Volume 17 Number 23 1989 Nucleic Acids Research

Interaction of a 3′ RNA region of the mustard *trnK* gene with chloroplast proteins

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Received September 7, 1989; Revised and Accepted November 1, 1989

ABSTRACT

The 3′ flanking region of the chloroplast *trnK* gene for $tRNA^{Lys}$ of mustard contains a palindromic sequence previously implicated with transcription termination and/or processing of the precursor RNA. Here we have investigated whether RNA sequences from the *trnK* 3′ region are capable of interacting with chloroplast proteins *in vitro*. We find specific binding to an RNA region which is located further downstream from the palindromic sequence. The approximate length and position of this 3′ binding region is reflected by a 41 nt spanning RNA segment which is protected against RNase T_1 digestion by chloroplast protein(s). Competition experiments and sequence analyses suggest that U residues play an essential role in the RNA-protein interaction. Only a small number of proteins, possibly one single species, is in contact with the *trnK* 3′ RNA.

INTRODUCTION

The two principal steps in RNA 3′ end formation, i.e. transcription termination and 3′ processing, have both been characterized in detail in procaryotes as well as in the nucleus of eucaryotic cells [1,2]. Despite much recent interest in 3′ end formation of chloroplast transcripts, little is known so far on the mechanisms involved. The 3′ flanking regions of many chloroplast genes reveal palindromic elements which could form stem-loop structures resembling factor-independent terminators of bacterial genes [3]. However, functional analyses of chloroplast 3′ regions have provided evidence that, at least in some cases, these potential stem-loop structures do not function as terminators during *in vitro* transcription [4,5] and in *E. coli* [6]. Instead, they seem to act by protecting upstream RNA sequences against nucleolytic attack [4], reminiscent of procaryotic REP sequences [7,8]. In addition, *in vitro* transcription of several chloroplast tRNA genes that lack significant secondary structure in their 3′ regions was shown to be terminated by chloroplast extracts [4], suggesting that *trans*-acting factors might be involved in RNA 3′ end formation in these cases.

We have previously analyzed a region on mustard (*Sinapis alba*) chloroplast DNA, which contains the split *trnK* gene for $tRNA^{Lys}$ [9]. This gene contains its own promoter and, 3′ to the coding region, a palindromic sequence, which might represent a putative transcription terminator or RNA processing signal [9]. The *trnK* gene is the only mustard chloroplast gene found to be transiently expressed during plastid development. Its (precursor) transcript shows peak levels at or before the time when transcripts of other chloroplast genes start to accumulate to high levels [10]. As part of our attempts to clarify the mechanisms involved in the *in vivo* expression of the *trnK* gene, we have investigated the possible role of 3′ sequences and their interaction with chloroplast proteins. Here we

present evidence that protein(s) of a chloroplast extract do not bind to the *trnK* 3′ palindromic sequence itself, but to an RNA region that is located 26 nt further downstream.

MATERIALS AND METHODS

Plasmid DNA

Plasmid pSPTH80/40 contains the last 16 bp of the *trnK* 3′ exon and 109 bp of the 3′ flanking region (positions 2631−2756; [9]). This plasmid was constructed by partial digestion of the 850 bp PstI insert of pSA364a-05 [11] with HinfI. After fill-in by Klenow enzyme (BRL) a 120 bp partial product containing two HinfI fragments 80 bp and 40 bp in length was inserted into the SmaI site of pUC12 [12]. A 170 bp EcoRI-HindIII region containing both insert and polylinker sequences of the intermediate plasmid was cloned into pSPT18 (Pharmacia). Plasmid pSPTBX140, which contains *trnK* intron sequences (positions 1125−1264), consists of the 145 bp BamHI-XbaI fragment of pSPTB0.5 [9] inserted into pSPT18. Plasmid pSPTHT150 contains the last 23 bp of the *psbA* coding region and 129 bp of the 3′ flanking region (positions 1040−1189). It was constructed by digestion of the 1.05 kbp insert of plasmid pSA452a [10] with HinfI and TaqI, fill-in by Klenow enzyme and ligation of a (blunt) 142 bp HinfI-TaqI fragment into the SmaI site of pSPT19 (Pharmacia). Plasmid pSPTH120 was constructed by placing the insert of plasmid pSA05-H120 containing the *psbA* promotor region [13] into pSPT18.

Preparation of RNA transcripts

Reactions (50 μl) contained 2 μg linearized template DNA, 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 50 units placental RNase inhibitor (Pharmacia) and 15 units of T7 or SP6 RNA polymerase (Boehringer, Mannheim). All four nucleoside triphosphates (NTP) were present at 0.6 mM for synthesis of unlabeled transcripts, while radioactive transcripts were prepared with 0.2 mM of either [α-^{32}P]UTP or [α-^{32}P]GTP (100 μCi; Amersham) and 0.6 mM of the three unlabeled NTPs. Samples were incubated at 37°C for 30 min and then digested with 1.5 μg DNaseI (Worthington DPFF) at 37°C for 10 min. RNA was then extracted and analyzed on 6% denaturating polyacrylamide gels. The areas containing the *in vitro* transcripts were excised and RNA was eluted [14].

Preparation of chloroplast RNA binding extracts

Chloroplasts were prepared from 4 day-old light-grown mustard seedlings (*Sinapis alba* L., var. albatros) and further purified by sucrose density gradient centrifugation [15]. They were then hypotonically lysed and a protein extract was prepared as described [16], except that the final pellet was resuspended and dialyzed against buffer lacking $MgCl_2$. Aliquots of the extract (20 mg protein/ml) were quick-frozen in liquid N_2 and stored at −80°C. RNA binding activity is stable for ≥ 6 months and samples may be thawed and quick-frozen repeatedly.

Gel retardation assay for RNA-protein binding

The 50 μl-reaction mixture contained 30 μg extract protein and 1,000 cpm (0.5−1.5 ng) ^{32}P-labeled *in vitro* transcripts in 30 mM Tris-HCl (pH 7.0), 5 mM mercaptoethanol, 5% glycerol, 5 mM $MgCl_2$, 50 mM KCl and 40 units RNase inhibitor. In competition experiments unlabeled competitor RNA was preincubated with the extract at 25°C for 10 min prior to addition of the labeled RNA transcript. After incubation of complete binding mixtures at 25°C for 15 min, samples were loaded onto a 5% nondenaturating polyacrylamide gel [13], which had been prerun at 25 mA for 2h. Electrophoresis was carried out at room temperature for 2½ h at 25 mA. The gel was dried and autoradiographed at −80°C.

RNase T_1 protection mapping

RNase T_1 protection mapping experiments were as described [17] with modifications. TH80/40 RNA that was labeled at G positions by transcription of pSPTH80/40 in the presence of [α-^{32}P]GTP was incubated with chloroplast extract in the standard binding mixture as described above, except that RNase inhibitor was omitted. After RNA-protein binding, 10 units of RNase T_1 (Sigma) were added and incubation was continued at 25°C for 10 min. Products were then electrophoresed on a nondenaturating gel and the band representing the RNase T_1-resistant complex cut out from the gel. The RNA component of this complex was eluted overnight at 4°C as described above and used for further RNase T_1 mapping. 500−1,000 cpm of this protected RNA region and of the original labeled TH80/40 transcript were each digested with 20 units RNase T_1 in 10 μl water for 30 min at 37°C. Samples were then prepared and run on a 20% denaturating polyacrylamide gel at 1,500 V for 4 h. ^{32}P-labeled RNA size markers were synthesized from SmaI-linearized pSPT18 (25 nt) and BamHI-linearized pSPT19 (41 nt) using T7 RNA polymerase.

UV crosslinking

Standard binding reactions with labeled TH80/40 RNA and chloroplast extract, but without RNase inhibitor, were carried out in 0·5 ml microfuge tubes for 15 min. Tubes were then covered with Saran wrap and placed at a distance of 4.5 cm under a reversed TS-36 UV Transilluminator (UVP, San Gabriel, CA) with max. emission wavelength 254 nm and max. intensity 7.0 mW/cm^2. After irradiation for 30 min, 20 units RNase T_1 were added and samples were incubated at 25°C for 10 min. They were then boiled in 10% glycerol, 1% SDS, 1% mercaptoethanol, 30 mM Tris-HCl, (pH 6.8) and 0.01% bromophenol blue for 4 min, quickly cooled, and electrophoresed on a 10% polyacrylamide/SDS gel [18]. The gel was dried and autoradiographed at −80°C.

RESULTS

Complex formation of trnK 3' sequence with chloroplast protein(s) detected by gel retardation assays

Fig. 1 depicts the region of the mustard *trnK* and *psbA* genes, indicating the positions of *in vitro* transcripts [19] that were used as test RNAs in gel retardation assays [20,21]. Each of these RNAs was incubated with a mustard chloroplast extract prepared as described [16], and the mixture was then subjected to gel analysis (Fig.2).

When the ^{32}P-labeled transcript TH80/40 of the *trnK* 3' region (Fig.1) was incubated with the extract (30 μg protein), the subsequent gel assay revealed two radioactive bands (Fig.2A, lane 2), both of which migrated with retarded mobility as compared to the position of the original free RNA (Fig.2A, lane 1). Neither of these retarded bands was seen with

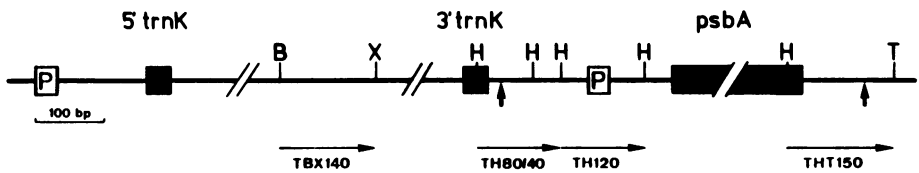


Figure 1. Organization of the *trnK−psbA* region of mustard chloroplast DNA [9,11]. Coding regions are shown as filled boxes. For each gene are indicated the position of the promoter ('P' box) and, on the 3' side, that of a potential stem-loop structure (vertical arrow). Restriction sites for construction of transcription vectors used are given in capital letters (B = BamHI, H = HinfI, T = TaqI, X = XbaI). Horizontal arrows delineate the *in vitro* transcripts used for RNA-protein binding.

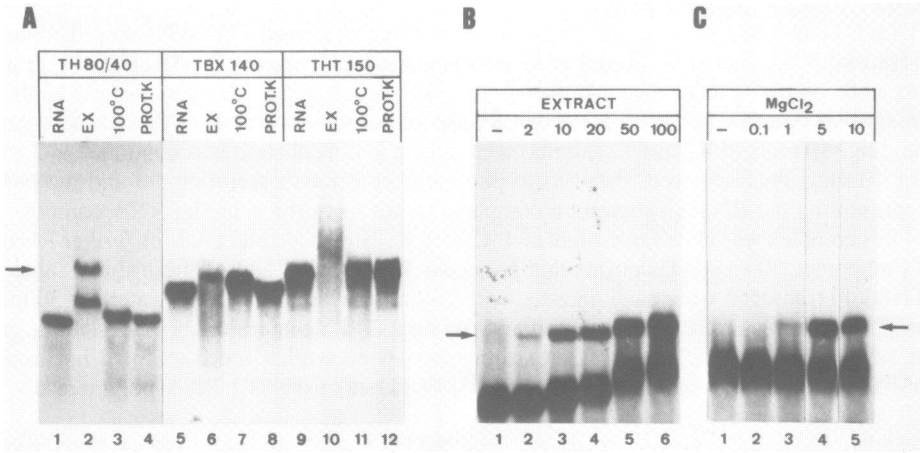


Figure 2. Gel retardation assay with *in vitro* transcripts of the *trnK–psbA* region.(A) ^{32}P labeled transcripts TH80/40, TBX140 or THT150 (see Fig.1) were incubated in binding buffer in the absence (lanes 1, 5, 9) or presence of chloroplast extract (lanes 2, 6, 10) and products were separated on a 5% non-denaturating polyacrylamide gel. Also shown are products obtained with extract denatured for 10 min at 100°C (lanes 3, 7, 11) or treated with proteinase K (4 mg/ml) at 37°C for 30 min (lanes 4, 8, 12). The arrow points to the upper retarded band that represents the putative TH80/40-protein complex (lane 2). (B) Gel retardation assay with transcript TH80/40 and increasing amounts of chloroplast extract as indicated above each lane (μg protein). (C) Effect of $MgCl_2$ on the formation of the putative TH80/40-protein complex (arrow). $MgCl_2$ concentrations (mM) used in each binding assay are indicated above each lane.

boiled or proteinase K-treated extract (Fig.2A, lanes 3 and 4), suggesting that protein(s) are involved in their formation. Variation of the extract concentration showed that with 2 μg protein in the assay the mobility of the faster-migrating band of this doublet was indistinguishable from that of free TH80/40 RNA (Fig. 2B, lanes 1 and 2). At higher extract concentrations, retardation of both the lower and upper band became noticeable. Furthermore, the intensity of the lower band decreased and that of the upper band increased with higher extract concentrations (Fig.2B, lanes 2–7). From these observations it appeared possible that the shifted position of the lower band (Fig.2A, lane 2) as compared to that of free TH80/40 RNA (Fig.2A, lane 1) was the result of unspecific interference by (bulk) proteins, while the upper band represents a specific TH80/40-protein complex.

To further test the possibility of specific binding, RNA retardation experiments were also carried out with transcripts TBX140 from the *trnK* intron (Fig.2A, lanes 5–8) and THT150 from the *psbA* 3′ region (Fig.2A, lanes 9–12). As with TH80/40, an extract-dependent band shift from the free RNA position towards lower mobility was observed in either case and, likewise, this shift did not occur with extract that had been treated with heat or protease. However, unlike with TH80/40, only one single retarded band was generated with TBX140 (Fig.2A, lane 6), which, based on its position, appears comparable to the lower retarded band seen with TH80/40 (Fig.2, lane 2). The absence of a prominent upper band in Fig.2, lane 6, suggests that the *trnK* intron transcript TBX140 does not specifically interact with extract proteins. Incubation of the extract with the *psbA* 3′ transcript THT150 (Fig. 2A, lane 10) gave a diffuse zone of retarded material, but no clearly resolved doublet comparable to that seen with TH80/40 (upper band in Fig.2A, lane 2). Attempts

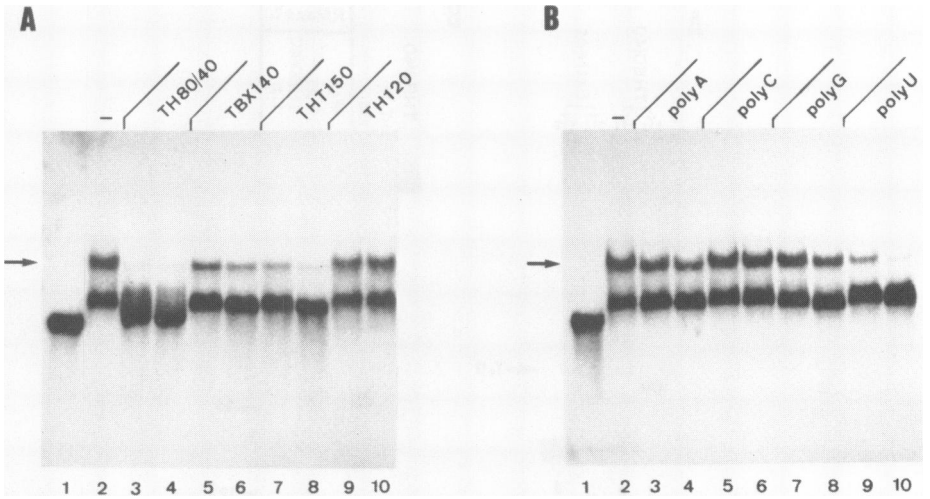
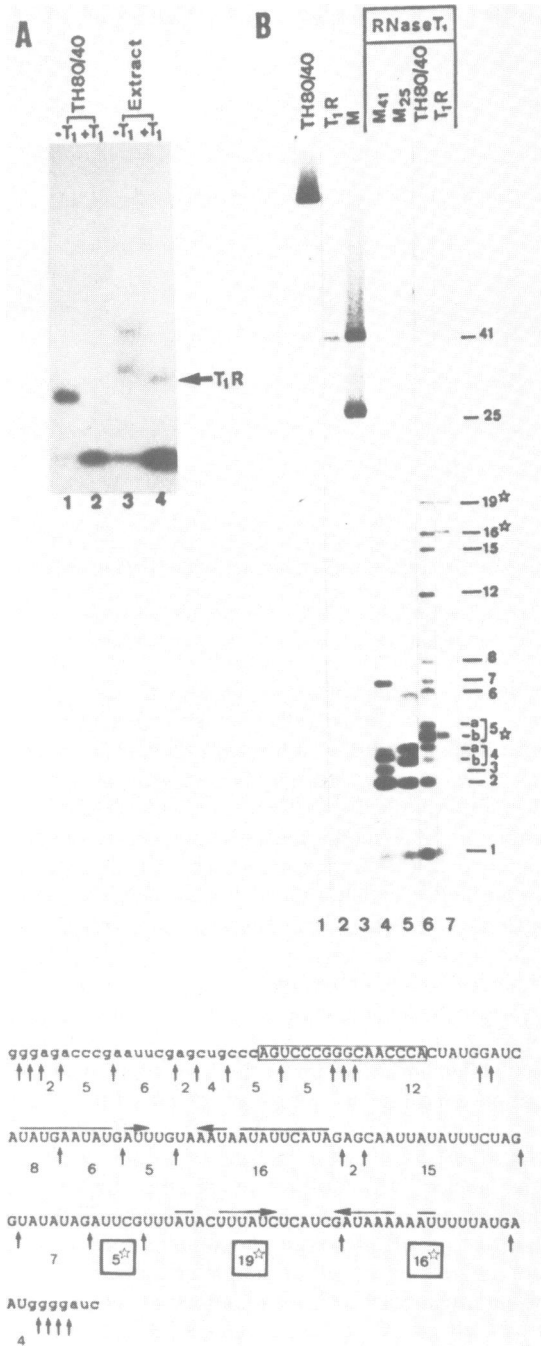


Figure 3. Gel retardation/competition experiments to test the specifity of TH80/40-protein complex formation. (A) Gel retardation assays were performed with labeled transcript TH80/40 and the unlabeled competitor RNAs (see Fig.1) indicated at the top. These were added in amounts of either 150 ng (lanes 3, 5, 7, 9) or 300 ng (lanes 4, 6, 8, 10). Controls are shown in lane 1 (free TH80/40) and lane 2 (complete binding mixture, but no competitor RNA). (B) Retardation assays as in (A), yet with indicated homopolymers as competitors.

to resolve this zone by varying the conditions for protein binding and electrophoresis were not successful (data not shown). Although these experiments do not exclude specific binding, they do not establish that the *psbA* 3′ transcript THT150 is capable of interacting with chloroplast proteins. Thus we have mainly concentrated on further characterizing RNA-protein complex formation with the *trnK* 3′ transcript TH80/40.

Several parameters of the binding reaction and gel retardation assay were tested. Formation of the putative TH80/40-protein complex was found to depend on the presence of $MgCl_2$, with an optimal concentration of 5 mM (Fig. 2C, lanes 1−5). Varying the KCl concentration from 0−500 mM had no effect on the relative intensity of retardation bands, suggesting that formation and/or stability of the TH80/40-protein complex is not likely to require a narrow range of optimal ionic strength. Addition of RNase inhibitor (40 units) helped to retain the intactness of the test RNA, but neither influenced the relative intensity nor the mobility of the RNA-protein complex bands (data not shown).

To obtain more detailed information on the specificity of TH80/40-protein complex formation, competition experiments were carried out with 100 and 200 fold excess of unlabeled (homologous and various heterologous) competitor RNAs. The presence of unlabeled transcript TH80/40 in the binding mixture resulted in almost complete disappearance of the labeled TH80/40-protein complex (upper band in Fig. 3A, lanes 3 and 4). Amongst heterologous RNA competitors, transcript TH120 from the *psbA* 5′ region (see Fig.1) had no effect on TH80/40-protein complex formation (Fig.3A, lanes 9, 10), while TBX140 from the *trnK* intron led to a slight decrease of the binding signal (Fig.3A, lanes 5 and 6). The significance of this weak competition effect at high excess of TBX140 is unclear, since with labeled TBX140 no evidence for specific RNA-protein complex formation was obtained (Figure 2A, lane 6). A stronger competition effect was found with


A
TH80/40
Extract
-T1 +T1 -T1 +T1
T1R
1 2 3 4
B
RNaseT1
TH80/40
T1R
M
M41
M25
TH80/40
T1R
41
25
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1 2 3 4 5 6 7
gggagacccgaauucgagcugccc AGUCCCGGGCAACCCA CUAUGGAUC
AUAUGAAUAUGAUUUGUAAAUAAUAUUCAUAGAGCAAUUAUAUUUCUAG
GUAUAUAGAUUCGUUUAUACUUUAUCUCAUCGAUAAAAAAUUUUUAUGA
AUggggauc

THT150 RNA from the *psbA* 3′ region (Fig.3A, lanes 7 and 8), suggesting that extract protein(s) which bind to TH80/40 also seem to interact, although more weakly, with THT150 (see also Fig.2A, lane 10). Amongst other nucleic acids that were tested as competitors neither *E. coli* tRNA (2 μg) nor DNA fragments representing the various test regions of the *trnK* and *psbA* genes (Fig.1) were found to be effective (data not shown). Presence of the homopolymers poly(A), poly(C) or poly(G) likewise did not affect the formation of the TH80/40-protein complex (Fig.3B, lanes 3−8). In contrast, poly(U) led to decreased intensity of the binding signal (upper band in Fig.3B, lanes 9 and 10).

RNase T_1 mapping of protected TH80/40 sequences

To locate the region(s) on TH80/40 that interact with extract protein(s), an RNase T_1 mapping procedure was applied [22,17]. The TH80/40 transcript labeled at guanosine positions by using [α-^{32}P]GTP was incubated with or without chloroplast extract, yet in the absence of RNase inhibitor. Addition of RNase T_1 resulted in complete digestion of TH80/40 control RNA incubated in the abscence of extract proteins (Fig.4A, lanes 1 and 2). The two bands formed in the presence of the chloroplast extract (Fig.4A, lane 3) both disappeared upon nuclease treatment and one single T_1-resistant band (T_1R) of higher mobility became visible (Fig.4A, lane 4).

When the RNA moiety of T_1R was reisolated and then electrophoresed, it gave rise to a single band, which comigrated with a 41 nt-marker RNA (M_{41}) obtained by run-off transcription of pSPT18/19 polylinker sequences [19] (Fig.4B, lane 2 and 3), indicating that an uninterrupted region within TH80/40 was protected by binding protein(s). To locate the protected region within TH80/40, the RNA component of T_1R (Fig. 4B, lane 2) and full-size TH80/40 (Fig.4B, lane 1) were each digested with RNase T_1 and the sizes of the (G-specific) cleavage products were compared to distances between G residues of the *trnK* 3′ sequence (Fig.4, bottom). Digestion of full-size TH80/40 resulted in fragments ranging from 19 nt to 1 nt in size (Fig.4B, lane 6). The expected TH80/40 cleavage products of most size classes could each be assigned to single bands, except for fragments of the 4 and 5 nt classes, each of which gave rise to two bands that slightly differed in their mobility ($5_{a/b}$ and $4_{a/b}$). This heterogeneity in the mobility of small equal-sized fragments [17] did not interfere with the present analysis, since comparison with the T_1 cleavage products of marker transcripts M_{41} and M_{25} (Fig.4B, lanes 4 and 5) allowed to distinguish fragments of the 5 nt class from those of the 4 nt class. Digestion of the isolated T_1R RNA revealed labeled fragments of 19 nt, 16 nt and 5 nt in size (Fig. 4B, lane 7), suggesting a length of approximately 40 nt for the entire protected region. This is in agreement with the value of 41 nt determined by sizing the T_1-resistant region of TH80/40 directly

Figure 4. RNase T_1 mapping of the TH80/40-protein complex. (A) Combined gel retardation/T_1 protection assays of TH80/40 labeled at guanosine positions, without (lanes 1 and 2) and with chloroplast extract (lanes 3 and 4). $-T_1$, products obtained without subsequent T_1 treatment (lanes 1 and 3); $+T_1$, treatment with 10 units RNase T_1 for 10 min (lanes 2 and 4). Arrow: RNase T_1 resistant complex (T_1R). (B) High resolution separation of T_1 resistant products on a 20% denaturating polyacrylamide gel. Intact TH80/40 (lane 1); isolated T_1R RNA (lane 2); marker transcripts M_{41} and M_{25} from the pSPT18/19 polylinker (lane 3); fragments resulting from RNase T_1 digestion of TH80/40 (lane 6), of T_1R RNA (lane 7), and of marker RNAs M_{41} and M_{25} (lanes 4 and 5). Fragment lengths (nt) are given at the right margin. 4a/b and 5a/b: equal-sized fragments with different mobility. Asterisks: oligonucleotide fragments of the T_1R complex. The nucleotide sequence of TH80/40 RNA is shown at the bottom, with bases of the polylinker regions indicated by small letters. The *trnK* 3′ exon is boxed and potential stem-loop forming regions are overlined. The expected RNase T_1 cutting sites at G residues are marked by arrows. Numbers beneath indicate sizes (nt) of the resulting oligonucleotides. Boxed numbers with asterisks represent oligonucleotides of the T_1R complex (see Fig. 4B, lane 7).

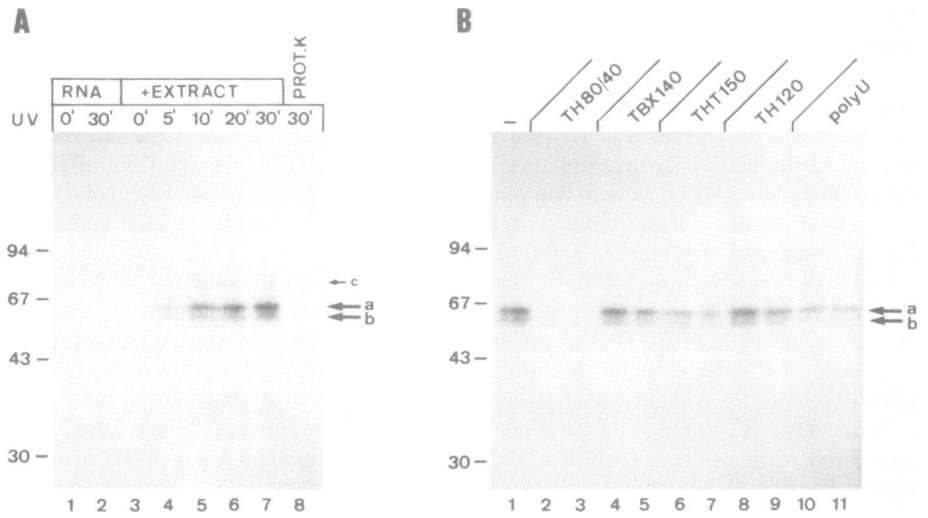

Figure 5. UV crosslinking of the TH80/40-protein complex. (A) UV crosslinking of chloroplast proteins to labeled TH80/40 RNA, treatment with RNase T_1, and separation of products on a 10% polyacrylamide/SDS gel. UV exposure times (min) are given at the top. Lane 1: RNA alone without UV irradiation. Lane 2: RNA with UV irradation for 30 min. Lanes 3–7: RNA incubated with chloroplast extract and exposed to UV irradiation for O, 5, 10, 20, 30 min. Lane 8: RNA incubated with proteinase K-treated extract (4 mg/ml, 30 min at 37°C). (B) Competition UV crosslinking (30 min) with labeled TH80/40 without (lane 1) and either 150 ng (lanes 2, 4, 6, 8, 10) or 300 ng (lanes 3, 5, 7, 9, 11) of indicated competitor RNAs. Molecular sizes (kDa) of marker proteins are given at the left margins. Arrows: UV crosslinked TH80/40-protein complexes of 62 kDa (a), 58 kDa (b) and 70 kDa (c).

(Fig.4B, lane 2). The *trnK* 3′ sequence (Fig.4, bottom) shows that only one single 19 nt fragment can be expected after RNase T_1 digestion of TH80/40. This fragment is flanked by 5 nt (upstream) and 16 nt (downstream) fragments, suggesting that it is this 40–41 nt region approximately 70 nt downstream of the *trnK* 3′ exon which is protected from RNase T_1 digestion by chloroplast protein(s).

UV crosslinking of polypeptides bound to the trnK 3′ transcript

Protein(s) of the chloroplast extract which interact with labeled TH80/40 were analyzed by UV crosslinking [23–25]. Following incubation of the test RNA with or without protein, the mixture was irradiated with UV and then treated with RNase T_1. The resulting labeled complexes consisting of proteins crosslinked to residual T_1-resistant RNA were separated on polyacrylamide/SDS gels. As shown in Fig.5A, only after incubation of TH80/40 in the presence of chloroplast extract and depending on UV irradiation (lanes 4–7), labeled bands of crosslinked material became visible, which migrated at approximately 70 kDa, 62 kDa and 58 kDa. These bands were not observed with protease-treated extract (Fig.5A, lane 8), suggesting that they indeed are the result of protein-RNA interaction. The 62 kDa species was the predominant band with a 3–4 fold higher intensity than that of the 58 kDa band, while the intensity of the 70 kDa band was lower by at least an order of magnitude and this band was not consistently observed in other experiments (see e.g. Fig. 5B).

The UV crosslinking analysis does not discriminate between the possibilities that either several different polypeptides are involved in complex formation with TH80/40 or, alternatively, one single binding protein might be attached to residual RNA fragments of

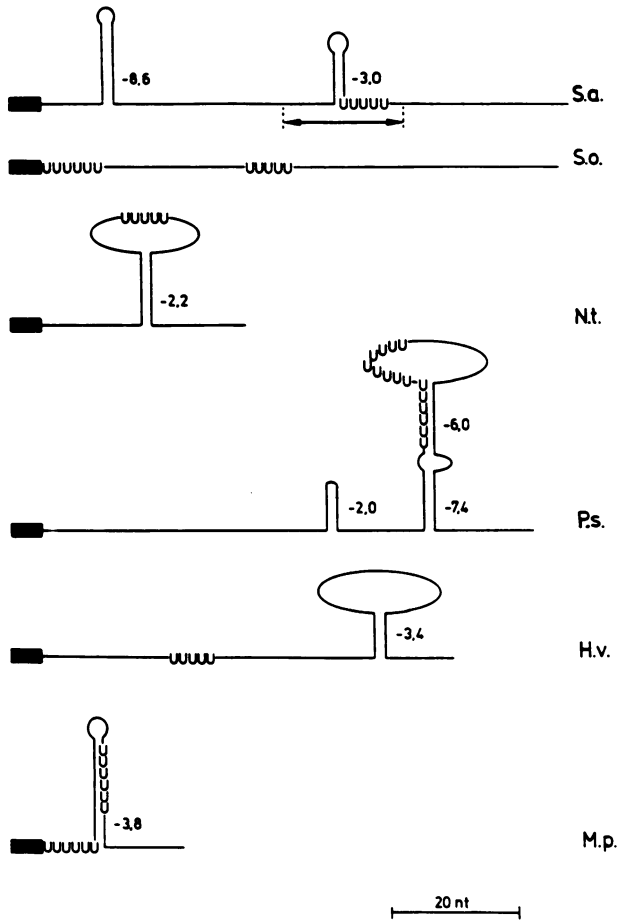

Figure 6. Scheme showing the region from the *trnK* 3′ exon (filled box at the left) to the *psbA* promoter (right end) of several plant species. Depicted are U tracks (UT) with at least 5 consecutive residues and palindromic regions (PR) which could be formed into stem-loop structures with $\Delta G \leq -2.0$ kcal [41] as indicated by the numbers next to the stems. Distances (nt) from the trnK 3′ exon are: S.a. (*Sinapis alba* [9]): 1st PR (11−40), 2nd PR (75−99), UT (99−103); S.o. (*Spinacia oleracea* [29]): 1st UT (1−6), 2nd UT (34−38); N.t. (*Nicotiana tabacum* [30]): PR (16−76), UT (42−46); P.s. (*Pisum sativum* [31]): 1st PR (48−68), 2nd PR (85−171), 1st UT (106−111), 2nd UT (113−121); H.v. (*Hordeum vulgare* [32]): PR (56−100),UT (22−26); M.p. (*Marchantia polymorpha* [33]): PR (8−42), 1st UT (1−8), 2nd UT (29−34). The arrow below the upper line marks the position of the T_1-resistant 41 nt-region within TH80/40 (see Fig.4).

different lengths. Although the results of T_1 mapping (Fig.4), which showed only one single protected region 41 nt in size, did not provide evidence for this latter possibility, it cannot be excluded that (conformation-dependent) differences in RNase T_1 sensitivity might be involved. To obtain more detailed information on the exact size and number of protein(s) interacting with TH80/40, further purification of the binding activity will be required.

To test the specifity of the RNA-protein complexes visualized by the UV crosslinking technique, competition experiments were carried out with excess of unlabeled competitor RNAs (Fig. 5B). Presence of unlabeled TH80/40 almost completely eliminated the binding signals (Fig. 5B, lanes 2 and 3), while neither TBX140 (lanes 4 and 5), nor TH120 (lanes 8 and 9) led to a significant decrease in the intensity of labeled bands. In contrast, THT150 (Fig.5B, lanes 4 and 5) and poly(U) (lanes 10 and 11) were both found to decrease the intensity of the binding signals, thus supporting the conclusion from the retardation competition experiments (Figure 3) that these RNAs might also interact, although more weakly, with TH80/40 binding protein(s).

DISCUSSION

Previous attempts to define the *in vivo* 3′ terminus of primary transcripts of chloroplast tRNA genes have proven difficult, while *in vitro* processing of synthetic precursors in chloroplast extracts has been successful [26−28]. In our present work we have established specific RNA-protein interaction between *trnK* 3′ RNA sequences and protein(s) of a chloroplast extract. This suggests that the primary transcript *in vivo* might extend at its 3′ side beyond the palindromic sequence, previously implicated with transcription termination and/or RNA processing [9], into the region 22−26 nt downstream that exhibits binding activity *in vitro*.

The competition experiments (Fig.3 and 5) have shown that RNA sequences representing other regions of the *trnK* and *psbA* genes vary over a wide range in their effectiveness as competitors. While there is no interference of *trnK* 3′ RNA-protein complex formation by RNA sequences from the *psbA* 5′ region (TH120) and little competition by *trnK* intron RNA sequences (TBX140), an RNA segment from the *psbA* 3′ region (THT150) acts as an efficient competitor, although less than the homologous competitor RNA from the *trnK* 3′ region (TH80/40). The degree of competition observed with this *psbA* 3′ RNA was unexpected, since labeled THT150 by itself did not give unambigous evidence for specific RNA-protein complex formation in gel retardation assays (Fig.2). It appears most likely that the requirements for THT150-protein complex formation are incompletely reflected by the *in vitro* system used, resulting in transiently and/or inefficiently formed complexes which interfere with the binding between labeled TH80/40 and chloroplast protein(s).

Among the four ribohomopolymers tested as competitors for TH80/40-protein interaction, only poly(U) revealed a significant competition effect comparable of that of THT150, indicating that uridine residues may play a functional role in the recognition of TH80/40 by chloroplast binding protein(s). This is supported by the results of RNase T1 mapping experiments (Fig.4), which showed that the 41 nt-region protected against RNase digestion by binding of extract protein(s) (T_1R RNA) is rich in U content (47·5% of all nucleotide positions). The RNA component of T_1R does not reveal any significant secondary structure, except for a weak potential stem-loop ($\Delta G = -3{,}0$ kcal). This sequence element is followed by a cluster of 5 uridine residues (Fig. 4, bottom, and Fig.6), reminiscent of procaryotic rho-independent terminators, but it lacks a typical GC-rich region preceding the U residues [1]. To define the sequence requirements for binding to the *trnK* 3′ RNA more precisely, it will be necessary to test appropriately designed mutants in binding assays.

In an attempt to detect common features, we have compared the *trnK* 3′ sequence from mustard with that of equivalent regions of chloroplast genes from other plants, using the Beckman MicroGenie programs [41,42] Except for a high content of A and T residues, none of these regions share any sequence elements or conserved secondary structure.

However, they all reveal the existence of clusters of at least 5 U residues, embedded in regions of variable nucleotide composition and secondary structure (Fig. 6). Taken together, this adds to the notion borne out by our *in vitro* binding studies that palindromic sequences are not critical determinants, while U tracks might be crucial for interaction of 3′ RNA sequences with chloroplast proteins.

It is well-established that multiple U residues play a role in RNA-protein interaction within the nucleo/cytoplasmic compartment of eucaryotic cells [34]. Evidence is available that U clusters are involved in the 3′ end formation of RNAs transcribed by both RNA polymerase I and II [35,36]. Even termination of transcription by RNA polymerase III requires U clusters of $\geq$ 4 nt within the 3′ flanking region of nuclear tRNA genes [37]. Recent studies have demonstrated that the nuclear 'La' phosphoprotein binds to the 3′ terminal U tracks of RNA polymerase III transcripts, thereby acting in termination of transcription [38,39]. The properties of the La protein appear particularly interesting in view of observations which suggest that chloroplasts might contain a polIII-like transcription system [40]. Purification and biochemical characterization of the RNA-binding protein(s), together with functional assays *in vitro*, will help clarify the mechanisms of RNA 3′ end formation and might allow studies on the role of these processes in plastid gene expression *in vivo*.

ACKNOWLEDGEMENTS

We wish to thank U. Müller and C. Wittig for skilled technical assistance S. Bülow and H. Neuhaus for initial help and discussion, and A. Scholz for construction of plasmid pSPTH120. This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie, FRG.

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