Identification of a maize nucleic acid-binding protein (NBP) belonging to a family of nuclear-encoded chloroplast proteins

William B. Cook* and John C. Walker

University of Missouri, Division of Biological Sciences, Columbia, MO 65211, USA

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ABSTRACT

A cDNA encoding a nuclear-encoded chloroplast nucleic acid-binding protein (NBP) has been isolated from maize. Identified as an *in vitro* DNA-binding activity, NBP belongs to a family of nuclear-encoded chloroplast proteins which share a common domain structure and are thought to be involved in post-transcriptional regulation of chloroplast gene expression. NBP contains an N-terminal chloroplast transit peptide, a highly acidic domain and a pair of ribonucleoprotein consensus sequence domains. NBP is expressed in a light-dependent, organ-specific manner which is consistent with its involvement in chloroplast biogenesis. The relationship of NBP to the other members of this protein family and their possible regulatory functions are discussed.

INTRODUCTION

The expression of chloroplast genes is subject to a variety of regulatory mechanisms. Variations in transcriptional activity caused by differences in promoter strength (1, 2), selective promoter use (3), variations in genome copy number (4), DNA conformation upstream of promoters (5) and the sequence context of the transcribed gene (6) have been demonstrated. However, marked differences between the transcription rates and the accumulation of mature mRNAs indicate that post-transcriptional mechanisms fulfill a primary role in the regulation of chloroplast gene expression. These discrepancies between the transcription rate, which is often constant and the level of message accumulation, which may vary dramatically, occur during the development of proplastids and etioplasts (7, 8 [spinach], 9 [barley]), between different organs (10, 11) and as adaptations to differences in light quality (12).

Post-transcriptional regulation of chloroplast gene expression may occur at the level of either message maturation or mRNA stability. Chloroplast precursor mRNAs undergo a variety of maturation events including *cis*- and *trans*-splicing (13, 14, 15), cleavage of polycistronic messages (16) and processing of 5' (17) and 3' (18, 19) ends. Mature mRNAs vary in their relative stabilities under different conditions and at different stages of

development. For example, the level of *psbA* message accumulation changes during the development from young to mature leaves in conjunction with a change in the half-life of the mature message measured *in vivo*. In contrast, the *rbcL* message does not change in level or in stability over the same period (20).

Plastid mRNAs generally possess inverted repeat (IR) sequences in the 3' untranslated region (21). The 3' IR sequences can fold into stable, RNase-resistant stem-loop structures (22) and act as processing signals (21, 22, 23, 24) but not as transcriptional terminators (18). However, the predicted thermodynamic stabilities of stem-loop structures can not account for the stability of the cognate mRNAs *in vitro* or the accumulation of mRNAs *in vivo*.

Developmental, organ-specific and light-dependent changes in the accumulation of specific mRNA species suggest that protein factors exist which modify the stabilities of mRNAs. Protein factors are known to interact with 3' IR sequences *in vitro* (19, 22). A variety of interactions have been described in which some proteins bind only to specific stem-loop structures or to mRNA precursors while others may bind to all 3' IR sequences.

Several examples exist in the green alga, *Chlamydomonas reinhardtii*, of nuclear mutants which fail to accumulate specific chloroplast mRNAs (25, 26, 27). The mRNAs are transcribed at normal rates relative to wild type and all other messages are present at wild type levels. It appears that, in the wild type, specific nuclear gene products stabilize the individual mRNAs which are absent from the mutants.

Recently, a chloroplast protein which appears to be involved in the processing of chloroplast transcripts has been isolated from spinach (28). 28rnp is a nuclear-encoded chloroplast protein which exhibits 3' processing activity and binds to the 3' IR structures of chloroplast mRNA precursors and mature messages *in vitro*. These activities suggest that 28rnp may have a role *in vivo* in 3' processing of precursors and/or stabilization of mature messages. The RNA binding activity of 28rnp does not exhibit sequence specificity *in vitro*, as it interacts with several different chloroplast transcripts. The accumulation of the nuclear-encoded 28rnp and its mRNA coincides closely with the accumulation of chloroplast mRNAs during light regulated chloroplast development.

* To whom correspondence should be addressed at Midwestern State University, Biology Department, Wichita Falls, TX 76308, USA

28rnp is a nuclear-encoded protein with an amino terminal acidic domain adjacent to a tandem pair of ribonucleoprotein consensus sequence (RNP-CS) type RNA-binding domains (28). The RNP-CS domain has been identified in a wide variety of proteins which interact with or are believed to interact with RNAs (29). RNP-CS containing proteins carry out many functions associated with the synthesis, processing and regulated expression of gene transcripts. The RNP-CS domain is a weakly conserved 80–100 amino acid region containing two more highly conserved sequences of eight (RNP1 or octomer sequence) and six (RNP2) residues (29, 30). The RNP-CS has been shown to be essential to RNA binding activity (31,32,33). X-ray crystallographic analysis of the U1A protein of the U1 snRNP has confirmed the roles of RNP1 and RNP2 in general RNA binding recognition (34).

Prior to the isolation of 28rnp, a group of three structurally homologous chloroplast proteins of 28kD, 31kD and 33kD were isolated from tobacco chloroplasts (35). The proteins were isolated on the basis of their single-stranded DNA binding activities. These nuclear encoded proteins also contain amino terminal acidic domains adjacent to tandem pairs of RNA-binding domains. They share global structural homology with 28rnp but they are not identical at the amino acid sequence level. In particular, the acidic domains of the four proteins are quite diverged. The proteins constitute a structurally homologous family of nuclear-encoded nucleic acid binding chloroplast proteins. The expression of each of the proteins is maximal in light-grown leaves suggesting that they may operate in the light-regulation of chloroplast gene expression.

This paper describes a Nucleic Acid Binding Protein (NBP) from maize. NBP was identified as a DNA binding activity. It bears the same structural organization as members of the protein family described above and it is expressed in a manner concordant with its involvement in chloroplast development.

MATERIALS AND METHODS

Plant growth and tissue culture

Maize B73 seedlings were grown in a soil mix in a growth chamber at 28°C with 14 h light and 10 h dark periods. Etiolated seedlings were germinated in plastic trays on wet paper towels with translucent covers. The seedlings were germinated in the dark but were exposed to subdued light daily which was sufficient to stimulate leaf expansion. Plants that were harvested at maturity were grown in three gallon pots in the greenhouse with supplemental overhead lighting. Maize Black Mexican Sweet (BMS) tissue cell cultures were established and maintained as previously described (48,49).

RNA isolation and cDNA library construction and use

Poly (A)⁺ RNA was isolated from various maize tissues by a phenol/chloroform extraction method as described (50). Oligo d(T) primed cDNA libraries were constructed and ligated into lgt11 using the Pharmacia cDNA synthesis protocol (Pharmacia). cDNA expression libraries were screened with a nick-translated DNA probe consisting of four copies of the maize ARE (51) fused to the CaMV 35S-90 promoter construction. The library was plated at 2–3 × 10⁴ p.f.u per 137 mm plate and screened essentially as described by Singh (52) using a binding buffer consisting of 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 5 μg·ml⁻¹ sonicated and denatured calf thymus DNA.

For hybridization screening, libraries were plated as above and plaques were grown for ~8 hours at 42°C before binding to BioTrace NT membrane. Bound plaques were denatured and neutralized and the filters were baked at 80°C for 2 hours. Baked filters were prehybridized for 3 hours in 50% formamide, 5 × SSC, 50 mM NaPO₄, 5 × denhardt's, 100 μg·ml⁻¹ salmon sperm DNA, 100 μg·ml⁻¹ yeast RNA and 0.2% SDS and hybridized in the same solution containing 10⁶ cpm·ml⁻¹ of a denatured, oligolabeled probe (53). Following 12 hours hybridization, filters were washed at room temperature for 20 min in 0.2 × SSC/0.1% SDS and 3 × 20 min in the same solution at 65°C. Washed filters were exposed to XAR-5 film at -70°C for ≥ 12 hours. Sequence analysis was performed using the Sequenase 2 protocol (US Biochemical).

DNA and RNA analysis

Northern analysis was performed using 2 μg each of poly (A)⁺ RNA from various maize tissues. RNAs were fractionated on 1% agarose/formaldehyde gel and blotted to Biotrace RP membrane (Gelman). Prehybridization, hybridization, washing and film exposure were carried out as described above.

Southern analysis was performed using 10 μg total maize B73 DNA. DNA was digested to completion and fractionated on 0.8% agarose gel. The fractionated DNA was denatured, neutralized and transferred to BioTrace RP membrane. After baking for 2 hr at 80°C under vacuum, the membrane was prehybridized, hybridized, washed and exposed to film as described above.

Figure 1. A comparison of amino acid sequences encoded by NBP1 and four related cDNAs. The amino acid sequences encoded by 28kd, 31kd, 33kd and 28rnp were compared to the sequence of NBP1 in a pairwise fashion with the Lipman-Pearson algorithm (55) using a MacIntosh software package from DNASTAR. Vertical lines indicate amino acid identity with NBP. Shared structural features are indicated above and below the sequences: Each protein possesses an N-terminal chloroplast transit peptide although the sequence of the 28rnp transit peptide is not shown. A highly acidic domain is present in each protein immediately following the transit peptide. The first residue in each acidic domain is underlined and indicated by an overhead arrow. The initial residue in the acidic domain of the mature NBP polypeptide is predicted. The boundaries of two RNP-CS domains are delineated by bold typeface and by left or right facing arrows. Highly conserved consensus sequences, RNP1 and RNP2, are bracketed. This pairwise comparison of sequences fails to illustrate a substantial degree of homology between 28kd and 31kd in the N-terminal portion of their transit peptides.

Chloroplast import

0.5 μ g of the NBP1 cDNA was transcribed using T7 RNA polymerase (Promega) and 4 μ l of the 25 μ l transcription reaction were translated using a wheat germ extract with 35 S-methionine as label. An entire translation reaction was used for import into isolated chloroplasts. Intact, import-competent chloroplasts were isolated from peas as described (54). Following import, the reactions were treated with thermolysin for 30 min. Proteins from thylakoid and stromal fractions were isolated and analyzed by 12% PAGE/fluorography. 5 μ l of the *in vitro* translation and the entire sample of import products were loaded onto the gel for analysis.

RESULTS

Identification of a maize nucleic acid-binding protein

NBP was initially identified as a DNA binding activity from a cDNA expression library. A λ gt11 library constructed with poly (A)⁺ RNA from maize BMS tissue culture cells was probed with a 359 bp double stranded DNA probe. The β -gal fusion protein encoded by one clone, designated G1a, bound the probe strongly through three rounds of screening and plaque purification. The G1a fusion protein exhibited no sequence specificity, binding several other unrelated DNA probes with comparable avidity (data not shown). Further analysis (reported below) indicates that the clone encodes a putative RNA-binding protein. The isolation of an RNA-binding protein in this screen was fortuitous as the binding buffer contained denatured calf thymus DNA to block single stranded DNA-binding activity. No other RNA-binding proteins were isolated in previous or subsequent screens. The EcoRI insert of λ G1a was subcloned into a Bluescript plasmid for further analysis.

cDNA sequence analysis

The nucleotide sequence of the G1a cDNA was determined and the deduced amino acid sequence was examined for protein structural motifs. The G1a amino acid sequence contains an amino terminal acidic domain and a tandem pair of RNP-CS domains at its carboxyl terminus but no ATG translational start codon. To obtain a full length sequence, a λ gt11 cDNA library was constructed with poly (A)⁺ RNA from green maize seedling leaves and screened with a 5' fragment of G1a which did not

contain the RNP-CS domain. Several clones were isolated which extended upstream from the 5' end of G1a. The longest cDNA isolated, NBP1, contained an in frame ATG 188 bp upstream of the G1a border. The sequence extends 123 bp 5' of the ATG. The entire NBP1 cDNA is 1265 bp in length including a string of 14 As at its 3' end.

Amino acid sequence analysis

NBP1 encodes a 303 amino acid protein comprised of four distinct domains. A 62 amino acid N-terminal domain contains a high proportion of non-polar residues (25%), neutral residues (14%) and prolines (14%) and a net charge of +7. The acidic domain, which formed the N-terminal portion of G1a, follows the NBP1 N-terminal domain. The acidic domain consists of 66 amino acids and has a net charge of -23. Neither the N-terminal domain nor the acidic domain bear homology to any sequence present in the protein sequence data banks. However, characteristics of the N-terminal domain are consistent with those of known chloroplast transit peptides (36). The carboxyl terminal 60% of the protein is made up of a pair of non-identical, RNP-CS-type RNA-binding domains.

The structure of NBP is very similar to the four nuclear-encoded chloroplast proteins 28rnp, 28kD, 31kD and 33kD. Each, in its mature form, has a highly acidic amino terminal domain adjacent to a pair of RNP-CS domains at the carboxyl terminus. Prior to import into the organelle, each also possesses a chloroplast transit peptide (see 28). A comparison of NBP1 to the four chloroplast proteins is shown (Fig. 1). NBP1 shares 65–70% amino acid sequence identity with the RNP-CS domains of 28kd, 31kd and 28rnp. Outside the RNP-CS domains the identity is lower so that the overall identity with NBP1 ranges from 28% for 33kd to 54–65% for 28kd, 31kd and 28rnp.

Genomic organization and RFLP mapping of NBP

The apparent conservation of structure between NBP1 and the four nuclear-encoded chloroplast proteins suggested that NBP1 may belong to a family of such proteins. To determine if there are genes related to NBP in the maize genome, total genomic maize DNA was probed with the entire G1a cDNA. Southern analysis yielded a single hybridization band when the DNA was cut with EcoRI and two bands when cut with HindIII or BamHI (Fig. 2). The two HindIII bands were subsequently explained by the presence of a HindIII restriction site within the second of three introns which interrupt the coding region. No BamHI

Figure 2. Southern analysis of NBP in the maize genome. 10 μ g of total maize DNA was digested to completion with BamHI, EcoRI or HindIII then probed with the entire G1a sequence as described in Materials and Methods. The sizes of DNA molecular weight markers are indicated at the right of the figure in kilobasepairs. B=BamHI, E=EcoRI and H=HindIII.

Figure 3. Structural map of NBP genomic clone and NBP1 cDNA. NBP genomic map indicates exon (E1–E4) and intron (I1–I3) locations, ATG translational start and TAA translational stop codons, and selected restriction enzyme recognition sites. The NBP1 cDNA map indicates the locations of major structural domains as well as the 3' untranslated region and locations of intron splicing. The two maps are drawn to different scales as indicated at the right of the figure. Restriction sites are represented by H=HpaI, S=SalI, K=KpnI, Hd=HindIII, X=XbaI and Ssp=SspI.

Figure 4. Northern analysis of NBP transcript accumulation in maize tissues. Poly (A)⁺ RNA isolated from various maize tissues was probed with the a 215 bp probe corresponding to the acidic domain of NBP as described in Materials and Methods. Labels above lanes indicate the tissues from which RNA was isolated: TC=Tissue Culture, R=Roots, ES=Etiolated Seedling, GS=Green Seedling, T=Tassel, ESh=Ear Shoot, S=Silk, HL=Husk Leaf, ML=Mature (fully expanded) Leaf.

restriction site occurs in the coding sequence or the introns of the genomic clone suggesting that two genes with homology to some portion of the G1a probe may be present in the maize genome.

NBP was located on the long arm of chromosome 7 by Recombinant Inbred RFLP analysis (37). DNA isolated from inbred progeny of parental lines Co159 and Tx303 was digested with HindIII and analyzed by Southern blot using the 5' fragment of G1a as a hybridization probe.

Genomic sequence analysis

A genomic clone which contained the entire NBP coding sequence was isolated and partially sequenced to determine the sizes and locations of three intervening sequences (Fig. 3). Three introns of ~1050, ~985 and 177 bp were found to interrupt the coding sequence at positions +467, +567 and +849.

Northern analysis

The similarity of NBP to the four nuclear encoded chloroplast proteins suggested that NBP may be a chloroplast protein involved in organellar biogenesis. If so, NBP would be expected to exhibit a light-dependent or developmentally regulated pattern of expression. To evaluate this possibility RNAs from a variety of maize tissues were probed with the 5' fragment of the G1a clone. Prominent hybridization was found only with poly (A)⁺ RNA from green seedling leaves (Fig. 4). A lower level of hybridization was observed with RNAs from mature leaves and husk leaves, suggesting that the expression of NBP is under developmental control. Little or no hybridization was found with RNAs from dark grown seedling leaves. This may be another manifestation of the developmental regulation of NBP expression or it may indicate that NBP expression is light-dependent. Hybridization between NBP1 and RNAs from reproductive tissues and roots was entirely absent demonstrating a strong tissue specificity for NBP expression. The fact that NBP is expressed primarily in light-grown seedling leaves is consistent with the hypothesis that NBP may be involved in chloroplast biogenesis.

Chloroplast import

The involvement of NBP1 in the regulation of chloroplast gene expression would require that it be imported into the chloroplast following translation in the cytoplasm. To determine whether the N-terminal domain functions as a chloroplast transit peptide, *in vitro* transcription/translation products of NBP1 were imported

Figure 5. Chloroplast import of NBP1 *in vitro* transcription/translation product. NBP1 cDNA was transcribed and translated *in vitro* in the presence of ³⁵S-methionine and incubated with isolated intact pea chloroplasts. Following incubation in the light the chloroplast/translation product was incubated with thermolysin for 30 min on ice. Chloroplasts were reisolated and lysed. Thylakoid membranes were removed by centrifugation and soluble stromal proteins were precipitated with 10% trichloroacetic acid. The entire samples of thylakoid and stromal proteins and one-tenth of an *in vitro* translation reaction were fractionated by electrophoresis through a 12% polyacrylamide gel and radiolabeled proteins were visualized by fluorography. The sizes of molecular weight markers are indicated at the right of the figure in kilodaltons. Precursor (P) and Mature (M) forms of the NBP1 translation product are indicated at the left of the figure. IVT=*in vitro* translation product, S=stromal proteins, T=thylakoid proteins.

into isolated intact chloroplasts. *In vitro* translation products produced in a wheat germ extract system were incubated with isolated chloroplasts and treated with thermolysin. Stromal and thylakoid fractions were separated and analyzed by polyacrylamide gel electrophoresis and fluorography (Fig. 5). The NBP1 translation product was imported into chloroplasts and concurrently processed to a mature protein of the expected size. The mature protein was localized in the stromal chloroplast fraction.

DISCUSSION

A great deal of progress has been made in understanding the mechanisms by which chloroplast gene expression is regulated (7–9, 21–24, 28, 38). The primary role of post-transcriptional regulation predicts the involvement of factors which are responsible for the differential processing and stabilization of transcripts. The nucleotide sequence of the entire chloroplast genome has been determined in several species and the regulatory proteins which are predicted to be present do not appear to be encoded by chloroplast genes. Therefore, most, if not all, of these factors are expected to be encoded in the nucleus and imported to the chloroplast. Evidence for such nuclear-encoded chloroplast factors has been available for many years with the characterization of mutants which are defective in particular chloroplast functions due to nuclear mutations (39, 40, 41). Several of these mutants have been shown to lack particular chloroplast functions due to the absence of a specific chloroplast-encoded component as the result of a specific nuclear mutation (25–27, 42).

Recently, a nuclear encoded chloroplast factor has been isolated and characterized. The spinach 28rnp binds to cis-acting regulatory stem-loop structures of chloroplast transcripts and also possesses an activity which is required for correct processing of the 3' ends of chloroplast transcripts *in vitro*. 28rnp consists of four distinct domains: An amino terminal transit peptide is located adjacent to a highly acidic domain which is followed at the carboxyl terminus by a tandem pair of RNP-CS type RNA binding domains (Fig. 1) Three tobacco chloroplast proteins share the structural organization of 28rnp. They are nuclear-encoded proteins which bind strongly to single stranded DNA and presumably to RNA as well. The remarkable structural homology

between the three tobacco proteins and 28rnp suggests that they may share at least a general functional similarity, i.e. they may perform the same processing function(s) but with different binding specificities or they may bind to the same spectrum of transcripts but perform other post-transcriptional processing functions. The subject of this paper, the maize NBP, which is capable of binding nucleic acids and possesses the same structural framework as the four chloroplast proteins, may also share the same or a related activity.

Although the five chloroplast proteins share a common structural design the level of amino acid sequence identity outside the RNP-CS domains is low (Fig. 1). This is not entirely surprising even if similar functions are predicted for the five proteins. The amino terminal transit peptides diverge substantially from one another. However, among several hundred different chloroplast transit peptides no significant sequence homology has been identified (36). It has been proposed, in light of this fact, that chloroplast import requires the presence of a transit peptide in the configuration of a random coil (43). Accordingly, it would be the secondary structure and not the primary structure of the peptide which is conserved.

A similar argument applies to the highly diverged acidic domains of these proteins. The function of these domains is not known, but they may be analogous to the 'acid blobs' found on some nuclear DNA transcriptional activation factors. The 'acid blobs' in these regulatory proteins are thought to be involved in protein-protein interactions during transcriptional activation (44). This interaction is dependent not on the primary structure, but on the charge density and secondary structure within the functional domain. The acidic domains of the five chloroplast proteins may also be involved in protein-protein interactions, acting as sequence-specific anchors with which processing proteins interact, or they may exhibit activities which are dependent only on charge density.

The level of identity among RNP-CS amino acid sequences of different proteins is generally quite low (29,31). The relatively high level of amino acid identity within these domains among the five chloroplast proteins suggests either a recent divergence or similarity of function which maintains some selective pressure on the degree of divergence. Minimal information is available about the mechanism by which RNA sequence specificity is achieved by the RNP-CS. The highly conserved RNP1 and RNP2 sequences appear to be involved in general RNA recognition (34) while another eight amino acid region near the RNP1 consensus is necessary and sufficient to confer sequence specificity on the U1A and U2B" proteins (32). Conclusions about the binding specificities of the chloroplast proteins await direct experimental evidence.

Despite the structural similarity of the five chloroplast proteins, they exhibit quite different expression patterns. The mRNAs of the three tobacco proteins are accumulated in roots as well as in leaves, although at substantially lower levels. Accumulation of 28kD is light dependent while 31kD and 33kD accumulation are unaffected by light/dark changes. The accumulation of the 33kD message is lower than that of 28kD or 31kD by a factor of ≥ 20 . The spinach 28rnp message is accumulated in young and mature leaves and, to a lesser extent, in etiolated cotyledons but not in roots. Accumulation of the maize NBP message appears to be dependent on light, as little occurs in etiolated seedlings. The NBP transcript is also accumulated in an organ specific manner: the message accumulates in leaf tissue but not in reproductive or root tissues and to high levels only in seedling

leaves not in mature leaves or husk leaves. This suggests that the five chloroplast RNP-CS proteins are likely to perform different functions or, if they perform the same functions, they do so in different temporal or spatial contexts.

A number of nuclear mutants of maize which are defective in chloroplast development have been induced by transposon mutagenesis and isolated (45). Many of these nuclear mutants appear to be defective in chloroplast development due to the absence of a particular chloroplast-encoded component. It is possible that some of these nuclear mutations affect factors which are involved in post-transcriptional regulation of chloroplast gene expression. Recently, four of the maize mutations were mapped to chromosome 7L (46), suggesting that one may be identical to the NBP locus. Should this be the case, characterization of the *in vivo* function of NBP would be greatly simplified. Possible homology between the NBP locus and one of the four mutant maize loci will be aided by the presence in the maize locus of a *Mu1* element of Robertson's *Mutator* transposable element system (47).

It is likely that additional members of the nuclear-encoded chloroplast nucleic acid-binding protein family will be identified. The five members of the family which have been described thus far were isolated by entirely different methods; 28rnp on the basis of its 3' processing activity, the three tobacco proteins by their single stranded DNA-binding activities and NBP by virtue of its DNA-binding activity. This suggests that members of the protein family exhibit a wide range of binding specificities. Other chloroplast proteins which share the same structural framework may not have been isolated by these means simply because they do not bind strongly to single- or double-stranded DNAs. Just as these proteins exhibit a range of nucleic acid binding specificities, a variety of functions may also be represented in the family. While 28rnp processes 3' ends and binds stem-loop structures, other post-transcriptional functions occur in chloroplasts and may be mediated by other members of the protein family. It is evident from mutant analyses that RNA processing or stabilizing proteins exist with very specific chloroplast targets. As these proteins are isolated and studied it may be that they will be found to be members of this family.

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