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Detection of a plant protein analogous to the yeast spliceosomal protein, PRP8

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We have investigated whether a spliceosomal protein analogous to the yeast protein, PRP8, was present in higher plants. A protein with a molecular weight > 200 kDa was detected in Western blots of tobacco (*Nicotiana tabacum* L.) nuclear extracts with affinity-purified antibodies, raised against four different β-galactosidase-PRP8 fusion proteins. The > 200 kDa protein was also immunoprecipitated by antibodies against the snRNA-specific trimethylguanosine cap structure and was, therefore, snRNP-associated. The presence of this protein in plants, in addition to yeast, *Drosophila* and humans, and the conservation of large size and epitopes highlights the importance of PRP8 in pre-mRNA splicing.

Pre-mRNA splicing; PRP8; snRNP; Spliceosomal protein; Tobacco; Affinity-purified antibodies

1. INTRODUCTION

Pre-mRNA splicing takes place in a multi-component ribonucleoprotein complex called the spliceosome, which consists of the pre-mRNA, small nuclear ribonucleoprotein particles (snRNPs) and other protein components (reviewed in [1,2]). In plants, while intron characteristics required for splicing have been defined and snRNA genes have been well analysed [3], very little is known about the snRNP and non-snRNP protein molecules involved. The current lack of a plant in vitro splicing system renders a direct analysis of the plant spliceosome per se impossible. To circumvent this problem, antisera, raised to proteins required for splicing in yeast, have been used to screen plant nuclear extracts for the presence of immunologically related proteins. In this paper the detection of a plant analogue of the yeast splicosomal protein, PRP8, is reported.

PRP8 is an extremely large protein (280 kDa) which is essential for pre-mRNA splicing [4–8]. It is specifically associated with U5snRNPs [5] and spliceosomal complexes [6], is required for the assembly of the U4/U6.U5 tri-snRNP particle and the spliceosome itself [7], and has been shown to contact the pre-mRNA substrate [8,9]. The *PRP8* gene has been cloned by complementation of the temperature-sensitive phenotype of *prp8–1* mutants of *Saccharomyces cerevisiae* [4]. Homologues of PRP8 have been identified immunologically in human [8,10,11] and *Drosophila* [12] underlining the importance of PRP8 in pre-mRNA splicing.

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2. MATERIALS AND METHODS

2.1. *Plant Material*

6–8-week-old tobacco plants (*Nicotiana tabacum* cv. Xanthi) were collected freshly and used as starting material for the isolation of nuclei.

2.2. *Isolation of nuclei from tobacco leaves and preparation of nuclear extracts*

Isolation of nuclei and their purification by Percoll density gradient centrifugation were carried out essentially according to the method of Willmitzer and Wagner [13], except that fresh leaf tissue was used and the enzymatic digestion step was omitted. Nuclei were also prepared according to Maier et al. [14]. Purified nuclei were resuspended in buffer D [15] and sonicated for 2 × 5 s (MSE Soniprep 150, maximum amplitude), then centrifuged at 14,000 × *g* for 10 min. The supernatant fraction was retained and used for subsequent investigation.

2.3. *Antibodies*

Four nonoverlapping regions of the *PRP8* gene were fused to the *lacZ* gene and expressed as β-galactosidase-PRP8 fusion proteins (8.1, 8.2, 8.3 and 8.4) [4,5]. Antibodies against each fusion protein and affinity purified against the corresponding TrpE–PRP8 fusion proteins as previously described [11]. The polyclonal antibodies against trimethylguanosine (m_3G) [16] were a kind gift from Prof. R. Lührmann, Marburg, Germany.

2.4. *Immunoprecipitation*

Immunoprecipitation (IP) was performed with antibodies to m_3G. IgG was bound to protein A-sepharose (Sigma) in NTN buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.1% v/v NP-40) and washed. Plant nuclear extract (280 μg protein) was added to IP buffer resulting in the following final concentrations: 12 mM HEPES-KOH (pH 7.0), 2.5 mM $MgCl_2$, 0.2% NP-40, 175 mM NaCl, 0.04 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF, 5% v/v glycerol and incubated with the protein A-sepharose bound IgG for 1 h, with mixing, at 4°C. The antibody

complexes were centrifuged, washed, removed from the beads of protein A-sepharose and analysed in SDS-polyacrylamide gels (8.5% polyacrylamide). Western blotting was carried out as described previously [5].

3. RESULTS AND DISCUSSION

Yields of 4–6 × 10^6 nuclei per g fresh leaf tissue were routinely obtained by both methods of nuclei preparation. The presence of intact snRNAs in the nuclear extracts was confirmed by high resolution Northern analysis of RNA prepared from the nuclear extract (results not shown). Tobacco nuclear extracts were tested by immunoblotting for the presence of proteins that are recognised by affinity-purified antibodies raised against β-gal-PRP8 fusion proteins. A protein with a molecular weight >200 kDa was specifically recognised by all four sera, (Fig. 1, lanes 1–4). The four different antibodies do not recognise epitopes in common and this large tobacco protein, therefore, possesses epitopes present in four distinct regions of yeast PRP8. The protein was not recognised by pre-immune anti-8.4 serum (Fig. 1, lane 6). Using the anti-8.2 and anti-8.4 antisera, a protein of > 200 kDa was also detected in nuclear extracts from pea (results not shown).

Antibodies raised against the snRNA-specific m_3G cap structure have been widely used to purify snRNPs [1] and have been successfully employed to precipitate snRNAs, including U5 snRNA, from a number of monocot. and dicot. plants [17,18]. Immunoprecipitation experiments were performed with polyclonal antibodies against m_3G. The precipitated fraction was separated by electrophoresis in SDS-polyacrylamide gels and tested by immunoblotting with the affinity-purified anti-8.3

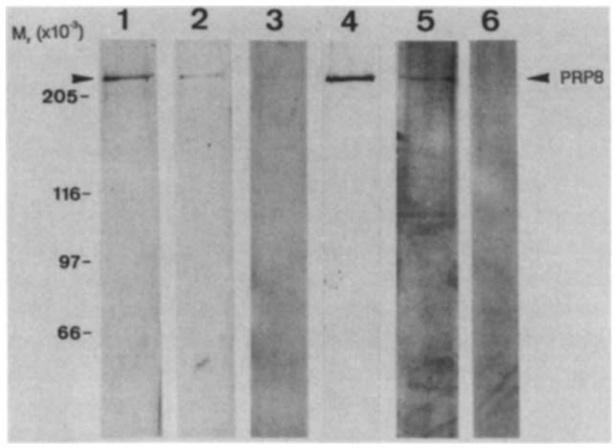

Fig. 1. Detection of a large tobacco protein immunologically related to PRP8. Aliquots of tobacco nuclear extract (100 μg protein) were analysed by Western blotting using affinity purified antibodies against β-gal-PRP8 fusion proteins. Primary antibodies used: Anti-8.1 (lane 1), anti-8.2 (lane 2), anti-8.3 (lane 3) and anti-8.4 (lane 4), non-affinity purified anti-8.4 antibodies (lane 5) and pre-immune anti-8.4 serum (lane 6). Molecular weight markers are indicated on the left hand side and the band corresponding to the PRP8 analogue on the right hand side.

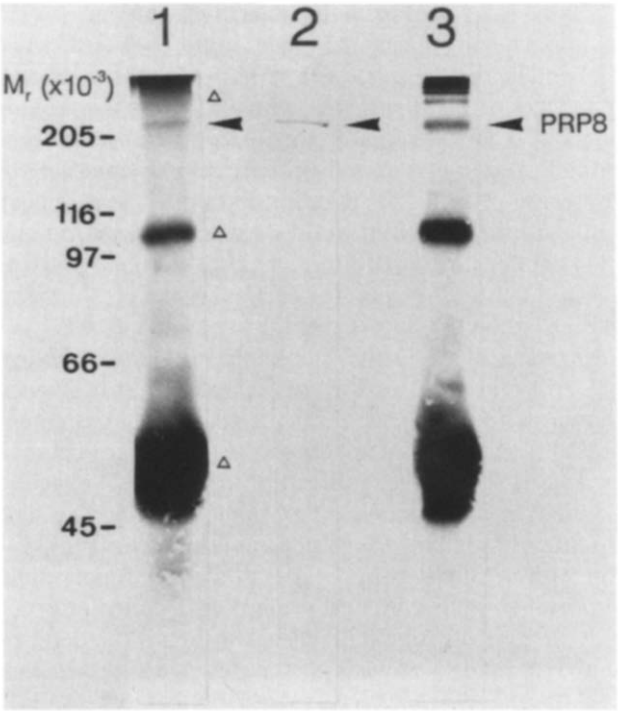

Fig. 2. Detection of the cross-reacting protein in anti-m_3G immunoprecipitates. A Western blot of anti-m_3G immunoprecipitaes from tobacco nuclear extract (lane 1), yeast splicing extract (lane 3) and non-immunoprecipitated total tobacco nuclear extract (42 μg protein) (lane 2) was probed with affinity-purified anti-8.3 primary antibodies. Open triangles indicate signals due to antibodies present in the gel from the anti m_3G antisera (lanes 1 and 3). Molecular weight markers are indicated on the left hand side and the band corresponding to the PRP8 analogue on the right hand side.

antibodies. The pattern of proteins detected in tobacco nuclear extract was very similar to a control precipitation with *S. cerevisiae* with the detection of a protein species with a molecular weight of > 200 kDa (Fig. 2, lanes 1 and 3 respectively). Thus, the large protein detected by the four anti-β-gal-PRP8 antibodies is snRNP-associated. The results reported here indicate that a protein analogous to the yeast splicing factor, PRP8, is present in plants. The protein contains four distinct epitopes in common with yeast PRP8 and is conserved with respect to large size (> 200 kDa) and snRNP association.

Proteins analogous to yeast PRP8 have been detected in HeLa cells [8,10,11], *Drosophila* [12] and now plant tissue. While PRP8 may have a number of functions in the splicing process [7], any or all of these must be of sufficient importance for distinct regions and the size of the protein to be conserved over a wide phylogenetic spectrum. The association of PRP8 with pre- and post-splicing complexes, active spliceosomes and the substrate pre-mRNA [5–9] and the conservation of its exceptionally large size, may indicate an architectural role for PRP8 [9], with multiple domains confering different functions or interactions with other splicing factors.

There is as yet no detailed analysis of the protein components of splicing in higher plants. However, given the similarities in splice site consensus sequences and sequences of plant snRNAs with those of other eukaryotes, it seems reasonable to expect that at least some snRNP and spliceosomal protein components will be conserved. Previous indications that this is the case have come from the identification of proteins immunologically related to the B/B′ and D core proteins and the U1-and U2snRNP-specific proteins, A and B″, of mammalian snRNPs, in polypeptides purified from *Vicia faba* nuclei by anti-m_3G immunoaffinity chromatography [18]. The basis of such conservation has only been shown at the sequence level for the U2B″ protein of potato, the only plant spliceosomal protein gene cloned to date [19]. The gene encoding PRP8 has yet to be isolated from a source other than *S. cerevisiae*. The cloning of the plant *PRP8* gene and sequence comparison with the gene from yeast may identify functionally important regions in this protein and contribute not only to the study of PRP8 itself, but to a more detailed analysis of the role of snRNPs in plant gene expression.

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