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cDNA cloning and sequencing of tobacco chloroplast ribosomal protein L12

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Tobacco chloroplast ribosomal protein L12 was isolated as a ssDNA–cellulose-binding protein from a chloroplast soluble protein fraction. Based on the N-terminal amino acid sequence of chloroplast L12, a cDNA clone was isolated and characterized. The precursor protein deduced from the DNA sequence consists of a transit peptide of 53 amino acid residues and a mature L12 protein of 133 amino acid residues. The chloroplast L12 protein was synthesized with a reticulocyte lysate and subjected to nucleic acid-binding assays. L12 synthesized in vitro does not bind to ssDNA, dsDNA nor ribonucleotide homopolymers, but it binds to cellulose matrix.

Chloroplast; Ribosomal protein L12; Tobacco

1. INTRODUCTION

Chloroplasts are photosynthetic organelles present in green plants which contain their own genome which is distinct from that in the nucleus. The chloroplast genome is generally comprised of single homologous circular DNA molecules of 120–160 kb in size [1,2]. Accumulating evidence indicates that the expression of many chloroplast genes is effectively controlled at the post-transcriptional level. In order to identify components involved in post-transcriptional regulation, we have applied ssDNA affinity chromatography to search for ribonucleoproteins (RNPs) from tobacco chloroplast lysates. Approximately 50 protein species were recovered from an ssDNA–cellulose column, and to date five of them have been identified as RNPs containing the consensus sequence-type RNA binding domains [3,4]. Surprisingly, one of the proteins which bound to the ssDNA-cellulose column was identified as chloroplast ribosomal protein L12.

Chloroplast ribosomes are 70 S in size and many structural similarities exist between the rRNAs and ribosomal proteins from chloroplasts and *E. coli* (see for example [5–8]). *E. coli* ribosomes contain four copies of ribosomal protein L7/L12 (for a review, see [9]). This L7/L12 protein is attached to the 23 S rRNA through L10 as an L10–$(L7/L12)_4$ complex [10]. L7/L12 binds to ribosomes via its N-terminal region, whereas the C-terminal domain is required for EF-G-dependent GTP hydrolysis. The C-terminal domain is the most conserved part of the protein [11]. Recently, Rice and Steitz have proposed that the C-terminal domain (residues 69–87) of L7/12 (120 amino acids long) forms a helix-turn-helix motif strikingly similar to those found in many DNA-binding regulatory proteins [12], however, no direct interaction between L7/L12 and RNA has been reported [9]. We have characterized a cDNA clone for tobacco chloroplast ribosomal protein L12 (CL12) and found a striking sequence homology between tobacco CL12 and *E. coli* L7/L12. We then attempted to confirm the binding to ssDNA–cellulose and the prediction of Rice and Steitz but after being synthesized in vitro, CL12 failed to bind to nucleic acids.

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2. MATERIALS AND METHODS

2.1. *Protein isolation and cDNA analysis*

Chloroplasts were isolated from tobacco leaves (*Nicotiana tabacum* var. Bright Yellow 4) and lysed essentially as described by Obokata [13], then chloroplast soluble proteins were fractionated by ssDNA–cellulose column chromatography [3]. In brief, isolated chloroplasts were lysed in 20 mM Tris–HCl (pH 8.0) containing 2 mM PMSF and 2 mM DTT and centrifuged for 30 min at 30,000×g. The soluble proteins were precipitated with 80% saturated ammonium sulfate and dissolved in 10 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM PMSF, 1 mM EDTA and 0.1 M NaCl. The sample was applied to an ssDNA–cellulose column (Sigma) and the bound proteins were eluted with 0.3, 0.6 and 2 M NaCl in the above buffer. The 0.3 M fraction was separated in a 7.5–20% polyacrylamide gradient gel containing 0.1% SDS, and transferred to PVDF membranes. The N-terminal amino acid sequences of the fractionated proteins were determined with an Applied Biosystems 470A sequencer.

A probe 5'-AC(T/C)TT(T/C)TCIGGIGC(T/C)TCIAACIGC-3' which corresponds to the N-terminal sequence (residues 8–1) of CL12 was synthesized with an Applied Biosystems 381A DNA synthesizer. Construction and screening of a tobacco (*N. sylvestris*) leaf cDNA library in λgt10 was essentially according to the instruction manual of an Amersham cDNA synthesis kit. DNA sequencing and cDNA cloning were carried out as previously described [3].

2.2. *Nucleic acid binding assays*

A DNA fragment coding for the mature portion of CL12 was prepared by PCR using its cDNA as template and two primers (a *Xho*I site for insertion is underlined):

exp. 10: 5'-GCTCGAGCCGTCGAAGCACCTGA-3'
1233: 5'-ACCGTTTCGAGCTGCTCTATAGACT-3'

The amplified fragment was inserted into an expression plasmid, pTMV, immediately following the TMW leader sequence, as previously described [14]. The resultant plasmid DNA was linearized with *Eco*RI and used to synthesize a capped transcript to direct in vitro protein synthesis as in [14] except that [^{3}H]leucine was used instead of [^{35}S]methionine. The N-terminal sequence of CL12 synthesized in vitro (the authentic N-terminal sequence is underlined) is: MARAAVEAPEKVV-. Nucleic acid binding assays were done as described [14].

3. RESULTS

3.1. *Characterization of chloroplast ssDNA-binding proteins*

Total soluble proteins from highly purified tobacco chloroplasts were subjected to ssDNA affinity chromatography. Approximately 5% of the applied proteins bound to ssDNA–cellulose in the presence of 0.1 M NaCl. The bound proteins were eluted stepwise with 0.3, 0.6 and 2 M NaCl as shown in Fig. 1. The 0.3 M fraction was pooled, concentrated and fractionated in a polyacrylamide gel (Fig. 2). About 24 bands were detected after staining with Coomassie R350. The N-terminal amino acid sequences of three bands of around 21 kDa were determined and found to be nearly identical. These proteins are not encoded by the tobacco chloroplast genome [15], indicating that they are of nuclear origin. *N. tabacum* is an amphidiploid, and nuclear-encoded proteins generally show polymorphism (e.g. [13]). A homology search revealed that the N-termini of these proteins show strong similarity to those of ribosomal protein L12 from spinach chloroplasts [6] and from *E. coli* [16].

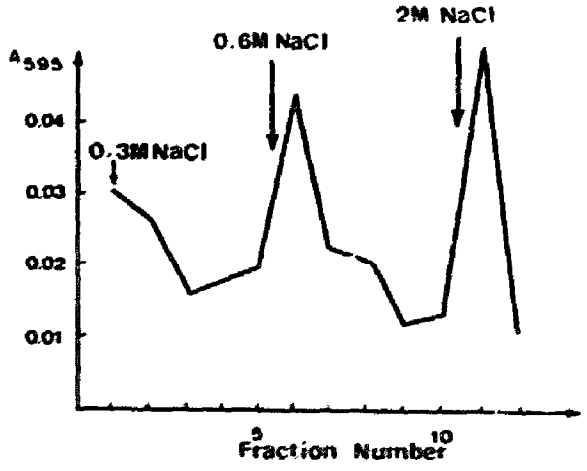

Fig. 1. Elution of chloroplast proteins from a ssDNA cellulose column. Protein concentration was determined according to [19]. Fractions 1–5 were pooled and concentrated as in [20].

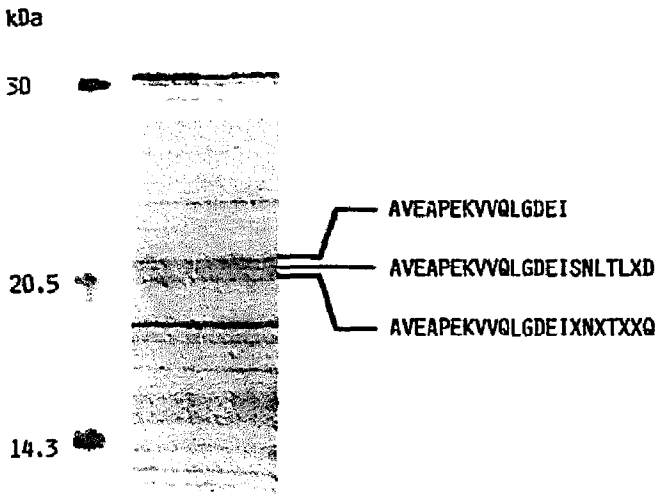

Fig. 2. SDS-polyacrylamide gel electrophoresis pattern of the pooled protein fraction eluted with 0.3 M NaCl. N-Terminal protein sequences are on the right.

3.2. *cDNA sequence of CL12*

We isolated a cDNA clone from an *N. sylvestris* leaf cDNA library with an oligonucleotide probe prepared based on the above N-terminal amino acid sequence (*N. sylvestris* is the female progenitor of *N. tabacum*). Sequencing this cDNA (clone λKNSCL12B) (766 bp) revealed a reading frame of 186 amino acids (Fig. 3), and its deduced amino acid sequence has striking homology (67%) to the spinach chloroplast L12 precursor [17] (Fig. 4). The 5' leader sequence of 48 bp is rich in C/T (88%). The spinach cDNA for L12 has been reported to contain a second initiation codon five condons downstream from the first ATG codon [17]. The

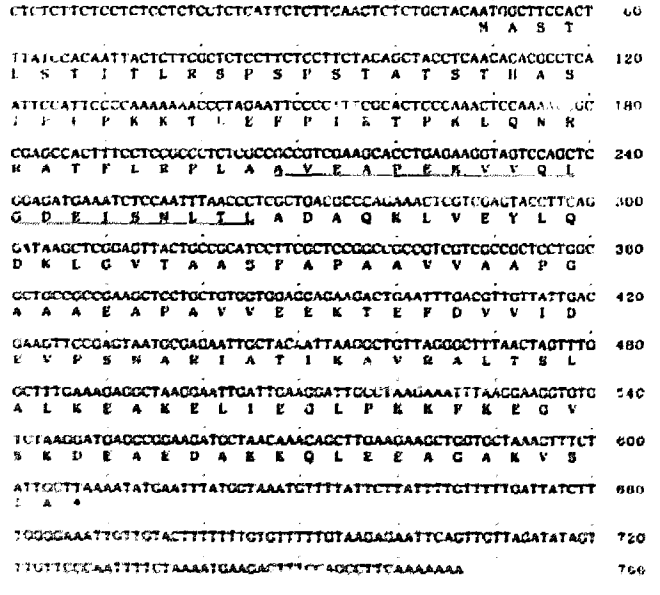

Fig. 3. The cDNA sequence and predicted protein sequence of *N. sylvestris* chloroplast ribosomal protein L12. The underlined amino acids were confirmed by protein sequencing.

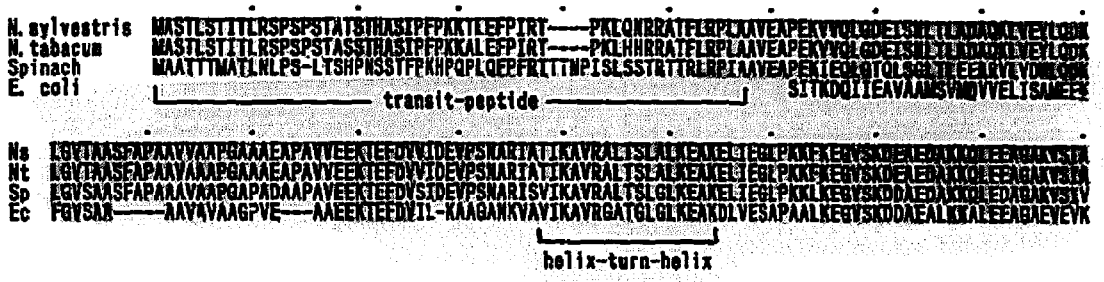

Fig. 4. Comparison of the amino acid sequences of ribosomal protein L12 from tobacco (*N. sylvestris* and *N. tabacum*) and spinach chloroplasts, as well as from *E. coli*. Identical amino acids are stippled. Dashes denote gaps introduced to optimize sequence alignment. The transit peptide and the putative helix-turn-helix motifs are indicated.

tobacco ATG corresponds to the first ATG of spinach but there is no other ATG in the tobacco reading frame (Fig. 3), suggesting that the first ATG is the functional initiator in spinach. The 3′ flanking sequence (143 bp) is rich in T and contains no apparent poly(A) signal, but a stretch of 7 A,s at the 3′ end. The first 53 amino acids serve as a transit peptide and show 58% homology with the transit peptide (56 residues) of the spinach chloroplast L12 precursor [17]. The mature protein is 133 amino acids long with a predicted molecular weight of 13,867 and is 80 and 43% homologous to those in spinach chloroplasts [17] and *E. coli* [16], respectively (Fig. 4). Recently the deduced amino acid sequence of the *N. tabacum* chloroplast L12 precursor was reported [18]. The mature portion of the molecule is identical to that from *N. sylvestris* and the transit-peptide has 92% homology with that of *N. sylvestris*. These results clearly indicate that the chloroplast ssDNA binding protein is ribosomal protein L12 from tobacco chloroplasts (CL12). As shown in Fig. 4, residues which can form a helix-turn-helix motif are highly conserved between *E. coli* and chloroplasts.

3.3. *Nucleic acids binding assays*

To confirm that CL12 binds directly to ssDNA, the mature form of CL12 was synthesized in a rabbit reticulocyte lysate. The resultant protein migrated at around 21 kDa (Fig. 5, lane I). CL12 synthesized in vitro was tested for binding to ss- and dsDNA–cellulose and cellulose was included as a control. A fraction of CL12 remained bound to ss-, dsDNA–cellulose as well as cellulose itself, indicating that the binding was not mediated through DNA (Fig. 5, lanes CL12). In a separate experiment, cp28 synthesized in vitro [14] was used as a positive control. This protein bound only to ss- and dsDNA (Fig. 5, lanes cp28), indicating that this test of binding is reliable. Therefore, we conclude that CL12 cannot bind to DNA and the observed binding to DNA-cellulose columns is due to binding to the cellulose matrix or due to the association of CL12 with other chloroplast ribosomal protein(s) which directly bind to ssDNA.

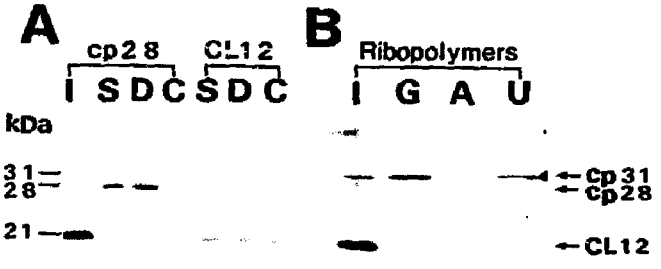

Fig. 5. Nucleic acid binding assays. (A) Binding of CL12 and cp28 to DNA. The [^{3}H]proteins were incubated with single-stranded calf thymus DNA–cellulose (S), double-stranded calf thymus DNA–cellulose (D) and cellulose (C) at 0.05 M NaCl. (B) Binding of [^{3}H]cp31 and [^{3}H]CL12 to ribonucleotide homopolymers. CL12 and cp31 were mixed and incubated with poly(U)-, poly(A)- and poly(G)-sepharose beads (U, A and G, respectively) at 0.1 M NaCl. The bound proteins were eluted and resolved on a 12.5% polyacrylamide gel containing 0.1% SDS. The gels were treated for fluorography and exposed at −70°C for 11–48 h. Lane I's are controls to show the proteins used.

We also examined CL12 binding to ribonucleotide homopolymers. In vitro-synthesized cp31 [14] was mixed with CL12 to serve as an internal positive control. As expected, cp31 bound to poly(G) and poly(U) but not to poly(A) (Fig. 5, lanes ribopolymers). However, CL12 failed to bind to any of these polymers, suggesting that CL12 is unable to bind to RNA.

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