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Transcriptionally active chromosomes (TACs) of barley chloroplasts contain the α-subunit of plastome-encoded RNA polymerase

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Abstract Transcriptionally active chromosomes (TACs) were isolated from mature chloroplasts of barley, from proplastids enriched in basal segments of barley primary foliage leaves, and from ribosome-deficient plastids of heat-bleached barley leaves. Immunological analysis with a specific antibody raised against the plastid *rpoA* gene product revealed that chloroplasts contain an immunoreactive protein of 38 kDa in the TAC fraction which appears to be identical to the α-subunit contained in the soluble RNA polymerase (sRNAP) fraction of the same chloroplasts. However, only traces of immunoreactive protein were detected in a TAC preparation derived from "proplastids". A positive correlation could be demonstrated between transcriptional activity and the amount of immunoreactive 38-kDa protein by analyzing different TAC fractions eluting at different times during gel filtration of a standard TAC preparation as well as in TAC preparations obtained under various detergent conditions.

Key words Barley chloroplasts · RNA polymerase α-subunit · Transcriptionally active chromosome (TAC)

Introduction

Chloroplast RNA polymerase activity is found in two biochemically distinct chloroplast fractions: a soluble extract usually purified by DEAE anion exchange chromatography (Gruissem et al. 1986) and a fraction of protein/DNA-complexes (termed the transcriptionally active chromosome, TAC) released from plastid membranes by Triton X-100 and separated from other plastid components by gel filtration (Hallick et al. 1976; Rushlow et al. 1980). While RNA polymerase activity in the soluble fraction depends on exogenously added DNA templates, the RNA polymerase in the TAC fraction is tightly bound to plastid DNA (plDNA) and only elongates transcripts already initiated *in vivo* (for reviews see Bogorad 1991; Igloi and Kössel 1992; Gruissem and Tonkyn 1993). The two RNA polymerase activities differ with respect to inhibition by heparin and in their salt and temperature optima (Gruissem 1989). In the case of *Euglena*, the RNA polymerase of the TAC fraction seems to transcribe only ribosomal RNA genes (Rushlow et al. 1980; Gruissem 1989). Recent analysis of transcripts synthesized by TAC preparations derived from barley plastids of different developmental stages, however, clearly demonstrated that the TAC-associated RNA polymerase is capable of transcribing all gene classes (Krupinska and Falk 1994). Dominant polypeptides of highly purified soluble RNA polymerase from maize chloroplasts have been characterized by microsequencing, which revealed the presence of all four polypeptides encoded by the plastid *rpo* genes (Hu and Bogorad 1990; Hu et al. 1991). These genes code for RNA polymerase subunits homologous to those of *E. coli* (Igloi and Kössel 1992). Antibodies raised against these polypeptides were shown to inhibit the activity of the soluble fraction but not of the TAC fraction (Little and Hallick 1988), suggesting that different enzymes are active in the two plastid fractions. Besides the polypeptides encoded by the plastid *rpo* genes, additional polypeptides are present in preparations of purified soluble RNA polymerase and sigma-like factors were identified among these (Tiller and Link 1993; Troxler et al. 1994).

Prior to the detection and isolation of plastid *rpo* genes the existence of a nuclear-encoded plastid RNA polymerase had been postulated (Bünger and Feierabend 1980; Siemenroth et al. 1981; Lerbs et al. 1985). This has gained further support by recent analyses of the transcriptional

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activities of ribosome-deficient plastids derived from heat-bleached barley leaves (Falk et al. 1993) and from white leaves of the barley mutant "albostrians" (Hess et. al. 1993). Strong support for this concept is also provided by the situation observed in the plastids of the parasite *Epifagus*, as they are transcriptionally active in spite of the absence of the entire *rpoB/C1/C2* operon (Morden et al. 1991). Taken together these observations strongly suggest that plastids contain at least two RNA polymerases coded in two different genetic compartments. However, genes for a nuclear-encoded enzyme have not yet been identified.

In contrast to the soluble RNA polymerase, the exact polypeptide composition of the enzyme contained in the TAC fraction is not known. The rather complex protein compositions of the TAC fractions analyzed so far (Briat and Mache 1980; Narita et al. 1985; Bülow et al. 1987; Rajasekhar et al. 1991) do not allow an identification of RNA polymerase components. It is therefore still not clear whether TAC fractions contain an enzyme completely different from that of the soluble extracts.

In this report, TAC fractions derived from barley plastids of two different developmental stages and a chloroplast fraction enriched in soluble RNA polymerase were immunologically analyzed with an antibody raised against the over-expressed α-subunit of maize plastids.

Materials and methods

Plant material. Barley (*Hordeum vulgare* L.) seedlings were grown for 5 or 7 days in moist vermiculite at 21°C in a daily light/dark-regime of 16 h light (8000 lux) and 8 h darkness. A plastid fraction enriched in proplastids but still containing residual amounts of young chloroplasts ("proplastids") was prepared from 0.5-cm leaf sections above the kernels of 5-day seedlings, and chloroplasts were isolated from apical segments of 7-day seedlings (Krupinska and Falk 1994). Ribosome-deficient plastids were prepared from 7-day-old seedlings grown at 34°C in continuous light as described by Falk et al. (1993).

Preparation of plastids and transcriptionally active fractions. Plastids were prepared as described by Poulsen (1983). Soluble fractions with RNA polymerase activity were prepared following the method of Gruissem et al. (1986). After DEAE cellulose chromatography the fraction was further purified by heparin-sepharose (Pharmacia, Uppsala, Sweden) chromatography according to Lerbs-Mache (1993). Fractions containing transcriptionally active chromosomes (TACs) were prepared as described previously (Krupinska and Falk 1994) according to a modified protocol of the method of Rushlow and Hallick (1982) and with further modifications. Gel filtration was either performed in the presence of 1% Triton X-100 (Krupinska and Falk 1994) or with 3% (v/v) Triton X-100. Usually TAC fractions were concentrated by ultracentrifugation after gel filtration. Alternatively, a TAC extract was subjected to a second round of gel filtration in the presence of a detergent mixture consisting of 1 mM of CHAPS [3-(3-cholamidopropyl)-dimethylammonio)-1-propane-sulphonate] and 2 mM of deoxycholate.

Analysis of TAC transcription. The transcriptional activity of TAC extracts was determined as described previously (Krupinska and Falk 1994); 1 fmol of a-^{32}P-UTP incorporated in a 7-µl standard assay with 2 µl of extract during a 30-min incubation at 30°C was defined as 1 unit of activity (Units$_a$). To analyze the composition of transcripts synthesized *in vitro* by TAC extracts ^{32}P-labelled transcripts were hybridized to DNA fragments representing specific plastid genes as described by Krupinska and Falk (1994).

Antibody preparation. A 1284-bp *Bam*HI/*Pst*I fragment of the maize plastome containing the complete *rpoA* gene except for the first seven codons (Ruf and Kössel 1988) was subcloned into the plasmid pT7-7 (Tabor and Richardson 1985). An *rpoA*-encoded polypeptide, deviating due to polylinker sequences from the maize *rpoA*-encoded polypeptide only in the seven N-terminal amino acids, was over-expressed in *E. coli*. After lysis of the cells the proteins were purified according to Marston (1987). Polyclonal antibodies were raised in the rabbit. For comparison a polyclonal antiserum specific for the β-subunit of plastid ATPase (Herrmann et al. 1985) was used.

Protein analysis. Polypeptides contained in the TAC fractions were analyzed by SDS polyacrylamide-gel electrophoresis on 10%(w/v) gels prepared according to Laemmli (1970). Staining was done either with Coomassie or with silver according to Blum et al. (1987). Similar gels were used for immunological analyses. After electrophoresis, proteins were either first stained with Coomassie Brilliant Blue or directly transferred onto PVDF (Immobilon, Millipore, Bedford Mass., USA) membranes. Immunological analysis was performed as described by Humbeck et al. (1994). After incubation with the primary antibody immunoreactive bands were visualized using a peroxidase-coupled (rabbit) anti-serum with chemiluminescence detection (ECL, Amersham, Braunscheig, Germany) following the manufacturer's instructions. Filters were stripped with 0.2 M NaOH and were re-used for further immunodecorations as described (Suck and Krupinska 1996). Fluorographic signals were measured using a video-based densitometer (1D-BAsys, Biotec Fischer, Reiskirchen, Germany).

Results

As shown in Fig. 1 the polypeptides of TAC fractions prepared from chloroplasts and from "proplastids" of barley primary foliage leaves were separated by polyacrylamide-gel electrophoresis. For comparison, the polypeptides of a soluble RNA polymerase fraction prepared from chloroplasts and purified by DEAE anion exchange and heparin chromatography (see Materials and methods) were separated on the same gel. The transcriptional activity of the two different TAC fractions was determined *in vitro* and expressed in Units$_a$ (see Materials and methods). From the "proplastid" (P) TAC 300 Units$_a$ and from the chloroplast (C) TAC 300 and 150 Units$_a$, respectively, were loaded onto the gel. As a control three different amounts [2.5 ng (1×), 5 ng (2×), 25 ng (10×)] of the over-expressed *rpoA* gene product were loaded in parallel lanes of the same gel. After transfer of the polypeptides onto a PVDF membrane the α-subunit of the plastid-encoded RNA polymerase was assayed using the antisera raised against over-expressed *rpoA* gene product. The fluorogram obtained after chemiluminescence detection of antibody-protein complexes shows that the antibody recognizes a 38-kDa protein in the soluble fraction of chloroplasts as well as in the TAC fraction derived from chloroplasts. In case of the TAC fraction derived from „proplastids" much less immunoreactive protein is detectable. Signals in the molecular-weight range of 60–70 kDa are artifacts which disappeared during repeated probing of filters with different antibodies (Suck and Krupinska 1996). The signals at 38 kDa were measured densitometrically and correlated with the transcriptional ac-

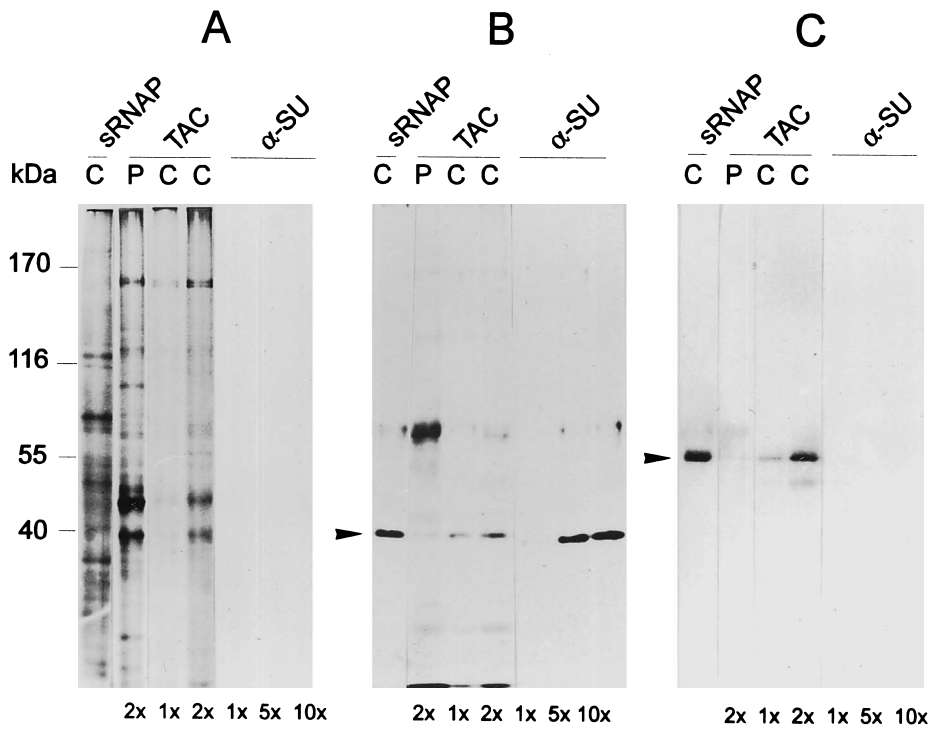

Fig. 1A–C Immunological analysis of TAC fractions derived from chloroplasts (*C)* and "proplastids" (*P*) in comparison with a soluble RNA polymerase (*sRNAP*) fraction derived from chloroplasts (*sRNAP, C*). For protein separation by SDS-PAGE, 150 (1×) and 300 $Units_a$ (2×) of the TAC fractions were loaded, respectively. Three different amounts [2.5 ng (1×), 12.5 ng (5×), 25 ng (10×)] of over-expressed a-subunit of sRNAP were loaded in parallel lanes of the gel. **A** Coomassie-stained gel. **B** Western-blot filter after immunodecoration with the antibody specific for the α-subunit of sRNAP. **C** Reprobing of the same filter with an antibody specific for the β-subunit of plastid ATPase

tivities of the respective TAC fractions. Based on the same transcriptional activity, the chloroplast TAC fraction contains more than twice as much immunoreactive 38-kDa polypeptide as the TAC fraction derived from "proplastids". Addition of the antibody specific for the α-subunit of plastome-encoded RNA polymerase during *in vitro* transcription had no effect on the transcriptional activity of the TAC extracts (data not shown).

In order to investigate whether the presence of the 38-kDa immunoreactive protein in the TAC fraction is due to loosely bound sRNAP co-purified with the TAC-associated RNA polymerase the following analyses were performed with TAC extracts derived from chloroplasts.

First the elution profile obtained during standard preparation of the TACs as described by Krupinska and Falk (1994) has been examined in detail. Fractions containing the same number of drops, respectively, were analyzed for their absorbance at 260 and 280 nm and for their transcriptional activity. As shown in Fig. 2 A transcriptional activity closely parallels the absorbance at 260 nm which is indicative of the DNA content of the fractions. Aliquots of these fractions were used for protein analyses by gel electrophoresis. As shown in Fig. 2 A and B fractions 11 and 12 have the highest protein content. Together with fraction 13 these two fractions have a common protein profile which differs from the composition of earlier- and later-eluting fractions. Immunological analysis clearly shows that the immunoreactive 38-kDa protein is contained in these three fractions. It is evident that the abundance of 38-kDa protein correlates with the fractions' transcriptional activity which is strictly dependent on endogenous DNA (Fig. 2 C). The activity cannot be enhanced by addition of exogenous DNA templates (data not shown). In contrast to the 38-kDa protein the β-subunit of plastid ATPase is about equally distributed among the various fractions and its abundance does not correlate with transcriptional activity of the fractions. Therefore it is interpreted as an impurity in the TAC fractions.

In addition to TAC extracts prepared by the standard procedure, two TAC extracts which have been prepared with the following modifications of the standard protocol were analyzed for their content of the 38-kDa immunoreactive protein. In one case 3% (v/v) Triton X-100 instead of 1% (v/v) was used during gel filtration. In the second case a TAC extract prepared with 1% (v/v) Triton X-100 was subjected to a second round of gel filtration in the presence of CHAPS and deoxycholate. Both variations caused changes in the protein composition of the TAC fractions (Fig. 3). Immunological analysis of the 38-kDa immunoreactive protein revealed that its level correlates with the transcriptional activity of the three different TAC extracts (Fig. 3). In contrast, the abundance of the β-subunit of plastid ATPase is clearly reduced in the TAC extract prepared in the presence of 3% (v/v) Triton-X 100 and does not correlate with the transcriptional activity of the three different TAC samples.

Finally the impact of the 38-kDa immunoreactive protein on transcription was investigated by comparing the relative transcriptional activities of different plastid genes in TAC extracts derived from proplastids and chloroplasts, respectively. For this purpose radiolabelled transcripts synthesized in TAC extracts *in vitro* were hybridized with DNA

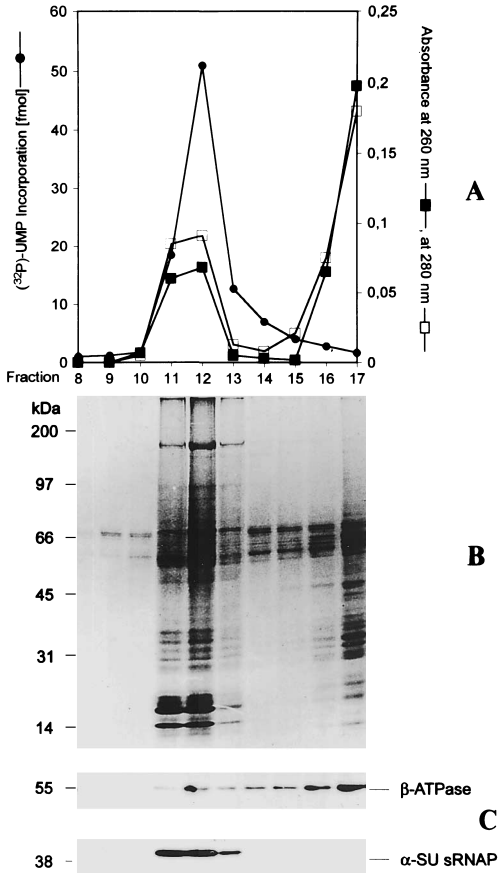

Fig. 2 A–C Analysis of different fractions with equal volumes collected during gel filtration of a chloroplast lysate containing 1% (v/v) Triton X-100. **A** Absorbance at 260 nm (-■-) and at 280 nm (-□-) compared to relative transcriptional activities of the fractions (-●-). **B** Coomassie-stained acrylamide gel obtained after electrophoretic separation of proteins in the fractions derived from the gel filtration column. **C** Immunological analysis of the proteins after transfer to a PVDF membrane. The filter was subsequently probed with an antibody specific for the β-subunit of plastid ATPase and the antibody specific for the α-subunit of sRNAP

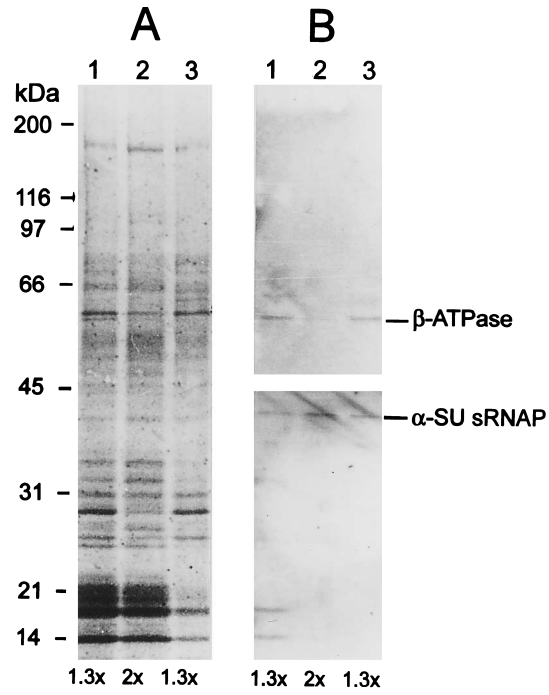

Fig. 3 A, B Analysis of proteins in different TAC fractions derived from chloroplasts. The first TAC fraction (*lane 1*) was prepared according to the standard protocol, the second TAC fraction (*lane 2*) was isolated in the presence of 3% (v/v) Triton X-100 and the third TAC fraction (*lane 3*) was subjected to a second round of gel filtration in the presence of CHAPS and deoxycholate. After gel electrophoresis, proteins were stained with Coomassie Brilliant Blue and then transferred to a PVDF membrane (**A**). Transcriptional activities of the samples are indicated below the filter [200 $Units_a$ (1.3×), 300 $Units_a$ (2×)]. The filter was subsequently immunodecorated with antibodies specific for the β-subunit of plastid ATPase and the α-subunit of sRNAP (**B**)

fragments representing specific plastid genes (Fig. 4). TAC transcripts derived from chloroplasts and "proplastids", respectively, were compared to transcripts synthesized by a TAC fraction from ribosome-deficient barley plastids (Falk et al. 1993). The relative transcriptional activities of various plastid genes differ between the TAC extracts derived from chloroplasts and proplastids as reported previously (Krupinska and Falk 1994). The main difference concerns the relative transcription of the *psbC/D* genes in comparison to ribosomal RNA genes. The ratio of transcripts hybridizing with *psbC/D* to transcripts hybridizing with rDNA is clearly higher in the case of the TAC transcripts derived from chloroplasts compared to "proplastids". The relative transcriptional activities of plastid genes in a TAC extract from ribosome-deficient barley plastids are similar to the case of a TAC derived from "proplastids". In both TAC extracts relatively high proportions of transcripts hybridizing to rDNA fragments are synthesized (Fig. 4).

Discussion

The data presented in Fig. 1 clearly show that a 38-kDa protein recognized by the antisera raised against the maize *rpoA* gene product is present in both the soluble RNA polymerase fraction (sRNAP) and the TAC fraction derived from barley chloroplasts. Analysis of different fractions collected during gel filtration clearly shows that the transcriptional activity of TAC extracts positively correlates with the amount of immunoreactive 38-kDa protein. In contrast the levels of other proteins contained in the TAC extracts, e.g. the β-subunit of plastid ATPase, do not correlate with transcriptional activity, demonstrating that these proteins are not constitutive parts of the RNA polymerase in the TAC fraction. Modifications in the conditions used for the preparation of TAC extracts were performed in order to investigate whether the 38-kDa immunoreactive protein is an intrinsic part of of the RNA polymerase in the TAC fraction or is indicative of residual sRNAP. Minor changes in the protein composition of the TAC fraction were observed in the presence of 3% (v/v)

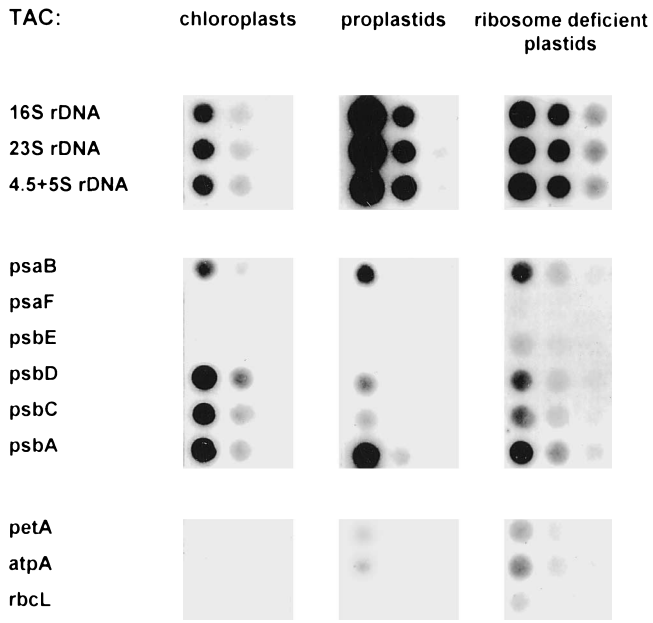

Fig. 4 Analysis of transcripts synthesized *in vitro* by TAC extracts derived from chloroplasts, proplastids and ribosome-deficient plastids, respectively. The ^{32}P-radiolabelled transcripts were hybridized to identical filters each carrying 328, 82 and 20.5 fmol (from the left to the right) of plasmid DNA from each recombinant pBluescript clone containing defined barley plDNA fragments (see Krupinska and Falk 1994)

Triton X-100, more distinct changes in the protein pattern were observed after a second round of gel filtration in the presence of CHAPS and deoxycholate. Additionally, TAC extracts were treated with high ionic strength according to Narita et al. (1985). Based on the transcriptional activities of the fractions, the level of immunoreactive 38-kDa protein was not affected by this treatment (data not shown). The results support the view that the immunoreactive protein is an intrinsic part of the RNA polymerase in the TAC fraction and does not belong to loosely bound sRNAP. It is reasonable to assume that the 38-kDa protein immunodetected in the chloroplast TAC fraction is identical with the α-subunit of plastid-encoded RNA polymerase which is already known to be present in the soluble fraction of chloroplasts (Ruf and Kössel 1988; Purton and Gray 1989; Hu and Bogorad 1990). However, it cannot be strictly excluded that the immunoreactive TAC polypeptide and the α-subunit, though of identical size, are immunologically related but structurally different. Disregarding this remote possibility and accepting that the α-subunit is indeed contained in the chloroplast TAC fraction, the occurrence of additional subunits, or even of the holoenzyme, of plastome-encoded RNA polymerase in the TAC fraction can be envisaged. An antibody raised against the over-expressed *rpoB* gene product gave rise to a signal in the sRNAP fraction but not in the TAC fraction (data not shown). Due to the low sensitivity of this antibody, it is not possible at present to decide whether the β-subunit is contained in the TAC fraction or not. If additional subunits of the sRNAP indeed were to be present in the TAC fraction this would imply that, at least in the case of chloroplasts, both plastid fractions active in transcription contain identical core components and that these are modulated to different states by additional components specifying the soluble or the TAC status, respectively.

It is evident that the "proplastid" fraction isolated from leaf bases does not exclusively contain proplastids but also an undefined portion of young chloroplasts. Hence it may be assumed that the proteins immunologically detected in the "proplastid" TAC fraction only reflect the α-subunits contained in the young chloroplasts and that proplastids themselves are devoid of this α-subunit. This supposition is supported by the observation that genes for the plastome-encoded RNA polymerase are scarcely expressed in meristematic tissue of barley and Sorghum plastids (Schrubar et al. 1990; Baumgartner et al. 1993). Rather, they are expressed during leaf development, when cells enter the zone of elongation, a finding that is in accordance with our own observations on maize plastids (Zeltz, unpublished).

The 38-kDa polypeptide is more abundant in TAC fractions derived from chloroplasts as compared with the proplastid-enriched fraction, although the two different TAC fractions exhibit comparable transcriptional activities. This leads us to the conclusion that the transcriptional activity of the "proplastid" TAC cannot be due solely to the plastome-encoded enzyme. It is therefore likely that, at least in the case of proplastids, TAC fractions contain a different, probably nuclear-encoded, RNA polymerase. According to recent studies with barley plastids deficient in ribosomes it is evident that plastids possess a nuclear-encoded RNA polymerase (Falk et al. 1993; Hess et al. 1993). A TAC fraction derived from these ribosome-deficient plastids transcribes all classes of plastid genes in a similar fashion, as does a TAC fraction derived from "proplastids" (Fig. 4). The hybridization experiments performed with TAC transcripts derived from chloroplasts, on one hand, and proplastids and ribosome-deficient plastids, on the other hand, show differences which might be explainable by differences in the composition of TAC-associated RNA polymerases. By immunological analysis of a TAC fraction prepared from ribosome-deficient plastids of heat-bleached barley no immunoreactive 38-kDa protein was detectable. At present it cannot be ruled out, however, that the failure to detect this protein is due to an insufficient amount of protein (data not shown).

To check whether the α-subunit of the plastome-encoded RNA polymerase has an influence on transcriptional activity in the TAC extract derived from chloroplasts the antibody was added during *in vitro* transcription assays. The fact that we could not detect any influence on transcriptional activity does not necessarily indicate that the α-subunit has no impact on transcription in the TAC extract. As already discussed by Little and Hallick (1988) the epitopes of the protein could be inaccessible to the antibody in the TAC fraction. It is still possible that an α-like subunit of the TAC-associated RNA polymerase is influencing transcriptional activity during chloroplast development. In this context it is of interest that a conjugation-

specific protein of the protozoan *Tetrahymena* has regions of similarity to the α-subunit of prokaryotic RNA polymerase. This protein may be involved in the control of development-specific gene transcription (Martindale 1990).

A recent report on two biochemically different RNA polymerases in the soluble fraction of mustard plastids, which differ in their relative amounts depending on the developmental stage of the plastids (Pfannschmidt and Link 1994), suggests a heterogeneous and variable composition of the plastid transcriptional apparatus. To investigate whether the chloroplast TAC fraction contains only the α-subunit of plastome-encoded RNA polymerase or the complete holoenzyme, similar immunological analyses should be employed using antibodies specific for the other components of this enzyme.

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