*-toluenesulfonyl fluoride, and 1% 2-propanol and centrifuged as described above shortly before use. The desalted extract was used directly or aliquoted and stored in liquid nitrogen.

DNA Synthesis in Chloroplast Extracts. DNA synthesis was performed at 37 °C in a total volume of 50 μL. An equal volume of chloroplast extract was added to a 2-fold concentrated reaction mix, yielding the following concentrations of the components: 45 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 10% glycerol, 20 mM phosphoenolpyruvate, 2 mM ATP, 0.2 mM each of CTP, GTP, and UTP, and dATP, dCTP, and dGTP at 50 μM each. In addition, 7.8 μM [^{3}H]TTP (specific radioactivity 12.8 Ci/mmol), 12 units/mL pyruvate kinase, 1 mM DTE, and phenylmethanesulfonyl fluoride and *p*-toluenesulfonyl fluoride, 25 μg/mL each, were also present. Plasmid DNA template was present in the standard assay at a final concentration of 50 μg/mL. At the end of incubation, 45 μL of the reaction mixture was spotted onto a GF/C filter, and acid-insoluble radioactivity was determined as described above.

Analysis of in Vitro Synthesized DNA by Restriction Endonuclease Digestion. (A) Isolation of DNA Synthesized in Isolated Chloroplasts. Chloroplasts were incubated in the presence of [α-^{32}P]TTP and [α-^{32}P]dCTP. The in vitro DNA synthesis reaction was stopped by addition of 5 mL (10 volumes) of ice-cold buffer B, and the chloroplasts were collected by centrifugation at 2500*g*, for 1 min at 4 °C. The pellet was washed with the same volume of buffer B and lysed in 0.4 mL of buffer C containing 200 μg/mL proteinase K and 0.5% sodium dodecyl sulfate as described above. The viscous lysate was phenol extracted, and the nucleic acids were precipitated by alcohol.

(B) Isolation of DNA Synthesized in Chloroplast Extracts. At the end of the in vitro DNA synthesis reaction, which was carried out in the presence of [α-^{32}P]TTP (as described in the legend to the figures), the volume was brought to 100 μL with 20 mM Tris-HCl and 1 mM EDTA. An equal volume of phenol/chloroform (1:1) saturated with 10 mM Tris-HCl, pH 7.9, 1 mM EDTA, and 5 μg of sheared yeast RNA was added to each sample. After the solution was mixed, the aqueous phase was removed and loaded onto a 1.5-mL Sephadex G-50 superfine column, previously equilibrated with 20 mM Tris-HCl, pH 8, and 1 mM EDTA in order to separate the high molecular weight [^{32}P]DNA from [^{32}P]TTP. The column was eluted with the same buffer, and Cherenkov radiation of each fraction was determined. The peak fractions of the labeled high molecular weight material, eluting in the void volume, were combined, and nucleic acids were precipitated by ethanol as described above. Analysis of in vitro synthesized DNA by electrophoresis on agarose gels was performed as described for restriction enzyme DNA fragments (see below).

Digestion by Restriction Endonucleases. Aliquots of the [^{32}P]DNAs were digested with the indicated restriction endonucleases in a volume of 100 μL (chloroplast DNA) or 10 μL (plasmid DNAs) under the conditions recommended by the suppliers. At the end of the digestion at 37 °C, the chloroplast DNA was treated with phenol/chloroform and precipitated by ethanol as described above, and the precipitate was dissolved in 20 μL of electrophoresis buffer (40 mM Tris–acetate, pH 7.8, 5 mM sodium acetate, and 1 mM EDTA) containing 2% ficoll and 0.05% orange G. The plasmid DNA reaction mix was diluted with 10 μL of water, and 2 μL of 20% ficoll and 0.5% orange G was added. The DNA fragments were separated by electrophoresis in 1% agarose gels for 14–16 h at 20–25 mA (45–65 V). The gels were stained for 30 min in 1 μg/mL ethidium bromide and photographed with shortwave UV illumination. Following this, the gels were dried with an automatic slab gel drier (Hoefer Scientific Instruments, San Francisco, CA) and exposed to Kodak X-Omat film at −70 °C, using an intensifier screen. For quantitative analysis of the ^{32}P-labeled DNA fragments, the DNA bands were localized with the aid of the exposed film and cut out of the dried gel, and their radioactivity was determined, after addition of LSC (Amersham), in a liquid scintillation counter.

Density Labeling of Chloroplast DNA. For in vitro density labeling of chloroplast DNA in isolated chloroplasts, the standard assay was scaled up 10 times. TTP was replaced by 50 μM bromodeoxyribouridine triphosphate, and 10 μM [^{3}H]dCTP (specific radioactivity 23.9 Ci/mmol) was substituted for unlabeled dCTP. After 60 min of incubation at 37 °C, the chloroplast DNA was isolated essentially as described for the in vivo [^{32}P]PO_4-labeled chloroplast DNA but with sheared calf thymus DNA as the carrier at a final concentration of 20 μg/mL, in the proteinase K incubation step. The DNA was finally precipitated with ethanol and dissolved in 20 mM Tris-HCl, pH 8, and 1 mM EDTA. Two equal aliquots of the DNA solution were brought to a volume of 1 mL with the same buffer, and NaCl was added to a final concentration of 100 mM. In vivo labeled ^{32}P-labeled chloroplast DNA (see above) was added to serve as an internal density marker. One sample was sonicated on ice 6 times for 8 s with a Branson sonifier (setting 4 low). For analysis, 4.95 g of CsCl was added, and the volume was brought to 5 mL with 20 mM Tris-HCl, pH 8, 100 mM NaCl, and 1 mM EDTA (resulting refractive index 1.4018–1.4020). The samples were centrigured at 40000 rpm for 16 h at 10 °C in a VTi 65 Spinco rotor. The tubes were punctured on the bottom, and the refractive index of the fractions was determined. Thirty micrograms of heat-denatured salmon sperm DNA carrier was added to each fraction and incubated overnight at 37 °C with 1 mL of 0.3 N NaOH to render any contaminating ^{32}P-labeled RNA acid soluble. DNA was precipitated by addition of 2 mL of 10% trichloroacetic acid/1% sodium pyrophosphate at 0 °C. The precipitate was collected on GF/C glass-fiber filters and washed with 10 mL of 10% trichloroacetic acid and 1% sodium pyrophosphate, then with 10 mL of 5% trichloroacetic acid, and finally with 10 mL of ethanol. The ^{3}H and ^{32}P radioactivity of the air-dried filters was determined after addition of scintillation counting fluid.

Isolation of Plasmid DNA. The DNA from plasmid pZmc100 (Bedbrook et al., 1977) was isolated essentially as described by Norgard et al. (1979). Briefly, lysozyme-treated cells were lysed with sodium deoxycholate and Brij 58. After sedimentation of chromosomal DNA by centrifugation at 100000*g*, the cleared lysate was extracted with chloroform/isoamyl alcohol (24:1), and nucleic acids were precipitated with ethanol. The precipitate was dissolved in 30 mM Tris-HCl, pH 8, and 1 mM EDTA, and RNase A was added to a final

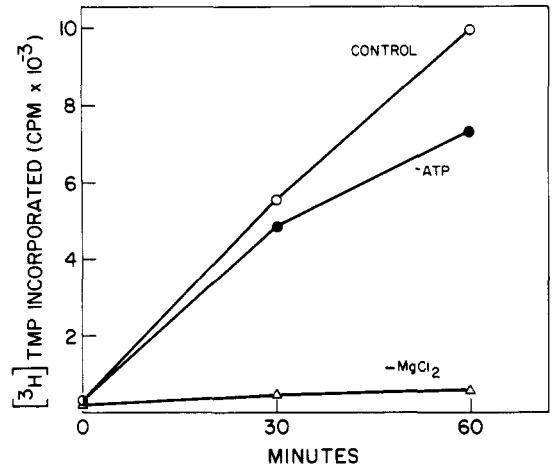

FIGURE 1: Kinetics of [^{3}H]TMP incorporation into acid-insoluble material in isolated chloroplasts. DNA synthesis was performed at 37 °C as described under Materials and Methods in the presence or absence of Mg^{2+} and ATP. The reaction was stopped at the indicated times by transfer of the sample onto ice. The final concentration of chloroplast protein in the assay was 310 μg/mL.

concentration of 25 μg/mL. After 30 min at 37 °C, the DNA solution was extracted twice with chloroform/isoamyl alcohol and DNA precipitated with ethanol as described above. The supercoiled form of the plasmid DNA was isolated by CsCl density gradient centrifugation in the presence of propidium iodide. The plasmid pZcp7 (see below) was prepared as described by Kopecko & Cohen (1975).

Construction of Recombinant Plasmids Containing PstI Fragments of Chloroplast DNA from Maize. *Pst*I-digested chloroplast DNA was ligated to *Pst*I-cut pBR322 to construct a library of plasmids containing corn chloroplast DNA fragments. So that self-ligation of the vector could be avoided, pBR322 linearized with *Pst*I was first treated with calf intestinal alkaline phosphatase to remove 5′-phosphate groups. Competent *Escherichia coli* RR1 cells (Peacock et al., 1981) were transformed with the ligation mixture. Tetracycline-resistant, ampicillin-sensitive colonies were grown up, and plasmid DNA was isolated according to Birnboim & Doly (1979). After electrophoresis on an agarose gel and transfer of the DNA to nitrocellulose according to Southern (1975), the chloroplast DNA containing plasmids were identified by hybridization to chloroplast DNA, which was labeled with [α-^{32}P]dATP by using random calf thymus DNA primers (Summers, 1975). The positive recombinant plasmids were digested with *Pst*I, and the inserted chloroplast DNA fragments were identified according to their molecular weight after electrophoresis on an agarose gel. The plasmid pZcp7 was found to contain a 4.54-kbp chloroplast DNA fragment.

Results

DNA Synthesis in Isolated Chloroplasts. Chloroplasts isolated from corn plant homogenates by differential centrifugation are capable of incorporating [^{3}H]TTP into acid-insoluble material in vitro for at least 60 min (Figure 1). Chloroplasts further purified on a percoll gradient, where starch grains, nuclei fragments, and broken chloroplasts can be separated from intact chloroplasts (Ortiz et al., 1980), exhibit the same amount of DNA synthesis per microgram of chloroplast protein and the same kinetics of [^{3}H]TTP incorporation (data not shown). To avoid the extensive loss of chloroplasts which occurs during percoll gradient centrifugation, we used partially purified chloroplasts, prepared as described under Materials and Methods, without a percoll gradient step, for the following experiments. Because intact

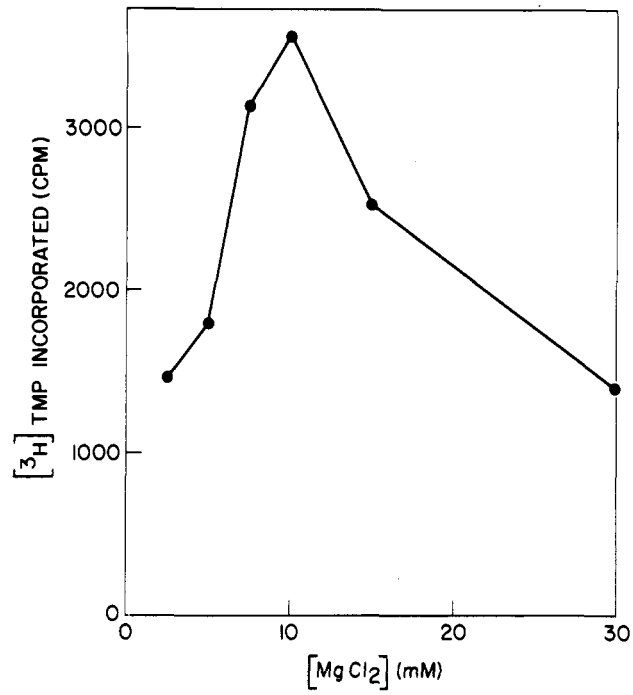

FIGURE 2: Influence of Mg^{2+} concentration on [^{3}H]TMP incorporation in isolated chloroplasts. Incubations were performed for 30 min at 30 °C as described under Materials and Methods in the presence of the indicated concentrations of $MgCl_2$. The concentration of [^{3}H]TTP was 2.2 μM (specific radioactivity 46 Ci/mmol). The concentration of chloroplast protein in the assay was 310 μg/mL.

chloroplasts have been reported not to be able to take up dTTP (Bohnert et al., 1974), the incorporation of the deoxynucleotides by our chloroplast preparations could be due to damage of the fragile chloroplasts during isolation or during in vitro incubation. Alternately, it is possible that intact corn chloroplasts do, in fact, incorporate deoxynucleotides at the level observed in these experiments.

Incorporation of [^{3}H]TTP into acid-insoluble material by chloroplasts increased linearly with the amount of chloroplasts added at least up to a final protein concentration, in the assay, of 310 μg/mL. Mg^{2+} ions were absolutely required for DNA synthesis (Figure 1, Table I), and the reaction was optimal at a Mg^{2+} concentration of 10 mM (Figure 2). Omission of the ribonucleoside triphosphates, except for ATP, from the assay mix had no significant effect (Figure 1, Table I). In the absence of ATP and the ATP regenerating system, the activity was reduced by 27% (Table I). When one or more deoxyribonucleoside triphosphates were omitted from the assay mix, incorporation of [^{3}H]TTP was decreased between 86 and 91% (Table I).

In vitro DNA synthesis in the isolated corn chloroplasts was found to increase linearly with temperature from 25 to 37 °C (Figure 3). Unless otherwise mentioned, a temperature of 37 °C was used for in vitro DNA synthesis assays. The reaction exhibited a very broad pH optimum between pH 7.5 and 8.1 (data not shown). In contrast to findings by Spencer & Whitfeld (1969) with chloroplasts isolated from spinach, KCl was inhibitory at all concentrations tested up to 200 mM. The inhibition by KCl is 21, 50, 60, and 80%, respectively, at KCl concentrations of 40, 80, 120, and 200 mM.

(A) Effect of Inhibitors. To further characterize the DNA synthesis in isolated corn chloroplasts, we studied the effect of different inhibitors.

Aphidicolin, which is a specific inhibitor of animal and plant DNA polymerase α (Huberman, 1981; Sala et al., 1980b), did not inhibit DNA synthesis up to 25 μg/mL (Figure 4).

Table I: Requirements for [^{3}H]TMP Incorporation into DNA in Isolated Chloroplasts or Chloroplast Extracts[a]

components	[^{3}H]TMP incorpn (% of control)	
	chloroplasts[b]	chloroplast extract[c]
complete system	100	100
–$MgCl_2$	4	0.3
–ribonucleoside triphosphates + ATP	91	103
–ATP (and ATP regenerating system)	73	207
–dATP	14	11
–dATP, –dCTP	11	4
–dATP, –dCTP, –dGTP	9	3

[a] Incorporation of [^{3}H]TMP in isolated chloroplasts and chloroplast extracts during a 60-min incubation at 37 °C was measured as described under Materials and Methods. The [^{3}H]TTP concentration in the assay with isolated chloroplasts was 10 μM (10 μCi/mmol). The final concentration of protein in the assay was 200 μg/mL (isolated chloroplasts) and 56 μg/mL (chloroplast extract). The complete system is detailed under Materials and Methods. [b] The 100% value is 0.98 pmol/60 min. [c] The 100% value is 1.0 pmol/60 min.

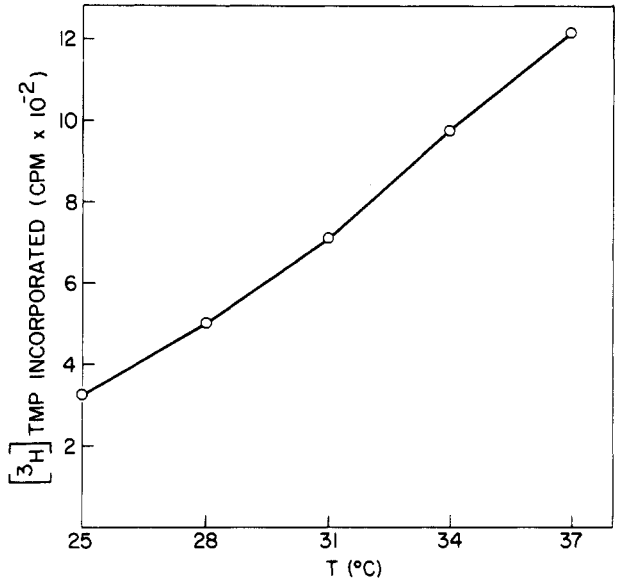

FIGURE 3: Influence of temperature on [^{3}H]TMP incorporation in isolated chloroplasts. Assays were performed for 30 min as described under Materials and Methods at the indicated temperatures. The concentration of [^{3}H]TTP was 2.2 μM (specific radioactivity 46 Ci/mmol), and the concentration of chloroplast protein in the assay was 930 μg/mL.

Nalidixic acid and rifampicin, which were reported to inhibit DNA synthesis in an in vitro system with chloroplasts from the algae *Chlamydomonas reinhardii* (Keller & Ho, 1981), also had no significant effect on the incorporation of [^{3}H]TTP in chloroplasts from corn (Table II). When dideoxythymidine triphosphate (dideoxy-TTP) was used, a 50% inhibition of DNA synthesis was observed at an inhibitor:substrate ratio of 12.5 (Figure 4). We do not consider this inhibition to be significant since it is known that dideoxy-TTP competitively inhibits DNA polymerases β and γ from animal cells at inhibitor:substrate ratios below 1 (Edenberg et al., 1978). DNA polymerase α is also partially inhibited by dideoxy-TTP but at much higher inhibitor concentrations (Zimmermann & Weissbach, 1981) which are comparable to the inhibitor concentrations we find are needed for 50% inhibition of the in vitro chloroplast DNA synthesis (Figure 4). This may be compared to the findings by Sala et al. (1980a), who showed that a DNA polymerase purified from spinach chloroplasts

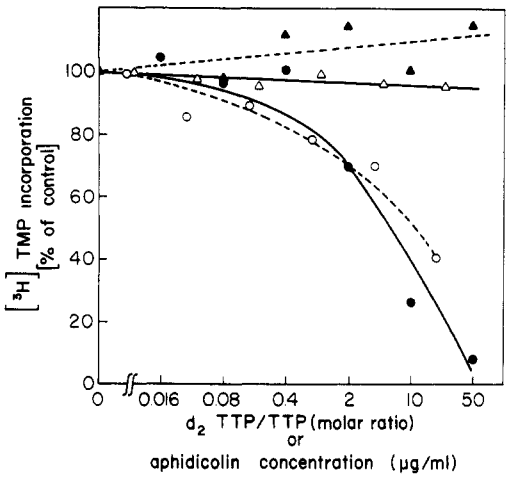

FIGURE 4: Influence of aphidicolin and dideoxythymidine triphosphate on [^{3}H]TMP incorporation in isolated chloroplasts and chloroplast extracts. Incorporation of [^{3}H]TMP in isolated chloroplasts and in chloroplast extracts during a 60-min incubation at 37 °C was measured in the standard assay (cf. Materials and Methods) with the indicated concentrations of the inhibitors. Aphidicolin was added from a stock solution in 50% dimethyl sulfoxide (Me_2SO), yielding a final Me_2SO concentration of 2.5% (v/v) (chloroplast experiment) and 5% (v/v) (chloroplast extract experiment). The final concentration of chloroplast protein in the intact chloroplast assay was 1070 μg/mL, and in the chloroplast extract assay it was 56 μg/mL. One hundred percent incorporation was 0.75 pmol in the aphidicolin experiment and 0.77 pmol in the dideoxythymidine triphosphate (d_2TTP) experiment for the intact chloroplast series, and 0.20 pmol (aphidicolin experiment) and 0.33 pmol (d_2TTP experiment) for the chloroplast extract series. Chloroplasts: (△) aphidicolin; (○) d_2TTP. Chloroplast extracts: (▲) aphidicolin; (●) d_2TTP.

Table II: Effect of Ethidium Bromide, *N*-Ethylmaleimide, Nalidixic Acid, and Rifampicin on [^{3}H]TMP Incorporation into DNA of Isolated Chloroplasts and Chloroplast Extracts[a]

		[^{3}H]TMP incorpn			
		chloroplast		chloroplast extract	
	concn	%	pmol	%	pmol
ethidium bromide	0	100	0.26	100	0.47
	0.8 μM	100		93	
	2 μM	10		50	
	20 μM	52		9	
	500 μM	23		2	
N-ethylmaleimide	0 mM	100	0.26	100	0.47
	0.5 mM	13		0.6	
	2 mM	10		0.6	
	8 mM	9		0.6	
nalidixic acid	0	100	0.98		
	1.6 μg/mL	100			
	40 μg/mL	99		nd[b]	
	1000 μg/mL	88			
rifampicin	0	100	0.21		
	0.16 μg/mL	100			
	4 μg/mL	107		nd	
	100 μg/mL	91			

[a] DNA synthesis assays were performed at 37 °C for 60 min as described under Materials and Methods. Inhibitors were added in 5 μL of water except for rifampicin which was dissolved in 50% ethanol. Control samples received an equal volume of the solvent alone. The final concentration of protein in the assay was between 855 and 1070 μg/mL in the chloroplast experiments. The assay with chloroplast extract contained 150 μg/mL protein. The numbers in the picomole columns are the picomoles of the [^{3}H]TMP incorporated in the controls. [b] nd, not determined.

is relatively insensitive toward dideoxy-TTP. Ethidium bromide, which inhibits DNA replication and transcription by intercalating between paired bases of DNA (Wang, 1974), reduced incorporation of [^{3}H]TTP in chloroplasts by 50% at

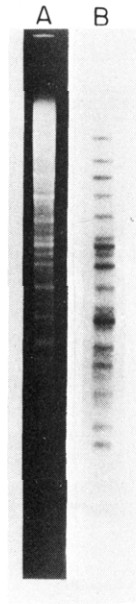

FIGURE 5: Restriction endonuclease digestion analysis of DNA synthesized in isolated chloroplasts. The standard assay (see Materials and Methods) was scaled up 5-fold to analyze the DNA synthesized in isolated chloroplasts. The dCTP and [^{3}H]TTP were replaced by 2 μM [α-^{32}P]dCTP (specific radioactivity 11 Ci/mmol) and 2 μM [α-^{32}P]TTP (specific radioactivity 16 Ci/mmol). The concentration of chloroplast protein was 1100 μg/mL. After incubation for 30 min at 30 °C, the DNA was isolated and digested with restriction endocnuclease *Bam*HI, and the DNA fragments were analyzed by electrophoresis in a 0.75% agarose gel (an aliquot of 8000 cpm out of a total of 24000 cpm fixed was used) as described under Materials and Methods. (A) Fluorescence pattern after ethidium bromide staining. (B) Autoradiography, 18-h exposure.

a concentration of 22 μM (Table II). The sulfhydryl group inhibitor *N*-ethylmaleimide inhibited DNA synthesis by 90% at 0.5 mM (Table II).

(B) Characterization of DNA Synthesized in Vitro by Chloroplasts. To analyze the nature of the in vitro synthesized DNA, we labeled isolated corn chloroplasts with [α-^{32}P]dCTP and [α-^{32}P]TTP for 30 min. Total chloroplast DNA was purified by proteinase K treatment and phenol extraction and digested with the restriction endonuclease *Bam*HI (Bedbrook & Bogorad, 1976). The resulting DNA fragments were separated by electrophoresis in a 0.75% agarose gel. In Figure 5, the fluorescence pattern of the ethidium bromide stained DNA is compared with the radioactivity of the DNA fragments. Most of the radioactive DNA precursor is incorporated into discrete fragments of chloroplast DNA (Figure 5B) despite the presence of significant amounts of what may be nuclear DNA in the crude chloroplast preparation which show up in the ethidium bromide fluorescence (Figure 5A). This interpretation is supported by the finding that, in the presence of 10 μg of aphidicolin/mL which should inhibit all DNA polymerase α activity arising from contaminating chromatin (Misumi & Weissbach, 1982), the same radioactive labeling pattern into discrete chloroplast DNA fragments is obtained (data not shown).

For estimation of the length of the DNA synthesized in isolated chloroplasts, the [^{3}H]TTP in the in vitro DNA synthesis assay was substituted by bromodeoxyuridine triphosphate (BrdUTP), and dCTP by [^{3}H]dCTP. The newly synthesized density labeled chloroplast DNA was purified by proteinase K treatment and phenol extraction and analyzed by density gradient centrifugation in CsCl before and after breakage of the DNA by sonication. ^{32}P-labeled chloroplast DNA isolated from corn seedlings, which were labeled with

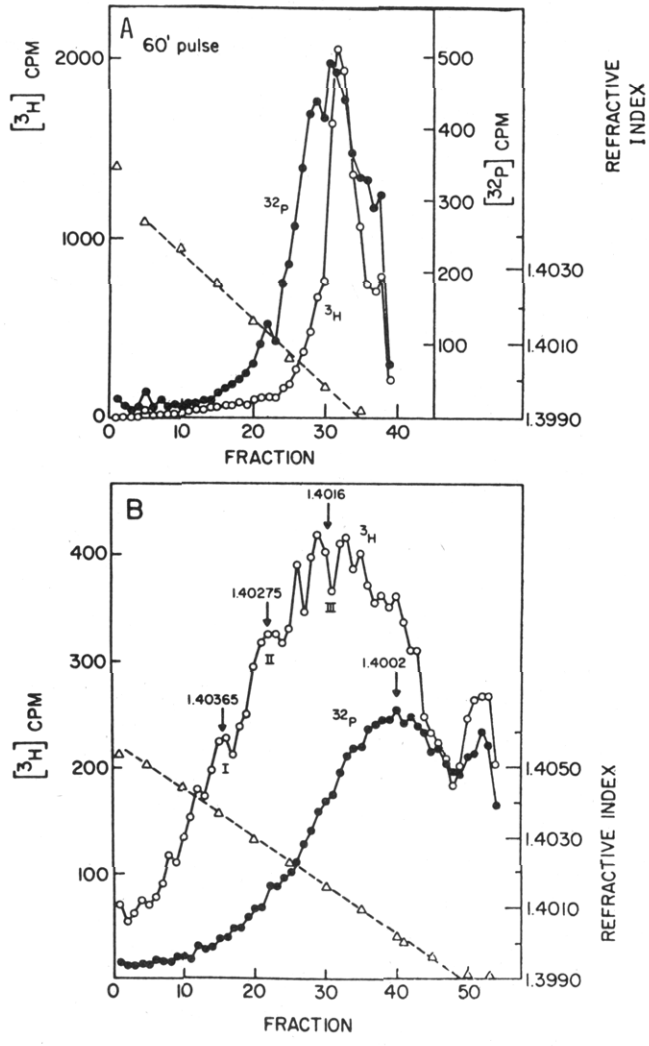

FIGURE 6: CsCl density gradient analysis of the chloroplast DNA synthesized in isolated chloroplasts in the presence of bromodeoxyribouridine triphosphate. In vitro density labeling, isolation, and CsCl density gradient analysis of chloroplast DNA were performed as described under Materials and Methods. The incubation time was 60 min, and a total of 25000 ^{3}H cpm was incorporated into DNA. As an internal density marker, 8000 cpm of ^{32}P-labeled chloroplast DNA, isolated from [^{32}P]phosphate-labeled corn plants, was added (see Materials and Methods). The density of the gradients increases from right to left, and selected densities are indicated by arrows. (○) ^{3}H-Labeled chloroplast DNA synthesized in vitro in the presence of BrdUTP. (●) ^{32}P-Labeled chloroplast DNA (marker). (A) Without sonication of DNA. (B) With sonication of DNA.

[^{32}P]orthophosphate, served as an internal density marker. With relatively intact chloroplast DNA, prior to sonication, no density shift between in vitro density ^{3}H-labeled DNA and marker [^{32}P]DNA could be observed in the density gradient centrifugation (Figure 6A). However, after sonication, which yielded DNA fragments of an average size of 0.5 kbp (as determined by electrophoresis in agarose gels), more than 50% of in vitro labeled chloroplast DNA was shifted to a higher density region of the CsCl gradient (Figure 6B). Upon recentrifugation of the material of higher buoyant density, the in vitro density labeled [^{3}H]DNA rebanded at the same high density, whereas most [^{32}P]DNA banded at the position of unsubstituted marker DNA (data not shown). From the average size of chloroplast DNA fragments, which was found to be the same throughout the gradient (data not shown), and the degree of density shift, a rate of DNA synthesis between 140 (peak III) and 340 (peak I) nucleotides/h could be calculated (Luk & Bick, 1977).

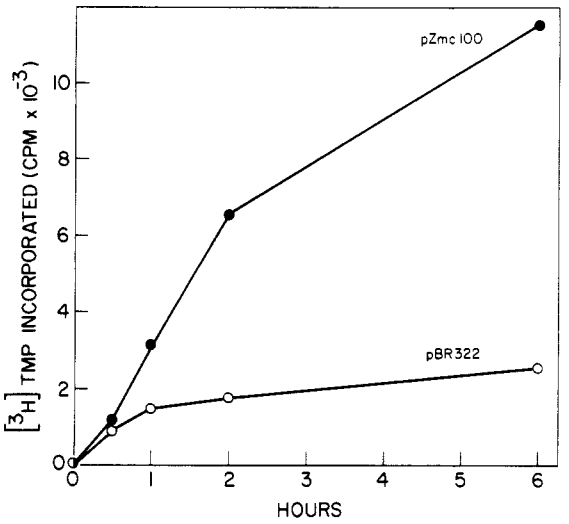

FIGURE 7: Kinetics of [^{3}H]TMP incorporation in chloroplast extracts. DNA synthesis in the chloroplast extracts (final protein concentration 28 μg/mL) for the indicated times with pZmc100 (●) or pBR322 (○) supercoiled plasmid DNAs as templates is shown. The incubation conditions are described under Materials and Methods. The concentration of both plasmid DNAs was 100 μg/mL.

DNA Synthesis in Chloroplast Extracts. Extracts, prepared from corn chloroplasts with high salt extraction as described under Materials and Methods, also catalyzed the incorporation of [^{3}H]TTP into acid-insoluble material in the presence of pZmc100 plasmid DNA as template. The recombinant plasmid pZmc100 consists of the bacterial plasmid pMB9 with an 11-kbp *Eco*RI fragment from corn chloroplast DNA, containing one set of ribosomal RNA genes of the maize chloroplast genome (Bedbrook et al., 1977). With this template incorporation of [^{3}H]TTP into DNA by chloroplast extracts is linear for 2 h and continues at a slower rate for at least 4 more h (Figure 7). Using the same amount of pBR322 plasmid DNA as template resulted in an initial incorporation rate similar to the reaction with the pZmc100 template. However, the rate of incorporation started to level off after 30 min (Figure 7). A similar selectivity was observed when the recombinant plasmid pZcp7 which also contains a chloroplast DNA fragment (cf. Materials and Methods) was used (data not shown). The incorporation of [^{3}H]TTP into DNA was proportional to the amount of extract up to a protein concentration of 285 μg/mL in the assay. The in vitro reaction was dependent on exogenously added DNA (Figure 8) since very little synthesis occurred in its absence. At a concentration of 100 μg of pZmc100 DNA/mL, a 10-fold stimulation of [^{3}H]TTP incorporation was observed as compared to that for the sample without added DNA.

(A) Other Requirements for DNA Synthesis by Chloroplast Extracts. In addition to a DNA template, Mg^{2+} ions were essential for in vitro DNA synthesis. When $MgCl_2$ was omitted from the reaction mix, the rate of [^{3}H]TTP incorporation dropped to 0.3% of the control value (Table I). The reaction was also dependent on deoxyribonucleoside triphosphate, and the omission of one or more of the deoxyribonucleoside triphosphates reduced the rate of DNA synthesis between 89 and 97% (Table I). On the other hand, ribonucleoside triphosphates in the presence of standard amounts of ATP did not influence the reaction (Table I). Surprisingly, the ATP generating system (phosphoenolpyruvate and pyruvate kinase) showed an inhibitory effect on DNA synthesis. In the absence of ATP and phosphoenolpyruvate, a 2-fold stimulation of incorporation of [^{3}H]TTP into acid-insoluble material was observed (Table I). The inhibition

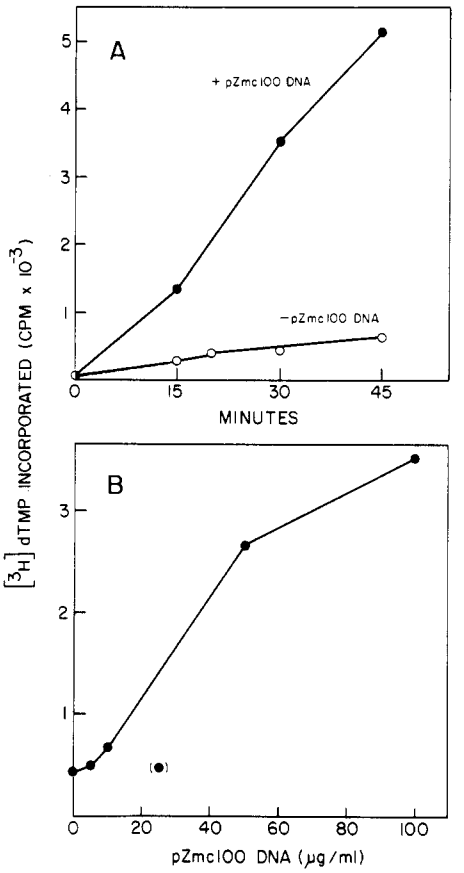

FIGURE 8: Requirement for exogenously added DNA for DNA synthesis in chloroplast extracts. The reactions with chloroplast extracts (final concentration of chloroplast protein was 580 μg/mL) were performed as described under Materials and Methods. The concentration of [^{3}H]TTP was 5 μM (specific radioactivity 20 Ci/mmol). (A) Kinetics of [^{3}H]TMP incorporation in the presence (●) or absence (○) of 100 μg of pZmc100 supercoiled plasmid DNA per mL. (B) Titration of DNA synthesis with pZmc100 plasmid DNA. The incubation was 30 min.

observed under standard assay conditions was due to the presence of ATP itself (data not shown). The same inhibitory effect was found when ATP was replaced by adenosine monophosphate or adenosine diphosphate. However, adenylyl imidodiphosphate, which is structurally related to ATP, inhibited [^{3}H]TTP incorporation much less. The slight inhibition of DNA synthesis by ATP in chloroplast extracts is not observed in the intact, isolated chloroplast whose DNA synthesis is stimulated by ATP (Table I).

To compare DNA synthesis observed in chloroplast extracts to the reaction in isolated chloroplasts, we studied the influence of different inhibitors. As we observed in isolated chloroplasts, aphidicolin had no effect on the [^{3}H]TTP incorporation in chloroplast extracts (Figure 4). On the other hand, DNA synthesis in chloroplast extracts was 3–4 times more sensitive toward dideoxy-TTP as compared to DNA synthesis in isolated chloroplasts. A 50% inhibition of [^{3}H]TTP incorporation was observed at a dideoxy-TTP:TTP ratio of 3.6 (Figure 4). In addition, DNA synthesis in chloroplast extracts with a supercoiled plasmid DNA template showed a higher sensitivity toward ethidium bromide. At a concentration of 2 μM ethidium bromide, the reaction was inhibited by 50% (Table II), a more than 10-fold increase in sensitivity toward this inhibitor than intact chloroplasts show. As found with DNA synthesis in isolated chloroplasts, [^{3}H]TTP incorporation in chloroplast extracts was inhibited greater than 90% by *N*-ethylmaleimide at a concentration of 0.5 mM (Table II).

Table III: In Vitro Labeling of Plasmid DNA Domains by Chloroplast Extracts[a]

plasmid DNA	ATP concn (μM)	restriction nuclease used	[α-^{32}P]TMP incorpn: size of fragment in base pairs	cpm	rel sp act.	DNA domain represented
pBr322	2000	*Hin*cII	3355	844	100	
			1007	313	109	
pBr322	2000	*Bgl*I	2319	543	100	
			1809	391	89	
			234	nd[b]		
pZcp7	25	*Pst*I	4540	4225	100	chloroplast
		*Bam*HI	3238	4070	137	vector
			1124	1377	122	vector
pZcp7	2000	*Pst*I	4540	6007	100	chloroplast
		*Bam*HI	3238	3100	73	vector
			1124	1285	88	vector
pZcp7	2000	*Pst*I	4540	3521	100	chloroplast
		*Sal*I	2961	1783	79	vector
			1401	740	64	vector

[a] For the calculation of the relative specific activity, the radioactivity of the DNA fragments was corrected for the blank value (50 cpm) and divided by their respective molecular weights. The specific radioactivity of the largest fragment was assigned a value of 100. [b] nd, not determined.

To further characterize the DNA synthesis in chloroplast extracts, we analyzed the reaction products formed with different plasmid DNA templates by electrophoresis in agarose gels. The plasmid supercoiled DNA template is very efficiently relaxed by chloroplast extracts, forming DNA molecules with reduced numbers of superhelical turns and completely relaxed and open circular forms. This is due to an apparent topoisomerase I activity which is present in the chloroplast extracts. In longer incubations, a small percentage of linear plasmid molecules could be observed, and these may result from the action of a nuclease. When the radioactivity incorporated into DNA during incubation of the chloroplast extract with [α-^{32}P]TTP as substrate was analyzed, only open circular or linear forms of the plasmid DNAs were labeled (Figure 9, bands c and d). When pZmc100 plasmid DNA was used as a template, we also observed the formation of a labeled molecular species with low mobility in agarose gels (Figure 9, bands a and b). No radioactive DNA precursor was observed to be incorporated into the supercoiled form of the plasmid until 60 min, when a trace of label was detected in the position of the supercoiled DNA (this is not apparent in Figure 9).

To determine if the observed DNA synthesis with the plasmid DNA templates starts at unique initiation sites or is random, we analyzed the reaction products made with either pBR322, pZmcp7, or pZmc100 DNA templates during different incubation times in the presence of high and low concentrations of ATP. Two different concentrations of ATP were used because of the observed inhibition of DNA synthesis in extracts by high concentrations of ATP (Table I). After in vitro synthesis, the plasmid DNA was isolated from the reaction mixture and cleaved with restriction endonucleases to determine if DNA synthesis had preferentially occurred on the chloroplast DNA segment of the pZmcp7 plasmid. When the specific radioactivity of DNA fragments of pBR322 derived from either *Hin*cII or *Bgl*I endonuclease cleavage was analyzed, a minor difference in the specific radioactivity of individual fragments was observed (Table III). This rather small difference did not change with pulse length (data not shown). Similar results were obtained when fragments derived from pZmc100 plasmid DNA template were analyzed by a double digestion with *Eco*RI and *Bam*HI (data not shown). However, when pZcp7 plasmid DNA which contains a 4.54-kbp *Pst*I fragment of corn chloroplast DNA was used, a preferential labeling of the pBR322 portion of the recombinant

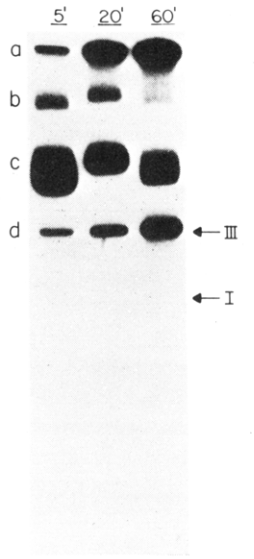

FIGURE 9: Agarose gel electrophoresis of [^{32}P]DNA synthesized in chloroplast extracts with pZmc100 plasmid DNA as template. For analysis of in vitro synthesized DNA, the standard assay (see Materials and Methods) was scaled up 4 times. [^{3}H]TTP was replaced by 5 μM [α-^{32}P]TTP (100 Ci/mmol), and samples were incubated at 37 °C for the indicated times. The final concentration of protein was 56 μg/mL. ^{32}P-Labeled plasmid DNA was isolated and analyzed by electrophoresis on a 0.6% agarose gel as described under Materials and Methods. The origin of the gel was 2 mm above bands a.

DNA molecule was observed in a 5-min incubation at an ATP concentration of 25 μM. In the presence of 2000 μM ATP, however, the inverse labeling pattern was observed. After a 5-min incubation, the chloroplast portion of pZcp7 DNA showed a 1.3-fold higher specific radioactivity. We, thus, conclude that if specific initiation is occurring, it is at a low level and may be obscured by the random DNA synthesis taking place on these plasmid DNA templates in the crude extract.

Discussion

The ability of intracellular compartments to synthesize DNA in vitro has previously been noted for both nuclei and mitochondria isolated from animal cells, and numerous studies have characterized these systems (Krokan et al., 1975; Zimmermann et al., 1980). Chloroplast DNA replication in the

unicellular organism *Chlamydomonas reinhardtii* has also been studied, and in vitro DNA synthesis by isolated chloroplasts has been achieved (Keller & Ho, 1981). However, little information is available about DNA synthesis in isolated plastids in higher plants, and it is not clear, in fact, whether true chloroplast DNA synthesis has ever been observed in vitro (Hermann & Possingham, 1980). The present study clearly shows that chloroplast DNA synthesis does occur in isolated maize chloroplasts or crude chloroplast extracts.

The characteristics of in vitro DNA synthesis by chloroplasts are similar to those observed for other isolated organelles in that Mg^{2+} and deoxynucleotide precursors are required. The marked dependence on ATP which isolated animal cell nuclei exhibit (Krokan et al., 1975) is not apparent in isolated chloroplast, and higher levels of ATP are, in fact, inhibitory to the ability of chloroplast extracts to synthesize DNA. We also observe a linear response of the isolated chloroplast to increasing temperature but a striking inhibition by KCl. It is also interesting that neither aphidicolin nor dideoxynucleoside triphosphates are potent inhibitors of chloroplast DNA synthesis. Since aphidicolin is a powerful inhibitor of plant and animal DNA polymerase α (Huberman, 1981; Sala et al., 1980b; Misumi & Weissbach, 1982), this result indicates that this enzyme is probably not involved in chloroplast DNA replication, a conclusion previously reached by Sala et al. (1980a). These workers also noted that a partially purified preparation of a new DNA polymerase found in chloroplasts was also not inhibited by dideoxy-TTP. In contrast to in vitro and in vivo DNA synthesis in chloroplasts from the algae *Chlamydomonas reinhardtii* and *Euglena gracilis* (Keller & Ho, 1981; Pienkos et al., 1974), DNA synthesis in isolated chloroplasts from maize is not inhibited by rifampicin or nalidixic acid (Table II). However, the sensitivity of the chloroplast systems to the intercalating agent, ethidium bromide, may be a useful tool, particularly in studies employing chloroplast extracts and exogenous supercoiled DNA templates. Thus, it is known that *Chlamydomonas* chloroplasts' in vitro ct DNA synthesis is very sensitive to ethidium bromide (Keller & Ho, 1981).

A primary objective of this study was to demonstrate that synthesis of chloroplast DNA was, in fact, occurring in vitro. The restriction endonuclease pattern of new DNA synthesized in the isolated chloroplast clearly shows this to be the case. These data, and the density label experiments, reveal that the size of the newly synthesized chloroplast DNA reaches several hundred nucleotides in length in 1 h, and it seems to be distributed throughout the chloroplast DNA. It is unclear, therefore, whether the DNA synthesis we observe reflects a replicative type of DNA synthesis or is a repair synthesis in which large stretches of DNA are replaced. This type of reaction is known to occur in chloroplasts of *Euglena gracilis*, where, during development of the chloroplasts, stretches of $(1.5–6) \times 10^3$ nucleotides are replaced without net synthesis of DNA (Walfield & Hershberger, 1978). In addition, our experiments with chloroplast extracts have not yet revealed any specific origins of DNA synthesis in the chimeric chloroplast plasmid DNAs used as templates, so this issue remains to be resolved. We do observe that DNA synthesis on the exogenous circular DNA template seems to take place in the open circular or linear forms of the plasmid DNA. This is not unexpected since DNA replication of circular plasmid or mitochondrial DNA is known to require opening or relaxation of the supercoiled circular form (Bogenhagen et al., 1978). In addition, we observe a topoisomerase activity in our chloroplast extracts which can relax supercoiled covalently closed circular DNA (data not shown). Our studies also reveal the formation of higher molecular weight labeled DNA products formed from the exogenous chimeric DNA template in the chloroplast extracts. Whether these are circular intermediates or larger DNA structures of another type remains to be elucidated. A more thorough understanding of the DNA synthesis observed with the isolated chloroplast or, equally interesting, in the soluble extracts obtained from chloroplasts remains a primary goal of our future studies.

References

Bedbrook, J. R., & Bogorad, L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4309–4313.

Bedbrook, J. R., & Kolodner, R. (1979) *Annu. Rev. Plant Physiol. 30*, 593–620.

Bedbrook, J. R., Kolodner, R., & Bogorad, L. (1977) *Cell (Cambridge, Mass.) 11*, 739–749.

Birnboim, H. C., & Doly, J. (1979) *Nucleic Acids Res. 7*, 1513–1523.

Bogenhagen, D., Gillum, A. M., Martens, P. A., & Clayton, D. A. (1978) *Cold Spring Harbor Symp. Quant. Biol. 43*, 253.

Bohnert, H. J., Schmitt, J. M., & Hermann, R. G. (1974) *Port. Acta Biol., Ser. A 14*, 71.

Bost, P., & Grivell, L. A. (1981) *Nature (London) 289*, 439.

Edenberg, H. J., Anderson, S., & DePamphilis, M. D. (1978) *J. Biol. Chem. 253*, 3273–3280.

Hermann, R. G., & Possingham, J. V. (1980) *Results Probl. Cell Differ. 10*, 65.

Huberman, L. A. (1981) *Cell (Cambridge, Mass.) 23*, 647–648.

Kasamatsu, H., Grossman, L. I., Robberson, D. L., Watson, R., & Vinograd, J. (1974) *Cold Spring Harbor Symp. Quant. Biol. 38*, 281.

Keller, S. J., & Ho, C. (1981) *Int. Rev. Cytol. 69*, 157–190.

Kolodner, R. J., & Tewari, K. K. (1975a) *J. Biol. Chem. 250*, 8840–8847.

Kolodner, R. J., & Tewari, K. K. (1975b) *Nature (London) 256*, 708–711.

Kolodner, R. J., & Tewari, K. K. (1975c) *Biochim. Biophys. Acta 402*, 372–390.

Kopecko, D. J., & Cohen, S. N. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1373–1377.

Krokan, H., Bjrklid, E., & Prydz, H. (1975) *Biochemistry 14*, 4227–4232.

Lewin, B. (1980) in *Gene Expression*, Vol. 2, Chapter 21, Wiley, New York.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Luk, D. C., & Bick, M. D. (1977) *Anal. Biochem. 77*, 346–349.

Misumi, M., & Weissbach, A. (1982) *J. Biol. Chem. 257*, 2323–2329.

Norgard, M. V., Emigholt, K., & Monahan, J. J. (1979) *J. Bacteriol. 138*, 270–272.

Olson, J. M. (1981) *Ann. N.Y. Acad. Sci. 361*, 8–19.

Ortiz, W., Reardon, E. M., & Price, C. A. (1980) *Plant Physiol. 66*, 291–294.

Peacock, S. L., McIver, C. M., & Monahan, J. J. (1981) *Biochim. Biophys. Acta 655*, 243–250.

Pienkos, R., Walfield, A., & Hershberger, C. L. (1974) *Arch. Biochem. Biophys. 165*, 548–553.

Robberson, D. L., Kasamatsu, H., & Vinograd, J. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 737–741.

Rochaix, J. D., & Malnoe, P. (1978) *Cell (Cambridge, Mass.) 15*, 661.

Sala, F., Amileni, A. R., Parisi, B., & Spadari, S. (1980a) *Eur. J. Biochem. 112*, 211.
Sala, F., Parisi, B., Burroni, D., Amileni, A. R., Pedrali-Noy, G., & Spadari, S. (1980b) *FEBS Lett. 117*, 93–98.
Schnepf, E. (1980) *Results Probl. Cell Differ. 10*, 1–27.
Southern, E. M. (1975) *J. Mol. Biol. 98*, 503–517.
Spencer, D., & Whitfeld, P. R. (1969) *Arch. Biochem. Biophys. 132*, 477–488.
Summers, J. (1975) *J. Virol. 15*, 946–953.
Walfield, A. M., & Hershberger, C. L. (1978) *J. Bacteriol. 133*, 1437–1443.
Wang, J. C. (1974) *J. Mol. Biol. 89*, 783–801.
Zimmermann, W., & Wissbach, A. (1981) *Mol. Cell. Biol. 1*, 680–686.
Zimmermann, W., Chen, S. M., Bolden, A., & Wissbach, A. (1980) *J. Biol. Chem. 255*, 11847–11852.

Antitumor Proteins of *Streptomyces macromomyceticus*: Purification and Characterization of Auromomycin, Macromomycin A, and Macromomycin D†

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ABSTRACT: Macromomycin A and the two related proteins auromomycin and macromomycin D were isolated from the culture filtrates of *Streptomyces macromomyceticus* by chromatography on columns of DEAE-cellulose, Amberlite XAD-7, and decylagarose. Antibodies prepared against macromomycin A showed antigenic identity by Ouchterlony double diffusion between the three purified proteins. This similarity was further demonstrated by their behavior on disc gel electrophoresis, the amino acid compositions, and comparative peptide mapping of the aminoethylated derivatives. They differed, however, in other chemical and biological properties. Auromomycin and macromomycin A, p*I* 5.4, have antibiotic activity, which is absent in macromomycin D, p*I* 5.2. This antibiotic activity was associated with chromophore groups that were extractable by methanol. High-pressure liquid chromatography of the methanol extracts gave difference profiles for each of the purified proteins. The differences in the three proteins extended to their ultraviolet–visible spectra, fluorescence and circular dichroism, and the changes of these properties with heating. The heat denaturation, with auromomycin and macromomycin melting at 70.5 °C and macromomycin D at 57.0 °C, was reversible. Changes were noted in the spectra both during and following heating at 80 °C; the antibacterial activity was lost in auromomycin and only partially reduced in macromomycin A. The properties of the three proteins support the general similarities in their polypeptide structures, modifications in the properties of which are endowed by the differences in the associated nonprotein chromophores.

Several distinguishable but related proteins with antibiotic and antitumor activity have been isolated from the culture filtrates of *Streptomyces macromomyceticus*. Macromomycin (Chimura et al., 1968), purified to chemical homogeneity (Yamashita et al., 1976; Im et al., 1978), was cytotoxic to a variety of tumor cells both in vivo and in vitro. It inhibited the incorporation of thymidine into DNA (Beerman, 1978; Im et al., 1978; Suzuki et al., 1978; Vandrê et al., 1979) and caused single-stranded cleavage of cellular DNA in vivo (Beerman, 1978; Suzuki et al., 1978; Kappen et al., 1979; Vandrê & Montgomery, 1979). A second antitumor protein, auromomycin,[1] with comparatively greater biological activity, has been obtained from *S. macromomyceticus* (Yamashita et al., 1979). This protein shares several chemical and biological properties with macromomycin and can be converted into macromomycin by chromatography on Amberlite XAD-7, with the loss of a chromophoric material that absorbed at 355 nm. As with the antitumor antibiotic neocarzinostatin (Napier et al., 1979, 1980; Koide et al., 1980), a methanol-extractable chromophore, having both antitumor and antibiotic activity, is also associated with auromomycin (Suzuki et al., 1980; Woynarowski & Beerman, 1980; Kappen et al., 1980b).

Both macromomycin and auromomycin cleaved cellular DNA in vivo, but it is not clear whether both are capable of the same reaction in vitro. Although auromomycin and its chromophore cleaved DNA in vitro under a variety of experimental conditions (Kappen et al., 1979, 1980b; Suzuki et al., 1979b, 1980), the cleavage of DNA by macromomycin under similar conditions was not observed. However, cleavage of DNA by macromomycin in vitro has been demonstrated with high concentrations of protein after previous reduction with sodium borohydride (Suzuki et al., 1979a). Some of the observed differences in the DNA strand breakage by macromomycin may be related to the method of purification. Macromomycin, prepared by elution from Amberlite XAD-7 with water, is devoid of biological activity but blocks the cleavage of DNA by auromomycin (Kappen & Goldberg, 1979; Napier et al., 1980; Kappen et al., 1980b), and it has been suggested that the biological activity present in other macromomycin preparations may be the result of contaminating auromomycin (Kappen et al., 1980b). However, it

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[1] A new nomenclature is used in this paper for the purified proteins isolated from culture filtrates of *S. macromomyceticus*. Macromomycin D is a biologically inactive protein with an isoelectric point of 5.2. Biologically active protein having no absorbance at 355 nm is referred to as macromomycin A and has the same properties as macromomycin purified from auromomycin-free culture filtrates (Im et al., 1978; Yamashita et al., 1976; Vandrê et al., 1979). Auromomycin is the biologically active protein with an absorbance at 355 nm and has the same properties as previously reported (Yamashita et al., 1979). The macromomycin prepared by Kappen et al. (1980b), having no biological activity, is designated as apomacromomycin.

0006-2960/82/0421-3343$01.25/0