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Transcript analysis of the tobacco plastid operon *rps2/atpI/H/F/A* reveals the existence of a non-consensus type II (NCII) promoter upstream of the *atpI* coding sequence

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Abstract The plastid ATP synthase complex is composed of nine subunits, of which six are encoded in the plastome. The plastid-encoded genes are arranged in two transcriptional units: *atpB*/*E* and *atpI*/*H*/*F*/*A*. We have recently reported that besides containing four −10 and −35 consensus-type (CT) promoters, the *atpB*/*E* operon also contains a non-consensus type (NCII) promoter that alone is responsible for its expression in non-photosynthetic plastids. As the functionality of ATP synthase requires expression of all nine subunits, NCII promoter-driven transcription of the *atpI*/*H*/*F*/*A* operon is to be expected in non-photosynthetic plastids. Therefore, a detailed transcriptional analysis of this operon was carried out using RNA samples from tobacco leaf, cultured cells (BY-2) and seedlings grown on streptomycin and spectinomycin; which contain chloroplasts, translationally active non-photosynthetic plastids and translationally inactive plastids, respectively. We identified a total of three transcription initiation sites (TIS) and four transcript processing sites in the non-coding regions of this operon. Our results also demonstrate that *rps2* is co-transcribed with the *atpI*/*H*/*F*/*A* genes. One of the TIS (−208 *atpI*) is characterized by an NCII type promoter, while other two primary transcripts (−131 *atpI* and −384 *atpH*) initiate from CT promoters. In non-photosynthetic plastids the *atpI*/*H*/*F*/*A*-specific transcript pool seems to be solely contributed by initiation at the −208 *atpI* (NCII type) promoter, because transcripts from CT promoters do not accumulate in these plastid types.

Key words *atpI*/*H*/*F*/*A* Operon · Differential promoter usage · NCII promoter · *Nicotiana tabacum* · Transcriptional analysis

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Introduction

The promoter regions of most plastid transcription units have been reported to consist of prokaryotic (σ^{70}-type) −10 and −35-like consensus sequences, referred to hereafter as consensus-type (**CT**) promoters. These promoters are utilized by a prokaryotic-type RNA polymerase that is at least partially encoded by the plastid genes *rpoA*, *rpoB*, *rpoC1* and *rpoC2* (reviewed in Igloi and Kössel 1992; Sugiura 1992; Gruissem and Tonkyn 1993; Link 1994). Besides this *E. coli*-like transcription system, several reports have suggested the occurrence of another transcription machinery in plastids that could involve a unique, entirely nuclear-encoded polymerase (reviewed in Igloi and Kössel 1992; Gruissem and Tonkyn 1993; Mullet 1993).

The recent demonstration of transcriptional activity in the ribosome-deficient plastids of barley *albostrians* mutants and artificially created tobacco *rpoB* deletion mutants, which lack the functional plastid-encoded polymerase, clearly established the existence of a **N**uclear-**E**ncoded **P**olymerase, **NEP** (Hess et al. 1993; Allison et al. 1996). Using a different approach, i.e., inhibition of plastid protein synthesis by spectinomycin and streptomycin (Spc/Str) we identified a novel class of non-consensus type (NCII) plastid promoters, which are specifically utilized by the entirely nuclear-encoded transcription system. We found that only genes involved in house-keeping processes, and not photosynthesis, are characterized by NCII-type promoters (Kapoor et al. 1997). It was also demonstrated that, in non-photosynthetic plastids in which accumulation of CT promoter-derived transcripts is almost negligible, transcripts from NCII-type promoters alone may provide the necessary mRNA pool for translation of the respective polypeptides. As one of the NCII promoters characterized in the above study was −290 *atpB*/*E*, we anticipated that similar promoter(s) would provide the necessary transcripts for the rest of the plastid-encoded ATP synthase polypeptides in non-photosynthetic plastids.

The *rps2/atpI/H/F/A* gene cluster codes for four ATP synthase subunits: the CF_1 α-subunit and CF_0 subunits I, III and IV (Hennig and Herrmann 1986). This operon has been reported to produce up to 20 transcripts ranging in length from 0.5 to 6.0 kb (Cozens et al. 1986; Hudson et al. 1987; Stahl et al. 1993; Stollar and Hollingsworth 1994). In spinach it has been found to be co-transcribed with an upstream ribosomal protein gene *rps2* (Stahl et al. 1993; Stollar and Hollingsworth 1994). However, there has been no reported example of developmental or tissue-dependent qualitative differences in the transcript populations so far.

Here we report a detailed investigation of transcripts derived from the *rps2/atpI/H/F/A* gene cluster in morphologically distinct plastid types from tobacco leaf, suspension culture cells (BY-2 cell line) and wild-type tobacco seedlings grown in the presence of Spc/Str. Our data revealed major differences in the *rps2/atpI/H/F/A*-specific transcript population among developmentally different plastids in leaves and cultured tobacco cells. These differences are at least in part due to cell type-dependent, selective promoter utilization.

Materials and methods

Plant materials

Tobacco (*Nicotiana tabacum* var. BY-4) seedlings were grown for 20 days at 28° C under 18 h light/6 h dark cycles in a growth chamber. *N. tabacum* BY-2 cells were cultured in MS medium (pH 5.4) containing 2,4-D (0.2 mg/l), thiamine-HCl (1 mg/l), NaH_2PO_4 (370 mg/l) and 3% sucrose for 2 days at 25° C at 100 rpm in the dark. Treatment of tobacco with spectinomycin and streptomycin was carried out as described (Kapoor et al. 1997).

Preparation of nucleic acids

DNA fragments derived from tobacco chloroplast genome clone pTBa4 (Shinozaki et al. 1986; Sugiura et al. 1986) were subcloned into pBluescript SK+ (Stratagene). The following oligonucleotides were used for primer extension and TAP-RLPCR analyses: Linker primer (5′ CGCTCTAGAACTAG-TGGATCC 3′); R2-1 (5′CCATGACCAAAATGAACTCC 3′); AI3(5′GTCTACTTGAATCGGAT ATTTTGAATACCAACTAAG-ATTTAGA 3′); AI-2 (5′GGCCTACTTCCACACCGGATATAT-CGTATAACCCC 3′); AI-1 (5′TGGCCTACTTCCACACCGGA 3′); AH-2 (5′ATTCACAGTCACAAGGGGCCGGAAGGACTT-C3′); AH-1 (5′ACGGAA GCGGCAGAAATCAG 3′); AF-2 (5′GAACTAACGAACTAGCCCTTTTATTG 3′). Total cellular RNA from tobacco leaves and BY-2 cells was extracted as described (Kapoor et al. 1997).

RNA analysis

Northern blot analysis, primer extension, ribonuclease protection assays and in vitro capping experiments were performed as described earlier (Sambrook et al. 1989; Vera and Sugiura 1992; Kapoor et al. 1994). The [α-^{32}P]UTP-labeled antisense RNA probes were prepared with a T7 or T3 in vitro transcription system (Stratagene), using DNA subcloned in pBluescript SK+ as template. In order to differentiate transcription initiation sites from processed 5′ termini we used the tobacco acid pyrophosphatase (TAP)-reverse ligation PCR (RLPCR) method as described (Fromont-Racine et al. 1993). A linker RNA molecule is ligated to the 5′ end of TAP-treated and untreated RNA samples. Then these RNA samples are used for cDNA synthesis by reverse transcriptase using an appropriate gene-specific primer. This is followed by PCR amplification of the cDNA using the gene-specific primer and a DNA primer that is complementary to the RNA linker, one of which is labeled with ^{32}P. The resultant products are analyzed on denaturing polyacrylamide gels. As the linker RNA can be ligated to 5′ monophosphate ends but not to primary transcripts, which contain triphosphates, the primary transcripts do not give rise to corresponding bands in the lanes containing RNA not treated with TAP. The linker RNA used in TAP-RLPCR analysis was synthesized with the T3 MEGAscript in vitro transcription kit (Ambion) using pBluescript SK+ linearized with *Xho*I as template. The transcription product was eluted after being fractionated on a 7% polyacrylamide gel containing 7 M urea.

Results

Northern blot analysis

We carried out a Northern blot analysis of transcripts of the *rps2/atpI/H/F/A* gene cluster to determine whether or not similar populations of RNA accumulate in developmentally different plastids. Total RNA from chloroplast-containing tobacco leaves and non-photosynthetic plastid-containing cells of the cultured line BY-2 was isolated, fractionated, blotted and hybridized to antisense gene-specific RNA probes for the *rps2*, *atpI*, *atpH*, *atpF* and *atpA* coding regions and the intron of *atpF* (Fig. 1). In agreement with earlier reports from spinach, pea and maize, more than 20 transcripts ranging in size from 0.4 to 5.7 kb were detected in leaf samples (lanes L, Fig 1). However, in BY-2 cells the longest transcript was approximately 3.1 kb long, which did not correspond to any transcript species accumulating in leaves. Among other transcripts, a 1.65-kb transcript was also found to be unique to the BY-2 cells. These results clearly demonstrate differential developmental accumulation patterns for *atpI/H/F/A* transcripts, which might be due to differences in transcriptional and/or post-transcriptional processes.

Analysis of transcript 5′ ends

As we have recently demonstrated, qualitative differences in transcript accumulation might result from developmentally specific differential promoter utilization by discrete RNA polymerase activities (Kapoor et al. 1997). Therefore, a detailed analysis of the 5′ ends of transcripts derived from the *atpI/H//F/A* gene cluster was carried out to distinguish between transcription initiation sites (TISs) and processed 5′ ends. First, the 5′ ends of the respective transcripts were precisely mapped using ribonuclease protection and primer extension analyses. Total RNA from Spc/Str-treated seedlings was also included in the primer extension analysis. The Spc/Str treatment inhibits plastid protein synthesis, thereby resulting in seedlings which are presumably incapable of synthesizing plastid-encoded RNA polymerase (see

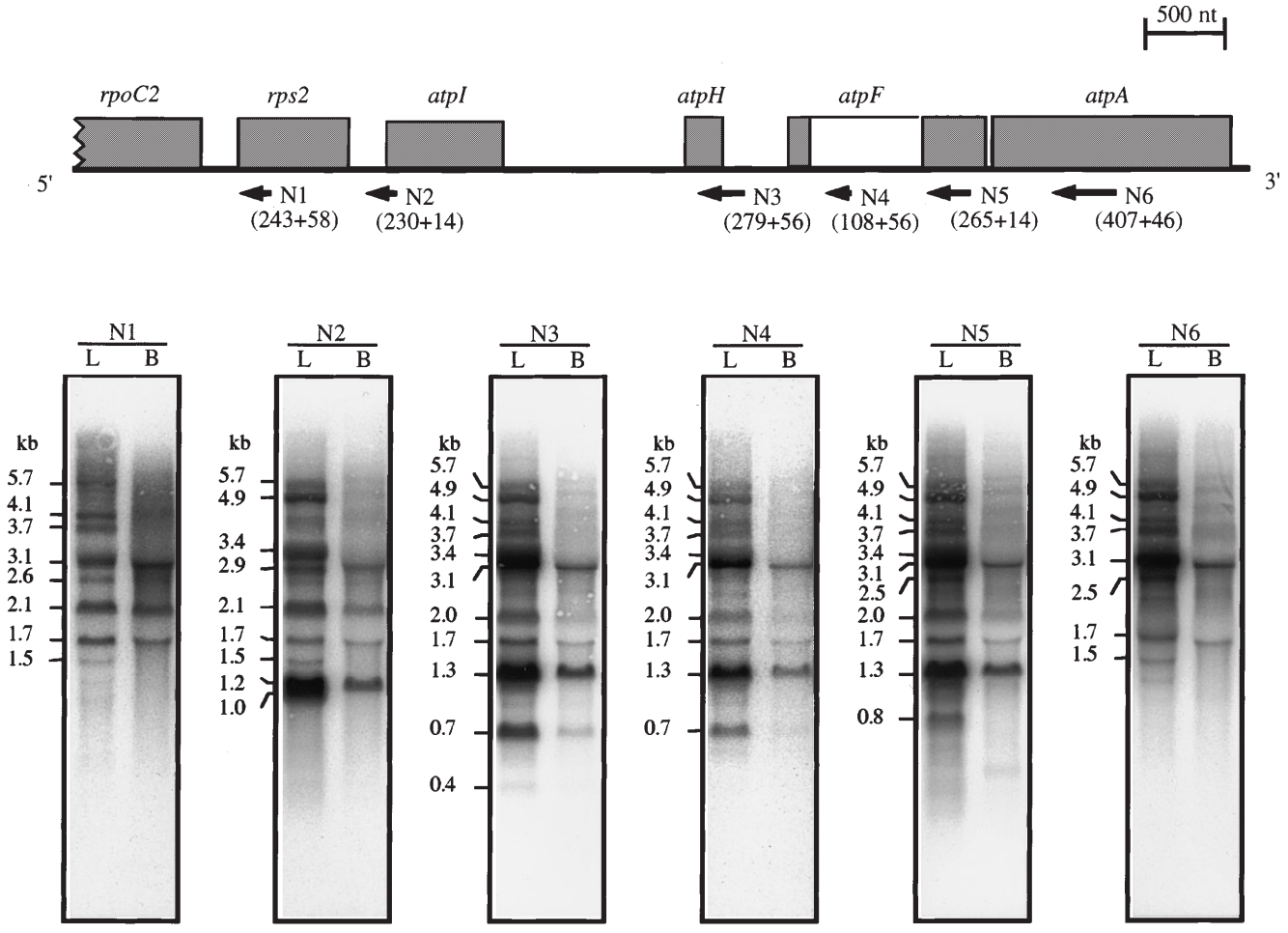


Fig. 1 Northern analysis of the *rps2/atpI/H/F/A* gene cluster. The upper panel shows a schematic representation of the genetic organization of the region, and the locations, sizes and orientations of the antisense RNA probes (N1–N6). Protein-coding regions are shown as *shaded boxes* and the intron with in *atpF* gene as an *empty box*. *Figures in parentheses* indicate lengths of fragments and vector sequences in the in vitro transcribed RNA probes, respectively. The lower panels show the autoradiograms of the transcript pattern obtained when total RNA from tobacco leaves (L) and BY-2 cells (B) was hybridized with the in vitro transcribed ^{32}P-labeled RNA probes indicated at the top of each panel. Calculated sizes of the transcripts are given on the left

Kapoor et al. 1997). Finally, the TISs were identified using TAP-RLPCR (see Materials and methods).

The region upstream of rps2. The ribonuclease protection assay using antisense RNA probe R1, resulted in two protected RNA fragments of 392 and 266 nucleotides (Fig. 2A, R1). These bands were observed both in leaf (lane L) and in BY-2 cells (lane B). The 266-nucleotide band was of the same intensity in both the lanes, but the intensity of the 392-nt band in leaf was only one-third of that in BY-2 cells. It was rather difficult to identify the *rps2*-specific band with this probe alone; therefore, these samples were analyzed with a smaller probe, R2 (Fig. 2C). With R2, instead of the 392-nt species, a 270-nt band is protected, while the 266-nt fragment remains as in the case of probe R1. Hence, the 266-nt band corresponds to the *rps2* mRNA and the 392- and 270-nt bands represent the 3′ ends of the *rpoC2* transcript. Primer extension analysis using total RNAs from leaf, BY-2 cells and spc/str treated seedlings (lanes L, B, and S, respectively) and primer R2-1 resulted in single bands that mapped to position −42 relative to the *rps2* translation initiation site (Fig. 2B, D).

To determine whether the −42 *rps2* transcript is a primary or processed transcript, we subjected this RNA to in vitro capping and TAP-RLPCR analyses. However, even after several attempts we did not succeed in obtaining any signal using either of these techniques. This negative result, though inconclusive, suggests that either the 5′ end of −42 *rps2* transcript is devoid of any free phosphate group or that these have been modified, rendering this RNA species incapable of RNA ligation. Whatever the case may be, it is evident that the 5′ end of the −42 *rps2* transcript is the product of some sort of post-transcriptional modification. The possibility that it might have arisen from processing of a longer transcript initiated 5′ to the *rpoC2* gene also cannot be totally ruled out. But the higher level of the *rps2*-specific RNA band (266 nt) relative to the 392- and 270-nt bands representing *rpoC2*, observed in ribonuclease protection as-

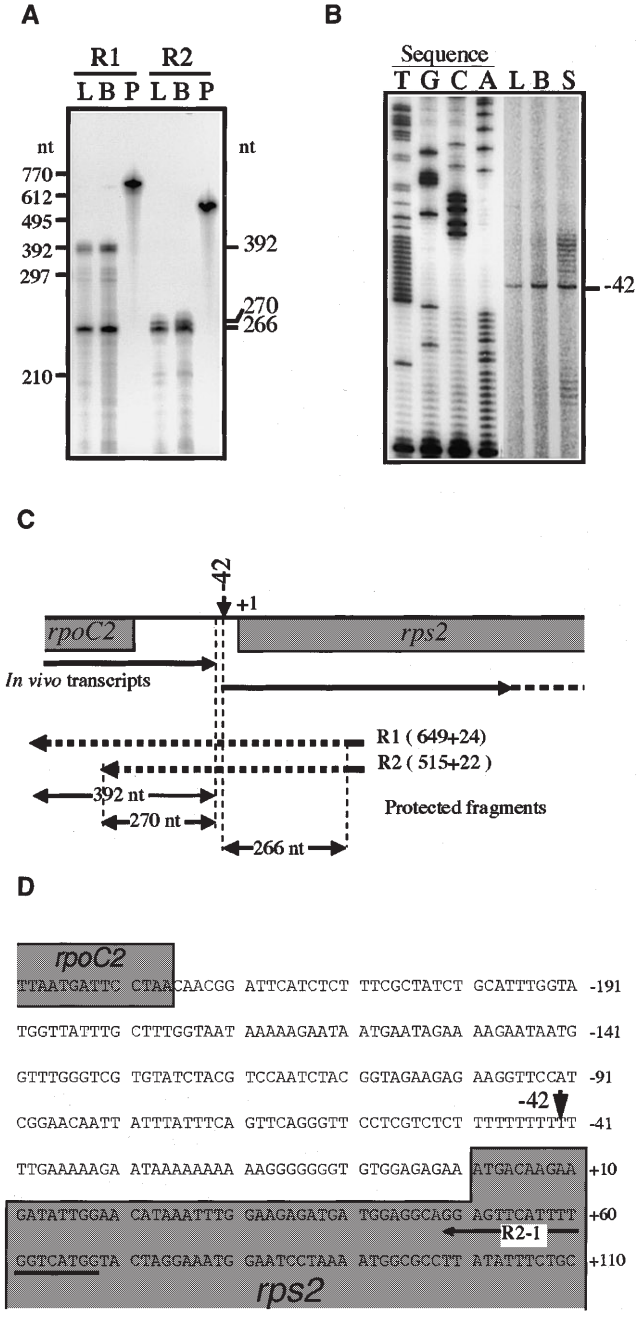


Fig. 2A–D Characterization of the region upstream of *rps2*. **A** Ribonuclease protection assays. The in vitro transcribed, ^{32}P-labeled antisense RNA probes R1 and R2 were loaded as such (P) or hybridized to total RNA from leaf (L) or BY-2 cells (B) and the hybrids digested with RNase before fractionating on a denaturing polyacrylamide gel. **B** Precise mapping of the 5′ end of the −42 *rps2* transcript by primer extension. Lanes L, B and S show the extension products of primer R2-1 (see panel C), obtained using total RNA from leaf, BY-2 cells and Spc/Str-treated seedlings, respectively. Lanes T, G, C and A contain dideoxy sequencing reactions obtained utilizing the same primer. **C** Schematic representation of the *rps2* 5′ region. The in vivo transcripts are shown as long rightwardly directed *arrows*. The *broken arrows* represent the antisense RNA probes. *Figures in parentheses* are lengths of fragments and vector sequences in the in vitro transcribed RNA probes, respectively. **D** Sequence showing the relative position of the 5′ end (*vertical arrow*) of the −42 *rps2* transcript with respect to the *rpoC2* and *rps2* coding regions

says (especially in leaf samples), argues in favor of a promoter activity in this region.

The region upstream of atpI. To analyze the region between *rps2* and *atpI*, a 726-nt antisense RNA probe encompassing the entire non-coding region and including parts of both coding regions was used in ribonuclease protection assays (Fig. 3A, D). The leaf RNA resulted in four protected fragments of 726 nt, 548 nt, 471 nt and 426 nt (lane L). The 726-nt band that represents the transcript initiating 5′ to the *rps2* coding region was also detected in BY-2 cells (lane B). The 548-nt fragment was also protected by BY-2 RNA but 471- and 426-nt fragments were not. The 5′ ends of the RNase-protected fragments were mapped by primer extension using primers AI-2 and AI-3 to −208, −131 and −85 relative to the *atpI* initiation codon (Fig. 3B, E). This analysis also confirmed the absence of −131 and −85 *atpI* 5′ ends in BY-2 cells. The primer extension product corresponding to the 548-nt fragment seen in the RNase protection assay was resolved into four distinct 5′ ends mapping at −205, −208, −211 and −214 relative to the *atpI* translation initiation site. However, in BY-2 cells, two (corresponding to −208 and −211) and in Spc/Str-treated seedlings only one 5′ end (−208) was observed. To avoid confusion in later discussion we have referred to this site as "−208 *atpI*".

The results of the TAP-RLPCR analysis to distinguish the primary transcripts from the processed species are shown in Fig. 3C. The use of primer AI-1 in conjunction with the linker primer resulted in four amplified products corresponding to −85, −131, −208 species and a 370-bp band of unknown identity (marked with an asterisk in Fig. 3C, left panel). This band, however, disappeared when, instead of the AI-1 primer, the AI-3 primer was used (Fig. 3E). Taken together, the results obtained using both these primers clearly demonstrate that −208 and −131 *atpI* transcripts have their triphosphate moiety intact, and thus represent primary transcripts. The band corresponding to the −85 transcript was detected in both TAP+ and TAP− lanes; therefore, it represents a processed transcript species with only one phosphate group at the 5′ end. The presence of the −208 primary transcript in the non-photosynthetic plastid-containing BY-2 cells, and in Spc/Str-treated seedlings in which plastid translation is inhibited (Fig. 3C; right panel, lanes B, S), suggests that this transcript might be synthesized by a non-plastid encoded transcription system that utilizes NCII promoters (see Kapoor et al. 1997). In contrast, the −131 *atpI* primary transcript was only found to accumulate in leaves, an accumulation pattern typically demonstrated by CT promoter-derived transcripts.

The region upstream of atpH. The ribonuclease protection assay using the R4 (514 nt) antisense RNA probe, consisting of 37 nt of the *atpH* coding region and 477 nt corresponding to the region upstream of the *atpH* initiation codon, resulted in three major protected fragments of 514, 421 and 82 nt. Another band of 490 nt was observed in both L and B lanes, but its authenticity was

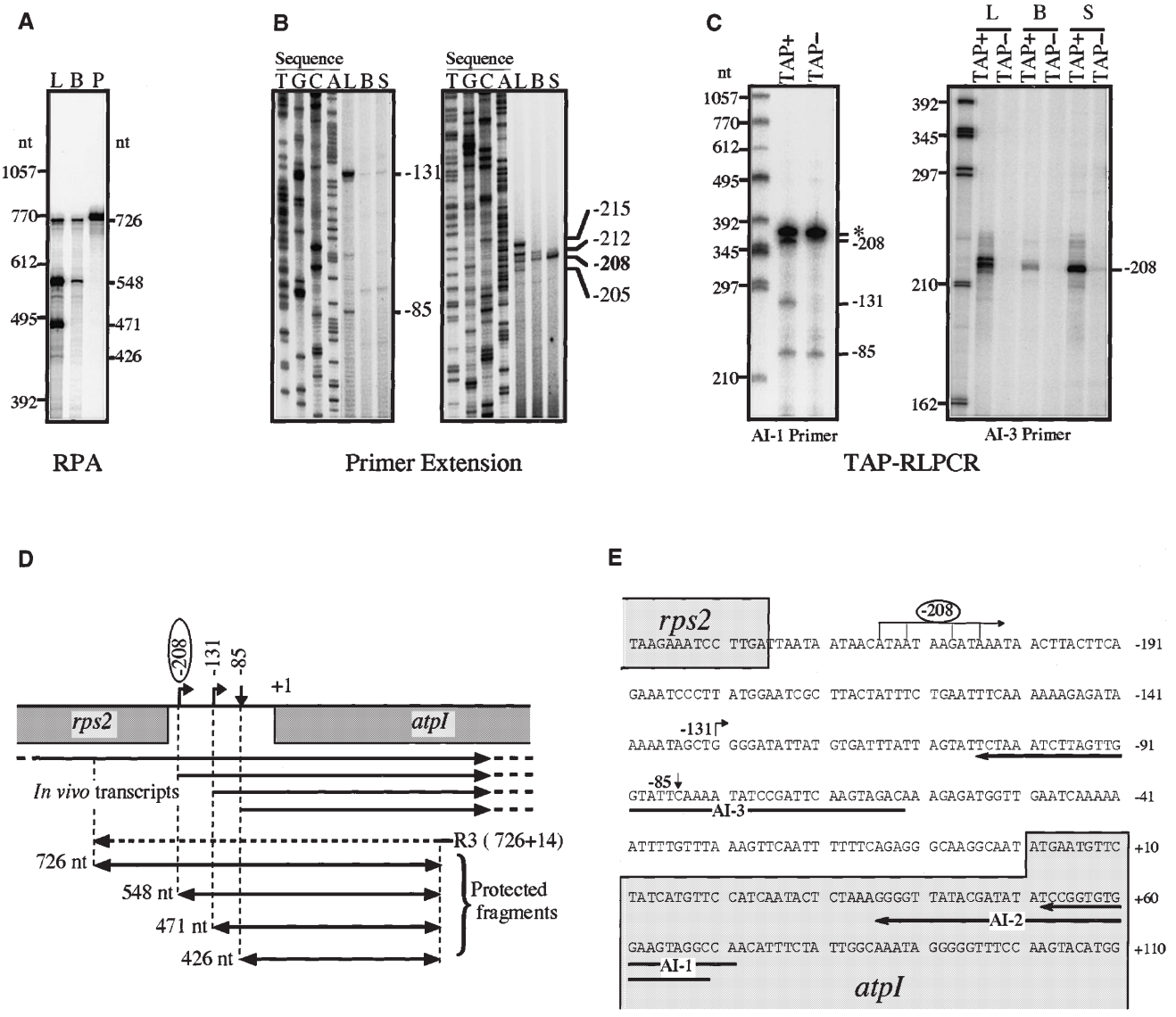


Fig. 3A–E Characterization of the region upstream of *atpI*. **A** Ribonuclease protection assay using the in vitro transcribed, ^{32}P-labeled antisense RNA probe R3 and total RNA from leaf (L) or BY-2 (B) cells. All other conditions were the same as in Fig. 2A. **B** Precise 5′ end mapping of the −85, −131 and −208 *atpI* transcripts by primer extension analysis using primer AI-2. For other details see Fig. 2B. **C** TAP-RLPCR analysis to distinguish TISs from processed 5′ ends. The linker RNA molecule is ligated to the 5′ ends of TAP-treated (TAP+) and untreated (TAP−) total RNA samples and cDNA is synthesized using AI-1 and AI-3 as primers. PCR amplification of the cDNA follows, using AI-1 and AI-3, and a ^{32}P-labeled oligonucleotide primer complementary to the RNA linker. Lanes L, B and S show the TAP-RLPCR products obtained using total RNA from leaf, BY-2 cells and Spc/Str-treated seedlings, respectively. **D** Schematic representation of the *atpI* 5′ region. The rightwardly directed *arrows* indicate the TISs, while a processed 5′ end is marked by a vertical arrow. The −208 site is *encircled* as it represents the NCII-type promoter element. Other details are as in Fig. 2C. **E** Sequence showing the relative positions of transcription initiation sites (*rightward arrows*) and a processed 5′ end (*vertical arrow*), with respect to *rps2* and *atpI* coding regions

not confirmed in primer extension assays; hence it might represent a degradation product. The 514-nt band corresponds to the completely protected probe and thus represents all the primary transcripts that initiate 5′ to *atpI* and *rps2* (Fig. 4A, D). The primer extension analysis using AH-1 and AH-2 primers demonstrated that the 421- and 82-nt bands represent two transcript 5′ ends at positions −384 and −45, respectively, relative to the *atpH* initiation codon. The −45 transcript was detected in all three RNA samples analyzed, whereas the −384 transcript was found to accumulate only in leaves (Fig. 4B). The TAP-RLPCR analysis utilizing primer AH-1 revealed that the −384 species is a primary transcript and the −45 transcript results from RNA processing (Fig. 4C, E).

The region upstream of atpF. The *atpF* gene is the intron-containing member of the *rps2*/*atpI*/*H*/*F*/*A* operon. The antisense RNA probe R5 (858 nt) was hybridized to total RNA from leaves and BY-2 cells and the RNase-protected fragments were analyzed. A total of three bands, of 858 nt, 610 nt and 362 nt, were observed (Fig. 5A). As shown in Fig. 5D, these data can be

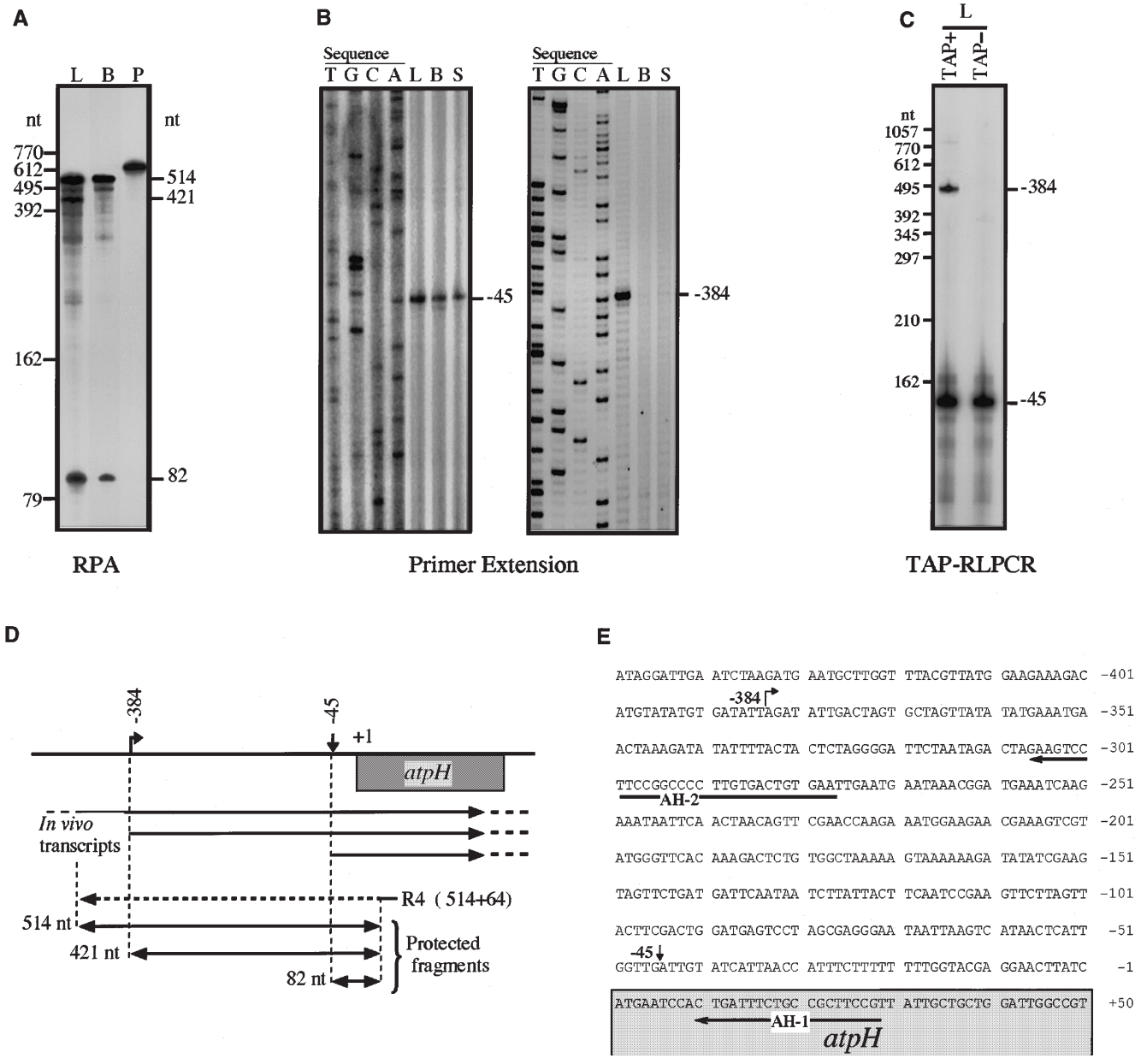


Fig. 4A–E Characterization of the region upstream of *atpH*. **A** Ribonuclease protection assay using the in vitro transcribed, ^{32}P-labeled antisense RNA probe R4 and total RNA from leaf (L) or BY-2 (B) cells. For other details see Fig. 2A. **B** Precise 5′ end mapping of the −45 and −384 *atpH* transcripts by primer extension analysis using primer AH-1. For details see Fig. 2B. **C** TAP-RLPCR analysis to distinguish the TIS (−384 *atpH*) from the processed 5′ end (−45 *atpH*). Other details are same as Fig. 3C. **D** Schematic representation of the *atpH* 5′ region. Other details are as in Fig. 3D. **E** Sequence showing the relative positions of the transcription initiation site (*rightward arrow*) and the processed 5′ end (*vertical arrow*) with respect to the *atpH* coding region

accounted for only if there is a transcript 5′ end corresponding to position −216 relative to the *atpF* translation initiation site. This hypothesis was confirmed by primer extension using primer AF-1. An extended product was visualized at position −216 in all three samples, i.e., from leaves, BY-2 cells and Spc/Str-treated seedlings. The TAP-RLPCR analysis, however, showed that this transcript 5′ end results from post-transcriptional processing.

The region between atpF and atpA. There is only a small stretch of 54 nt between the coding regions of the *atpF* and *atpA* genes. The ribonuclease protection and primer extension analysis did not detect any transcript species that either initiated or terminated in this region (data not shown). Hence this region was not analyzed further.

Discussion

We have recently reported that in plastids there exists a distinct class of promoters (NCII) that does not possess

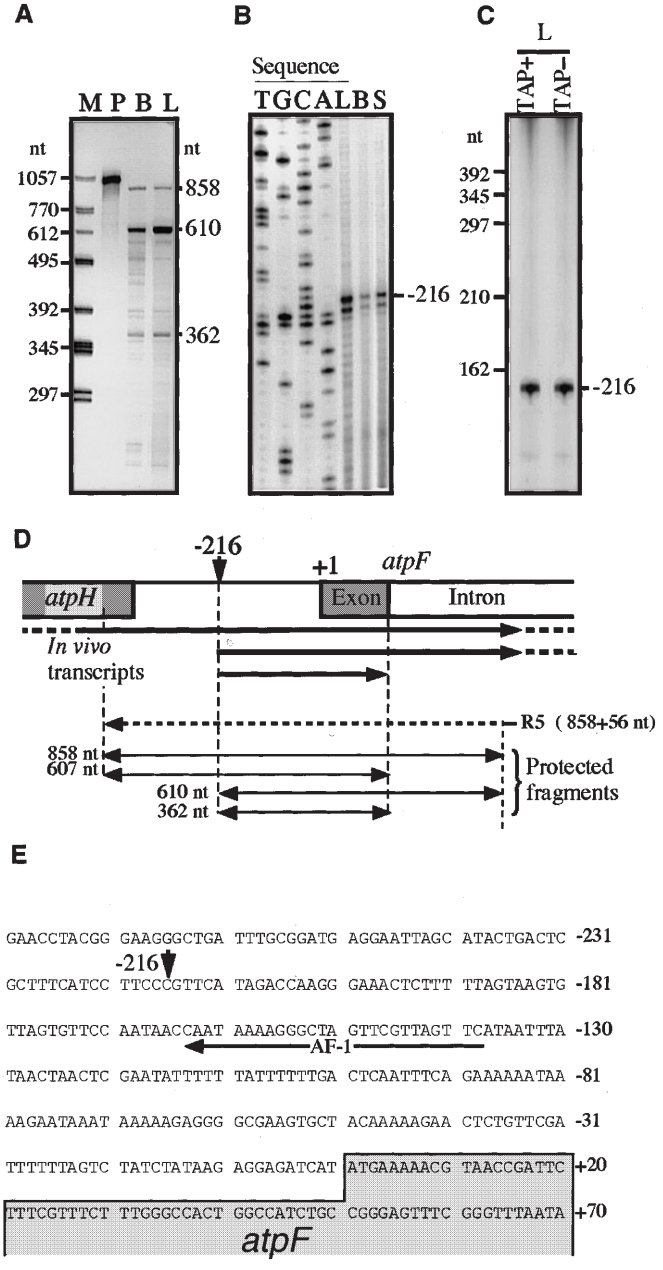
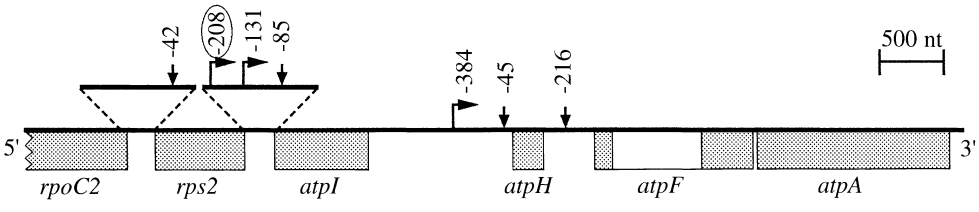


Fig. 5A–E Characterization of the region upstream of *atpF*. **A** Ribonuclease protection assay using the in vitro transcribed, ^{32}P-labeled antisense RNA probe R5 and total RNA from leaf (L) or BY-2 (B) cells. For other details see Fig. 2A. **B** Precise 5′ end mapping of the −216 *atpF* transcripts by primer extension using primer AF-1. Other details are as in Fig. 2B. **C** TAP-RLPCR analysis showing that the −216 *atpF* site results from RNA processing. Other details are as in Fig. 3C. **D** Schematic representation of the *atpF* 5′ region. Other details are as in Fig. 3D. **E** Sequence showing the position of the processing site (*vertical arrow*) with respect to the *atpF* coding region

any sequence similarity to −10 or −35 elements of σ^{70}-type prokaryotic promoters. The accumulation of transcripts originating from these promoters is not affected even in the absence of plastid protein synthesis, indicating that these promoters are utilized by a polymerase that is not encoded in the plastid. Moreover, these promoters have only been detected in the upstream regions of plastid genes involved in housekeeping functions (e.g. *atpB/E rpl32* and *rrn16*) and not in the process of photosynthesis (Kapoor et al. 1997). The accumulation of *rrn16* NCII promoter-specific transcripts in *rpoB*-deleted plastid mutants of tobacco also confirmed the utilization of this promoter type by a non-plastid encoded polymerase (Allison et al. 1996).

It is obvious that transcription from the *rps2/atpI/H/F/A* operon must be required to complement the expression of the *atpB/E* operon for the establishment of a functional ATP synthase complex. Therefore, in non-photosynthetic plastid types where transcript accumulation from CT promoters is almost negligible, the existence of NCII promoter(s) for the larger *atp* operon was postulated. A detailed transcript analysis of the *rps2/atpI/H/F/A* operon in tobacco revealed at least three transcription initiation sites (TISs) and four processed 5′ ends (Fig. 6). Two of the TISs were found upstream of the *atpI* gene (−208 and −131), while the third one was localized to a position 384 bp upstream of *atpH*. In spinach and pea, putative TISs upstream of *rps2*, *atpI* and *atpH* had been predicted during sequence analysis of the genes belonging to this operon (Hennig and Herrmann 1986; Hudson et al. 1987). Later, two transcript 5′ ends were also mapped using S1 nuclease mapping to positions −21 to −13, and −340, relative to the *atpH* initiation codon (Hutly et al. 1990). In maize however, S1 analysis revealed transcript 5′ ends at positions −18 and −51 relative to *atpH* and *atpA* translation initiation sites (Rodermel and Bogorad 1987). However, attempts to characterize the primary transcripts associated with this operon, by hybridizing capped total spinach and pea RNA to PCR-generated specific intergenic fragments, revealed only two TISs – upstream of *atpH* and *rps2* but not *atpI* (Stollar and

Fig. 6 A summary of all the sites of transcript initiation and 5′ end processing characterized in the present investigation. Protein-coding regions are shown as *shaded boxes* and the intron within the *atpF* gene as an *empty box*. The positions are given as numbers of base pairs upstream of the respective genes. The rightwardly directed *arrows* represent the TISs, while sites of 5′ end processing are marked by *vertical arrows*. The −208 *atpI* site is *encircled* as it represents the NCII type promoter element



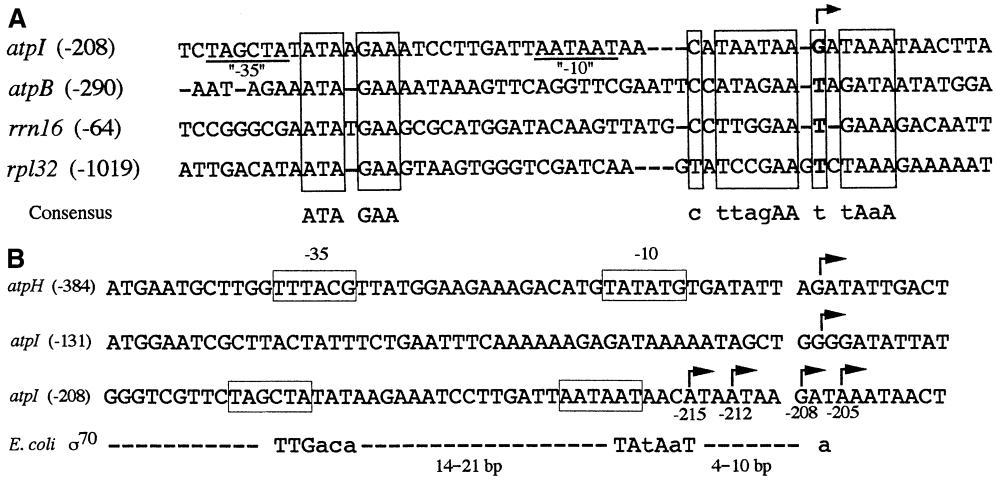


Fig. 7A, B Comparison of the −208 *atpI* promoter region with other known examples of NCII promoters (A) and of −208 *atpI*, −131 *atpI* and −384 *atpH* with the *E. coli* consensus promoter elements (B). The transcription start sites (*arrow*) and regions of high homology (*boxed*) are indicated

Hollingsworth 1994; Woodbury et al. 1989). Initially, we also tried to characterize the TISs specific to *rps2*/*atpI*/H/F/A operon by using well established methods, i.e., in vitro capping coupled with ribonuclease protection assays (Vera and Sugiura 1992; Kapoor et al. 1994). However, probably due to the low levels of transcripts and/or rapid processing of the primary transcripts, we could detect only one capped and protected fragment which corresponded to −384 *atpH* species (data not shown). Therefore, we turned to a more sensitive PCR-based technique, TAP-RLPCR (Fromont-Racine et al. 1993), which, besides confirming the −384 *atpH* site, revealed two more TISs at positions −208 and −131 relative to the *atpI* initiation codon. Of these three TISs, transcripts from only one (−208 *atpI*) were found to accumulate in the non-photosynthetic plastids of BY-2 cells and in seedlings germinated and grown on spectinomycin- and streptomycin-containing medium. Accumulation of the −208 *atpI* primary transcript in plastids that are incapable of translation was a clear indication that this promoter belongs to the category of NCII promoters. Comparison of the −208 *atpI* promoter region with the other known NCII-type promoters revealed considerable sequence similarity (Fig. 7A). Interestingly, however, this region also possessed a high degree of homology to −10 and −35 consensus-type elements as well (Fig. 7B). There is thus a possibility that this promoter is utilized by both polymerase systems. That would explain the multiple −208 *atpI* TISs that we observed in leaf RNA samples; in Spc/Str-treated seedlings and BY-2 cells (in which the plastid-encoded polymerase is either absent or present in very low quantities) fewer TISs were seen. However, whether −208 *atpI* represents a double promoter, and how it affects *atpI*/*H*/*F*/*A* transcript pool in different plastid types needs to be investigated in greater detail.

Expression from other two TISs (−131 *atpI* and −384 *atpH*), however, was found to be typical of other CT promoters, i.e., high-level transcript accumulation in leaves and very low or undetectable levels in non-photosynthetic plastids of BY-2 cells and Spc/Str-treated seedlings (see Kapoor et al. 1997). Moreover, the sequences 5′ to the −384 *atpH* TIS were found to show considerable homology to −10 and −35 elements; thus, it could easily be categorized as a CT promoter (Fig. 7B). Interestingly however, the sequences upstream of −131 *atpI* showed no similarity either to CT or NCII promoters (Fig. 7B). The only other known example of such a promoter is the light-inducible promoter of the *psbD*/*C* operon in barley and wheat (Berends-Sexton et al. 1990; Christopher et al. 1990; Wada et al. 1994). An attempt to compare the promoter region of −131 *atpI* with that of 4a *psbD*/*C* (barley) revealed no significant similarity (see Christopher et al. 1990; Allison and Maliga 1995). Whether such non-consensus type light-regulated promoters are utilized by a minor variant (in terms of σ factors) of the plastid-encoded RNA polymerase (see Tiller et al. 1991) or by another as yet uncharacterized transcription system, remains to be investigated.

As far as *rps2* is concerned, the only transcript 5′ end (−42 *rps2*) detected in the 5′ region of this gene seems to result from RNA processing. Taken together, our data suggest that the distribution of TISs in tobacco plastids seems to be different from that in pea and spinach. However, lack of a NCII-type promoter upstream of *atpI* in pea and spinach would necessitate a functionally similar promoter upstream of *rps2* that is capable transcribing all the genes of the *rps2*/*atpI*/*H*/*F*/*A* operon. Therefore, it would not be surprising if the capped transcript initiating upstream of *rps2* reported in pea and spinach (Stollar and Hollingsworth 1994; Woodbury et al. 1989) is also found to be of NCII type. In tobacco too, the *rps2*-specific transcript accumulates both in photosynthetic and non-photosynthetic tissues; this strongly indicates that the transcript initiates from an NCII type promoter which might be present either in the non-coding region between *rpoC2* and *rps2* or else further upstream of the *rpo* genes.

Moreover, our data demonstrate that all the transcript species with processed 5′ ends accumulate also in cell types other than that which contains chloroplasts. However, as stated earlier, transcripts from TISs other than −208 *atpI* TIS were not found to accumulate in non-photosynthetic plastids. Hence, the transcripts that initiate at −208 *atpI* seem to be the source of all the *atpI*/*H*/*F*/*A*-specific transcript species in non-photosynthetic plastids.

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