Association between 36- and 13.6-kDa α-Like Subunits of Arabidopsis thaliana RNA Polymerase II*

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Two subunits in RNA polymerase II (e.g. RPB3 and RPB11 in yeast) and two subunits common to RNA polymerases I and III (e.g. AC40 and AC19 in yeast) contain one or two motifs related to the α subunit in prokaryotic RNA polymerases. We have sequenced two different cDNAs (AtRPB36a and AtRPB36b), the two corresponding genes from Arabidopsis thaliana that are homologs of yeast RPB3, and an Arabidopsis cDNA (AtRPB13.6) that is a homolog of yeast RPB11. The B36a subunit is the predominant B36 subunit associated with RNA polymerase II purified from Arabidopsis suspension culture cells, and this subunit has a stoichiometry of about 1.

Results from protein association assays showed that the B36a and B36b subunits did not associate, but each of these subunits did associate with the B13.6 subunit in vivo and in vitro. Two motifs in the B36b subunit related to the prokaryotic α subunit were shown to be required for the in vitro interactions with the B13.6 subunit. Our results suggest that the B36 and B13.6 subunits associate to form heterodimers in Arabidopsis RNA polymerase II like the AC40 and AC19 heterodimers reported for yeast RNA polymerases I and III but unlike the B44 homodimers reported for yeast RNA polymerase II.

Eukaryotes contain three classes of nuclear RNA polymerase, referred to as RNA polymerases I or A, II or B, and III or C. Each class of RNA polymerase is a multimeric enzyme composed of two unique large subunits in excess of 100 kDa that are related to β' and β subunits of Escherichia coli RNA polymerase and 10 or more smaller subunits (reviewed in Refs. 1–5). In the yeast Saccharomyces cerevisiae five or more of these smaller subunits are common to RNA polymerase I, II, and III, and seven subunits are common to RNA polymerases I and III (3, 4). Subunits of about 40 kDa (e.g. yeast AC40 and B44 or RPB3) and 12.5–19 kDa (e.g. yeast AC19 and B12.5 or RPB11) in RNA polymerase I, II, and III have limited amino acid sequence homology with the α subunit of the prokaryotic RNA polymerase (6–8). The localized amino acid sequence homology between the eukaryotic α-like subunits and the α subunit in prokaryotic RNA polymerases has been referred to as the “α motif” (2, 9).

The yeast AC40 and AC19 subunits are common to RNA polymerase I and III, and the related subunits, B44 or RPB3 and B12.5 or RPB11, are unique to RNA polymerase II (6, 8, 10, 11). Bacterial RNA polymerase has an α subunit with a stoichiometry of 2, and the core enzyme is composed of αβαβ' (12). The yeast B44 subunit is reported to have a stoichiometry of 2 in RNA polymerase II (13), but the AC40 and AC19 subunits in RNA polymerases I and III have apparent stoichiometries of 1 (3, 14). The stoichiometry of the yeast B12.5 subunit has not been reported.

Yeast RNA polymerase II contains a total of 12 subunits, and each of these is encoded by a single copy gene (reviewed in Refs. 3 and 4). All of the RNA polymerase II subunit genes in yeast have been sequenced. Only a limited number of RNA polymerase II subunit genes in other eukaryotes have been cloned and sequenced (4). With the exception of genes encoding the largest subunit of RNA polymerase II in soybean and trypanosomes (15–17), those RNA polymerase II subunit genes that have been identified in organisms besides yeast are reported to be single copy genes.

Nuclear RNA polymerase subunit-subunit interactions, subunit functions, and assembly pathways are only beginning to be unraveled. For example, the AC40 and AC19 subunits of yeast RNA polymerase I and III have been shown to associate with one another in a yeast two-hybrid system (9). Extrageneric suppression of mutations in the AC40 and AC19 subunit genes confirmed the interaction between these two subunits and a third subunit, ABC10γ (9). Studies on mutations in the three largest subunits of yeast RNA polymerase II indicate that the B44 subunit associates with second largest subunit (i.e. B150 or RPB2), which in turn complexes with the largest subunit (i.e. B220 or RPB1) to facilitate assembly of the enzyme (18).

Here, we report on the cloning and sequencing of genes and/or cDNAs for the 36-kDa (B36a and B36b) and 13.6-kDa (B13.6) subunits in Arabidopsis RNA polymerase II, which are homologs to yeast B44 and B12.5 (i.e. encoded by the RPB3 and RPB11 genes in S. cerevisiae), respectively, to determine the stoichiometry of the B36 subunit in the enzyme and investigate its self-association and its association with the B13.6 subunit.

MATERIALS AND METHODS

Antibody Screening of an Arabidopsis cDNA Library—An Arabidopsis thaliana (ecotype Columbia) cDNA library in YEp3 (19) was used for antibody screening. Approximately 5 × 106 plaque-forming units were plated on an E. coli Y1090 lawn at moderate density and blotted onto Immobilon-NC (HATF) membranes (Millipore, Bedford, MA). Replica filters were probed with affinity-purified antibody (20, 21) raised against the 40-kDa subunit of cauliflower (Brassica oleracea) RNA polymerase II (60 ng of IgG/ml in Tris-buffered saline and 1% nonfat dry milk) at room temperature for 24 h. Filters were washed with three changes of Tris-buffered saline and then reacted with goat anti-rabbit IgG conjugated to alkaline phosphatase for 90 min at room temperature. Secondary antibody and reaction with alkaline phosphatase were carried out according to the supplier’s instructions (5 Prime→3 Prime, Boulder, CO).

Isolation of AtRPB36b and AtRPB13.6 cDNA clones—AtRPB36a and AtRPB36b cDNA inserts were used to screen another 5 × 106 plaque-forming units of the

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The Arabidopsis cDNA library (19). Six positive clones were selected, purified, and sequenced. Five of the six clones contained identical sequence (of varying length) to the AtRPB36a cDNA clone, and the remaining clone, AtRPB36b, contained a similar but distinct sequence.

An EST cDNA clone (GenBank™ accession number Z47635) from an Arabidopsis cell suspension library (22) was identified which had homology to the B36 subunit (8). The EST sequence (22) was reported as an alignment of the sequence of a full-length cDNA clone. The complete sequence of this cDNA clone was obtained from the EST cDNA clone, which was provided by Dr. Gary Phillips (Laboratoire de Biologie Moléculaire de Plantes, CNRS, Strasbourg Cedex, France). We refer to this clone as AtRPB36b. Both AtRPB36a and AtRPB36b cDNA clone inserts were used to screen an A. thaliana (ecotype Columbia) EMBL3 genomic library (provided by Harry Klee, Monsanto Chemical Company, St. Louis, MO). Genomic clones were selected, purified, and with mapping and restriction enzymes. Restriction fragments corresponding to genomic fragments of AtRPB36a and AtRPB36b were subcloned into pBluescript (Stratagene, La Jolla, CA) vectors or pMOB (23) and sequenced using a Tn1000 kit (Gold Biotechnology, St. Louis, MO).

DNA Sequence Analysis—Oligonucleotides used for sequencing and cloning procedures were synthesized at the University of Missouri DNA Core Facility. Sequencing was performed manually using Sequenase (US Biochemical Corp.) and by automated sequencing using the DyeCycle (Applied Biosystems, CA). Computer analysis was performed using the BLAST family of programs (24) and the E-mail BLAST server at National Center for Biotechnology Information, the Genomics Computer Group package (Genomics Computer Program, Madison, WI), and IBM Pustell Sequence Analysis software (International Biotechnologies, Inc., New Haven, CT). Protein sequence alignments were done using GAF, BESTFIT, and RILEUP programs from the GCG package with the Gap Weight and Gap Length Weight parameters 3.0 and 0.1, respectively.

Genomic Southern and Northern Analyses—A. thaliana (ecotype Columbia) genomic DNA was isolated, treated with restriction enzymes, and blotted onto nylon membranes using standard methods (20, 25, 26). Hybridization was carried out in 6 × SSC (1 × SSC is 0.15 M NaCl + 0.015 sodium citrate) containing 1% SDS and 100 μg/ml herring sperm DNA at 60 °C, and washings were conducted in 2 × SSC at 55 °C.

Northern blotting was carried out with 2 μg of poly(A)* RNA isolated from Arabidopsis suspension culture cells (27). RNA was isolated by a standard protocol (25), denatured with glyoxal and Me2SO, subjected to electrophoresis on 1.4% agarose gels, and transferred to a nylon membrane (26). AtRPB36a and AtRPB36b cDNAs were labeled with 32P using the Prime-a-Gene labeling system (Promega Corp., Madison, WI). A mixed probe was used for hybridization in 6 × SSPE (26), 1% nonfat dry milk, 1% SDS, and 0.5 mg/ml denatured herring sperm DNA at 68 °C. Washings were in 2 × SSC and 0.1% SDS at 15 °C, 25 °C, 0.1% SDS at 15 min at 25 °C, and 0.2 × SSC and 1% SDS for 1 h at 50 °C.

Expression of Cloned cDNAs in E. coli—Small N-terminal portions of open reading frames of AtRPB36a and AtRPB36b were isolated using specific primers, Pfu DNA polymerase (Stratagene), and polymerase chain reaction. These were cloned in-frame to the HistagPET-16b expression vector (Novagen, Madison, WI). In order to avoid mistakes introduced by polymerase chain reaction, most of the ORFs (with the exception of small N-terminal regions) were cloned by using corresponding restriction fragments from the original cDNAs. The N-terminal regions made by polymerase chain reaction were verified by sequencing. Expression of the cloning was done by the addition of IPTG to mid-log cultures of the BL21(DE3) strain of E. coli. After 2–3 h of induction, cells were harvested and sonicated. Fusion proteins were purified from inclusion bodies under denaturing conditions as described by the supplier (Novagen).

In Vitro Translational and Polycyclamide Gel Electrophoresis—ORFs from AtRPB36a, AtRPB36b, and AtRPB13.6 cDNAs were fused to the T3 phage promoter in a PBluescript II KS* or SK* vectors (Stratagene) with a TMV β translational enhancer (28). RNA was synthesized using the Ribobug™ T3 system (Promega, Madison, WI). The purified RNA polymerase II was isolated from vitro synthesized RNA used to program a FlexiRabbit™ rabbit reticulocyte in vitro translation system (Promega, Madison, WI) with [35S]methionine (DuPont NEN). Translation products were monitored on 10% SDS-polyacrylamide gels (29), 10% Tricine-SDS (30), or non-denaturing 7.5% polyacrylamide gels and subjected to autoradiography.

1 The abbreviations used are: ORF, open reading frame; HA, hemagglutinin; kb, kilobase pair(s).

Nondenaturing gels were constructed and subjected to electrophoresis at 4 °C using the Laemmli system (29) minus SDS.

Epitope Tagging and Immunoprecipitation—A double-stranded oligonucleotide was cloned upstream of the start codon for a given ORF to create an in-frame influenza hemagglutinin (HA) epitope tag. These constructs encoded proteins with a 12-amino acid extension, MGYP-KQKKGQGLQAAG (K. Sfakianakis, personal communication). Epitope-tagged and untagged cDNAs were co-translated in vitro (as described above), and the fusion-labeled in vitro translation products were immunoprecipitated with 12CA5 monoclonal antibodies (Berkeley Antibody Company, Richmond, CA), which were immobilized on Protein A-agarose (Sigma). Five μl of immobilized antibody was added to 10 μl of translation mix and adjusted to 200 mM Tris (phosphate-buffered saline containing 0.05% Tween 20). Immunoprecipitation was carried out at 4 °C for 12 h in a rotator. After incubation, the resin was washed five times with 1 ml of ice-cold PBS/Tween, and immunoprecipitates were eluted from the resin in 50 μl of SDS sample buffer (29). Five μl samples were resolved on 10 or 15% SDS gels and subjected to autoradiography.

Yeast Two-hybrid System—A full-length AtRPB36.1.6 ORF was cloned in-frame in a GAL4 DNA-binding domain into a pA51 vector, and full-length AtRPB36a and AtRPB36b ORFs were cloned in-frame with the GAL4 activation domain into pACTII (31). Both plasmids were sequentially introduced into yeast strain J C981 (an Ade' derivative of Y187; obtained from J. R. Cannon, University of Missouri; Ref. 31), and the transformed yeast cells were grown in Trp-{1-[L-Leu]} sucrose-complete medium (25). Mid-log cultures were harvested, and β-galactosidase activity was assayed by a nitroceollose filter/liquid N2/S-bromo-4-chloro-3-indolyl β-d-galactosidase method or assayed directly in lysates as described by Ausubel et al. (25) using a chemiluminescent assay, GalactoLight™ (Tropix, Bedford, MA).

N-terminal and C-terminal truncations of the B36b Subunit—Truncations in the N-terminal or C-terminal portions of the B36b subunit were made using a series of restriction endonuclease sites. The B36b subunit was chosen for truncations because of some convenient restriction endonuclease sites not found in the B36a subunit cDNA. A chimeric construct was made that consisted of amino acids 249–319 of the B36b subunit fused to the C terminus of GH2/4 (32). The GH2/4 cDNA encodes a glutathione S-transferase (33).

Purification of Arabidopsis RNA polymerase II and Subunit Analysis—A. thaliana (ecotype Columbia) cell cultures were grown in liquid media (27) as 1-litter cultures in 4-liter flasks with constant agitation on a rotary shaker at 25 °C. Eight days after subculture, cells were harvested and sonicated. Frozen in liquid N2 and stored at -80 °C prior to RNA polymerase II purification.

For purification of RNA polymerase II, 200 g of cells were thawed and suspended in 200 ml of grinding buffer (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 60 mM ammonium sulfate, EST, 0.1 mM benzamidine, 0.5 mM ethylene glycol and 20% (v/v) ethylene glycol) containing 1 mM Pefabloc SM (Boehringer Mannheim) 10 μg/ml aprotinin, 1 μg/ml pepstatin, 300 μg/ml benzamidine, and 10 μg/ml leupeptin. All purification steps were carried out at 4 °C.

Cells were broken by grinding for 2 min using full speed with a Polytron PT20ST and subsequently with 15 30-s bursts and 90-s intermittent periods with a Bead-Beater and 100 g of acid-washed glass beads (425–600 microns; Sigma). The homogenate was filtered through two layers of Miracloth and centrifuged at 10,000 g for 20 min. The supernatant was collected, and RNA polymerase II was purified by precipitation with and elution from Polymin P, ammonium sulfate precipitation, and chromatography on DEAE cellulose and phosphocel- lulose. The source was described by J. Endriss and J. Durieux (34). The phosphocel- lulose fraction was dialyzed against 20 mM HEPES (pH 7.8), 0.1 mM EDTA, 0.5 mM dithiothreitol, and 50% glycerol, and dialyzed was stored frozen at -80 °C. Wheat germ RNA polymerase II was purified using the methods of J. Endriss and K. Burgess (34) with final chromatography on heparin-Sepharose (23).

The B36b subunit was judged to be nearly homogeneous on 15% SDS-polyacrylamide gels (29) when compared with purified wheat germ RNA polymerase II subunit. Subunit stoichiometries were determined for the largest subunits (205 ± 175, 135-, and 36-KD subunits) in purified Arabidopsis RNA polymerase II using 7.5 and 15% SDS gels. Peak areas for the three largest subunits were measured for Coomassie Blue-stained gels using Image I Software (Universal Image, Corp., Westchester, PA). Quantification of 35S incorporation into the three largest subunits was carried out with a Fuji BAS1000 instrument and MacBAS1000 software (Fuji Medical Systems, Stamford, CT).
RESULTS

Cloning the Arabidopsis RPB36 Subunit cDNAs—A polyclonal antibody raised against the 40-kDa subunit of cauliflower RNA polymerase II (21) was affinity-purified on a wheat germ RNA polymerase II Affi-Gel resin (see "Materials and Methods" and Ref. 20). The affinity purified antibody was used to screen an Arabidopsis cDNA expression library as described previously (20). Seventeen positive clones were selected from primary screening, and five were purified to homogeneity. One of the purified clones, AtRPB36a, was sequenced and found to be related in amino acid sequence to yeast RPB3 (10) and human hRPB33 (Ref. 35; Fig. 1). The Arabidopsis cDNA had an insert of 1.3 kb that contained an ORF encoding 319 amino acids with a predicted molecular mass of 35.5 kDa and an estimated pI of 4.4. The Arabidopsis B36a amino acid sequence showed 39% identity to the RPB3 subunit in yeast RNA polymerase II and 44% identity to the hRPB33 subunit in human RNA polymerase II but only 31% identity to the AC40 subunit in yeast RNA polymerases I and III (11).

Southern blot analysis of Arabidopsis genomic DNA suggested that more than one copy of this subunit gene was present in the Arabidopsis genome because a variety of restriction endonucleases produced multiple restriction fragments (of varying intensities) that hybridized to the AtRPB36a cDNA probe (Fig. 2A). To determine if more than one gene encoded the 36-kDa RNA polymerase II subunit, we screened 5 \times 10^5 plaque-forming units of the λYES cDNA library with the AtRPB36acDNA and selected six positive clones. Each purified clone was partially sequenced. Five of these were identical in sequence to AtRPB36a with the exception of the position of the poly(A) tail in the 3'-untranslated region (data not shown), reflecting heterogeneity in the site selection for poly(A) addition. One of the clones contained a full-length cDNA that was related, but distinct from AtRPB36a. This 1.2-kb cDNA clone, AtRPB36b, contained an ORF encoding 319 amino acids with 88% identity to the amino acid sequence in AtRPB36a and 37% identical to yeast RPB3 (Fig. 1). The predicted pI of the B36b protein was 4.7. Within the ORFs, AtRPB36a and AtRPB36b showed 91% identity in nucleotide sequence, and in the untranslated regions, the two cDNA clones were 82% identical (data not shown). A Northern blot with a mixed AtRPB36a and AtRPB36b probe revealed only one size mRNA of 1.5 kb (Fig. 2B). We have not attempted to quantitate the relative amounts of the individual AtRPB36a and AtRPB36b mRNAs.
The Genes for AtRPB36a and AtRPB36b—To determine the gene structure and promoter sequences for B36a and B36b subunits, we isolated the genes corresponding to the two Arabidopsis AtRPB36 cDNAs. The two cDNA were used to screen an Arabidopsis λEMBL genomic library. Ten genomic clones were selected, purified, and mapped with restriction enzymes.

Fig. 3. Genomic structure of AtRPB36a and AtRPB36b. Exons are indicated by the closed boxes, 3′- and 5′-untranslated regions by the open boxes, and introns, promoters, and 3′ regions of the genes by the thick lines. The percentage identity of nucleotide sequences in different regions of the two genes is indicated. ATG and TGA indicate the translation start and termination codons, respectively. A 3-kb size marker is shown below the two genes.

2 T. Guilfoyle and T. Ulmasov, unpublished results.
subunits or recombinant His-tagged subunits to the third largest subunit of Arabidopsis RNA polymerase II revealed that the B36a subunit was the predominant third largest subunit in the enzyme purified from suspension culture cells. A subunit with the predicted mobility of the B36b subunit was not detected in Coomassie Blue-stained gels (Fig. 4B, left panel) or by autoradiography with 35S-labeled Arabidopsis RNA polymerase II (Fig. 4B, right panel). From this analysis, it is not clear whether the B36b subunit is a minor component of RNA polymerase II in suspension cells, is associated with a form of the enzyme that fails to purify using the methods employed, is modified in vivo so that it migrates identically to B36a, or fails to associate with the enzyme.

The third largest subunits in S. pombe and S. cerevisiae RNA polymerase II are reported to have a stoichiometry of 2 in the purified enzymes (13, 36), while the homologous subunits in plant and animal RNA polymerase II are reported to have a stoichiometry of one (39–43). To determine the stoichiometry of the B36a subunit in Arabidopsis RNA polymerase II, we measured the peak areas for the 205 + 175, 135-, and 36-kDa subunits by imaging (Image I Software) 7.5% Coomassie Blue-stained gels (Fig. 4B, left panel). This analysis indicated that the stoichiometries of the three largest subunits were 1, 1.1, and 1.3 (i.e. using the largest subunit, 205 + 175, as the baseline), for the 205 + 175-, 135-, and 36-kDa subunits, respectively. As a second method for determining stoichiometry of these subunits, we quantitated the 35S incorporation in the three largest subunits in Arabidopsis RNA polymerase II that had been labeled in vivo with [35S]methionine. Since the amino acid sequences for the 205- (15), 135- (38), and 36-kDa subunits (see Fig. 1) are known, the number of methionines in each subunit could be divided into the 35S incorporated into each subunit (i.e. determined by phosphor imaging) to determine the subunit stoichiometries. The stoichiometry for each subunit was near unity. The relative stoichiometries obtained for three independent experiments are shown to the right of each subunit in Fig. 4B (right panel). With results from Coomassie Blue staining and 35S labeling taken together, the best estimate for stoichiometry of the B36 subunit in Arabidopsis RNA polymerase II is 1.

The Arabidopsis B13.6 Subunit—Because the stoichiometry of the B36 subunit appeared to be 1 in purified Arabidopsis RNA polymerase II and because a second subunit with homology to the prokaryotic α subunit of RNA polymerase II has been identified in yeast (RPB11; Ref. 8) and human RNA polymerase II (hRPB14; Ref. 44), we searched the database for a plant homolog to yeast RPB11. An EST from an Arabidopsis cell suspension cDNA library (Ref. 22) was identified (GenBank accession number Z47635). Several additional AtRPB13.6 cDNA clones were isolated from the Arabidopsis cell suspension cDNA library, and all of these were identical in sequence (with the exception of variable lengths in the untranslated regions). We sequenced a full-length AtRPB13.6 cDNA clone, and the derived amino acid sequence indicated that the Arabidopsis homolog (B13.6) is a 13.6-kDa subunit with pI of 6.5 (Fig. 5). The B13.6 subunit has 41 and 42% amino acid sequence identity with yeast RPB11 and human hRPB14, respectively (Fig. 5). The Arabidopsis B13.6 subunit, like the yeast RPB11 and human hRPB14 subunits, contains the "α motif" and a second leucine-rich C-terminal motif that may be related to the α subunit in prokaryotic RNA polymerases (see also Fig. 9). Preliminary evidence, based on Southern analysis and cDNA cloning, indicates that the gene encoding B13.6 is single copy in the Arabidopsis genome.3

* Arabidopsis B36a and B36b Subunits Associate with the B36 Subunit in Vivo—The unit stoichiometry of the B36 subunit in Arabidopsis RNA polymerase II and the identification of a second smaller subunit, B13.6, containing the "α motif" suggested that these two subunits might associate as heterodimers in the Arabidopsis enzyme. Lalo et al. (9) have previously shown that two yeast subunits, AC40 and AC19, which are common to RNA polymerases I and III, associate in vivo in a yeast two-hybrid system. The yeast AC40 and AC19 subunits contain the "α motif" as do the yeast RPB3 (also Arabidopsis B36a and B36b) and RPB11 (also Arabidopsis B13.6) subunits. Two determine if the B36a and B36b subunits associated with themselves, with one another, or with the B13.6 subunit, we tested these subunits in a yeast two-hybrid system. Table I shows results that indicate that the B36a and

3 R. Larkin, unpublished results.
B36b subunits do not associate as homodimers or heterodimers in the two-hybrid system. On the other hand, the B36a and B36b subunits do associate with the B13.6 subunit. This interaction was specific because the GAL4-B13.6 fusion protein failed to interact with out-of-frame B36a and B36b clones or with an unrelated SNF4 clone (45). Likewise GAL4-B36a and GAL4-B36b fusion proteins failed to interact with an SNF1 clone, while SNF1 and SNF4 did interact in this system (45). These results with the B36 and B13.6 fusion proteins are similar to those found by Lalo et al. (9) for yeast AC40 and AC19 and suggest that B36a-B13.6 and B36b-B13.6 associate as heterodimers in vivo.

In Vitro Protein-Protein Interactions with the B36a, B36b, and B13.6 Subunits—To confirm the interactions among the Arabidopsis B36a, B36b, and B13.6 subunits, we carried out in vitro protein-protein interaction analyses. One of these analyses involved immunoprecipitation (with HA monoclonal antibody) 35S-labeled in vitro co-translated subunits fused with or without HA epitope-tags. To test whether the B36a and B36b subunits could associate with themselves or with one another in vitro, we used an epitope-tagged B36b subunit. Only the epitope-tagged B36b subunit was immunoprecipitated whether it was translated along with the untagged B36a or the untagged B36b subunit (Fig. 6A). The untagged B36a and untagged B36b subunits could be distinguished from the epitope-tagged B36b subunit because of their different mobilities in SDS-polyacrylamide gels. These results indicated that the B36 subunits did not form stable associations with themselves or with one another in vitro. On the other hand, the B36a and B36b subunits were immunoprecipitated when co-translated with an epitope-tagged B13.6 subunit (Fig. 6B). A subunit related to the B36 subunits in Arabidopsis RNA polymerase I and III, AC42 (46), was not immunoprecipitated with epitope-tagged B13.6. As an additional control, we used an IAA4/5 polypeptide was epitope-tagged in lanes 1–6 and the IAA4/5 polypeptide was epitope-tagged in lanes 1–6, and the IAA4/5 polypeptide was epitope-tagged in lanes 7 and 8. In vitro translation products were resolved on 10% Tricine-SDS-polyacrylamide gels. Positions of subunits are indicated adjacent to the autoradiograms.
trophoresis as in a complex. Panel B: gel.

translated subunits resolved on a nondenaturing 7.5% polyacrylamide gel. The B36b and B13.6 subunits were co-translated and subjected to nondenaturing gel electrophoresis as in panel A, lane 2. The gel lane was excised, laid on its side (lane 2), and subjected to SDS-polyacrylamide gel electrophoresis. Lane 1, co-translated B36b and HA epitope-tagged B13.6 (i.e., an aliquot of the translation products was applied to the first dimension, nondenaturing gel); lane 3, immunoprecipitate of translation products shown in lane 1 using an HA monoclonal antibody. The arrow indicates the position of the B13.6 subunit that migrates in the B36b-B13.6 complex (C) resolved from free (F) B36b on the nondenaturing gel. Some smearing or tailing of the B36b-B13.6 complex is evident in the nondenaturing gel.

unit or in the absence of HA antibody (data not shown).

A second in vitro approach that showed B36b interaction with B13.6 was obtained by resolving in vitro co-translated subunits by electrophoresis in polyacrylamide gels under nondenaturing conditions. When the B36b subunit was co-translated with the B13.6 subunit, the B36b subunit showed a mobility shift in the gels (Fig. 7). The gel lane (lane 2) containing a band with decreased mobility was excised and subjected to electrophoresis in an SDS-polyacrylamide gel. The SDS gel showed that the mobility shift in the nondenaturing gel was due to association of the B13.6 subunit with the B36b subunit.

The N-terminal and C-terminal Truncations in B36b α Motifs. Prevent Association with B13.6. The C-terminal region in the Arabidopsis B36 subunits contains a motif related to the prokaryotic RNA polymerase α subunit (see Fig. 1). This motif is rich in leucine, and it has been suggested that this motif in S. pombe RPB3 and homologous subunits in other RNA polymerases consists of a putative leucine-zipper motif that might be involved in subunit-subunit interactions (36). To test whether this C-terminal motif in Arabidopsis B36b might be involved in interactions with B13.6, we tested several C-terminal truncations using the immunoprecipitation assay with in vitro co-translated subunits as described above. Fig. 8A shows a schematic diagram of these truncations, and the precise positions of the truncations are shown in Fig. 1. Truncations at the extreme C terminus of B36b did not prevent association with B13.6 (Fig. 8C, lanes 1-4); however, truncations that deleted a portion of the leucine-rich motif (C288; Fig. 8C, lane 5) or the entire leucine-rich motif (C300; Fig. 8C, lane 6) resulted in B36b subunits that did not associate with B13.6. These results are consistent with the C-terminal motif in B36b being important for subunit-subunit interactions. C-terminal truncations in B36a subunit have not been tested, but this C-terminal region in B36a is strongly conserved with that in B36b (see Fig. 1).

We did not observe interactions between the leucine-rich C-terminal α-like motif in B36b and B13.6 when the C-terminal motif of B36b was tested as a fusion protein in isolation from the remainder of the B36b subunit (GST-N249) (Fig. 8C, lane 8). In this case, amino acids 249-319 in B36b were fused to a GH2/4 protein at its C terminus (32). The GH2/4 cDNA encodes a glutathione S-transferase (33). Failure to observe interaction between the fused B36b C-terminal motif and B13.6 could result if more than one interaction motif or a more extensive interaction motif is required for the stable association of B36b and B13.6. Since the α motifs in the N-terminal regions of RPB3 and AC40 in yeast appear to be important for subunit interactions and enzyme assembly (9, 18), we made an N-terminal truncation that removed a portion of the α motif in B36b. This truncated subunit was not immunoprecipitated with epitope-tagged B13.6 in co-translation experiments (Fig. 8C, lane 7). This result is consistent with there being two motifs or one extended motif (i.e. including both α-like motifs in

FIG. 8. C-terminal and N-terminal truncations that prevent B36b and B13.6 interactions. Panel A, schematic diagrams of the N-terminal and C-terminal truncations in the B36b subunit. The position of the putative zinc-finger is indicated above construct 1. The hatched box and black box indicate the positions of the N-terminal α motif (α motif 1) and leucine-rich C-terminal α-like motif (α motif 2), respectively. The stippled box represents the GH2/4 GST fusion protein. HA-13.6 is a schematic of the HA epitope-tagged B13.6 subunit showing the N-terminal position of the epitope-tag (open diamond) and the α-like motifs (hatched and black boxes). The N refers to N-terminal, and the C refers to C-terminal truncations at the amino acid positions indicated by the number. Panel B, autoradiograms of in vitro translation products used for immunoprecipitation assays. HA epitope-tagged B13.6 was co-translated with B36b or a B36b C-terminal truncation, and translation products were resolved on 10% Tricine-SDS-polyacrylamide gels. The B36b subunit or truncations tested were untruncated (319 amino acids) (lane 1); C319 (314 amino acids with 5 amino acids missing from C terminus) (see Fig. 1); C310 (lane 3); C300 (lane 4); C288 (lane 5); C244 (lane 6); N47 (272 amino acids with 47 amino acids missing from N terminus) (lane 7); C terminus (amino acids 249-319) fused to the C terminus of the GST protein GH2/4 (39) (lane 8). Panel C, immunoprecipitations of samples shown in panel B.
FIG. 9. The N-terminal “α motif” and a leucine-rich C-terminal α-like motif in B36a, B36b, B13.6, and homologous subunits in other eukaryotic RNA polymerses. A, The N-terminal “α motif” (α motif 1) in large and small α-like subunits in RNA polymerases I, II, and III from Arabidopsis, yeast, and human. B, the leucine-rich C-terminal α-like motif (α motif 2) in large and small α-like subunits in RNA polymerases I, II, and III from Arabidopsis, yeast, and human. Amino acid alignments are shown with conserved amino acids shaded black for identity (the predominant amino acid at each position) and gray for similarity (BOXSHADE program, Kay Hofman, Bioinformatic group, Lausanne, Switzerland). Leucines and isoleucines that predominate at specific positions are indicated at the top. B36a, B36b, B13.6, AC42, AC43, and AC14 are Arabidopsis subunits. Yeast subunits are indicated by a y, and human subunits by an h. EcRpoA is the E. coli α subunit, and TobCt is the tobacco chloroplast α-like subunit. The N-terminal amino acid position for the conserved domain in each subunit is indicated at the lower left of the sequence.

DISCUSSION

Our results have shown that Arabidopsis contains two genes that encode the third largest subunit of RNA polymerase II. This differs from other RNA polymerase II genes in Arabidopsis and in most other organisms studied, where the genes are single copy. The two genes are expected to encode the third largest subunit in yeast and human RNA polymerase II and lesser homology to a related subunit in yeast and mouse RNA polymerases I and III. This is supported by our cloning of two additional cDNAs from Arabidopsis that encode proteins that show stronger homology to the yeast and mouse AC40 subunits in RNA polymerases I and III than to the yeast RPB3 or human hRPB33 subunit in RNA polymerase II (46).

Therefore, it appears that Arabidopsis expresses two genes for the 36-kDa subunit in RNA polymerase II and two genes for the 42/43-kDa subunit in RNA polymerase I and III. All four polypeptides encoded by these genes contain the N-terminal “α motif” (7) and a leucine-rich C-terminal α-like motif (7, 36), which are located in amino acid sequence to the prokaryotic RNA polymerase α subunit. Two other cDNAs from Arabidopsis that contain these α-like motifs in their ORFs have been cloned. One of these, described above, encodes a protein of 13.6 kDa (B13.6) that is a homolog of the yeast RNA polymerase II B12.5 (RPB11) subunit. The second encodes a protein of 14 kDa, which is a homolog of the yeast RNA polymerase I and III subunits.
AC19 subunit.\textsuperscript{4}

The different mobilities of the B36 subunits in SDS-polyacrylamide gel electrophoresis allowed us to distinguish the B36a from the B36b subunit. RNA polymerase II purified from Arabidopsis suspension culture cells contains a third largest subunit with an apparent molecular mass of 40 kDa, which migrates identically to in vitro translated B36a in SDS-polyacrylamide gel electrophoresis. If the B36b subunit is associated with purified RNA polymerase II, then it is present in amounts not detectable by Coomassie Blue staining or by autoradiography in \textsuperscript{35}S-labeled enzyme. The reason for predominance of the B36a subunit in the purified enzyme is not clear because the promoters for both ATRPB36 genes are active in transgenic tobacco tissues undergoing cell division and in transfected protoplasts from carrot suspension culture cells.\textsuperscript{2} Furthermore, based on cDNA cloning, both ATRPB36 genes are expressed in the rapidly dividing Arabidopsis suspension culture cells from which the RNA polymerase II was purified. While we have not quantitated the relative amounts of B36a and B36b mRNAs in suspension culture cells, the fact that six out of seven cDNA B36 cDNA clones isolated were B36a suggests that the B36a mRNA is more abundant than the B36b. Our results on in vivo and in vitro protein-protein interactions suggest that the B36b subunit is not defective in assembly (i.e. at least assembly with the B13.6 subunit). The high conservation in amino acid sequence between B36a and B36b suggests that both subunits should be capable of assembly into RNA polymerase II. It is possible that the B36b subunit is expressed at much lower levels or assembles into RNA polymerase less efficiently than the B36a subunit in cell suspension cultures and that this subunit has simply escaped our detection. The B36b subunit may be more abundant in Arabidopsis RNA polymerase II found in specific tissues or specific developmental stages. It is worth noting that heterogeneity in the size of the third largest subunit in RNA polymerase II has been reported for enzymes purified from wheat and rye embryos (39, 42), suggesting that more than one gene encodes this subunit in other plant species.

Based upon the yeast two-hybrid system and immunoprecipitation experiments with epitope-tagged in vitro translated subunits, the B36a and B36b subunits fail to stably associate with themselves or one another but do associate with the B13.6 subunit. Our in vivo results are similar to the in vivo results of Lalo et al. (9), who used the yeast two-hybrid system to show that the yeast RNA polymerase AC40 and AC19 subunits associate with one another as a heterodimer, but that the AC40 subunit fails to homodimerize. Our in vitro protein-protein interaction results confirm the in vivo results. Based upon these in vivo and in vitro protein-protein interaction results and the apparent unit stoichiometry of the Arabidopsis B36 and yeast AC40 subunits, it is possible that heterodimers, Arabidopsis B36/B13.6 and yeast AC40/AC19, are the equivalent of an $\alpha_2$ homodimer in prokaryotic RNA polymerase. A stoichiometry of 1 for the third largest subunit of other plant and animal RNA polymerase II enzymes has been documented previously, based upon the intensity of subunit staining with Coomassie Blue (39–43, 47). Unit stoichiometry for the third largest subunit in Arabidopsis RNA polymerase II is supported by the relative intensity of Coomassie Blue staining and by the ratio of $\textsuperscript{35}S$ incorporated to the number of methionines in this subunit compared with the two largest subunits. In contrast to our results with Arabidopsis RNA polymerase II, the RPB3 subunits in S. pombe and S. cerevisiae RNA polymerase II are reported to have a stoichiometry of 2 (13, 36), and evidence has been presented that is consistent with a S. cerevisiae RNA polymerase II assembly pathway initiating with the homodimerization of RPB3 and subsequent interaction with RPB2 and RPB1 (18). While this proposed assembly pathway for yeast RNA polymerase II resembles that reported for bacterial RNA polymerase (48), there is no direct evidence for the homodimerization of yeast B44 (RPB3) subunits. It remains possible that yeast B44 and B12.5 (RPB11) form heterodimers like Arabidopsis B36 and B13.6. On the other hand, it is possible that the subunit stoichiometries and assembly pathways differ in RNA polymerase II from yeast and plants.

Lalo et al. (9) showed that a number of mutations in the "$\alpha$ motif" (see Fig. 9) of yeast AC40 were lethal. On the other hand, similar mutations within the "$\alpha$ motif" of yeast AC19 produced only minor growth defects. These results suggest that the "$\alpha$ motif" in yeast AC40 and AC19 may not be functionally equivalent (i.e. in enzyme assembly or activity). Other results have indicated that mutations in the "$\alpha$ motif" of the $\alpha$ subunit of E. coli RNA polymerase and the yeast RNA polymerase II RPB3 subunit produce defects in enzyme assembly (18, 49, 50). Our results with the Arabidopsis B36b subunit suggest that the N-terminal "$\alpha$ motif" and a second $\alpha$-like motif in the C terminus of this subunit may both be important for association with the B13.6 subunit. Similar to the N-terminal "$\alpha$ motif," the second C-terminal $\alpha$-like motif appears to be conserved in the larger (i.e. 36–44-kDa) and smaller (i.e. 12.5–19-kDa) RNA polymerase subunits related to the prokaryotic RNA polymerase $\alpha$-subunit. It is possible that both $\alpha$-like motifs (i.e. the "$\alpha$ motif" and the C-terminal leucine-rich motif) may contribute to subunit-subunit interactions and enzyme assembly. Recent results with the $\alpha$ subunit in E. coli RNA polymerase indicate that both $\alpha$ subunit motifs (shown in Fig. 9) that are conserved in eukaryotic $\alpha$-like subunits are important for $\alpha$ dimerization (51, 52). It is interesting that the C288 truncation (see Figs. 1, 8, and 9) in the B36b subunit, which is the shortest truncation tested that resulted in a loss of association between the B36b and B13.6 subunits, is located within two amino acids (i.e. see the alignment of leucine-rich C-terminal $\alpha$-like motifs in Fig. 9) of an insertion mutant that renders the E. coli $\alpha$ subunit inactive in dimerization (52). Additional experiments will be required to confirm the importance and specificity (i.e. specificity of AC subunit interactions versus B subunit interactions, specificity of plant subunit interactions versus animal or yeast subunit interactions) of the $\alpha$-like motifs in these subunit interactions.

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