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Sigma-like activity from mustard (*Sinapis alba* L.) chloroplasts conferring DNA-binding and transcription specificity to *E. coli* core RNA polymerase

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Abstract

A protein fraction which lacks DNA-binding activity itself, but confers enhanced protein-DNA complex formation to *E. coli* core RNA polymerase, was obtained from mustard chloroplasts by heparin Sepharose chromatography. Gel retardation and competition assays as well as DNase I footprinting experiments with a chloroplast DNA fragment containing the *psbA* promoter indicate that this reflects sequence-specific binding. Transcription of the *psbA* template by *E. coli* core enzyme in the presence of the chloroplast fraction results in enhanced formation of transcripts of the size expected for correct initiation at the *in vivo* start site. We conclude that the chloroplast fraction reveals sigma-like activity with *E. coli* RNA polymerase and thus might contain factor(s) of equivalent function in chloroplast transcription.

Introduction

The chloroplast transcription apparatus has been studied extensively during the past few years [6, 15, 42] and there have been several reports on highly purified RNA polymerase from chloroplasts [20, 22, 23, 38, 41]. These multisubunit enzymes, which were obtained from various plant species by using different purification schemes, often have proved to be very unstable, which has hampered attempts to define the precise function of individual components of the chloroplast transcription apparatus.

In vitro studies with crude chloroplast lysates have shown that these are capable of initiating transcription accurately at the same position as *in vivo* [3, 4, 16, 24, 32]. Upstream from the initiation site most chloroplast genes have sequence elements very similar to the prokaryotic "–10" and "–35" promoter regions [21, 42].

Reading frames that might code for polypeptides with homology to *E. coli* RNA polymerase α, β and β' subunits have been located on chloroplast DNA [9, 30, 31, 36, 37] and antibodies directed against the β and β' subunits of *E. coli* RNA polymerase have been shown to cross-react with putative subunits of the chloroplast enzyme [22]. These findings point to general similarities of the chloroplast and bacterial transcription apparatus and, furthermore, raise the possibility that protein(s) equivalent to bacterial σ factor(s) [7, 26] might be involved in promoter recognition and correct initiation of chloroplast transcription as well. A protein fraction, implicated with sigma-like activity on the basis of its ability to confer rifampicin insensitivity to core RNA polymerase, was obtained from *Chlamydomonas reinhardii* [40], and a maize chloroplast S factor [19] was shown to preferentially stimulate transcription of cloned chloroplast genes by chloroplast RNA polymerase.

Here we describe a protein fraction from mustard chloroplast that reveals sigma-like activity, i.e. enhancement of sequence-specific DNA binding and correct transcription initiation by *E. coli* core RNA polymerase.

Materials and methods

DNA fragments

A 120 bp Hinf I fragment containing approximately 50 nucleotides of the 5′ portion of the mustard chloroplast *psbA* gene as well as the promoter and additional 5′ flanking sequence [24] was isolated from plasmid pSA364a-05 [25], made blunt, and inserted into the Sma I site of pUC 13 to give plasmid pSA05/H120. This plasmid was linearized with either Eco RI or Hind III and subsequently 3′ end-labelled with α-^{32}P-dATP (Amersham, 410 Ci/mmol) using the fill-in reaction of Klenow fragment (BRL) [27]. Following digestion with either Hind III or Eco RI, the resulting 165 bp fragments 3′ labelled at the Eco RI or Hind III site ("H120") were separated in a 6% polyacrylamide gel and eluted [28]. The 460 bp Bam HI fragment (B0.5) used as competitor contains intron-specific sequences of the mustard *trnK* gene [29].

Preparation of E. coli *core RNA polymerase*

E. coli core enzyme ($\alpha_2\beta\beta'$) was prepared by phosphocellulose chromatography of holoenzyme ($\alpha_2\beta\beta'\sigma$) (Boehringer), following standard procedures [1, 7]. Core enzyme preparations contained low residual levels of sigma (less than 2 percent as compared to holoenzyme).

Isolation of chloroplast protein fraction SLF

Chloroplast crude lysates from 250 g of cotyledons from 4-day-old light-grown mustard (*Sinapis alba* L.) seedlings [33] were adjusted to 0.1 M $(NH_4)_2SO_4$ and 15 mM $MgCl_2$ and treated with 50 μg/ml DNase I (Worthington DPFF) for 30 min at room temperature [5]. The lysate was then passed through a 3 ml column of heparin Sepharose CL-6B (Pharmacia) equilibrated with buffer A (50 mM Tris/HCl pH 7.6, 0.1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 10% glycerol, 0.1 M $(NH_4)_2SO_4$). The column was washed extensively with buffer A and then eluted with a 40-ml linear 0.1–1.0 M $(NH_4)_2SO_4$ gradient at a constant flow rate of 0.1 ml/min.

Two-ml fractions were collected and 5-μl portions were assayed directly for DNA-binding activity in gel retardation experiments (see below) [11, 13] without prior adjustment of salt concentration. Peak fractions conferring enhanced gel retardation activity to *E. coli* core RNA polymerase were pooled, dialysed against buffer A containing 10 mM $(NH_4)_2SO_4$ and 50% glycerol, and stored at −20 °C (fraction SLF). DNase I activity was monitored by incubation with 3′ labelled H120 fragment and subsequent electrophoresis of the DNA on 8% polyacrylamide sequencing gels.

Gel retardation assay [11, 13]

Protein fractions were incubated with 2.5 ng of 3′ labelled H120 fragment and 0.5 μg *E. coli* core RNA polymerase in 50 μl of 30 mM Tris/HCl pH 7.0, 5 mM β-mercaptoethanol, 0.5 mM EDTA, 5% glycerol at 20 °C for 15 min. Samples were then loaded onto a 5% polyacrylamide gel (30:0.8 acrylamide: bisacrylamide) containing 0.5 M Tris/HCl pH 8.8 and electrophoresed in 25 mM Tris, 192 mM glycine at room temperature. Wet gels were autoradiographed at 4 °C by using Kodak XAR-5 films.

DNase I footprinting [12].

Protein-DNA binding was carried out in the reaction mixture described above for protein gel retardation assays, except that 7.0 ng of 3′ end-labelled DNA fragments were used. After 15 min at 20 °C, 10 μl DNase I (Worthington DPFF; 25 μg/ml) in 30 mM $MgCl_2$ were added and incubation continued for 60 s. Reactions were stopped with 50 μl of a mixture containing 200 μg/ml proteinase K (Merck), 200

μg/ml *E. coli* tRNA, 25 mM EDTA, 100 mM Tris/HCl pH 8.0, 2% SDS, and incubated for 10 min at 37 °C. DNA was then isolated by phenol/chloroform extraction and ethanol precipitation, boiled in 80% formamide for 30 s, quickly chilled, and loaded onto 0.2-mm-thin 8% sequencing gels covalently bound to one of the glass plates [14, 34].

In combined footprinting/gel retardation experiments ("retardation footprints") [8], DNase I reactions were stopped with 2.5 μl 250 mM EDTA and samples electrophoresed on native 5% polyacrylamide gels. Radioactive bands representing protein-DNA complexes (pooled bands from 10 lanes) were cut out and DNA was eluted as above and prepared for electrophoresis on sequencing gels. Chemical sequencing reactions were carried out as described [28].

In vitro *transcription*

Reaction mixtures contained 50 mM Tris/HCl pH 8.0, 50 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 600 μM each of ATP, CTP, and GTP, 10 μCi α-^{32}P-UTP (Amersham, 820 Ci/nmol), and 50 ng H120 fragment in a total volume of 100 μl. Following incubation with protein fractions for 20 min at 30 °C, 5 μl of 10 mM UTP were added and incubation was continued for 10 min. After addition of 5 μg *E. coli* tRNA, transcripts were prepared [24] and electrophoresed on 8% sequencing gels.

Results

Gel retardation analysis of DNA-protein complexes

DNase I-treated lysates [5] of mustard chloroplasts were subjected to heparin Sepharose chromatography [2] and fractions were assayed for DNA-binding activity by a gel electrophoresis ("gel retardation") technique [11, 13], using a DNA fragment that contains the mustard chloroplast *psbA* promoter [24, 25]. A low level of "retardation activity" (i.e., formation of protein-DNA complexes migrating slower than free DNA) is present in fractions eluting at 0.20–0.45 M $(NH_4)_2SO_4$ (Fig. 1A, lanes 4–6).

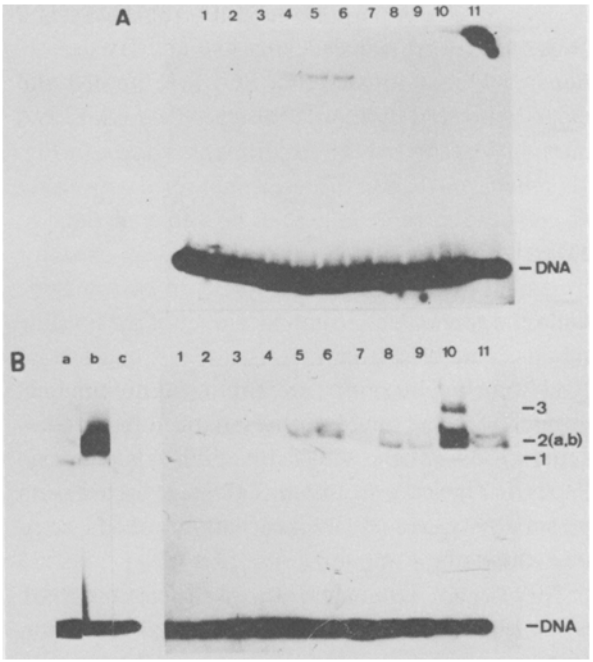

Fig. 1. Gel retardation analysis of mustard chloroplast proteins separated by chromatography on heparin Sepharose CL-6B. Protein-DNA complexes generated by incubation of column fractions with 3′ labelled H120 fragment in the absence (A) or presence (B) of 0.5 μg *E. coli* core RNA polymerase were detected by their decreased mobility during native polyacrylamide gel electrophoresis (A: upper band in lanes 4–6; B: bands 1–3). The position of unbound DNA fragment is indicated at the right (DNA). $(NH_4)_2SO_4$ concentrations of column fractions in lanes 1–11 were as follows: lane 1 = flowthrough (0.10 M), 2 (0.15 M), 3 (0.17 M), 4 (0.20 M), 5 (0.35 M), 6 (0.42 M), 7 (0.52 M), 8 (0.60 M), 9 (0.70 M), 10 (0.80 M), 11 (0.90 M). B, left panel: Controls showing retardation pattern obtained with 0.5 μg *E. coli* core enzyme alone (a), 0.5 μg holoenzyme alone (b), 0.5 μg core enzyme supplemented with 10 μg DNase I (c). Exposure times were 3 days in A and in B, lane a, and 1 day for all other experiments.

These fractions were found to contain RNA polymerase activity, although in low and variable amounts, indicating instability of these putative chloroplast RNA polymerase fractions. Attempts to obtain increased DNA binding by adding fractions that elute at higher salt concentrations were not successful (data not shown). However, when column fractions are added to *E. coli* core RNA polymerase, enhanced retardation activity giving rise to several labelled bands of lower mobility is detected with fractions eluting at 0.35–1.0 M $(NH_4)_2SO_4$ (Fig. 1B). These bands are designated 1–3 in the order of

decreasing electrophoretic mobility (bands 2a and 2b are not always resolved, see e.g. Fig. 2). Peak fractions at 0.75–0.95 M $(NH_4)_2SO_4$ were pooled and dialysed to give fracton SLF (see "Materials and methods"). The control experiments shown in Fig. 1B, lanes a and b, give the results obtained with either *E. coli* core enzyme (lane a) or holoenzyme (lane b) alone. Addition of DNase I, which was used for treatment of the chloroplast lysate, to core enzyme (lane c) does not lead to enhancement of the binding signals, suggesting that neither any residual DNase I itself nor any impurity present in this commercial preparation contribute to the enhanced retardation activity observed with the high-salt heparin Sepharose fractions. Enhancement of retardation activity by fraction SLF is heat-labile, but RNase A-insensitive (data not shown).

The effect of varying the ratio of SLF to core RNA polymerase on protein-DNA complex formation was examined by titration experiments. Figure 2 (left panel) shows that SLF itself does not bind efficiently to the *psbA* promoter fragment even at the highest concentrations used. Retardation activity, as monitored by the intensity of bands 1–3, does not seem to increase proportionally with the amonts of SLF added to core enzyme (Fig. 2, middle panel) and formation of the three complexes is affected differently. While there is only a slight increase in intensity of complex 1 at higher SLF concentration, intensities of the slower migrating protein-DNA complexes, bands 2 and 3, increase several-fold, thus leading to a retardation pattern resembling more closely that obtained with a mixture of *E. coli* RNA polymerase core and holoenzyme rather than with core enzyme alone (Fig. 2, right panel) [17]. These experiments were carried out at a 100-fold molar excess of core enzyme over H120 DNA fragment. A comparable degree of enhancement (activity ratio in the presence and absence of 1.2 μg SLF) was found at core enzyme/DNA ratios ranging from 20 to 150 (data not shown), indicating that the basic level of retardation

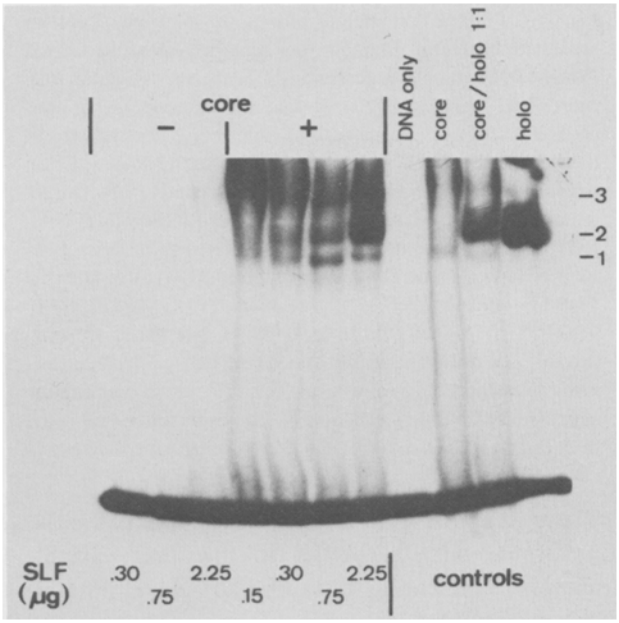

Fig. 2. Gel retardation assay for SLF/core-DNA complex composition with increasing SLF concentration. Indicated amounts of SLF were incubated with labelled H120 fragment in the absence (lanes 1–3) or presence (lanes 4–7) of *E. coli* core RNA polymerase (1.0 μg). Controls show H120 fragment alone, incubated in complete binding buffer (lane 8), with core enzyme (1.0 μg, lane 9), with core/holoenzyme 1:1 (0.5/0.5 μg, lane 10), with holoenzyme alone (1.0 μg, lane 11). 1–3: SLF/core-DNA complexes (band 2 is a doublet, see Fig. 1B).

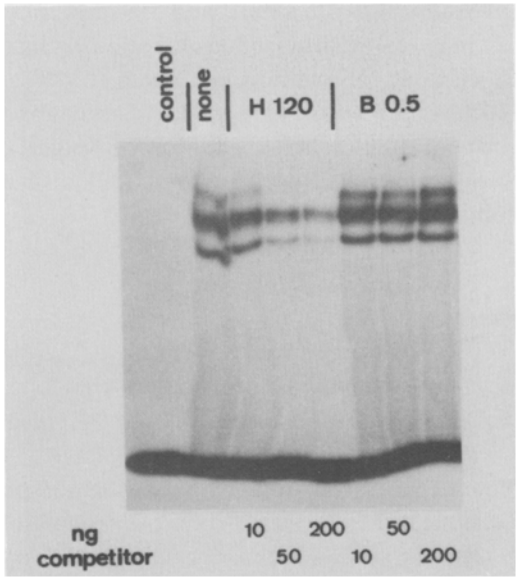

Fig. 3. Competition gel retardation assay. Protein-DNA complexes generated by 1.5 μg SLF and 1.0 μg core enzyme with 3′ labelled H120 fragment (2.5 ng) in the presence of indicated amounts of unlabelled H120 or fragment B0.5. Controls include labelled H120 fragment alone incubated in binding buffer (control), and SLF/core-H120 complexes formed in the absence of competitor DNA (none).

activity (in the absence of added SLF) is a function of the amount of core enzyme present in the assay, while the degree of enhancement appears to depend on the amount of SLF.

Figure 3 shows the results of competition retardation experiments in the presence of an excess of unlabelled DNA fragment. The competitor DNA used was either the same H120 fragment containing the *psbA* promoter or a DNA fragment (B0.5) containing intron sequences of the mustard chloroplast *trnK* gene [29]. While there is a significant decrease in relative intensity of binding signals with the H120 competitor fragment, no such decrease is evident with the *trnK* intron fragment, thus suggesting sequence-specific formation of stable SLF/core-DNA complexes. This is further supported by gel retardation assays with labelled *trnK* fragments comprising either the 5′ exon and 5′ flanking sequences including the *trnK* promoter (5′ *trnK*) or the 3′ exon and intron sequences (3′ *trnK*) [29]. It was found that the 5′ *trnK* fragment, but not the 3′ *trnK* fragment gives rise to bands of retarded mobility (S. Bülow, A. Scholz and G. Link, unpublished data).

DNase I footprinting

In an attempt to define the region(s) that interact

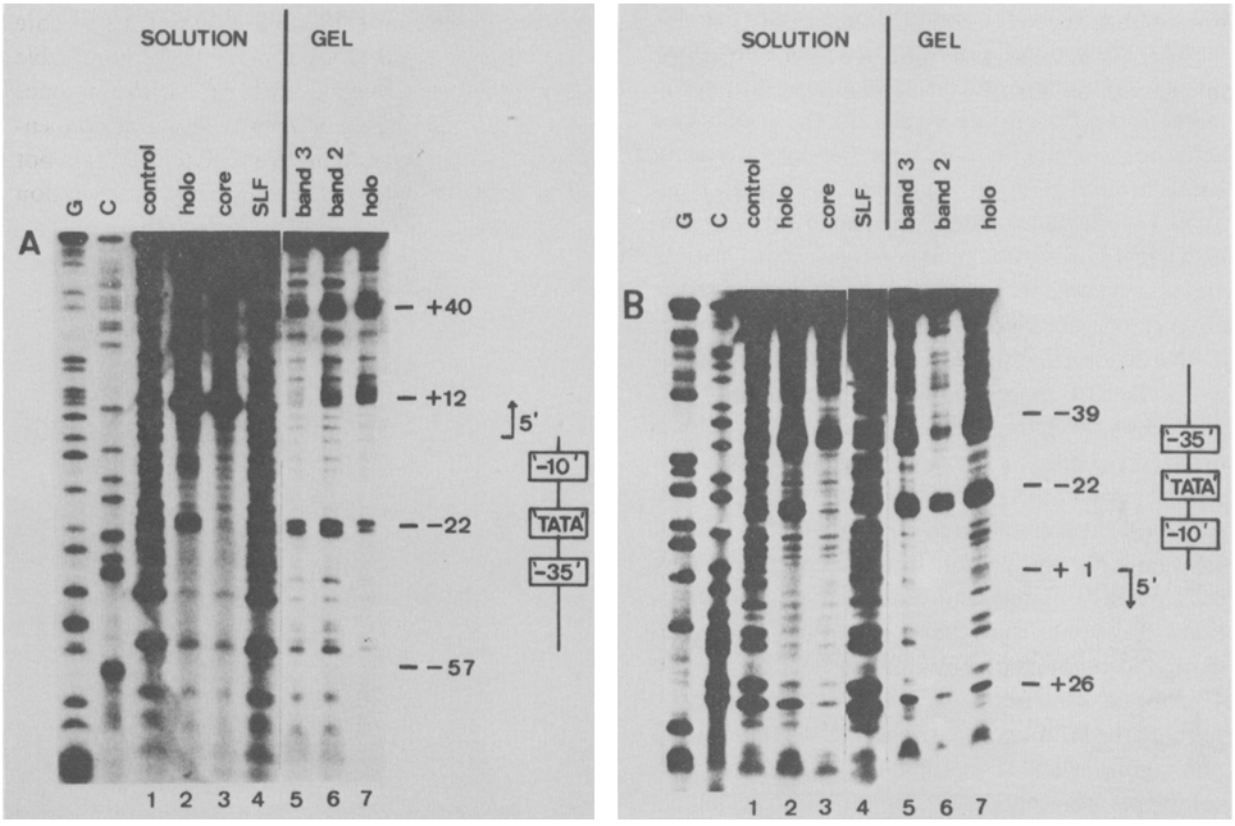

Fig. 4. DNase I footprinting of SLF/core-H120 complexes. (A) Analysis of 3′ labelled H120 coding strand, (B) non-coding strand. Lanes G, C: H120 cleavage products of chemical sequencing reactions [28]; "Standard" DNase I footprints obtained directly after protein-DNA binding; DNase I-protected products of fragment H120 incubated without protein (lanes 1), with 5 μg *E. coli* RNA polymerase holoenzyme (lanes 2), 3.5 μg core enzyme (lanes 3), 1.5 μg SLF (lanes 4); "Gel retardation" footprints obtained with the purified protein-DNA complexes (for details, see Materials and methods): Products of SLF/core-H120 complex 3 (lanes 5) and complex 2 (lanes 6), and of holoenzyme-H120 complexes from 20 μg of holoenzyme (lanes 7). Putative *psbA* promoter elements as well as several nucleotide positions, including the transcription start site, are indicated at the margins [24, 25].

with *E. coli* RNA polymerase in the presence or absence of SLF, we analysed DNA-protein complex formation with the promoter-containing H120 fragment [24, 25] by DNase I footprinting [12]. "Standard footprints" (i.e. direct electrophoretic analysis of DNase I cleavage products) with *E. coli* holoenzyme show a large protected region covering approx. 65–75 basepairs, the 5′ border of which is at least 10 nucleotides upstream of the "–35" element and the 3′ border approximately 25–30 nucleotides downstream of the transcription start site both on the H120 coding strand (Fig. 4A, lane 2) and non-coding strand (Fig. 4B, lane 2) [10, 18]. Interspersed are enhanced ("hypersensitive") bands around positions –22 ("TATA"-like region; ref. [24]) and +12 on the coding strand (Fig. 4A, lane 2) and at positions –22 and –39 on the non-coding strand (Fig. 4B, lane 2). Phosphocellulose-purified core RNA polymerase (containing only residual amounts of sigma factor) gives footprints similar to those obtained with holoenzyme, but lacking the hypersensitive bands around position –22 (Figs. 4A and B, lanes 3). SLF alone (lanes 4) does not lead to any appreciable DNase I protection or hypersensitivity of distinct regions as compared to the cleavage pattern in the absence of added protein (lanes 1).

To exclude effects of possible contaminants present in the SLF preparation and to reduce the background of DNA fragments that did not participate in the initial binding reaction, we purified DNase I-treated protein-DNA complexes by retardation gel electrophoresis (see Materials and methods). DNA cleavage products present in these complexes were subsequently eluted and fractionated. "Retardation" footprints that closely resemble the "standard" (solution) footprints shown in Figs. 4A and 4B, lanes 2, were obtained with *E. coli* holoenzyme for both the H120 coding strand (Fig. 4A, lane 7) and non-coding strand (Fig. 4B, lane 7). SLF/core-DNA complexes representing either retardation gel band 3 (lanes 5) or band 2 (lanes 6) give retardation footprints almost identical to those obtained with holoenzyme (lanes 7). The only apparent differences are the somewhat less protected bands around position –35 in both lanes 5 and 6 of Fig. 4A (complexes 3 and 2, coding strand) and the partial disappearance of the hypersensitive bands at position +12 in lane 5 of Fig. 4A (complex 3, coding strand) and at –39 in lane 6 of Fig. 4B (complex 2, non-coding strand). Core enzyme-DNA complexes and SLF/core-DNA complex 1 (compare Fig. 1B) did not give sufficient amounts of radioactive material upon elution from the retardation gel, likely due to either inefficient formation or decreased stability under footprint conditions in the presence of magnesium ions (not shown).

In vitro *transcription*

Run-off transcription was carried out using purified H120 fragment as template, and transcripts were analysed by electrophoresis on sequencing gels (Fig. 5). *E. coli* holoenzyme (lane 1) generates approxi-

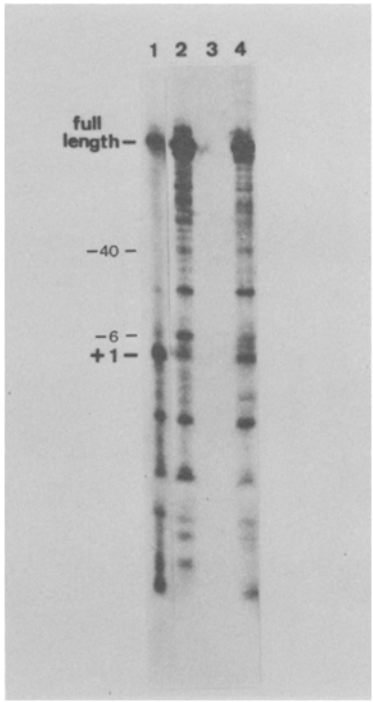

Fig. 5. In vitro transcription of the H120 fragment. Transcripts with: 5 μg *E. coli* RNA polymerase holoenzyme (lane 1), 3.5 μg core enzyme (lane 2), 1.5 μg SLF alone (lane 3), 1.5 μg SLF and 3.5 μg core enzyme (lane 4). Transcripts were analysed on sequencing gels. Indicated are the positions of full-length H120 transcripts and of transcripts with a size expected for correct initiation at the *in vivo* start site (+1). Two additional nucleotide positions of a H120 sequence ladder are given as approximate transcript size markers (–40 and –6).

mately equal amounts of two major transcripts, the full-length H120 transcript and a 63 nucleotide transcript, i.e. the size expected for correct initiation at the *in vivo* transcription start site of the *psbA* gene (+1 band) [25]. Several additional transcripts of various sizes are present in lower amounts, which might reflect unspecific initiation and/or premature termination by *E. coli* holoenzyme on the heterologous chloroplast DNA template [3, 24]. These additional bands are more prominent among the transcripts synthesized by core enzyme (Fig. 5, lane 2), while the relative intensity of the (+1) band corresponding to the correct initiation site is decreased. (Complete disappearance of this band would not be expected due to residual σ polypeptide present in the core preparation).

Addition of SLF to core enzyme (lane 4) results in higher relative levels of the correctly sized (+1) transcript and reduced levels of several other transcripts (e.g. bands that might reflect initiation near −6, −40, and further upstream). Fraction SLF alone does not reveal any transcription activity (lane 3).

The total amount of RNA synthesized was not appreciably affected by SLF in these experiments. Usage of native T4 DNA [7] as template led to six times higher incorporation of ribonucleotides into TCA-precipitable material with *E. coli* holoenzyme than with core enzyme (1 μg each), while addition of 1.5 μg SLF to 1 μg core enzyme led to 1.5-fold stimulation of total incorporation (data not shown).

Discussion

In the present study we have described a protein fraction (SLF) from mustard chloroplasts that confers enhanced specificity to *E. coli* core RNA polymerase both with regard to DNA binding and transcription. With increasing SLF concentrations, the pattern of *E. coli* core RNA polymerase-DNA complexes in gel retardation assays becomes more similar to that observed for holoenzyme [17, 39]. This SLF-mediated interaction with the DNA template appears to be sequence-specific, as indicated by the results that complex formation with a DNA fragment containing the promoter for the chloroplast *psbA* gene is competed by the same (unlabelled) fragment, but is resistant to competition with an unrelated (non-promoter) fragment.

It is interesting to note that the apparent binding activity in the gel retardation assay was always lower than might be expected from the high protein/DNA molar ratios used. Even *E. coli* RNA polymerase holoenzyme retarded no more than 60−70% of the labelled H120 fragment as judged from the relative intensities of radioactive bands from the gel (Fig. 2, right-hand lane). This could probably be partly assigned to the presence of inactive enzyme molecules and/or physically inaccessible DNA fragments. It is, however, reasonable to assume that this also might reflect structural features of the chloroplast promoter-bearing DNA fragment which are not fully compatible with high-efficiency binding of the heterologous RNA polymerase. In addition, protein/DNA complexes with limited half-life might dissociate during sample layering and electrophoresis and thus escape detection [13]. The half-life of complexes formed with core enzyme is considerably shorter than that of specific complexes with holoenzyme [26], which would be consistent with the low recovery of core/DNA complexes in the retardation assay, while these complexes lead to detectable DNase I footprints and, upon stabilization by added ribonucleotides, give rise to *in vitro* transcripts.

DNase I footprinting experiments have further substantiated the specificity of protein-DNA interaction conferred by SLF. Footprints of the region containing the *psbA* promoter obtained with either *E. coli* holoenzyme or SLF/core complexes are very similar, although not identical, and both differ from the footprints obtained with core enzyme alone, suggesting that the formation of SLF/core-DNA complexes might involve sequence-specific recognition and binding events, although the precise position of the critical sequence elements has yet to be established. It is interesting to note that there is hypersensitivity within the "TATA"-like element of the *psbA* promoter region [24]. This is reminiscent of footprints patterns of prokaryotic promoters [10, 18] and a chloroplast tRNA gene [35], which likewise show hypersensitivity at corresponding positions with *E. coli* holoenzyme, suggesting that this region might play a role in DNA binding. Further clarification of

the possible involvement of this and other regions awaits footprinting of variant *psbA* promoter fragments (A. Scholz and G. Link, in preparation).

In addition to binding specificity, fraction SLF appears to confer enhanced transcription specificity to core DNA polymerase, as indicated by the result that *psbA* transcripts of a size expected for correct initiation at the *in vivo* start site [24, 25] increase to higher relative levels when core enzyme is complemented with SLF. Based on the results of the present DNA binding and transcription experiments it is thus suggested that the chloroplast fraction SLF contains polypeptide(s) with properties comparable to prokaryotic sigma factors. It now appears possible, following further purification of the chloroplast SLF polypeptides, to identify the active species and define its (their) functional properties.

In conclusion, the present results extend the available evidence that chloroplasts possess sigma-like activity capable of complementing *E. coli* core RNA polymerase. This adds additional arguments to the view that the transcription machineries of chloroplasts and prokaryotes are structually and functionally related. Further purification and characterization of plastid sigma-like factor(s) will help to clarify the mechanisms of organelle transcription and the role of transcriptional regulation in plastid gene expression.

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