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Preferential transcription of cloned maize chloroplast DNA sequences by maize chloroplast RNA polymerase

(S factor/plasmids/supercoiled DNA)

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ABSTRACT *Zea mays* **chloroplast DNA-dependent RNA polymerase *in vitro* preferentially transcribes maize chloroplast DNA sequences incorporated in cloned chimeric bacterial plasmids. Preferential transcription is dependent on the presence of a 27.5-kilodalton polypeptide, the S factor, which has been purified from maize chloroplasts, and also on the template's being in the supercoiled form.**

Zea mays chloroplast chromosomes occur largely or entirely as 91.1 × 10^6-dalton supercoiled circles (1) *in vivo*. To study the effect of the physical form of the template on specificity of transcription by maize chloroplast (cp) RNA polymerase (RNA-P), we constructed chimeric bacterial plasmids (2–4) containing small defined maize cp DNA sequences and cloned them in *Escherichia coli* to use as templates for the polymerase. This is a step towards developing an *in vitro* system capable of mimicking transcription of chloroplast genes as it occurs *in vivo*.

Expression of the chloroplast gene for the large subunit of ribulose bisphosphate carboxylase is transcriptionally regulated in the differentiation of bundle sheath from mesophyll cells in *Zea mays* leaves (5) and the expression of the chloroplast chromosome's photogene 32 is transcriptionally controlled during photoregulated plastid development (6, 7). cp RNA-P transcriptional activity also rises sharply during light-induced plastid maturation (8, 9). Reconstituted systems capable of selective transcription of chloroplast genes *in vitro* would greatly facilitate analyses of these (5, 6) developmental programs for which transcriptionally regulated genes have been cloned.

Maize plastid RNA polymerase, in a highly purified form (10, 11), is comprised of polypeptides of about 180, 140, 100, 95, 85, and 40 kilodaltons (kDal). We describe here a 27.5-kDal polypeptide (designated S) from maize plastids that accelerates transcription of circular DNA by maize plastid RNA-P and promotes this enzyme's preferential transcription *in vitro* of maize cp DNA sequences inserted into bacterial plasmids. Selectivity depends upon the template DNA's being in a supercoiled form.

MATERIALS AND METHODS

DNA. The plasmid pZmc134 is a chimera of pMB9 plus *Eco*RI fragments *a* and *1* of maize cp DNA (3, 12, 13). *Eco*RI fragment *a* contains cp rRNA genes plus unidentified sequences. The plasmid pZmc150 is a chimera of pMB9 and maize cp DNA *Eco*RI fragment *1*. Supercoiled plasmid DNA was purified from bacterial lysates by $CsCl_2$/ethidium bromide density gradient centrifugation (13). Work with recombinant plasmids was carried out at the P2 level of physical containment as specified in National Institutes of Health Guidelines on Recombinant DNA Research. Calf thymus DNA fraction I was obtained from Sigma.

Endonucleases, Agarose Gel Electrophoresis, and Hybridization. Restriction endonucleases from New England Biolabs or Bethesda Research Laboratories were used as specified by the suppliers. DNA digested with restriction endonucleases was fractionated by agarose gel electrophoresis (12). DNA fragments were transferred (14) from agarose gels to strips of Millipore filter paper (HAWP00010). Hybridization with radioactive RNAs was in 0.3 M NaCl/0.03 M Na citrate at 66°C for 16 hr. Hybridization was revealed by autoradiography.

Quantitative Hybridization. pZmc150 or pMB9 DNA was denatured in 0.9 M NaCl/0.09 M Na citrate/0.5 M NaOH (1 μg of DNA per 50 μl) at 37°C for 1 hr and diluted in 0.9 M NaCl/0.09 M Na citrate/3 mM $MgSO_4$ to 1 μg of DNA per ml. DNA was loaded under gravity onto nitrocellulose filters (S&S, BA85) presoaked in 0.9 M NaCl/0.09 M Na citrate. Both sides of the filters were washed with 50 ml of 0.9 M NaCl/0.09 M Na citrate and they were baked at 80°C for 2 hr under decreased pressure. Three discs were cut from each filter with a no. 4 corkborer (0.385 cm^2). Discs were placed in vials containing preheated [^{3}H]RNA (80°C for 5 min) in 100 μl of 50% formamide in 0.3 M NaCl/0.03 M Na citrate. Hybridization was at 39°C for 18 hr under mineral oil. Filters were washed with chloroform, 0.6 M NaCl/0.06 M Na citrate, and 0.3 M NaCl/0.03 M Na citrate (1 ml/wash, five times) at 60°C. After one wash with 0.3 M NaCl/0.03 M Na citrate at room temperature, filters were treated with RNase (20 μg/ml in 0.3 M NaCl/0.03 M Na citrate) at room temperature for 30 min and were further washed with 0.225 M NaCl/0.0225 M Na citrate at room temperature three times. Filters were dried and counted in Scintiverse. These procedures are adapted from Roon *et al.* (15) and Mullinix *et al.* (16).

Solubilization and Preparation of cp RNA-P and Crude S Factor. Maize (*Zea mays* FR9cms × FR 37, Illinois Foundation Seeds, Inc., Champaign, IL) seeds were germinated and grown in vermiculite in a darkroom at 28°C. After 7 days, the plants were illuminated for 16 hr by fluorescent lamps. Intact chloroplasts were isolated and purified by sucrose gradient centrifugation as described (10, 17). Plastid fragments were washed twice with magnesium-free buffer containing 25 mM Tricine·KOH (pH 8.5), 40 mM 2-mercaptoethanol, and 50 μg of phenylmethylsulfonyl fluoride ($PhMeSO_2F$) per ml. The pelleted plastid fragments were suspended in an equal volume of the above buffer. RNA-P was solubilized by incubating the plastid suspension at 37°C for 20 min with occasional agitation. After centrifugation at 27,000 × *g* for 30 min, the supernatant was loaded directly onto a DEAE-cellulose column for further purification of RNA-P and preparation of factor S.

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Abbreviations: RNA-P, DNA-dependent RNA polymerase; cp, chloroplast; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; kDal, kilodaltons; kbp, kilobase pair(s).

RNA-P Assay. Each 0.1 ml standard assay mixture contained 5 μmol of Tris·HCl (pH 8.0); 4 μmol of 2-mercaptoethanol; 2.5 μmol of $MgCl_2$; 0.1 μmol each of ATP, CTP, and GTP; 10 or 5 nmol of [^{3}H]UTP (0.2 or 0.4 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) or [^{32}P]UTP; DNA as indicated; and 10 μl of RNA-P solution. After incubation at 37°C for 20 min, the reaction was stopped by the addition of 3 ml of cold 5% trichloroacetic acid in 20 mM sodium pyrophosphate. The acid-precipitable material was collected and assayed for radioactivity as described (17).

RESULTS

Isolation of Maize cp RNA-P S Factor. As shown in Fig. 1, cp RNA-P eluted from DEAE-cellulose with about 0.2 M KCl. The activity with calf thymus DNA as a template (about 10 pmol/μg of protein per hr) was about 50 times greater than when supercoiled plasmid DNA was the template.

Addition to the RNA-P mixture of a 10-μl aliquot of the fraction that elutes from DEAE-cellulose with a 0.5 M KCl step stimulated the transcription of plasmid DNA, but not of calf thymus DNA (Fig. 1 and Table 1). The material in this fraction is designated the S factor.

Purification and Properties of S Factor. The S factor-containing fraction was essentially free of RNA-P activity and DNA on elution from DEAE-cellulose. The complete reaction mixture containing S led to the incorporation of 2598 cpm of [^{3}H]UMP in 30 min at 37°C; minus S, incorporation was 570 cpm; minus DNA, 117; minus polymerase, 101 (assay conditions were as described in Table 1 with pZmc134 DNA). Sometimes, particularly when the scale of purification was increased, preparations contained small amounts of DNA. Rechromatography of the S factor-containing fraction through a DEAE-cellulose column and elution by a step gradient generally removed nearly all DNA from the S factor preparation.

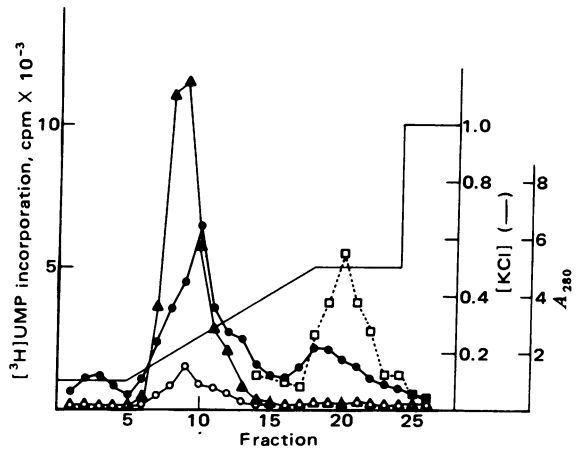

FIG. 1. Elution of maize cp RNA-P and S polypeptide during DEAE-cellulose column chromatography. Protein solubilized from chloroplasts from 600–700 g of leaves was applied to a 0.6 × 10 cm DEAE-cellulose column that had been equilibrated with 50 mM Tricine·KOH (pH 8.5), 40 mM 2-mercaptoethanol, 50 mM KCl, 30% glycerol, 5 mM $MgCl_2$, and 50 μg of $PhMeSO_2F$ per ml. After the column was washed with two vol of equilibration solution, it was developed with a linear gradient of 0.05–0.5 M KCl (7.5 ml total) in the same solution. Fractions of 0.5 ml were collected. RNA-P in the region of peak activity was collected and dialyzed overnight against 1 liter of buffer (50 mM Tris·HCl, pH 8/0.1 mM EDTA/20 mM 2-mercaptoethanol/50% glycerol). RNA-P was stored at −80°C without loss of activity. For S assay, 10 μl of dialyzed RNA-P (from elution peak) was included in each reaction. ▲, 10 μg of calf thymus DNA template; ○, 5 μg of supercoiled pZmc134 DNA template; □, S assay (5 μg of supercoiled pZmc134 template plus 10 μl of RNA-P solution); ●, A_{280}.

Table 1. Effect of S factor on transcription of various DNAs by maize cp RNA-P

DNA	pmol [^{3}H]UMP incorporated/ 30 min at 37°C +S	−S	Ratio (+/−)
Supercoiled pZmc134*	41.7	8.1	5.1
Circular pZmc134*	34.8	7.2	4.8
Denatured pZmc134†	63.4	62.7	1.0
Fragments of pZmc134‡	78.6	49.0	1.6
"Native" calf thymus§	460.0	450.0	1.0
Denatured calf thymus†	399.0	389.0	1.0
T-4	12.1	3.4	3.6

The standard assay mixture was used with 5 μmol of KCl, 5 nmol of [^{3}H]UTP (0.4 Ci/mmol), 5 μg of DNA (except 1.8 μg for T-4 DNA), 10 μl of cp RNA-P, and 10 μl of S solution (DEAE-cellulose fraction) where indicated.

* See text. Circular DNA: relaxed plus open circular.

† Heated at 100°C for 10 min and quickly cooled.

‡ After digestion with *Eco*RI and *Bam*HI, fragments of pZmc134 were extracted with phenol, washed twice in ether, precipitated with ethanol, dried, and dissolved.

§ Sigma.

(Omission of magnesium from the elution buffer increased the yield of DNA-free S factor but the recovery of RNA-P activity was lower.)

For further purification, S factor eluted from DEAE-cellulose (e.g., in about 1.5 ml) was dialyzed against buffer containing 50 mM Tris·HCl (pH 8), 50 mM KCl, 5 mM $MgCl_2$, 40 mM 2-mercaptoethanol, 50 μg of $PhMeSO_2F$ per ml, and 30% glycerol and applied to a small (0.6 × 5 cm) phosphocellulose column equilibrated with the same solution. S factor eluted at or shortly behind 1 column vol.

Additional purification of S factor was by filtration through Sephadex G-150 (0.8 × 115 cm column equilibrated with 50 mM Tris·HCl, pH 8.0/50 mM $MgCl_2$/40 mM 2-mercaptoethanol/10% glycerol/50 μg of $PhMeSO_2F$ per ml. One milliliter of S-containing solution eluted from phosphocellulose was applied to the Sephadex column calibrated with blue dextran, bovine serum albumin (68 kDal), ovalbumin (43 kDal), carbonic anhydrase (29 kDal), and cytochrome *c* (12.4 kDal). S eluted at about the 26-kDal position. Electrophoresis on a denaturing polyacrylamide gel revealed a single major band of approximately 29 kDal (Fig. 2). The mean value, 27.5 kDal, is used in this text.

S is completely inactivated by Pronase or by boiling for 5 min but is not inactivated by heating to 50°C for 5 min at pH 8.

As shown in Table 2, S does not replace the σ factor of *E. coli* RNA-P nor does the bacterial σ factor replace the S in the maize cp RNA-P transcription rate assay.

Purified rho factor from *E. coli* catalyzes RNA-dependent hydrolysis of ribulonucleoside triphosphate to the diphosphate and inorganic phosphate (21). Maize S displays no ATPase activity in a test with poly(C) and [γ-^{32}P]ATP.

That S does not affect transcription by permanently altering the DNA template was shown by incubating pZmc134 DNA with or without this polypeptide or without cp RNA-P at 37°C for 60 min and then analyzing the DNA by gel electrophoresis on 0.75% agarose. The proportions of supercoiled and circular (relaxed plus open circular) DNA were unchanged regardless of the presence of RNA-P or S in the incubation mixture. In another experiment supercoiled pZmc134 DNA (13 μg) was preincubated with or without S in 80 μl of buffer containing 4 μmol of Tris·HCl (pH 8.0), 2 μmol of $MgCl_2$, 4 μmol of KCl, and 3.2 μmol of 2-mercaptoethanol. After 30 min at 37°C, DNA was extracted with phenol and then ether, precipitated

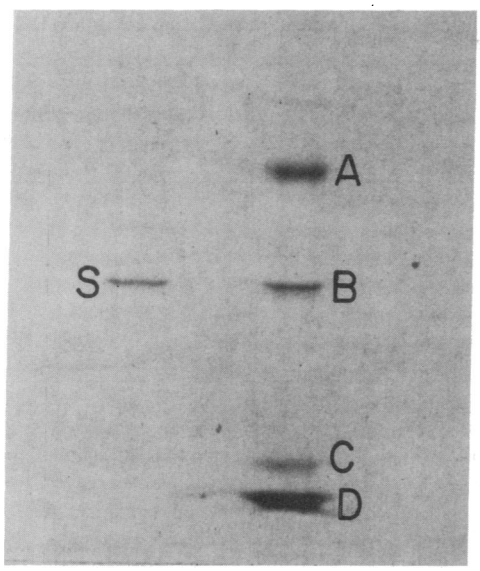

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis (18) of S factor purified through DEAE-cellulose (Fig. 1), phosphocellulose, and Sephadex G-150. S factor was dialyzed against 5 mM ammonium bicarbonate; lyophilized; dissolved in 62.5 mM Tris·HCl, pH 6.8/0.7 mM 2-mercaptoethanol/10% glycerol/3% $NaDodSO_4$; and heated at 90°C for 2 min. The 12% polyacrylamide gel was stained with Coomassie brilliant blue (19). Molecular weight standards: A, ovalbumin (43 kDal); B, carbonic anhydrase (29 kDal); C, lysozyme (14.3 kDal); D, RNase A (13.7 kDal).

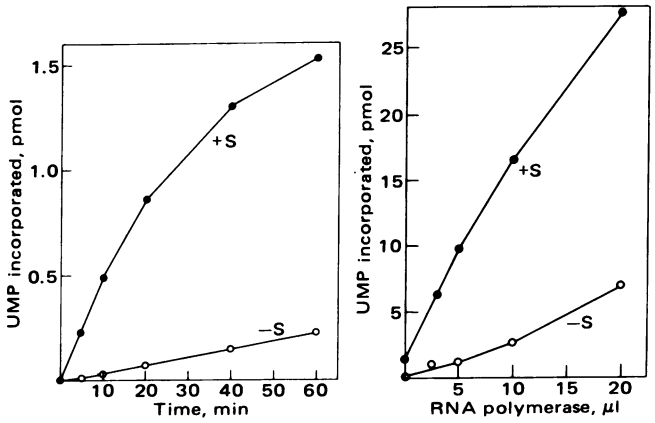

FIG. 3. (*Left*) Time course of [^{3}H]UMP incorporation into RNA with and without S. Reaction mixtures (150 μl) contained 5 μg of supercoiled pZmc134 DNA, 7.5 μmol of Tris·HCl (pH 8.0), 3.75 μmol of $MgCl_2$, 6 μmol of 2-mercaptoethanol, 37.5 nmol each of ATP, GTP, and CTP, 15 nmol of [^{3}H]UTP (1.33 Ci/mmol), 10 μl of cp RNA-P (DEAE-cellulose fraction), and either 10 μl of the S solution (DEAE-cellulose fraction) or 0.5 M KCl in the column elution buffer. At the times shown, 20-μl aliquots were precipitated with trichloroacetic acid and assayed for radioactivity. (*Right*) Effect of S on [^{3}H]UMP incorporation at different cp RNA-P concentrations. Reaction mixtures contained 7.5 μg of supercoiled pZmc134 DNA in the standard assay with different amounts of cp RNA-P added. The cp RNA-P was used after DEAE-cellulose chromatography. Each microliter of cp RNA-P solution contained 0.4 μg of protein.

with ethanol, and dissolved, and each DNA solution was divided into two portions and used as template in transcription reactions under the conditions for transcription described in Table 1. With pZmc134 DNA preincubated with S: transcription −S, 381 cpm; +S, 2025 cpm. With pZmc134 not preincubated with S: transcription −S, 538 cpm; +S, 2078 cpm. These data confirm the conclusion that S preparations do not permanently alter (e.g., partially digest) the DNA template. The effects of S on some characteristics of the cp RNA-P catalyzed reaction are shown in Fig. 3.

Table 1 shows the effect of S on transcription of various DNAs by cpRNA-P. Supercoiled pZmc134 DNA was separated from relaxed and open circular forms by $CsCl_2$/ethidium bromide density gradient centrifugation (13). Each fraction was estimated to be about 95% pure by agarose gel electrophoresis. Supercoiled and circular DNAs were transcribed equally rapidly and the rate was increased by S to the same extent. Transcription of denatured and of endonuclease-digested pZmc134 DNA was rapid but unaffected by S. Transcription of "native" or denatured calf thymus DNA was not affected by S.

Highly purified maize nuclear RNA-P II (22) transcribed

Table 2. Effects of maize cp RNA-P S factor and *E. coli* RNA-P σ factor on transcription

RNA polymerase	Factor	Rate*
E. coli (1 μg core)†	–	238
	σ (1 μg)	516
	S (5 μl)	237
Maize chloroplast	–	0.98
(8 μg)‡	σ (2 μg)	0.56
	σ (5 μg)	0.81
	S (5 μl)	4.65
	S (10 μl)	10.16

* pmol of [^{3}H]UMP incorporated for 30 min at 37°C. Template, 1 μg of T-4 DNA per reaction.
† *E. coli* RNA-P assay from Burgess and Travers (20).
‡ cp RNA-P assay as in Table 1.

supercoiled chimeric DNA more rapidly than it transcribed calf thymus DNA but its activity with either template was not influenced by S.

Selective Transcription of Cloned Supercoiled Maize cp DNA Sequences. One approach to the study of transcriptional specificity was by analyzing products of *in vitro* RNA synthesis through hybridization with fragments of templates. pZmc134 DNA was fragmented by the restriction endonucleases *Eco*RI and *Bam*HI or *Eco*RI and *Hin*dIII. The fragments were separated by agarose gel electrophoresis and transferred (14) to nitrocellulose filter sheets. [^{32}P]AMP-labeled RNA produced by cp RNA-P in the presence or absence of S with pZmc134 DNA as a template was hybridized to the DNA fragments on the filters.

Fig. 4 (*Eco*RI/*Bam*HI) shows that, when supercoiled pZm134 DNA was the template for cp RNA-P, RNA sequences complementary to both vehicle and cp DNA fragments were produced by the enzyme in the absence of S. However, some portions of cp DNA were preferentially transcribed (compare i, ii, and L) and transcription of vehicle DNA was relatively suppressed when S was included in the reaction mixture. When circular pZmc134 DNA (not supercoiled) was provided as the template, S had little effect on the specificity of transcription. Comparable results (compare 2 vs. 3 and 4) were observed when pZmc134 digested with *Eco*RI and *Hin*dIII was used in analytical hybridizations of the *in vitro* products.

The hybridization pattern shown in Fig. 4 demonstrates that S factor-promoted transcription is primarily from the cp DNA sequences in the chimeric plasmid and that the specificity is greater when supercoiled rather than circular plasmid DNA is the template. These conclusions were supported in quantitative experiments.

The S-influenced difference in transcription of various regions of the 14.7-kilobase pair (kbp) cp DNA sequence in pZmc134 (Figs. 4 and 5) indicated the desirability of using a relatively small cp DNA sequence as a template to study preferential transcription quantitatively. [^{3}H]UMP-RNA was

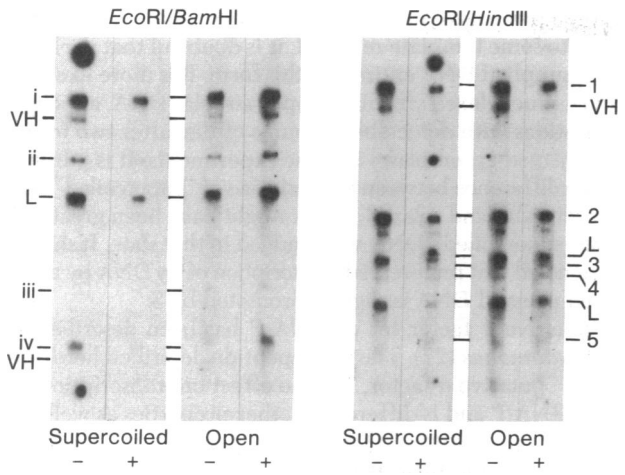

FIG. 4. Hybridization of RNA transcribed from pZmc134 DNA to fragments of the template. pZmc134 was hydrolyzed with restriction endonucleases *Eco*RI and *Bam*HI or *Eco*RI and *Hin*dIII. The DNA fragments (0.2 μg per gel) were separated by electrophoresis in 1.5% agarose gels (0.75 mA per gel for 16 hr) and transferred to Millipore filter strips (14). [γ-^{32}P]RNA was made during 30 min at 37°C in a standard reaction mixture but with 1 nmol of [γ-^{32}P]UTP (100 Ci/mmol) and 10 μl of cp RNA-P in the presence or absence of S. Supercoiled or circular pZmc134 DNA (5 μg) was used as a template. The amount of acid-precipitable products were: supercoiled DNA template, −S = 158 and +S = 1523; relaxed plus open circular DNA template, −S = 222 and +S = 1950 (all × 10^3 cpm). After extraction with phenol and ethanol precipitation, RNA was dissolved in 0.3 M NaCl/0.03 M Na citrate. About 80–120 × 10^3 cpm of each RNA was used for hybridization to a nitrocellulose filter strip. i, ii, iii, iv: Fragments generated by digestion of *Eco*RI fragment *a* from pZmc134 with *Bam*HI; 1–5: fragments produced by *Hin*dIII digestion. The locations of i–iv and 1–5 in fragment *a* are shown in Fig. 5. VH, fragments produced from the vehicle pMB9; L, *Eco*RI fragment *l* and its derivatives.

synthesized *in vitro* by cp RNA-P in the presence or absence of S by using supercoiled pZmc150 as the template. This 8.2-kbp chimeric plasmid is composed of the vehicle pMB9 plus cp DNA sequence *1*. Denatured pZmc150 or pMB9 DNA was fixed to nitrocellulose filter discs. A constant amount (the same number of counts) of [^{3}H]UMP-RNA was hybridized to a series of filters containing different amounts of DNA of the two types. *Eco*RI cp DNA fragment *1* is 2.2 and pMB9 is 6.0 kbp long. As shown in Fig. 6, 215 cpm of [^{3}H]RNA produced by cp RNA-P in the absence of S hybridized to pMB9 DNA and 280 cpm of product hybridized to pZmc150 DNA. The ratio of transcription of the cp DNA sequence to vehicle pMB9 DNA was calculated as: [RNA (in cpm) hybridized to pZmc150 DNA] minus [RNA (cpm) hybridized to pMB9 DNA]/[size of cp DNA fragment in kbp] to [RNA (cpm) hybridized to pMB9 DNA]/[size of pMB9 in kbp]. This ratio was 0.8 (65/2.2:215/6.0) in the

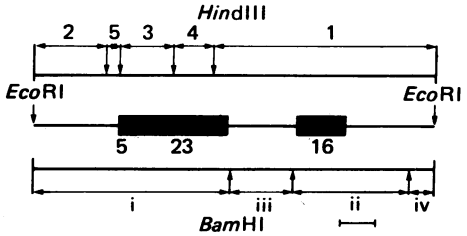

FIG. 5. Restriction map of *Eco*RI fragment *a* of pZmc134 DNA. The known recognition sites for endonucleases *Eco*RI, *Bam*HI, and *Hin*dIII are shown by arrows. The crosshatched regions represent the locations of genes coding for 16S, 23S, and 5S cp rRNAs (3). Bar, 1000 base pairs.

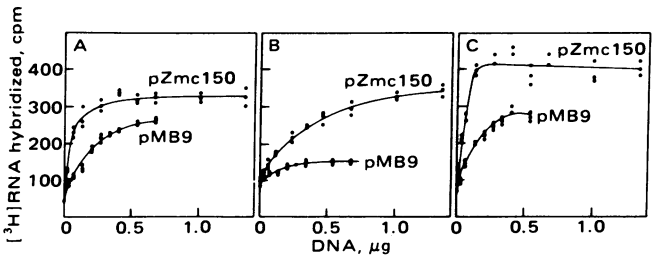

FIG. 6. Quantitative hybridization of RNA-P products formed in the presence or absence of S to pZmc150 or pMB9 DNA. [^{3}H]RNA was made *in vitro* by using supercoiled pZmc150 DNA as the template with or without S. About 1 ng of [^{3}H]RNA (500 cpm) was hybridized to the indicated amounts of pZmc150 or pMB9 DNA fixed on nitrocellulose filters. (*A*) cp RNA-P without S. (*B*) cp RNA-P with S present during transcription. (*C*) As in *A* but with *E. coli* RNA-P holoenzyme; S has no effect.

absence of S and 8 (190/2.2:65/6.0) in the presence of S (Fig. 6 *A* and *B*). This order of magnitude difference in transcriptional preference is about the same or greater than the stimulatory effect of S on RNA synthesis (Table 1). *E. coli* RNA-P acts similar to cp RNA-P minus S on pZmc150 DNA (Fig. *6C*); the transcription ratio was 1.6.

To control the state of the DNA template better than in the experiments shown in Fig. 4, we incubated supercoiled pZmc150 DNA with *E. coli* ω protein (23) until all the DNA was relaxed as assayed by gel electrophoresis. S accelerated the transcription of the enzymatically relaxed circular and supercoiled DNAs by cp RNA-P to about the same extent. However, quantitative hybridization showed that, by using enzymatically relaxed circular pZmc150 DNA as a template, the transcription ratio of fragment *1* to pMB9 was 1:1 without S and 2.5:1 with S (Fig. 7)—i.e., the transcription of *1* in the relaxed plasmid was favored only slightly by S compared with the effect on supercoiled pZmc150.

The effect of S on the specificity of DNA strand transcription by cp RNA-P was studied as follows: Fragment *1* from pZmc150 was purified by sucrose density gradient centrifugation and denatured, and the two strands were separated by agarose gel electrophoresis (24). The separated DNA strands were transferred to nitrocellulose filter strips (14) and hybridzed to [^{32}P]RNA transcribed from pZmc150 DNA by maize cp RNA-P in the presence or absence of S or to RNA isolated from

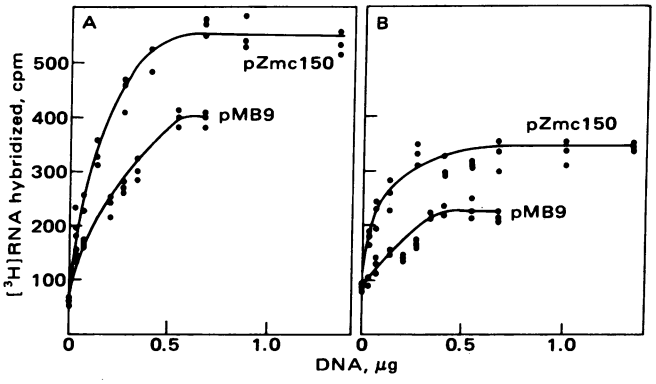

FIG. 7. Relaxed plasmid DNA as a template for cp RNA-P with or without S. Supercoiled pZmc150 DNA was treated with *E. coli* ω protein and the enzyme was removed by chromatography on DEAE-cellulose 52 (23). The relaxed DNA was used as template for transcription without (*A*) or with (*B*) S. Hybridization procedure was as in Fig. 6. pZmc150 DNA (10 μg) was treated with 3 μg of ω protein as described (23). About 3 μg of relaxed (ω-treated) pZmc150 was used as template.

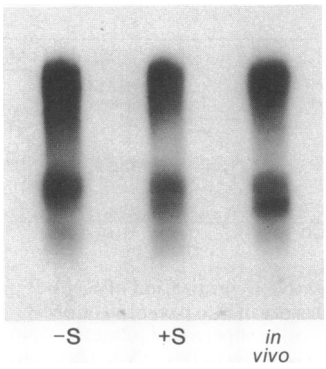

FIG. 8. Influence of S factor on specific transcription of DNA strands. Purified fragment *1* (1 μg) isolated from pZmc150 by sucrose gradient centrifugation (8) was denatured at room temperature for 10 min with 40 μl of 0.5 M NaOH/6.2 mM EDTA/6.25% glycerol. After 5 μl of 0.005% bromophenol blue was added, the 15-μl sample containing 0.3 μg of DNA was loaded on a neutral 1% agarose gel for electrophoresis (24). The DNA was transferred to nitrocellulose filters. [γ-^{32}P]UMP-RNA was made *in vitro* with or without S. Total cp RNA was labeled *in vitro* (12).

maize chloroplasts and labeled with ^{32}P *in vitro* (12). More of the RNA isolated from chloroplasts hybridized to the faster than to the slower migrating strand of fragment *1* DNA. *In vitro* only the slower migrating strand of *1* was transcribed by cp RNA-P without S present. S enhanced the transcription of the faster migrating DNA strand; RNA synthesized by cp RNA-P *in vitro* in the presence of S hybridized about equally to both strands (Fig. 8). It is possible that if the RNA-P were saturated with S, the hybridization pattern might approximate the *in vitro* RNA pattern more closely, but such experiments remain to be done.

DISCUSSION

The reconstituted RNA-synthesizing system comprised of DEAE-cellulose-purified cp RNA-P plus the S polypeptide from maize chloroplasts preferentially transcribes chloroplast genes from supercoiled chimeric plasmid DNA propagated in *E. coli*. The S factor affects the transcription of some cp DNA sequences within *Eco*RI fragment *a* more than others (Fig. 4). Thus, it would not be surprising to find other specificity-stimulatory factors in the chloroplast. The total range of effectiveness of S on cloned segments of the chloroplast genome remains to be explored but S does promote the preferential transcription by cp RNA-P of the gene for the large subunit of ribulose-bisphosphate carboxylase in a chimeric plasmid (unpublished results).

The experiments described show that, at least with this polymerase and the genes studied here, transcription is more highly selective for homologous genes when the template is supercoiled than when it is relaxed. These data are in line with the observation that inhibitors of DNA gryase differentially affect production of polypeptides from DNA templates *in vitro* when an *E. coli* S-30 extract (25) is used, although *E. coli* RNA polymerase can correctly initiate transcription from a fragment of phage λ (26). It is possible that the supercoiled versus relaxed state of the relatively small chloroplast genome can play a role in regulating gene transcription.

We have no ready explanation for the observation that S stimulates the rate of transcription of relaxed circular DNA without exerting a strong influence on transcriptional preference. It remains to be determined whether there is an optimal stoichiometry between the concentrations of S and RNA-P and whether initiation on this *in vitro* system is at precisely the same point as *in vivo*.

Although we have used the term "supercoiled" DNA in referring to some template material, it is doubtful that we have always supplied DNA entirely in this form. It is more likely to include some relaxed and nicked open circular DNA, although preparations with detectable amounts of the latter two forms were rejected as templates in these experiments. It is possible that the difference between transcription of "supercoiled" and "open circular and relaxed" DNA might have been greater if all the "supercoiled" DNA were indeed in that state. It should also be noted that preferential transcription of cp DNA by using relaxed plasmid DNA is slightly promoted by S.

A putative σ factor for cp RNA-P has been described in *Chlamydomonas* (27). The S polypeptide described here, unlike the "putative σ factor," has no effect on transcription by *E. coli* RNA-P and is different in other properties as well.

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