Light Regulation of the Abundance of mRNA Encoding a Nucleolin-Like Protein Localized in the Nucleoli of Pea Nuclei[1]

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A cDNA encoding a nucleolar protein was selected from a pea (*Pisum sativum*) plumule library, cloned, and sequenced. The translated sequence of the cDNA has significant percent identity to *Xenopus laevis* nucleolin (31%), the alfalfa (*Medicago sativa*) nucleolin homolog (66%), and the yeast (*Saccharomyces cerevisiae*) nucleolin homolog (NSR1) (28%). It also has sequence patterns in its primary structure that are characteristic of all nucleolins, including an N-terminal acidic motif, RNA recognition motifs, and a C-terminal Gly- and Arg-rich domain. By immunoblot analysis, the polyclonal antibodies used to select the cDNA bind selectively to a 90-kD protein in purified pea nuclei and nucleoli and to an 88-kD protein in extracts of *Escherichia coli* expressing the cDNA. In immunolocalization assays of pea plumule cells, the antibodies stained primarily a region surrounding the fibrillar center of nucleoli, where animal nucleolins are typically found. Southern analysis indicated that the pea nucleolin-like protein is encoded by a single gene, and northern analysis showed that the labeled cDNA binds to a single band of RNA, approximately the same size as the cDNA. After irradiation of etiolated pea seedlings by red light, the mRNA level in plumules decreased during the 1st hour and then increased to a peak of six times the 0-h level at 12 h. Far-red light reversed this effect of red light, and the mRNA accumulation from red/far-red light irradiation was equal to that found in the dark control. This indicates that phytochrome may regulate the expression of this gene.

Nuclear rRNA synthesis occurs in the nucleolus and is the first step in ribosome biogenesis. A number of nucleolar proteins are thought to play important roles in rRNA synthesis, early rRNA processing, and ribosome assembly. Among the best characterized of these is nucleolin, a multifunctional protein typically concentrated in the transition zone between the fibrillar center and the dense fibrillar component of nucleoli (Scheer et al., 1993).

Most of what is known about the structure and function of nucleolin comes from animal studies. The most recent study of a plant nucleolin-like protein is that of Bögre et al. (1996), who cloned several nucleolin-like cDNAs in alfalfa and identified a 95-kD nucleolin-like protein by immunoblotting. The transcript of one of the cDNAs, *nucMs1*, was characterized to be developmentally and mitogenically regulated. A related, earlier study is that by Martin et al. (1992), who used heterologous antibodies (against hamster nucleolin) to identify a putative 64-kD nucleolin homolog in onion root meristems. Nucleolin genes from several other species such as African clawed frog (Caizergues-Ferrer et al., 1989; Rankin et al., 1993), chicken (Maridor and Nigg, 1990), human (Srivastava et al., 1989), and mouse (Bourbon et al., 1988) have also been cloned and characterized. Additionally, there is a nucleolin-like protein, NSR1, in yeast (Lee et al., 1991; Kondo and Inouye, 1992).

Light increases the rate of nuclear rRNA gene transcription in several plants, and the light receptor for this response is the photoreversible pigment phytochrome (Thien and Schopfer, 1975, 1982). The synthesis of rRNA is obviously a crucial early step in the assembly of ribosomes, and nucleolin appears to play an important role in this event (Belenguer et al., 1989), as well as in later processing steps (Olson, 1990). This raises the question of whether light up-regulates the gene for nucleolin while stimulating the transcription of rRNA as part of the overall process of promoting ribosome assembly. In an effort to answer this question and to learn more about the structure of plant nucleolins, we recently selected an apparently full-length cDNA from a pea plumule library that encodes a nucleolin-like protein. Here we report on the sequence of that cDNA and the structural features of the deduced protein it encodes. We also used the cDNA as a probe to monitor light-induced changes in the abundance of its cognate mRNA, and we used antibodies to the protein encoded by the cDNA to localize it in nucleoli of pea plumule cells.

MATERIALS AND METHODS

The seedlings of pea (*Pisum sativum* L. cv Alaska) were grown in the dark for 7 d at $22 \pm 3°C$. These seedlings were used for the isolation of pea nuclei and pea nucleoli, for genomic DNA isolation, for isolation of the RNA used in the various northern analyses, and for localization studies.

Abbreviations: FR, far-red light; IPTG, isopropyl-1-thiol-β-D-galactopyranoside; NLS, nuclear localization signal; R, red light; RRM, RNA recognition motif.

Production of Polyclonal Antibody pc481

Nuclear proteins were extracted by 0.3 M NaCl from chromatin isolated from the nuclei of etiolated pea plumules according to the method of Chen et al. (1986), except that the nuclei were exposed to white light before they were treated with detergent. The salt-extracted proteins were separated by SDS-PAGE and stained with Coomassie blue, as previously described and illustrated (Li et al., 1991). A distinct protein band near 47 kD, later inferred to be a proteolytic breakdown product of nucleolin (see "Results" and "Discussion"), was excised from the gels and sent to Pocono Farms (Canadensis, PA) to be used for the inoculation of guinea pigs. The immune serum that was generated was termed polyclonal antibody preparation pc481. Unless otherwise noted, the pc481 and its preimmune serum used in all protocols was affinity purified through a protein A-sepharose affinity column using the procedure described in Martin (1982).

Nuclei and Nucleoli Preparation

Nuclei were isolated from the plumules of 7-d-old dark-grown pea seedlings by the method of Datta et al. (1985). The purity of the nuclear preparations was judged to be greater than 85% by microscopic assays. Nucleoli were purified from isolated nuclei, according to the protocol described by Dickinson and Kohwi-Shigematsu (1995). The nucleolar preparations were judged to be free of significant nuclear or other large organellar contamination by light microscopy. All of the steps in the nucleoli isolation were performed at 4°C, and, unless otherwise noted, all solutions contained a protease inhibitor cocktail, consisting of 1 mM pepstatin, 0.5 mM benzamidine, 0.2 mM Nα-p-Tosyl-L-Arg methyl ester, and 10 mg mL^{-1} trypsin inhibitor. The nucleoli-enriched fraction was prepared by resuspending isolated nuclei in TBS (10 mM Tris-HCl, pH 7.4, 10 mM MgCl$_2$, and 150 mM NaCl) plus protease inhibitor cocktail and sonicating the nuclei four times for 30 s, each time interspersed with 10 s in an ice bath, and then treating the resulting suspension with 0.02 mg mL^{-1} DNase I (type II, from bovine pancreas) for 1 h. This preparation was then carefully layered on the top of a 0.88 M Suc cushion and centrifuged at 800g for 15 min. The pellets were used as nucleolar protein preparations.

Immunoblotting

Protein samples assayed by immunoblot included total extracts of purified pea nuclei and nucleoli and of Escherichia coli cells expressing the nucleolin-like cDNA. Fifteen micrograms of sample protein was separated on either 10% SDS-PAGE (for nuclear and nucleolar proteins) or 8% SDS-PAGE (for E. coli proteins) and electroblotted onto a nitrocellulose membrane. The membrane was incubated in a blocking solution of PBS (0.14 M NaCl, 3 mM KCl, 1.5 mM KH$_2$PO$_4$, and 8 mM Na$_2$HPO$_4$·7 h$_2$O, pH 7.5) plus 5% nonfat dry milk at room temperature for 1 h, then incubated overnight at 4°C with pc481. After being washed three times by 2% nonfat dry milk in PBST (0.1% Tween 20 in PBS), the immunostain was developed by x-ray film

(RX, Fuji, Tokyo, Japan) and chemiluminescent western blotting detection reagents (Amersham) with horseradish peroxidase-labeled secondary antibodies.

Immunolocalization

Plumules were fixed in 2% paraformaldehyde in distilled water or 0.1 M Na phosphate (pH 7.5) for 30 min at room temperature. During fixation the tissue was cut into small pieces with a scalpel and evacuated to promote penetration of the fixative. Tissue was rinsed in buffer A (0.1 M Na phosphate, pH 7.5, and 0.15 M NaCl), and cells were gently teased apart from the fixed tissue in a drop of buffer A on slides that had been pretreated with poly-L-Lys (Sigma). Often, broken cells released intact nuclei during this process, and most of the cells and the nuclei released from them bound ionically to the slide surface.

Slides were placed in a moist chamber (Petri dish with wet filter paper) to prevent dehydration of the samples. Samples were then treated with a blocking buffer (1% nonfat dry milk in buffer A) for 1 h before being incubated overnight at 4°C with pc481 or preimmune serum. Both antibodies were used at either a 1:100 or 1:20 dilution. After several washes with the blocking buffer, the samples were incubated with rabbit anti-guinea pig IgG tetramethylrhodamine isothionate conjugate antibody (Sigma) at a 1:100 dilution for 1 h at room temperature in the dark, then washed several times again with the blocking buffer followed by several washes with buffer A. The final rinse was with distilled water. Stained samples were mounted in Immu-Mount (Shandon, Pittsburgh, PA) and analyzed on either a confocal laser scanning microscope (MRC 600, Bio-Rad) or a confocal laser scanning microscope (TCS 4D, Leica) equipped for epifluorescence, with the filter system appropriate for rhodamine fluorescence. Samples treated with the preimmune serum or without the first antibody treatment were used as controls.

Silver- and Bismuth-Staining on Western Blots

Ten micrograms of pea nucleolar protein loaded onto each lane was separated by SDS-PAGE and transferred onto nitrocellulose membrane. The blots were assayed either by the silver-staining method as described by Lischwe et al. (1981) or by the bismuth-staining method according to the protocol of Gas et al. (1984).

Screening of cDNA Library

The cDNA library was constructed by Stratagene in ZAP II from mRNA isolated from etiolated pea plumules. The library was screened with pc481, which had been preabsorbed with E. coli and phage lysate. A total of approximately 2.5 × 10^5 plaque-forming units were screened. Plaques were lifted onto nitrocellulose membranes (BA85, Schleicher & Schuell). The membranes were washed three times in buffer B (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20), transferred to a blocking solution (buffer B plus 5% nonfat dry milk), and incubated at room temperature for 1 h. The membranes were then incubated overnight at 4°C with preabsorbed pc481. After they were

washed three times in buffer B, the membranes were incubated at room temperature for 1 h with goat anti-guinea pig secondary antibodies (H+L; Kirkegaard and Perry Lab., Inc., Gaithersburg, MD), using 1000× dilution in fresh blocking solution. The membranes were washed three times in buffer B and once in buffer B minus the Tween 20. Finally, the membranes were incubated with a substrate solution (10 mL of 0.1 M Tris, pH 7.5, 1 mL of nitroblue tetrazolium, and 1 mL of 5-bromo-4-chloro-3-indolyl phosphate). Eight positive clones from the first-round screening were further purified until they were separated into single plaques. The cDNA clone containing the largest insert was named NA481-5. Recombinant pBluescript SK phagemids were excised in vivo from the Zap II vector by infecting 200 μL of XL-Blue cells with 100 μL of phage stock (3.5 × 10^{10} plaque-forming units 100 μL^{-1}) and 1 μL of ExAssist Helper phage (1.0 × 10^{10} plaque-forming units mL^{-1}), according to the manufacturer's protocol (Stratagene). Phagemid DNA was then isolated and prepared for sequencing and restriction mapping.

Overexpression of NA481-5 in *E. coli*

Cells of *E. coli* strain XL1-Blue containing NA481-5 cDNA in pBluescript SK II vector and containing the same vector only were grown to mid-log phase (A_{600} = 0.60) in Luria broth with 50 μg mL^{-1} carbenicillin at 37°C. To induce the protein expression, IPTG was added in a final concentration of 10 mM. Cell samples (0.5 mL) were taken at the time points of 0 and 3 h after the addition of IPTG. The cells were pelleted by a brief centrifugation, resuspended in 70 μL of the SDS-PAGE loading buffer, and boiled just prior to SDS-PAGE electrophoresis and immunoblotting.

Extension and Amplification of the 5' cDNA End

A total RNA preparation (15 μg) isolated from etiolated pea plumules was denatured at 70°C for 10 min and then incubated in 62.5 mM MeHgOH for 10 min before it was subjected to the procedure of extension and amplification of the 5' cDNA end according to the protocol of Schuster et al. (1992), with minor modifications. Gene-specific primers were used: GSP1 (5' 124 TTGTCGTTTCGCCAGTCAAGT-CT 148 3') and GSP2 (5' 105 AAGGTTGATGCTGCTCC 121 3'). Two anchored primers were also used: (5' GCATGCG-CGCGGCCGCGGAGGCCCCCCCCCCCCCCC 3') and (5' G-CATGCGCGCGGCCGCGGAGGCC 3'). The products were then ligated into the Bluescript plasmid SK (Stratagene). Following transformation, recombinant colonies were identified and plasmid DNA was prepared for sequencing.

DNA Sequencing

The cDNA insertions and the PCR products were ligated into the Bluescript SK plasmid and were sequenced by the dideoxy chain-termination method with modified T7 polymerase (Sequenase 2.0) using a sequencing kit (United States Biochemical). The cDNA insertions were successively sequenced with synthesized primers.

Genomic DNA Isolation and Southern Analysis

Genomic DNA was isolated from etiolated seedlings following the method of Murray and Thompson (1980). The crude DNA-precipitated bycetyl trimethyl ammonium bromide precipitation buffer (1% cetyl trimethyl ammonium bromide, 50 mM Tris-HCl, pH 8.0, and 10 mM EDTA, pH 8.0) was further purified by centrifugation on a CsCl gradient. For genomic Southern analysis, the procedure of Sambrook et al. (1989) was used, with 10 μg of restriction fragments loaded per lane. The samples were electrophoretically separated in a 0.7% agarose gel with 1× TBE buffer (89 mM Tris-borate, pH 7.4, and 2 mM EDTA) and blotted onto a Zeta-Probe membrane (Bio-Rad) with 0.4 M NaOH. The membrane was hybridized to a labeled 525-bp PCR fragment, which was generated by using two primers from the cDNA sequences and the plasmid containing the cDNA as a template. The two primers that were used for generating the fragment for probing were: (5' 43 GCTTC-CGCAGCTTCTT 58 3') and (5' 554 AGATGAGAAACCTG 567 3'). The hybridization was carried out in a solution containing 1 mM EDTA, 0.25 M Na$_2$HPO$_4$, pH 7.2, and 7% SDS according to the supplier's protocol (Bio-Rad).

Irradiations

The R and FR sources used were those described by Kim et al. (1989). Fluence rates of R were 4 to 6 μm^{-1} m^{-2} s^{-1}, and fluence rates of FR were 7 to 10 μm^{-1} m^{-2} s^{-1}. The dark-grown seedlings were irradiated by R alone or by R followed by FR. After each irradiation treatment seedlings were returned to the dark, harvested at different time points as indicated in the Figure 7 legend, and frozen with liquid nitrogen for RNA isolation.

RNA Isolation and Northern Blotting Analysis

Plumules from light- and dark-grown plants were frozen in liquid nitrogen and ground in a mortar. The resulting powder was used for total RNA isolation according to the method of Wadsworth et al. (1988). Total RNA from different light treatments was used for analysis of nucleolin mRNA expression. The RNA was electrophoretically separated in a 1.2% agarose gel containing 6% formaldehyde, blotted onto Zeta-Probe nylon membrane (Bio-Rad), irradiated with short-wavelength UV for 2 min, and incubated in a prehybridization solution (0.25 M Na$_2$HPO$_4$, pH 7.2, 1 mM EDTA, and 7% SDS). Hybridization to a ^{32}P-labeled cDNA insert was performed at 65°C for 16 h. The washing procedure was carried out at high-stringency conditions following the supplier's protocol (Bio-Rad). The wet membrane wrapped with plastic wrap was exposed to x-ray film (New RX, Fuji) with an intensifying screen at −70°C or placed in a phosphor imager (model 445S1, Molecular Dynamics, Sunnyvale, CA).

RESULTS

Origins of the pc481 Antibody

The pc481 antibody preparation was raised to a 47-kD nuclear protein band. The original rationale for raising

antibodies to a protein of this size was to obtain immuno-probes for studying a 47-kD nuclear NTPase, which is known to be regulated by light and calmodulin (Chen et al., 1986). However, for reasons noted in "Discussion," it is likely that the 47-kD band used to raise pc481 was lacking NTPase but included a 47-kD proteolytic breakdown product of nucleolin. The discovery that pc481 did not recognize the NTPase led us to investigate this anomaly further. We used the pc481 antibodies in additional immunoblot assays and in immunocytochemical assays, and to screen a pea plumule cDNA library. The results of all of these assays, summarized below, indicated that pc481 recognized a pea nucleolin-like protein.

Immunoblot Analysis of Pea Nuclear Proteins by pc481 Antibody

After their electrophoresis on a SDS polyacrylamide gel and transfer by electroblot to nitrocellulose, fresh preparations of pea total nuclear and nucleolar proteins had one band immunostainable by pc481 at 90 kD (Fig. 1A). When left at room temperature for 12 h (Fig. 1A,), the 90-kD polypeptide degraded into many smaller peptides, which
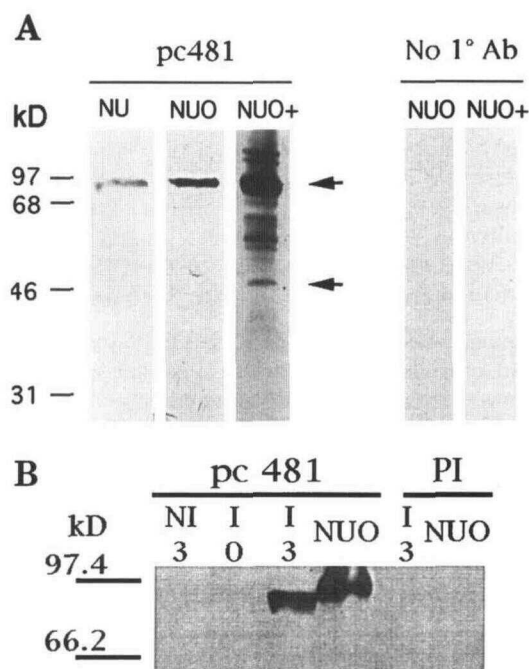

Figure 1. A, Western blot of total proteins of pea nuclei (NU), nucleoli (NOU), and nucleoli incubated at room temperature for 12 h (NUO+) (15 µg of protein loaded in each lane). B, *E. coli* cells expressing the pea nucleolin-like protein encoded by cDNA NU481–5. NI, No insert; cells transformed with plasmid only, extracted 3 h after treatment with IPTG; I, cells transformed with plasmid containing NU481–5 insert, extracted 0 and 3 h after treatment with IPTG; NUO, control lane containing proteins from purified pea nucleoli; pc481, lanes immunostained with pc481 as the primary antibody; and PI, lanes treated with preimmune serum as the primary antibody. Antibodies used for the immunostains in A and B were IgG-purified from pc481. The treatments with no-first antibody and pre-immune serum were used as the negative controls.

were also recognized by pc481, including one at 47 kD, the same molecular mass as the polypeptide used for raising this antibody. Total protein extracts of IPTG-induced *E. coli* cells expressing the nucleolin-like cDNA had one band immunostainable by pc481 at 88 kD (Fig. 1B). Extracts from noninduced cells showed no immunostainable band (Fig. 1B). Neither the no-first-antibody control nor the preimmune serum control showed any staining in any of the immunoblots (Fig. 1).

Immunolocalization

Nucleolar proteins are characterized in part by the subnucleolar domains they occupy, so to extend the immunoblot results and learn which subnucleolar domain was occupied by the antigen recognized by pc481, an immunocytochemical assay of pea plumule cells was carried out. An area of enhanced immunostaining consistently observed by this assay was a circular region surrounding the fibrillar center of the nucleolus (Fig. 2). Often, a uniform stain throughout the nucleolus was also apparent. Staining was predominately nucleolar, although a low level of staining outside the nucleolus was detected. The control samples (preimmune and no-first-antibody) showed essentially no fluorescence staining (Fig. 2C). Differential interference contrast microscopy of the immunostained sections confirmed that the stained nucleolar-like structures were enclosed within the nuclei (Fig. 2B).

Silver- and Bismuth-Staining on Western Blots

Animal nucleolin characteristically binds with a high affinity to silver and bismuth. Valdez et al. (1995) have shown that the silver binding probably arises from the Asp-rich sequences that reside in the acidic domains at the N terminus. The pea nucleolin-like protein has similar sequences (see below). Bismuth is believed to associate with highly phosphorylated proteins, including animal nucleolin (Locke and Huie, 1977; Gas et al., 1984). The pea nucleolin-like protein also carries many sites, with a likely potential to serve as phosphorylation sites (see below). A 90-kD protein band was detected in purified pea nucleoli by both staining approaches, and a protein band of this same molecular mass was positively immunostained by pc481 (Fig. 3). This indicates that pc481 recognizes a 90-kD pea nucleolar protein, which shares characteristic metal-staining properties in common with animal nucleolin.

Isolation of Nucleolin cDNA and Structure of Its Translated Sequence

Eight positive clones were selected from 2.5×10^5 plaque-forming units by screening with the polyclonal antibody pc481. The inserts ranged in size between 0.4 and 2.2 kb, as judged by restriction digestion and agarose electrophoresis. The three largest clones, 1.9, 2.1, and 2.2 kb, were individually sequenced (both strands) and were all found to share identical sequences. The actual length for the largest clone, named NA481–5, was 2126 bp. Using the cDNAs as probes, a 2.3-kb single band was identified on a northern blot. Since the cDNA may not represent the full
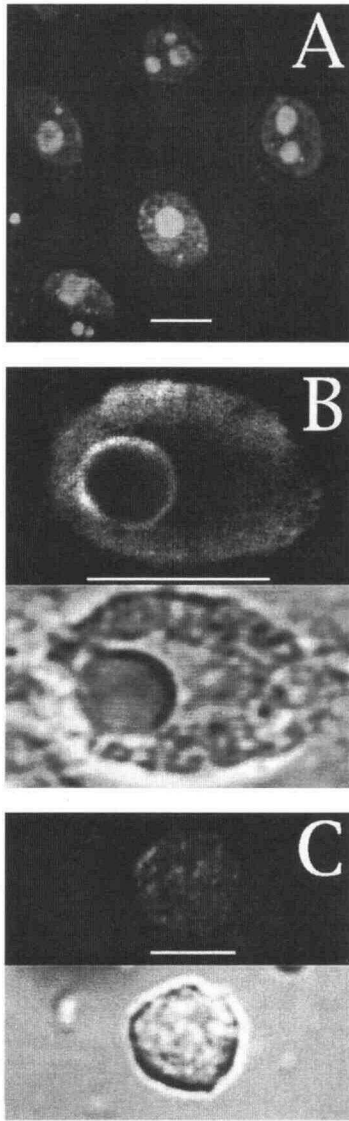
Figure 2. Immunocytochemical localization of a nucleolin-like protein in cells teased from fixed pea plumule tissue and bound to polylysine-coated slides using pc481 or preimmune serum as the primary antibody. A, Field showing a number of cells. Nucleoli within the cell nuclei are particularly strongly stained. Bar = 10 μm. B, Top, Immunostaining with pc481 of single nucleus with a single prominent nucleolus. The immunostain is mainly nucleolar in a peripheral area, which is believed to be in the transition zone between the fibrillar center and the dense fibrillar component where most nucleolins are usually detected. Bottom, Image of same nucleus as in top frame, viewed by differential interference contrast microscopy. Bar = 5 μm. C, Top, Immunostaining with preimmune serum of a cell that shows a weak, diffuse stain throughout the nucleoplasm of its nucleus. Bottom, Image of same cells as in top frame, viewed by differential interference contrast microscopy. Bar = 5 μm.

length, a 5′ rapid amplification of cDNA ends technique was employed to confirm the start codon of the open reading frame and to obtain an additional 48 bp of the 5′ end sequence. The complete nucleotide and deduced amino acids sequences of the cDNA obtained are shown in Figure 4. An open reading frame of 1833 bp was found
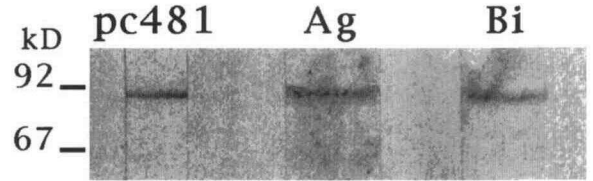

Figure 3. Analysis of electrophoretically separated nucleolar proteins after transfer to nitrocellulose by immunostaining with pc481, silver-staining (Ag), and bismuth-staining (Bi).

starting at a presumptive ATG start codon 75 bp downstream of the first nucleotide of the full-length sequence. The 264-bp untranslated region at the 3′ end includes the polyadenylation signal TATAAA at the nucleotide position 2149, and this signal is identical to that found in human nucleolin cDNA (Srivastava et al., 1989).

Based on the derived amino acid sequence of the pea nucleolin-like cDNA, the protein is a 611-amino acid
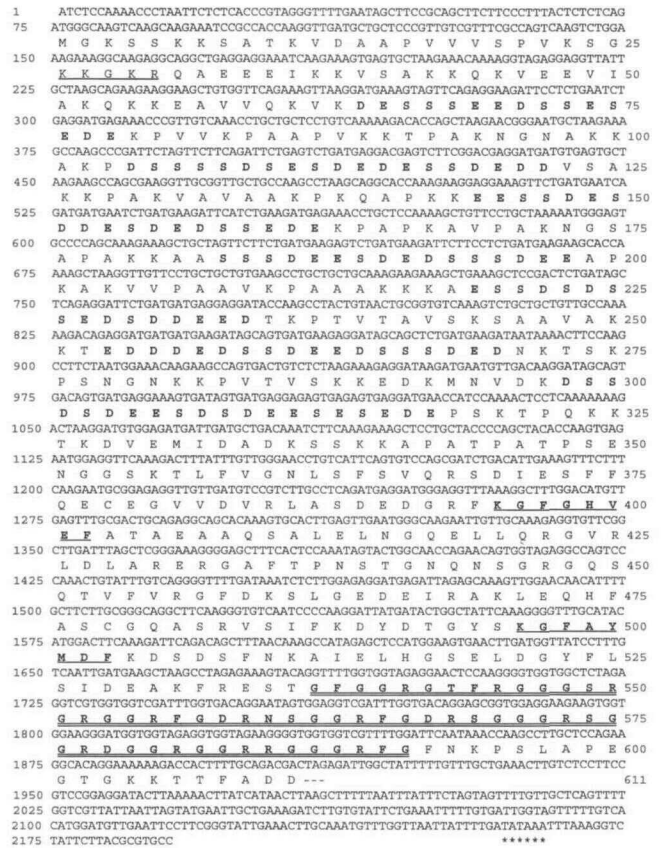

Figure 4. Nucleotide sequence and the deduced amino acid sequence of the pea nucleolin-like protein. The numbers in the left margin refer to the nucleotide sequence, whereas the numbers in the right margin refer to the amino acid residues. The potential NLS, similar to the prototype signal of the SV40 T antigen, is underlined in the N-terminal region of the protein sequence. Seven acidic motifs in the N terminus are in boldface. The two RRMs, centered on the amino acids residues at positions 398 and 499, are in boldface and single-underlined. The Gly- and Arg-rich domain in the carboxyl terminus is in boldface and double-underlined. The polyadenylation signal is underscored with asterisks on the nucleotide residues near position 2159.

polypeptide having a calculated molecular weight of 64,778. The amino acid sequence in the coding region is 66% identical and 75% similar to that of the alfalfa nucleolin homolog (Bögre et al., 1996), 28% identical and 50% similar to the yeast nucleolin homolog, NSR1 (Lee et al., 1991), and 31% identical and 45% similar to that of African clawed frog (*Xenopus laevis*) nucleolin (Caizergues-Ferrer et al., 1989). In addition, the sequence has the following distinct structural features of animal nucleolins: (a) highly charged acidic stretches at the amino terminus with characteristic repeats; (b) two RRMs, one of them (RNP-1) a highly conserved octamer and the other (RNP-2) a less-highly conserved hexamer; and (c) a conserved Gly- and Arg-rich carboxy-terminal sequence, designated the GAR domain. However, the pea nucleolin apparently has some different evolutionary constraints. The pea nucleolin has more acidic domains (seven instead of four in human and chicken and five in African clawed frog), and all of these domains are shorter than those of animals and contain

A

COMPARISON OF ACIDIC DOMAINS

Pea Nucleolin Homologue

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56  EAVVQKVK - DESSSEEDSSESEDE - KPVVK
96  GNAKKAKP - DSSSSDSESDEDESSDEDD - VSAKK
136 KPKQAPKK - EESSDESDDESDEDSSEDE - KPAPK
175 SAPAKKAA - SSSDEESDEDSSSDEE - APKAK
211 PAAAKKKA - ESSDSDSSEDSDDEED - TKPTV
245 SAAVAKKT - EDDDEDSSDEEDSSSDED - NKTSK
290 EDKMNVDK - DSSDSDEESDSDEESESEDE - PSKTP
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Alfalfa Nucleolin Homologue

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54  KALKVVKK - EESSSEESSEESEDE - KPVAK
94  GNVKKAQP - ETTSEESDSDDISSDEEE - VKKPA
132 NGSAPVKK - DDESSEEDSDESSESSDED - KKPAA
170 NGSAPAKK - DDSSEEEDSEDSDEDE - KPAAK
204 NGSVSAKK - DDSSSDEDSESSDEED - KKPAA
239 SAPTKKAA - SSSDEESDEESDEDED - AKPVS
271 AVAKKSKK - DSSDSDDEDDDSSSDED - KKPVA
299 VASKKEVS - ESESDSSDDD - HKMNI
316 DHKMNIDK - DSSDSDESEEESEDE - PLKTP
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Human Nucleolin

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15  KKMAPPPK - EVEEDSEDEEMSEDEEDDSSGEE - VVIPQ
135 KNGKNAKK- EDSDEEEDDDSEEDEEDDEDEDEDEDEIE - PAAM
177 AAAAAPAS - EDEDDEDDEDDEDDDDDEEDDSEEE - AMETT
236 PVKAKNVA - EDEDEEEDDEDEDDDDDEDDEDDDDEDDEEEEEEEEEE- PVKGA
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B

COMPARISON OF RNA RECOGNITION MOTIFS

PROTEIN	DOMAIN	RNP-2	RNP-1
Nucleolin Homologue	1	LFVGNL	KGFGHVEF
(*Pisum & Medicago*)	2		KGFAYMDF
NSR1	1	LFVGRL	RGYGYVDF
(*Sacchromyces*)	2	LFLGNL	KGFGYVQF
Nucleolin	1	LF I GNL	KKFGYVDF
(*Xenopus*)	2	LANKNL	KG I AYVEF
	3	LVLSNL	KGFAF I EF
	4	LFVKGL	KGFGFVDF
RNP CONSENSUS		LFVGNL	KGFGFVXF

Figure 5. A, The sequences of seven repeats in the N terminus of the pea nucleolin-like protein, alfalfa nucleolin homolog, and human nucleolin. In pea nucleolin the repeats range from 40 to 44 amino acids in length and are characterized by a central region containing exclusively Ser and acidic residues (Asp and Glu), flanked on each side by regions dominated by Ala, Lys, Pro, and Val. Similar repeats in human nucleolin and in an alfalfa nucleolin homolog are also shown. B, The alignment of the conserved RNP-1 and RNP-2 motifs found in pea nucleolin-like protein with alfalfa nucleolin homolog, *X. leavis* nucleolin, and yeast NSR1.

more Ser residues (Fig. 5A). However, like those in animals, all of the pea acidic domains are separated by basic sequences. Another notable difference is that the pea nucleolin only has two RRMs instead of the four found in animal nucleolins. A nucleolin-like protein in yeast, NSR1, also contains only two RRMs (Lee et al., 1991).

The alignment of the RNA-binding domains of pea nucleolin with those of *X. laevis* nucleolin and NSR1 in yeast is shown in Figure 5B. The second RRM of the pea nucleolin-like protein lacks an RNP-2 motif, which is believed to be less conserved than the RNP-1 motif. The alfalfa nucleolin homolog also lacks this second RNP-2 motif. The two RRMs are separated by 100 amino acids, which is very close to the distance (90 amino acids) defined by Bandziulis et al. (1989) for most animal nucleolins.

Animal nucleolin is known to be a major phosphoprotein in nucleoli. When analyzed for potential protein phosphorylation sites, the derived structure of the pea nucleolin-like protein has at least 48 possible casein kinase II phosphorylation sites, 2 cAMP-dependent protein kinase phosphorylation sites, 12 protein kinase C phosphorylation sites, and 1 unusual Tyr phosphorylation site.

The pea protein also has four potential Asn-glycosylation sites (at the residues 173, 271, 361, and 438), as also described for animal nucleolins. The carboxy-terminal GAR domain in pea nucleolin (53 amino acids) is about the same size as in alfalfa (55 amino acids), *X. laevis* (61 amino acids), Chinese hamster ovary cell (53 amino acids), human (50 amino acids), and mouse (49 amino acids) nucleolins, but is longer than that found in yeast NSR1 protein (36 amino acids). The overall composition in the pea GAR domain is roughly conserved, except for the presence in pea of a more diverse array of amino acid residues, other than Gly, Arg, and Phe, than is usually found in animal nucleolins.

Genomic Southern Analysis

The number of genes encoding the nucleolin-like protein in pea genome was estimated by Southern analysis (Fig. 6A) using high-stringency hybridization and washing conditions. A 525-bp fragment generated by PCR from the 5′ coding region of the NA481–5 cDNA was used to probe genomic DNA restricted with three different restriction endonucleases. The probe hybridized to a single fragment in each lane. This result indicates that pea nucleolin-like protein is derived from a single copy gene as in the mouse nucleolin gene (Bourbon et al., 1988). This result differs from the nucleolin homolog of cultured alfalfa cells, which is derived from a multiple gene family (Bögre et al., 1996).

Northern Analysis Reveals Light Regulation of mRNA Abundance for the Pea Nucleolin-Like Protein

In a northern assay of total RNA prepared from etiolated pea plumules, a single band of 2.3 kb was detected when hybridization was carried out using the entire sequence of NA481–5 cDNA as the probe (Fig. 6B). To test whether phytochrome was involved in the light-regulated expression of the pea nucleolin-like gene, dark-grown pea seedlings were irradiated with R and FR, as described above. As
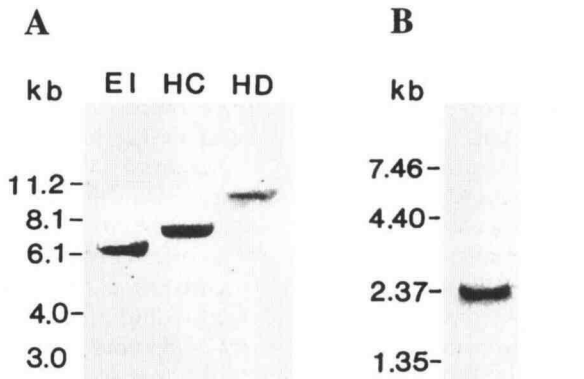
A

B

Figure 6. A, Genomic Southern analysis of the pea nucleolin-like protein. Genomic pea DNA (10 μg) was digested with three different restriction enzymes: *Eco*RI (EI), *Hinc*II (HC), and *Hind*III (HD), separated in a 0.7% agarose gel, and then blotted onto a nylon membrane. A 525-bp fragment generated by PCR, as described in "Materials and Methods," was used as a probe. Hybridization and washing were carried out at 65°C. B, Northern-blot analysis of the pea nucleolin-like protein. Total RNA (10 μg) was electrophoretically separated in a 1.2% agarose gel, blotted onto a nylon membrane, and then hybridized with NA481–5. Hybridization and washing were carried out at 65°C in aqueous conditions. The size markers (DNA ladder in A and RNA ladder in B) were purchased from Gibco.

shown by northern analysis, the abundance of a pea nucleolin-like mRNA was greatly decreased by R within 1 h after the light treatment, but began to increase after 5 h and reached at least 6× the dark control after 12 h (Fig. 7A). When a FR treatment was given after R irradiation, it reversed the R effect (Fig. 7B), which strongly suggests that the photoreceptor for this light response is phytochrome. Both the R induction of changes in the abundance of nucleolin mRNA and the FR reversal of the R effect have been observed three times, all qualitatively and quantitatively similar to the results shown in Figure 7.

DISCUSSION

The isolation of the cDNA for nucleolin using an antibody screen was the end result of an effort to evaluate an anomolous finding obtained while characterizing the pc481 antibodies. These antibodies were raised to a 47-kD nuclear protein band expected to be enriched in a light- and calmodulin-regulated NTPase, yet they recognize nucleolin, not NTPase. It is useful here to consider why pc481 does not recognize the 47-kD NTPase and why the 47-kD band contained a nucleolin peptide. Recent results indicate that light drastically reduces the level of the nuclear NTPase (C. Thomas and S.J. Roux, unpublished results). Thus, it is probable that the 47-kD band extracted from light-treated nuclei and used as the antigen was lacking NTPase. Furthermore, protease inhibitors must be used during nuclear isolation and extraction to prevent nucleolin breakdown (see below), and since the 47-kD protein band was extracted from nuclei in a buffer that did not contain protease inhibitors, it is probable that the extract containing this band had proteolytic breakdown products of nucleo-

lin, one of which is known to have an apparent molecular mass of 47 kD (Fig. 1A).

Despite its serendipitous origin, pc481 appears to be highly specific for nucleolin. Evidence for this specificity is 4-fold: In a library screen it selected only clones harboring the cDNA for nucleolin; in immunocytochemical assays it stained primarily nucleoli and primarily that region of nucleoli where animal nucleolin is concentrated; in immunoblots of protease-protected pea nuclear extracts it stained only one band near 90 kD, the expected molecular mass of higher plant nucleolin (Bögre et al., 1996); and in immunoblots of crude extracts of *E. coli* overexpressing the nucleolin cDNA, it stained one band near 90 kD.

The cDNA selected from a pea plumule library using pc481 clearly encodes a nucleolin-like protein (Fig. 5). As evaluated by a northern assay, the length of this cDNA is close to the full length of the mRNA it represents, although the mRNA may be 50 to 100 bp longer. The protein encoded by the cDNA has a high homology to the nucleolin homolog identified in alfalfa (Bögre et al., 1996). It is also similar to animal nucleolins in the database, showing over 50% identity in the N-terminal acidic/Ser-rich region and in the Gly/Arg-rich C-terminal regions. It is more similar to animal nucleolins than the most nucleolin-like protein yet detected in yeast, NSR1. Hamster nucleolin has an estimated molecular mass of 100 kD, based on its mobility on SDS-PAGE, but only 77 kD based on its derived amino acid sequence (Lapeyre et al., 1987), whereas in yeast the molecular masses are 67 kD from SDS-PAGE and 44 kD from the derived protein sequence (Lee et al., 1991), and in pea the SDS-PAGE molecular mass is 90 kD and the mass derived from the translated amino acid sequence is 65 kD. Thus, a large increase in SDS-PAGE molecular mass over cDNA-derived molecular mass would seem to be characteristic of all nucleolins. In peas this difference is unlikely to be due primarily to posttranslational modifications, as
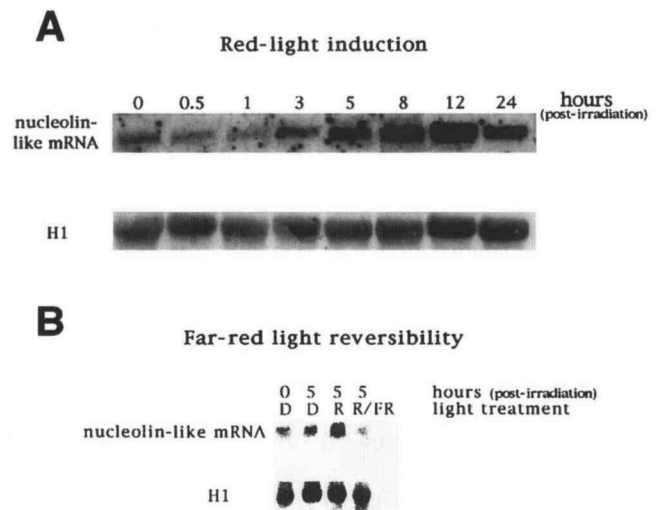
A

B

Figure 7. Northern-blot analyses of the time course of the effects of R on the abundance of the mRNA of pea nucleolin-like protein (A), and the reversibility of the effects of R by FR at 5 h after R irradiation (B). The quantity of RNA loaded was 10 μg in A and 20 μg in B. The H1 loading control is the gene for 28s rRNA.

speculated for alfalfa nucleolin by Bögre et al. (1996), since when the pea nucleolin-like protein is expressed in *E. coli*, it also migrates on SDS-PAGE with an anomalously high molecular mass of 88 kD. Nonetheless, the 2-kD size difference between the pea and *E. coli* versions of the proteins could reflect some posttranslational differences between them. Other proteins like nucleolin, with a high percentage of acidic and basic residues, have been observed to migrate with an apparent molecular mass larger than their amino acid sequence would predict (Meier and Blobel, 1992).

To obtain a single 90-kD stained band in immunoblots of nucleolar proteins, it was crucial to isolate the nucleoli under conditions that minimized proteolysis (use of protease inhibitors and rapid purification protocol). Allowing nucleoli to incubate at room temperature for 12 h generated many additional, lower-molecular-mass bands that stained positive with pc481, including a band at 47 kD (Fig. 1). Animal nucleolins yield a stable 48-kD proteolytic product, both from endogenous (nucleolar) and exogenous (trypsin) proteases (Sapp et al., 1989). The upper bands that show a positive immunostain in Figure 1 might represent protein aggregates.

The animal nucleolins are phosphoproteins, which are likely to be substrates for casein kinase II (Belenguer et al., 1989; Warrener and Petryshyn, 1991) and cdc2 kinase (Peter et al., 1990). It has been proposed that phosphorylation of nucleolin regulates the maturation of the protein into defined subfragments (Bourbon et al., 1983; Suzuki et al., 1985). This maturation process has been suggested to play a role in controlling the transcription of the genes for pre-rRNA (Bouche et al., 1984). The derived structure of the pea nucleolin-like protein contains many motifs that are characteristic of substrates for several kinases. In fact, it has many more potential casein kinase II phosphorylation sites (48) than are predicted for hamster nucleolin (5) (Caizergues-Ferrer et al., 1987). Recently, a pea nuclear casein kinase II has been isolated and characterized (Li and Roux, 1992), so it should be possible to test how many (if any) of the potential casein kinase II phosphorylation sites on the pea nucleolin-like protein are actually phosphorylated by casein kinase II.

A number of studies provide indirect evidence that animal nucleolins are multifunctional proteins. Besides its originally described roles of regulating rRNA processing and ribosome maturation in the nucleolus, the animal nucleolin has also been identified as a nuclear shuttling protein (Borer et al., 1989) that can move to the cell surface (Semenkovich et al., 1990). In primary neurons it can be found on the cell surface associated with laminin, a major extracellular matrix protein (Kleinman et al., 1991), and its migration to this site can serve as a signal to promote the proliferation of nerve cells (Kibbey et al., 1995).

Studies of a yeast nucleolin-like protein, NSR1, indicate that it may also be multifunctional. The yeast protein is induced by cold shock (Kondo et al., 1992) and is a NLS-binding protein (Lee et al., 1991). It is thought to be needed for the pre-rRNA processing steps involved in ribosomal assembly (Kondo and Inouye, 1992; Lee et al., 1992) and also for maintenance of steady-state levels of ribosomal

subunits (Lee et al., 1992). Because of its structural similarity to NSR1, hamster nucleolin was tested and also found to recognize the NLS (Xue et al., 1993). For both proteins, the recognition region was in the N-terminal acidic domain (Xue et al., 1993; Yan and Melese, 1993). This suggests a role for nucleolin in import of proteins into the nucleus, consistent with an earlier report that nucleolin was among the nucleolar proteins that shuttle between the nucleolus and cytoplasm (Borer et al., 1989). Alternatively, Xue and Mélese (1994) point out that many ribosomal proteins have NLS-like basic domains, and the binding of nucleolin to basic domains may simply reflect its key role in the assembly of ribosomal proteins into pre-ribosomes. When Xue et al. (1993) expressed the hamster nucleolin in a NSR1 mutant, they found that it failed to complement the mutant. They concluded that these two proteins are not interchangeable, even though they show overall sequence homology. These results suggest caution in proposing a functional similarity between the pea nucleolin-like protein and animal or yeast nucleolins.

The immunolocalization results indicating that pc481 stains primarily the region surrounding the fibrillar center of nucleoli (called the dense fibrillar component) in pea nuclei are similar to those reported by Martin et al. (1992) and Minguez and Morena Diaz de la Espina (1996), who probed onion cells with antibodies against animal nucleolins, and by Bögre et al. (1996), who examined alfalfa root tips with an antibody against a 12-amino-acid-long peptide corresponding to the predicted C terminus of the NucMs1 protein. Animal nucleolin is also localized in the dense fibrillar component region of nucleoli, a region where pre-rRNA transcripts are thought to transiently accumulate and primary processing events of pre-ribosomes occur.

The nucleolin homolog in cultured cells of alfalfa belongs to a multiple gene family (Bögre et al., 1996). In our genomic Southern blots a single band was detected using three different restriction digestions, with a 525-bp probe derived from the 5' end of the cDNA. This result indicates that the pea nucleolin-like protein is derived from a single gene.

The pattern of an R-induced initial decrease in the nucleolin mRNA level within the 1st h followed by an increase by 5 h is unusual. This pattern might result in part from a light-initiated endogenous rhythm that could produce a down-and-up variation, but the high mRNA level recorded at 24 h in contrast to the low level at 1 h makes this explanation seem unlikely. Because the R/FR treatments reversed the effects of R and yielded a nucleolin mRNA level near that of the dark control, the likely photoreceptor for this response is phytochrome, but an action spectrum would be needed to be confident of this conclusion.

In dark-grown mustard seedlings an increased accumulation and synthesis of rRNA in cotyledons was observed 6 h after a R irradiation (Thien and Schopfer, 1975, 1982). The R-induced rise of the mRNA level of nucleolin-like protein after 5 h could suggest that R regulates rRNA levels in part by controlling the biosynthesis of a nucleolar protein thought to play a critical role in rRNA transcription and/or processing.

Börge et al. (1996) correlated nucleolin mRNA levels in cells with their mitotic activity. In this context it is relevant to note that R photoreversibly up-regulates cell division rates in apical internodes of peas (Thompson, 1959) and induces an increase in nuclear number per unit weight of pea plumules (Baerson and Kaufman, 1990). Thus, the R-induced increase in nucleolin mRNA level in pea plumules reported here may be one aspect of the larger syndrome of an R-induced increase in the rate of cell division in this tissue.

ACKNOWLEDGMENTS

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