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Selective In Vitro Transcription of Chloroplast Genes

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Transcription of Euglena gracilis chloroplast genes has been investigated by using in vitro transcription systems. A DNA-dependent RNA polymerase responsible for the transcription of rRNA genes has been isolated as a nucleoprotein complex (transcriptionally active chromosome). The RNA polymerase remains tightly bound to the chloroplast DNA template and does not initiate transcription with cloned chloroplast genes. A transcriptionally active extract has been prepared from intact Euglena chloroplasts. The soluble RNA polymerase in this extract recognizes cloned chloroplast tRNA genes and tRNA-sized products have been detected after transcription. The tRNA-sized molecules specifically hybridize to the tRNA genes in the plasmid DNA. At least five tRNA-sized products have been identified from transcription of a trnY1-trnH1-trnM1-trnE1-trnW1-trnG1 cluster. Evidence is also presented that processing enzymes in the chloroplast-extract can recognize a polycistronic $tRNA^{Val}$-$tRNA^{Asn}$-$tRNA^{Arg}$ precursor and process it into tRNA-sized molecules. Truncated templates have been used to demonstrate that the chloroplast tRNA genes are actively transcribed. From a comparison of 5′ flanking sequences in chloroplast tRNA genes, a consensus sequence which might function as a promoter, has been identified. The properties of the RNA polymerase involved in the transcription of chloroplast rRNA genes and tRNA genes have been investigated and compared.

Key words: Euglena gracilis, ct TAC, ct tRNA genes, transcription, RNA polymerases, processing

The chloroplast DNA is a circular, double-stranded molecule of 60-160 kbp in size (for review see [1,2]). In Euglena gracilis, the major transcripts of the 145 kbp chloroplast genome are the 16S and 23S ribosomal RNAs [3,4], 5S rRNA [5], and at least 25 tRNAs [6,7]. These stable, abundant RNAs account for one-fourth of the total mass of RNA in light grown Euglena [8,9]. Chloroplast DNA therefore encodes the stable RNAs involved in the chloroplast translation apparatus. Present within the chloroplast stroma are autonomous systems for transcription of chloroplast DNA as well as 70S ribosomes active in chloroplast protein synthesis. The total number of

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proteins encoded in the chloroplast genome is not known, but in terms of chloroplast DNA complexity and extent of transcription, the number of protein coding loci is probably in the range of 50 to 100. Several chloroplast protein genes have been well characterized and their sequence is known (for review see [1,2]).

Despite our knowledge of the location and sequence of chloroplast ribosomal genes, transfer RNA genes, and protein coding genes, very few details are known about the regulation and expression of these genes. A thorough study of chloroplast gene expression requires a detailed knowledge of the transcriptional apparatus. A major goal in this study is to obtain highly purified DNA-dependent transcription systems that selectively initiate and transcribe chloroplast genes in vitro. The transcription system of Euglena gracilis chloroplasts is especially amenable for work on RNA synthesis for several reasons. The chloroplast DNA is well characterized, with the genes for 16S and 23S rRNAs [10–12], 5S rRNA [5], and tRNAs [13–15] all mapped and localized. Also, the DNA has been the subject of in vivo transcription studies [8,9,16,17]. In addition, a tightly bound chloroplast DNA-dependent RNA polymerase can be isolated as a transcriptionally active chromosome (TAC) from Euglena chloroplasts [18]. TAC transcription occurs selectively from the endogenous chloroplast DNA template and RNA molecules are initiated and elongated in vitro. The in vitro transcripts hybridize predominately to ribosomal RNA genes [19]. Little or no hybridization was found to tRNA genes or protein coding loci.

To study the transcription from tRNA genes and protein coding genes on the Euglena chloroplast genome, we have developed a soluble in vitro transcription system from chloroplasts. The Euglena chloroplast extract contains a DNA-dependent soluble RNA polymerase that faithfully transcribes chloroplast tRNA genes in vitro. By using cloned chloroplast tRNA genes we demonstrate that the genes are faithfully transcribed, and the transcripts are processed into mature tRNAs. We report the characterization of ribosomal RNA transcripts from the transcriptionally active chromosome and tRNAs that are transcribed in the chloroplast extract. The properties of the RNA polymerase activities involved in the transcription of each set of RNA molecules are described and compared. The development of chloroplast in vitro transcription systems also allows us to localize the promoter region for the ribosomal RNA genes and to test a consensus sequence located 5′ upstream of Euglena chloroplast tRNA genes, which has been proposed as a putative promoter region.

MATERIALS AND METHODS

Isolation of Euglena chloroplasts

Euglena gracilis Klebs, Pringsheim strain Z cells, American Type culture collection #12894, were grown to a density of 0.9–1.3 $\times$ 10^6 cells/ml at 25°C with continuous illumination as described [19]. Cells were harvested by centrifugation at 12,000 rpm in a Sorvall SS-34 rotor equipped with a Scent-Gyorgi and Blum continuous flow system. For the isolation of the Euglena transcriptionally active chromosome, the washing of cells, cell lysis, and chloroplast purification via differential centrifugation and flotation of chloroplasts were accomplished by previous published methods [21]. For the preparation of a chloroplast extract, chloroplasts were purified by differential sedimentation on silica gradients [22]. It should be noted that we find the chloroplasts from the Euglena strain described above to be very fragile, and only

a small portion of the isolated chloroplasts were intact after purification on silica gradients.

Isolation of the Euglena Transcriptionally Active Chromosome (TAC) and Preparation of a Chloroplast Extract

Twice floated chloroplasts were lysed and the TAC chromatographically purified according to the procedure outlined by Rushlow and Hallick [23] with the following modifications. First, the salt concentration of the Triton X-100 buffer was increased to 0.5 M with ammonium sulfate. This dissociates more protein away from the DNA and allows for increased specific activity of the TAC which remains bound to the DNA at high salt. In addition, heparin was added to the Triton X-100 buffer at a final concentration of 100 μg/ml to dissociate additional proteins.

For the preparation of a Euglena chloroplast extract purified intact chloroplasts were resuspended in 4 vol 10 mM Tris-Cl pH 7.9, 1 mM EDTA, 5 mM dithiothreitol, kept on ice for 30 min, and homogenized in a Dounce homogenizer every 10 min. The preparation of the extract was then accomplished by a method previously described for the preparation of a chloroplast extract from intact spinach chloroplasts [20].

In Vitro Transcription and RNA Characterization

The purified transcriptionally active chromosome is active in RNA synthesis in vitro in the presence of Mg^{2+} and the four nucleoside triphosphates, ATP, CTP, GTP, and UTP. RNA was synthesized under standard conditions as outlined by Rushlow et al [19]. For further characterization, labeled RNA synthesized by the TAC was purified by chromatography through Sephadex G-25 [19], and fractions containing RNA were pooled and used directly. RNA was hybridized to restriction nuclease fragments transferred from agarose gels to cellulose nitrate filters [24] as described [19].

Plasmid DNAs were transcribed in an in vitro reaction mixture containing 10 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 10 mM $MgCl_2$, 1.5 mM dithiothreitol, 40 mM KCl, 20 mM creatine phosphate and 10% glycerol. The concentration of nucleotides was 500 μM for ATP, GTP, CTP, and 25 μM UTP containing 20 μCi [α-32p] UTP (New England Nuclear; 760 Ci/mmole). Chloroplast extract (12.5 μl) was added to reactions of 25 μl final volume with 60 μg/ml DNA as standard concentration. The reaction mixture was incubated at 25°C for various times. Transcription was terminated by addition of proteinase K/sodium dodecyl sulfate at 37°C, and subsequent phenol-chloroform extraction. RNAs were separated by electrophoresis through a 10% polyacrylamide-8 M urea gel. For hybridization and 2-dimensional gel electrophoresis in vitro transcribed RNAs were separated by preparative gel electrophoresis and RNAs were isolated from the gel as previously described [25]. Hybridization was performed in 0.2% Sarkosyl, 6xSSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7) for 24 hr at 68°C. Two-dimensional gel electrophoresis of 75 nucleotide RNAs transcribed from pPG76 was performed according to Kuntz et al [26].

tRNA Processing

The 268 nucleotide polycistronic $tRNA^{Val}$-$tRNA^{Asn}$-$tRNA^{Arg}$ precursor was transcribed from pPG14 by RNA polymerase III in a HeLa extract [25]. The labeled

primary transcript was purified by preparative gel electrophoresis and isolated from the gel. The RNA was reincubated in an in vitro reaction mixture containing chloroplast extract as described above.

RESULTS

Properties and Specificity of the High Salt TAC

In previous reports the properties and selectivity of the transcriptionally active chromosome (TAC) of Euglena ct have been described [18,19]. The enzyme-DNA complex is stable and is comparable in transcriptional specific activity to E coli RNA polymerase. The RNA produced from the chromosome is exclusively ribosomal in nature. Strand-specific expression from the 16S, 23S, and 5S genes is obtained. Discrete sized mature products are not seen in gel analyses of the transcripts presumably because of a lack of processing enzymes in the TAC.

The TAC has unusual properties when compared to other known RNA polymerases. Activity is resistant to high levels of rifampicin, heparin, and α-amanitin which is distinct from prokaryotic or eukaryotic RNA polymerases. Also, the TAC activity is very salt resistant, with the enzyme remaining active at 0.75 M ammonium sulfate.

For previous studies TAC was isolated in the presence of 0.1 M ammonium sulfate. Here we have isolated an enzyme at higher salt (0.4–0.5 M), to investigate if the more purified enzyme would exhibit different transcriptional properties. The pattern obtained from a Southern-type hybridization experiment using in vitro labeled RNA (Fig. 1) is identical to results from previous experiments with TAC isolated at lower salt concentrations. Therefore, no apparent differences in properties or specificities between the low and high salt TAC can be detected.

Transcription of Euglena Chloroplast Genes in a Homologous Chloroplast Extract

A transcriptionally active chloroplast extract was prepared from isolated chloroplasts as described in Materials and Methods. The transcription in this extract is dependent on cloned chloroplast genes added as DNA templates to the reaction mixture (Fig. 2). Evidently, endogenous DNA is not present as a template for in vitro transcription, in contrast to the transcriptionally active chromosome.

Different cloned Euglena chloroplast tRNA gene clusters were incubated in the extract. The resulting transcripts of 75 and 85 nucleotides together with higher molecular weight RNAs could be separated on a polyacrylamide-8 M urea gel (Fig. 2). We have transcribed four tRNA gene clusters. The plasmid pPG14 contains the trnV1-trnN1-trnR1-trnL1 cluster from EcoG [13]. Seventy-five and 85 nucleotide tRNA-sized products would be expected if all genes were transcribed and the transcripts processed into mature $tRNA^{Val}$, $tRNA^{Asn}$, $tRNA^{Arg}$, and $tRNA^{Leu}$ (Fig. 2, lanes 2–4). The plasmid pPG76 contains the trnY1-trnH1-trnM1-trnW1-trnE1-trnG1 cluster from EcoV-H [14]. The expected transcripts would include $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Met}$, $tRNA^{Trp}$, $tRNA^{Glu}$, and $tRNA^{Gly}$ (Fig. 2, lanes 5–7). Plasmid pEZC300, which contains the trnT1-trnG2-trnM2 and trnS1-trnQ1 loci in EcoQ [15] was also transcribed. $tRNA^{Thr}$, $tRNA^{Gly}$, $tRNA^{Met}$, $tRNA^{Ser}$, and $tRNA^{Gln}$ are the expected products (Fig. 2, lanes 8–9). The plasmid pEZC514 contains the trnL2 loci from the Euglena chloroplast EcoI fragment [27]. The sequence of the trnL2 locus has been determined [Karabin and Hallick, unpublished observations]. The 85 nucleotide RNA

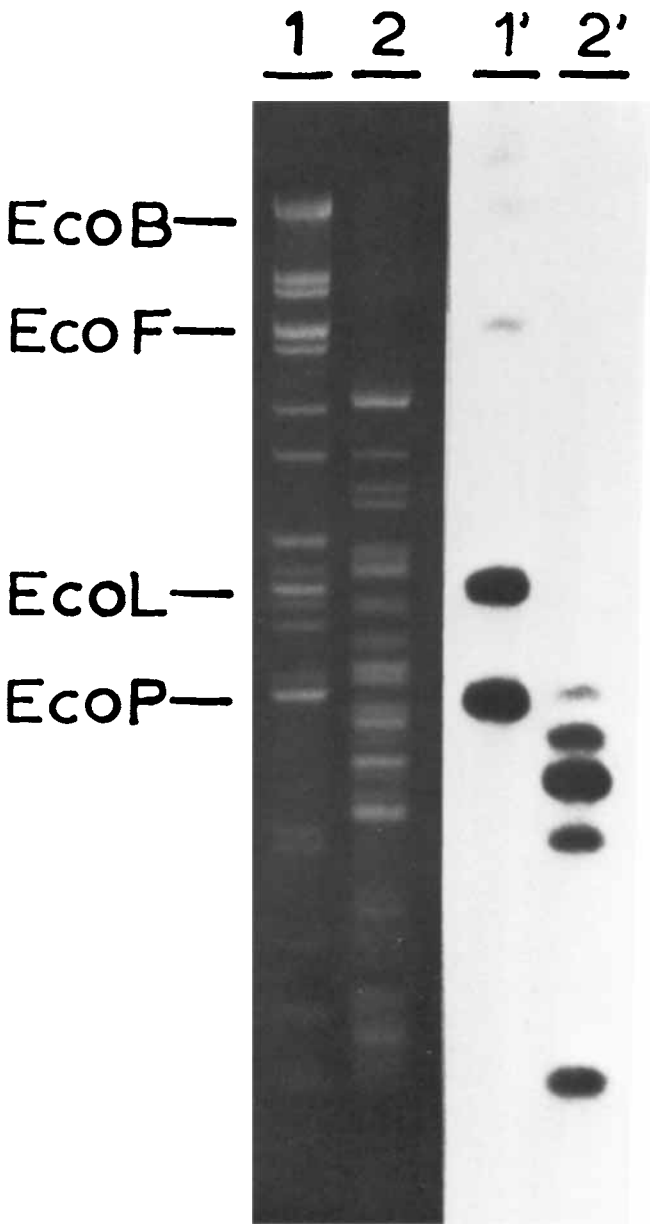
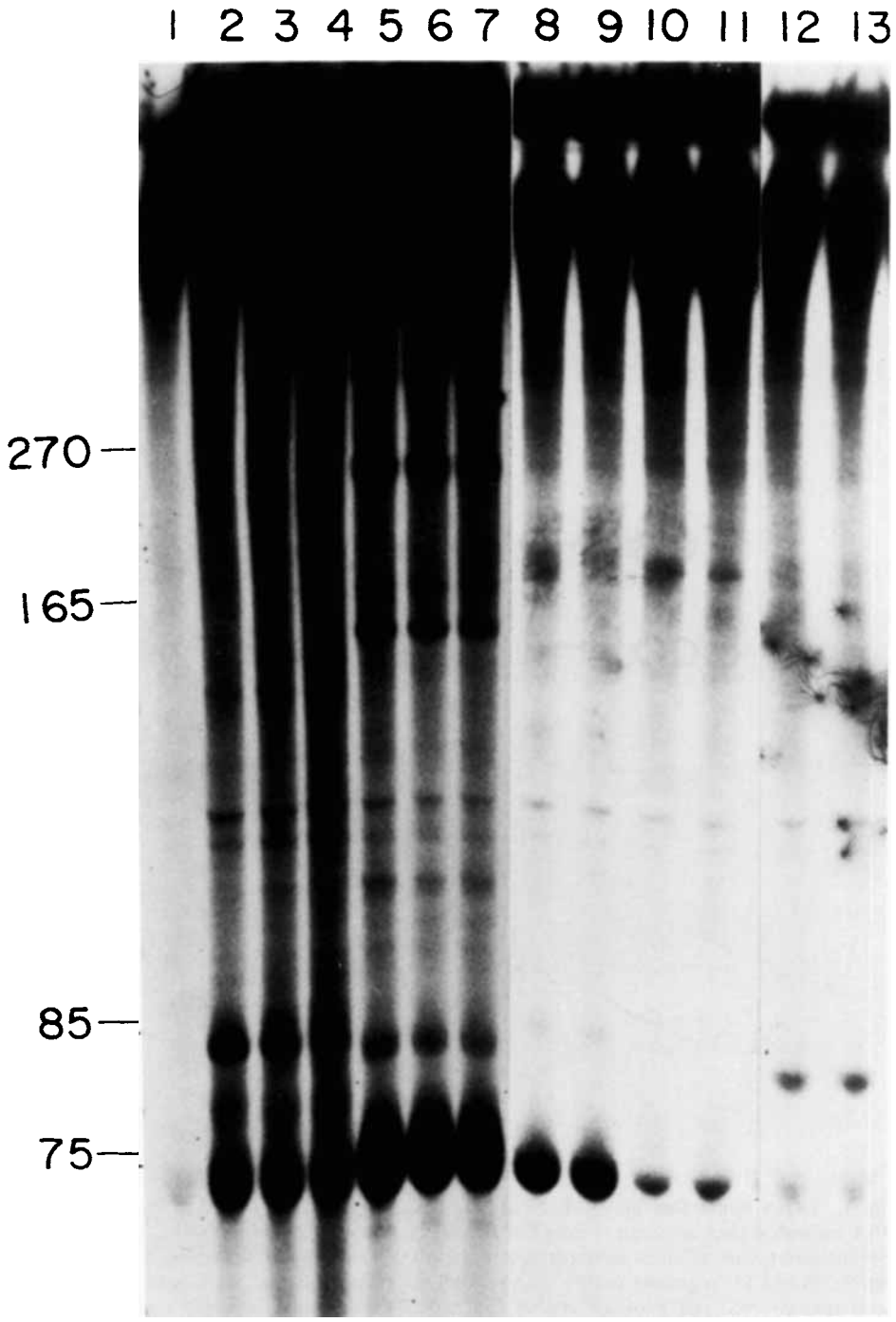


Fig. 1. cRNA synthesized by 0.4M $(NH_4)_2SO_4$ TAC. RNA was hybridized to Euglena chloroplast DNA cut with restriction enzymes EcoRI or HindIII (1, 1' and 2, 2', respectively). The only apparent hybridization occurs to bands corresponding to the ribosomal RNA tandem repeat. EcoP (16S and 5'-end of 23S and 5S) is present in three copies, EcoL (3'-end of 23S and 5S) is present in two copies, EcoB (pseudo 16S) and EcoF (3'-end of 23S and 5S) are present as single copies. The HindIII hybridization is consistent with TAC transcription of the entire rRNA region.


1 2 3 4 5 6 7 8 9 10 11 12 13
270
165
85
75

detected on the gel after transcription is in good agreement with the size for a mature $tRNA^{Leu}$ (Fig. 2, lanes 12–13). The EcoJ′ fragment in pPG689 also contains tRNA genes [38]. The sequence of these genes has not been determined, but tRNA-sized products are evident (Fig. 2, lanes 10–11). The higher molecular weight transcripts found for pPG14 and pPG76 could be possible precursor molecules from multicistronic transcripts of the tRNA gene cluster in EcoG and EcoV-H.

Characterization of tRNA-sized Transcripts

Since the nucleotide sequences for the tRNA genes in EcoG (pPG14) and EcoV-H (pPG76) are known, we began to characterize the products transcribed from these fragments in more detail. The transcribed locus from pPG76 was identified by Southern hybridization. The tRNA-sized transcripts were radioactively labeled in vitro and purified by gel electrophoresis. These RNAs were hybridized to membrane-filter blots of pPG76 restriction fragments. Hybridization is confined to the 3.2 kbp BamHI-SalI fragment, which defines the limiting boundaries of the transcribed locus (Fig. 3A). There is also predominantly hybridization to the AvaII-fragment, which contains the trnH1-trnM1-trnW1-trnE1 cluster and the 5′ part of the trnG1 locus. No hybridization could be detected to the non-coding MspI-SalI fragment. In vitro labeled, tRNA-sized transcripts (75 nucleotides) from pPG76 were also separated by 2D-electrophoresis. After autoradiography, four spots could be detected (Fig. 3B). The 85 nucleotide $tRNA^{Tyr}$ would not be present in the 2D-gel sample, since it can be separated from the other tRNAs on the 10% arylamide-8M urea gel. Based on the report of Kuntz et al [26], we can provisionally assign identities to the tRNAs separated by 2D-gel electrophoresis (Fig. 3B). The further characterization of the tRNA-sized products derived from pPG76 is currently underway.

To provide evidence that the observed tRNA-sized molecules are transcripts from the Euglena chloroplast trn loci, transcription experiments with restriction endonuclease-treated DNA templates were performed. The trnV1-trnN1-trnR1-trnL1 cluster in pPG14 was cut with BamHI, PvuI, or HhaI (Fig. 4). BamHI cleaves the plasmid DNA vector in the tet^R locus, and PvuI cleaves the central trnN1 locus in the anticodon stem sequence. HhaI cleaves the trnL1 locus downstream from the 5′ end of the gene. The BamHI-linearized pPG14 DNA is transcribed with almost the same efficiency as form I DNA (Fig. 4, lane 7). Therefore, the topological structure of the plasmid DNA molecule must be of minor relevance for transcription. Transcription of the PvuI-digested pPG14 DNA results in the loss of tRNA-sized transcripts 75 nucleotides in size (Fig. 4, lane 6). Transcription from the trnL1 locus seems to be unchanged with this template, since an 85 nucleotide RNA can still be detected that is the appropriate size for the mature $tRNA^{Leu}$. This RNA molecule is no longer present when the HhaI-digested pPG14 DNA is used for transcription in the ct extract,

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Fig. 2. Transcription of Euglena chloroplast tRNA genes in the chloroplast extract. Recombinant plasmid DNAs containing chloroplast tRNA genes were transcribed under standard conditions in the chloroplast extract, and the products were separated on a 10% acrylamide-8 M urea gel. Lane 1) no DNA; lanes 2–4) pPG14 DNA containing the trnV1-trnN1-trnR1-trnL1 cluster in EcoG; lanes 5–7) pPG76 DNA containing the trnY1-trnH1-trnM1-trnW1-trnE1-trnG1 cluster from EcoV-H; lanes 8,9) pEZC300 DNA containing the trnT1-trnG2-trnM2 and trnS1-trnQ1 loci in EcoQ; lanes 10,11) pPG689 DNA; lanes 12,13) pEZC514 DNA containing the trnL2 gene locus from EcoJ′. The DNA concentration was 40 μg/ml in all reactions except lanes 10–13, where the concentration was 20 μg/ml.

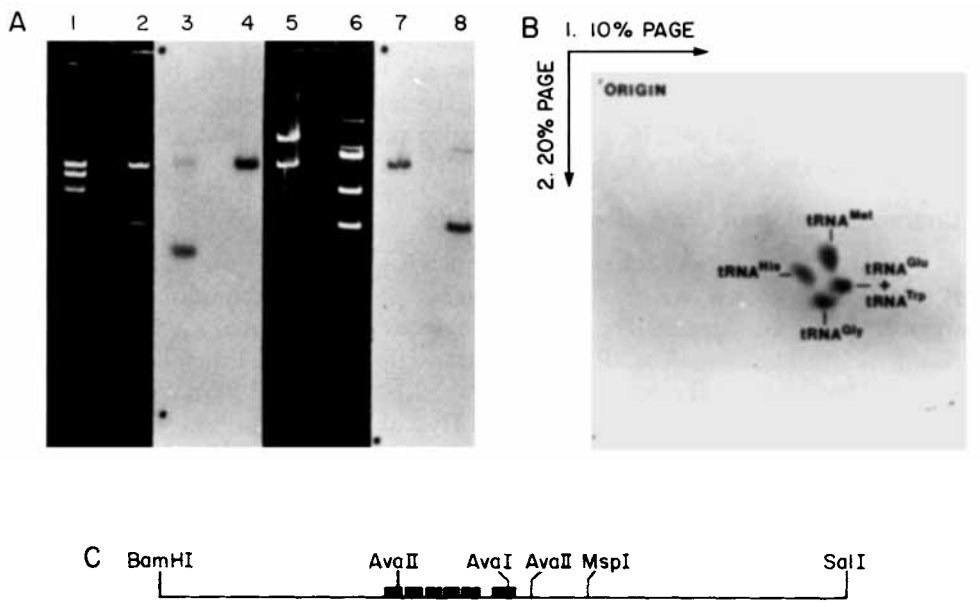


Fig. 3. Characterization of tRNA-sized transcription products. pPG76 DNA (60 μg/ml) was transcribed in the presence of α-^{32P}-UTP, and the transcription products were separated on a 10% acrylamide-8 M urea gel. tRNA-sized molecules (75 nucleotides) were eluted from the gel as described in Materials and Methods and used for further analysis. A) RNAs were hybridized to membrane filter blots of pPG76 restriction fragments: Lanes 1,3) Ava II; lanes 2,4) MspI; lanes 5,7) BamHI-SalI; lanes 6,8) BamHI-AvaI. B) 2D-gel electrophoresis of 75 nucleotide RNAs. Radioactively labeled RNAs were separated on a 10% polyacrylamide gel in the first dimension and then subjected to electrophoresis on a 20% polyacrylamide gel in the second dimension as described in Materials and Methods. C) Restriction map of the BamHI-SalI fragment from EcoV-H in pPG76 of restriction enzymes used in (A).

but transcription from the trnV1-trnN1-trnR1 locus of tRNA molecules 75 nucleotides in size seems to be restored with this template (Fig. 4, lane 5).

Similar results were obtained when restricted templates of the trnY1-trnH1-trnM1-trnW1-trnE1-trnG1 cluster in pPG76 were used for transcription (Fig. 4). The pPG76 DNA linearized with BamHI is almost as efficiently transcribed as form I DNA (Fig. 4, lane 3). No transcript of the size of a mature $tRNA^{Tyr}$ can be detected, when a pPG76 plasmid DNA digested with AvaI was used for transcription (Fig. 4, lane 2). AvaI cuts the trnY1 locus in the anticodon stem region, but has no cleavage site in the other tRNA genes of the gene cluster. When HhaI-digested pPG76 DNA is used for transcription the amount of the tRNA sized molecules 75 nucleotides in length is dramatically reduced, indicating that this restricted template changes, but does not abolish the transcriptional properties of the trn cluster in vitro. The remaining transcripts could arise from trnM1, trnH1, trnE1, or trnG1 loci.

From these data we conclude that cloned Euglena chloroplast trn clusters in pPG14 and pPG76 are preferentially transcribed by a chloroplast RNA polymerase present in a Euglena chloroplast extract. These transcripts give rise to mature tRNA-sized products, indicating the possible existence of tRNA processing enzymes in this extract.

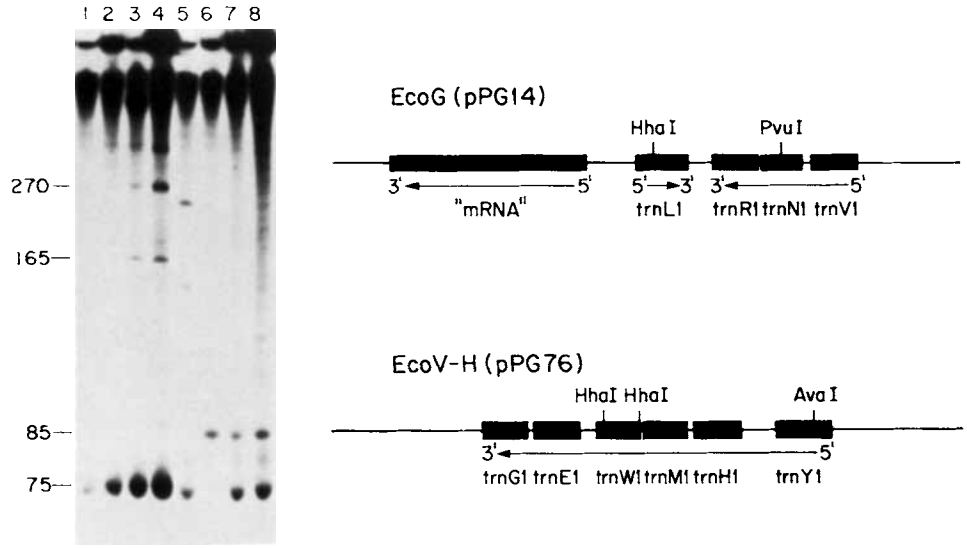
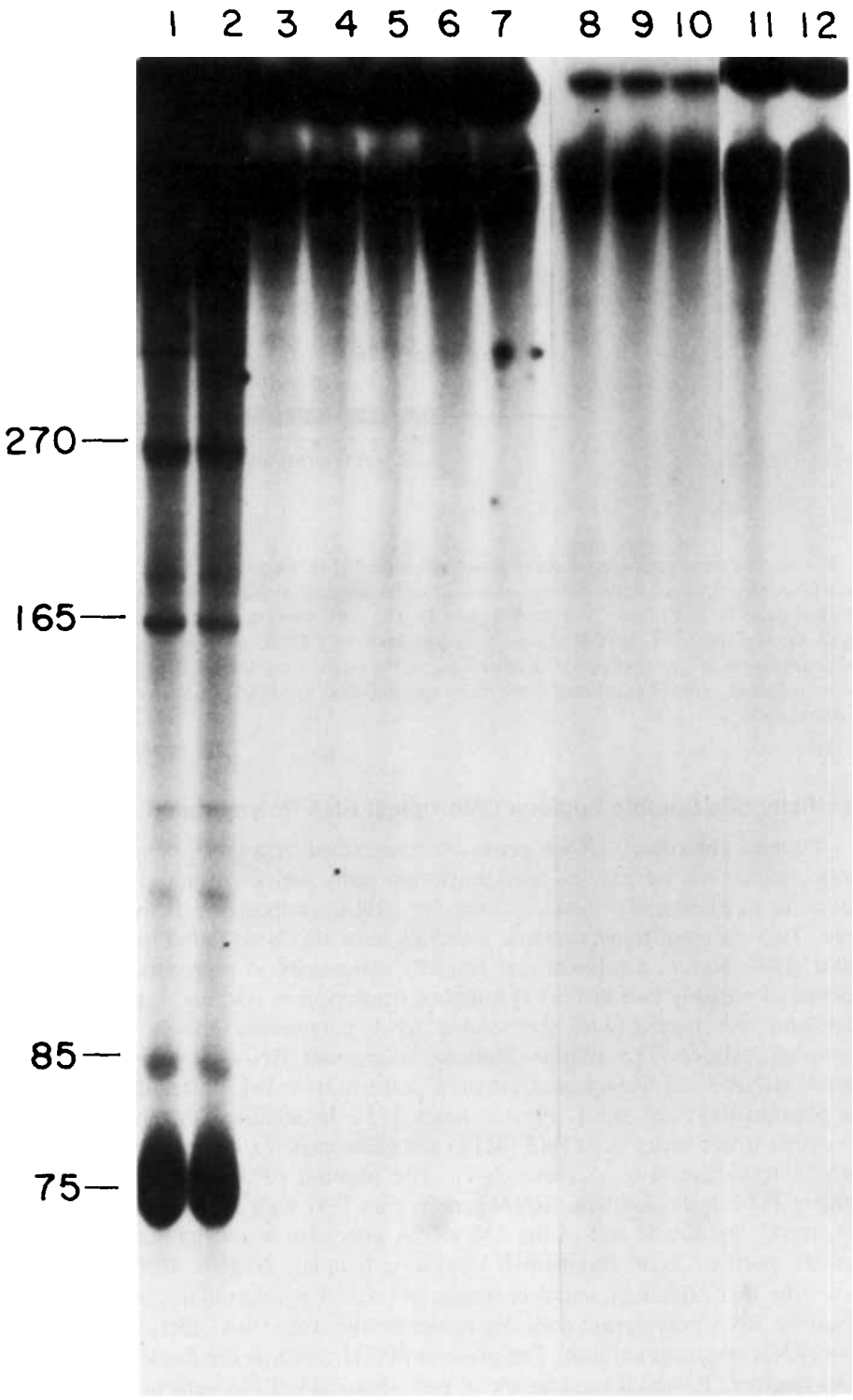


Fig. 4. In vitro transcription products of intact and truncated tRNA gene cluster. Lanes 1–4) pPG76 form I DNA was incubated in the chloroplast extract under standard conditions (lane 4) or after digestion with HhaI (lane 1), Ava I (lane 2) or BamHI (lane 3). The restriction sites are indicated in the physical map of the trn cluster in EcoV-H. Lanes 5–8) pPG14 form I DNA was incubated under standard conditions (lane 8) or after digestion with HhaI (lane 5), PvuI (lane 6), or BamHI (lane 7). The restriction sites are indicated in the physical map of the trn cluster in EcoG. The DNA concentration was 20 μg/ml in each reaction.

Specificity of a Soluble Euglena Chloroplast RNA Polymerase

Euglena chloroplast rRNA genes are transcribed by a DNA-bound RNA polymerase, which can be isolated as a transcriptionally active chromosome [18]. This activity is biochemically distinct from the tRNA synthesizing activity described above. Two different transcriptional activities have also been found in spinach chloroplast ([20]; Narita, Gruissem and Hallick, manuscript in preparation). With this evidence of possibly two distinct chloroplast transcription systems, it is important to understand the specificity of the soluble RNA polymerase present in a Euglena chloroplast extract. The soluble Euglena chloroplast RNA polymerase does not generate tRNA-sized transcripts from an E coli trnD1-trnW1 cluster in the recombinant plasmid pMY228 ([30], Fig. 5, lanes 3–5). In addition, the incubation of a Drosophila trnR1 locus in pYH48 [31] in the chloroplast extract does not result in a $tRNA^{Arg}$ transcript (Fig. 5, lanes 6–7). The plasmid pPG50 contains the EcoRI fragment P from the Euglena rRNA gene region [34] with the loci for 16S rRNA, trnI1, trnA1 and the 5′ end of the 23S rRNA gene. No transcripts can be detected when the purified EcoP fragment is used as a template (Fig. 6, lanes 8–10). It is noteworthy that, although active in transcription of Euglena chloroplast trn cluster, the soluble RNA polymerase does not recognize the trnI1-trnA1 loci, which are part of the rRNA transcription unit. The plasmid pPG11 contains the Euglena chloroplast EcoRI fragment F, which includes the 3′ part of the 23S rRNA gene and the gene for 5S rRNA in the third of three tandem rDNA repeats [33]. Upon incubation of pPG11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in the chloroplast extract no mature 5S rRNA or rRNA precursor molecule can be detected, indicating that the 5S rRNA gene is probably co-transcribed with the other ribosomal genes and not initiated separately by the soluble chloroplast RNA polymerase.

Comparison of Euglena Chloroplast RNA Polymerase Activities

A reasonable interpretation for the data described above is that two different transcription systems exist in Euglena chloroplasts with different specificities for the transcription of ribosomal RNA genes and the trn loci. At this point it is unknown if these two activities are related to two different enzymes, or an identical core enzyme with different regulatory subunits. Therefore, experiments were initiated to characterize these RNA polymerase activities in more detail. Table I summarizes the properties of the RNA polymerase active in the transcription of ribosomal RNA genes in the TAC and the soluble RNA polymerase activity found in the chloroplast extract, which directs the specific transcription of cloned chloroplast tRNA genes. Both enzymes are insensitive to high concentrations of α-amanitin, which therefore makes them clearly distinguishable from RNA polymerase II and III activities in the nuclear compartment. Although unlikely, we cannot completely exclude a possible contamination of the chloroplast extract with the nuclear RNA polymerase I activity. However, such a contaminant would not be expected to transcribe tRNA genes.

Both Euglena chloroplast RNA polymerase activities are also clearly distinct from prokaryotic RNA polymerase enzymes with respect to their resistance to high concentrations of rifampicin. The two RNA polymerase activities differ in their sensitivity to heparin. The RNA polymerase activity in the chloroplast extract is inhibited at even low concentrations of heparin. By contrast, the RNA polymerase in the TAC-complex is not sensitive to very high concentrations of heparin, and reinitiation of the 16S rRNA gene in the presence of heparin can be demonstrated in vitro. Finally, the RNA polymerase activity in the chloroplast extract is sensitive to high salt concentrations. Transcription of Euglena trn loci is almost eliminated in reaction mixtures containing 100 mM potassium chloride. In contrast, the TAC RNA polymerase activity is only partially inactivated by higher salt concentrations.

Processing of a Polycistronic Chloroplast tRNA Transcript

Since most of the transcripts synthesized in the Euglena chloroplast extract are tRNA-sized molecules, even though polycistronic transcription units were used as templates, we predicted that the chloroplast extract is also active in tRNA processing. Reaction conditions that led to an accumulation of unprocessed primary transcripts in the chloroplast extract have not yet been determined. We therefore initiated experi-

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Fig. 5. Characterization of the Euglena chloroplast soluble RNA polymerase. Lanes 1 and 2) pPG76 DNA (40 μg/ml) was transcribed under standard conditions in the Euglena chloroplast extract. Lanes 3–5) The E coli trnD1-trnW1 cluster in pMY228 was added to the reaction. DNA concentrations were 20 μg/ml (lane 3), 40 μg/ml (lane 4), and 80 μg/ml (lane 5). Lanes 6,7) A Drosophila trnY1 locus in pYH48 was incubated in the chloroplast extract. DNA concentrations were 40 μg/ml (lane 6) and 80 μg/ml (lane 7). Lanes 8–10) The Euglena chloroplast EcoP fragment was isolated from the plasmid pPG50 and the purified fragment was added to the transcription reaction. DNA concentrations were 10 μg/ml (lane 8), 20 μg/ml (lane 9), and 50 μg/ml (lane 10). Lanes 11,12) the plasmid pPG11 contains the ct EcoF fragment. DNA concentrations in the reactions were 20 μg/ml (lane 11) and 40 μg/ml (lane 12).

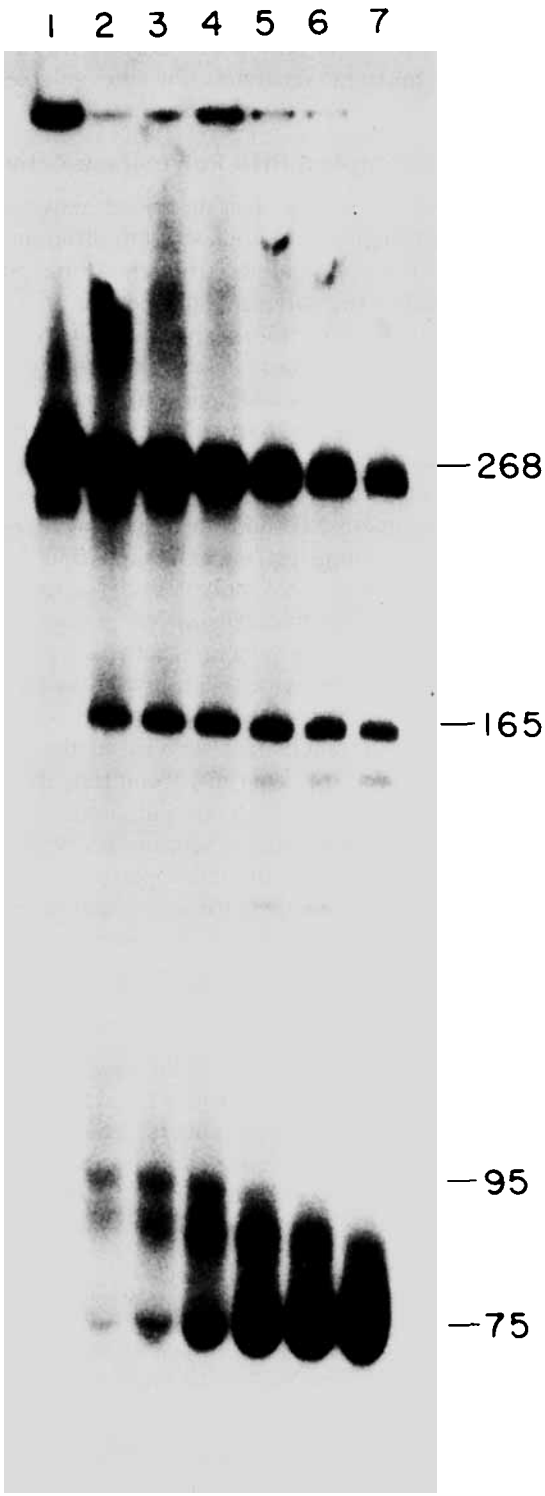
1 2 3 4 5 6 7
268
165
95
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TABLE I. Comparison of Biochemical Properties of Two Distinct RNA Polymerase Preparations of Euglena gracilis Chloroplasts

	Percent control activity	
Treatment	TAC RNA polymerase[a]	Soluble RNA polymerase[b]
α-amanitin		
5 μg/ml	100	100
50 μg/ml	100	100
200 μg/ml	100	100
Rifampicin		
50 μg/ml	100	100
100 μg/ml	100	100
200 μg/ml	100	100
Heparin		
2 μg/ml	100	0
20 μg/ml	98	0
KCl		
140 mM	65	3.5
240 mM	34	0

[a]RNA polymerase isolated as a transcriptionally active chromosome (TAC) for rRNA transcription.
[b]RNA polymerase present in a chloroplast transcription extract and inactive in rRNA transcription.

ments to prove that a polycistronic precursor transcribed from the clustered trn loci in pPG14 can be processed into mature tRNA molecules in the Euglena chloroplast extract. The 268 nucleotide polycistronic $tRNA^{Val}$-$tRNA^{Asn}$-$tRNA^{Arg}$ precursor was transcribed by RNA polymerase III in a HeLa-cell free extract [25]. The RNA was purified by gel electrophoresis and isolated as described in Materials and Methods. The polycistronic transcript was then incubated in the chloroplast extract for various times (Fig. 6). After 2.5 min, some of the precursor is cleaved, and tRNA-sized products can be detected. Most of the precursor is converted into tRNA-sized products after 60 min, and two intermediate precursors, approximately 160 and 95 nucleotides long, can be detected. Therefore processing enzymes, present in the Euglena chloroplast extract, are able to recognize and cleave the polycistronic precursor. Since the tRNA products of the processing reaction are of exactly the same length as the tRNA-sized molecules obtained from transcription of pPG14 in this extract, it is likely that the tRNAs are correctly processed from the polycistronic transcript.

DISCUSSION

We have shown that chloroplast genes can be selectively transcribed in vitro by homologous RNA polymerases. The selective transcription of the sense strand of

Fig. 6. Processing of a 268-nucleotide $tRNA^{Val}$-$tRNA^{Asn}$-$tRNA^{Arg}$ polycistronic precursor in the chloroplast extract. The 268-nucleotide precursor was transcribed from pPG14 by RNA polymerase III in the HeLa cell extract, and the primary transcript was purified as described in Materials and Methods. The precursor was then reincubated in the Euglena chloroplast extract under standard conditions but without α-^{32}P-UTP and plasmid DNA. Reactions were stopped after various times with proteinase K and samples were run on a 10% polyacrylamide-8 M urea gel. Lanes 1-7) Reincubation of the pPG14 primary transcript for 0, 2.5, 5, 10, 20, 30, and 60 min, respectively.

rDNA transcription units by the transcriptionally active chromosome (TAC) in vitro is consistent with the known in vivo transcription program of the chloroplast genome [9]. Also, with the more purified RNA polymerase in this complex, the in vitro transcripts consist predominantly of ribosomal RNAs. As was previously reported, the selective in vitro transcription of chloroplast DNA includes both initiation and elongation events, which involve the same RNA population [18]. Interestingly, the RNA polymerase involved in the transcription of the rDNA is insensitive to heparin, although initiation occurs in vitro [19]. In addition, cloned chloroplast genes are not recognized and transcribed in the presence of the TAC (data not shown). Therefore, we have developed a homologous in vitro transcription system from intact Euglena chloroplast. A soluble RNA polymerase present in a chloroplast extract recognizes and selectively transcribes chloroplast tRNA genes. Most of the trn loci used in these studies have been sequenced [13–15,27]. Since the structure of the transcription units in pPG14 and pPG76 are known, the specificity of the chloroplast RNA polymerase for tRNA genes could be addressed with experiments involving Southern [24] hybridization, two-dimensional gel analysis [26], and restriction endonuclease modified templates. Hybridization of the tRNA-sized transcription products from pPG76 is confined to the trnH1-trnM1-trnW1-trnE1-trnG1 locus, and at least four tRNA transcripts could be tentatively identified from their location on the two-dimensional gel. Additional evidence from experiments involving truncated DNA templates support the conclusion that selective RNA synthesis occurs from the chloroplast trn loci in vitro. First, BamH1 treated form III DNA or form I DNA from pPG76 and pPG14 gave comparable levels of tRNA-sized products. Therefore, transcription by the chloroplast RNA polymerase in vitro is not dependent on the topological structure of the plasmid DNA. Second, HhaI-digested pPG14 did not support transcription of a $tRNA^{Leu}$-sized transcript, but transcription of 75 nucleotide RNAs from the intact trnV1-trnN1-trnR1 locus did not seem to be impaired. Interestingly, these RNAs can no longer be detected when a PvuI-digested pPG14 DNA template is used for in vitro transcription (Fig. 4). Similar results were obtained when restriction endonuclease modified DNA templates of the trnY1-trnH1-trnM1-trnW1-trnE1-trnG1 cluster in pPG76 were used for transcription. It has also been demonstrated using a chloroplast extract prepared from intact spinach chloroplast that a soluble RNA polymerase selectively and faithfully transcribes Euglena and spinach chloroplast tRNA genes [20]. The in vitro transcribed tRNAs are correctly processed and have been shown to be mature at their 5′ and 3′ termini, a 3′-CCA_{OH} added by a nucleotidyl-transferase in vitro [28,29]. We would predict that the trn cluster in pPG14 and pPG76 are initially transcribed into long polycistronic precursors by a Euglena chloroplast RNA polymerase, but longer precursor RNA transcripts have not yet been characterized. However, processing enzymes in the Euglena chloroplast extract recognize and process a 268-nucleotide polycistronic chloroplast $tRNA^{Val}$-$tRNA^{Asn}$-$tRNA^{Arg}$ precursor. The tRNA-sized processing products are indistinguishable in size from the in vitro transcripts.

It is believed that the chloroplast genome encodes its own set of tRNAs, and that all of the trn loci are expressed. Since the expression of Euglena cloned chloroplast tRNA genes can be demonstrated in vitro, work was initiated to identify DNA sequences on the chloroplast chromosome recognized by the enzyme for transcription initiation. Sequences 5′ upstream from all Euglena trn genes were searched for possible common sequences. One possible consensus sequence,

$$5'-GT\frac{A}{T}NTA\frac{AA}{TT}A-3'$$

has been identified [Gruissem and Hallick, unpublished results]. This sequence can be located 5′ upstream from the start of the trnL1 (40 bp), trnY1 (11 bp), trn T1 (42 bp), and trnQ1 loci, as well as the trnV1 (26 bp) and trnH1 loci, which are the first tRNA genes in the trnV1-trnN1-trnR1 (EcoG) and trnH1-trnM1-trnW1-trnE1-trnG1 (EcoV-H) clusters, respectively. This sequence could therefore serve as a possible promoter for initiation of the tRNA transcription in Euglena chloroplasts. Several lines of evidence are consistent with the proposal that the consensus sequence might play a role in transcription of chloroplast tRNA genes. First, no tRNA-sized transcripts are synthesized from the trnI1 and trnA1 loci in EcoP, which are located in the spacer between the 16S rRNA and the 23S rRNA gene and believed to be co-transcribed with the rRNA genes. The sequence in this gene region is known [32] and the consensus sequence cannot be detected 5′ upstream of the trnI1 locus. Second, it was reported that a 'Pribnow-box'-like sequence and a -35-like sequence could be identified upstream of the trnV1 and trnM1 loci in spinach chloroplast [34]. However, no tRNA-sized transcripts are synthesized from the E coli trnD1-trnW1 locus in pMY228 [30], although the LacUV5 promoter could be utilized for transcription initiation by the soluble chloroplast RNA polymerase. Finally, the fact that we cannot detect a transcript from the Drosophila trnR1 locus [31] suggests that initiation and transcription by the chloroplast RNA polymerase is highly specific for chloroplast tRNA genes. The availability of the Euglena chloroplast in vitro transcription system together with BAL 31 restricted templates containing tRNA genes should allow us to define the gene control sequences for transcription initiation of the chloroplast tRNA genes.

The RNA polymerase involved in the transcription of Euglena chloroplast tRNA genes was found to be different in its biochemical properties from the enzyme present in the TAC. It is possible, therefore that two different transcription systems exist in Euglena chloroplasts which are involved in the transcription of rRNA and tRNA genes. This is consistent with our observation that the soluble RNA polymerase does not initiate transcription with the cloned 16S rRNA in pPG50 [32] and no tRNA-sized transcripts are synthesized from cloned tRNA genes in the presence of the TAC. However, we cannot rule out that a secondary structure in the DNA template might be a prerequisite for rRNA transcription, since it was demonstrated for spinach that the purified RNA polymerase from the TAC is less active in a reconstituted system [35]. The secondary structure in the DNA molecule could be preserved in the TAC with the RNA polymerase tightly bound to this structure. A soluble RNA polymerase has been isolated from spinach [20] and maize chloroplast [36]. The soluble spinach chloroplast RNA polymerase also had different biochemical properties than the TAC counterpart [35,37]. The characteristics of this enzyme are most similar to the RNA polymerase activity isolated from maize chloroplast. At present it is unknown if the two RNA polymerase activities in the Euglena TAC and chloroplast extract are two different and independent enzymes or if they only differ in certain subunits of an otherwise similar core structure. The development of the chloroplast extract and the isolation of a TAC should allow us to approach this problem biochemically, since the RNA polymerase enzymes can be purified from preparations. These in vitro transcription systems therefore prove to be powerful tools to identify RNA polymerases

involved in transcription of chloroplast genes, and also to elucidate the promoter sequences of chloroplast genes actively transcribed in vitro.

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