Calmodulin mRNA in Barley (*Hordeum vulgare* L.)[1]

APPARENT REGULATION BY CELL PROLIFERATION AND LIGHT

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ABSTRACT

Calmodulin is encoded by a 650-nucleotide mRNA in higher plants. This messenger was identified in barley and pea by a combination of *in vitro* translation and blot hybridization experiments using anti-sense RNA produced from an eel calmodulin cDNA probe. In all plant tissues tested, calmodulin mRNA represents between 0.01 and 0.1% of the total translatable mRNA population. Calmodulin mRNA levels are three- to fourfold higher in the meristematic zone of the first leaf of barley. At all other stages of leaf cell differentiation, calmodulin mRNA levels are nearly identical. During light-induced development in barley leaves, the relative proportion of translatable calmodulin mRNA declines about twofold. Cytoplasmic mRNAs that may encode calmodulin-like proteins were also detected. The levels of several of these putative Ca^{2+}-binding protein mRNAs are modulated during the course of light-induced barley leaf cell development.

Calcium ions act as second messengers in eukaryotic cells by virtue of changes in their concentration in the cytoplasm. A primary intracellular target for Ca^{2+} acting as second messengers is a group of proteins known as Ca-modulated proteins (reviewed in Van Eldik *et al.* [20]). This family of molecules binds Ca^{2+} reversibly, with dissociation constants in the micromolar to nanomolar range. Members of this family include calmodulin, troponin C, parvalbumin, S-100, and two as yet unidentified polypeptides (4, 18). All of these proteins share extensive amino acid sequence homology, at least part of which reflects their common ability to selectively bind Ca^{2+} over other divalent cations.

The most widely distributed of the Ca-modulated proteins is calmodulin, which has been found in every eukaryote examined (for reviews, see Refs. 3, 9, and 20). Calmodulin is an acidic, 148-residue polypeptide that was discovered as a heat-stable activator of cyclic nucleotide phosphodiesterase in animals. It was subsequently shown to be a regulator of NAD kinase in plants. Calmodulins isolated from a variety of organisms share a remarkable degree of amino acid sequence homology (3). Recent studies on the spinach leaf protein (14) show that, with the exception of the amino terminal tripeptide whose sequence is not unequivocally established, calmodulins isolated from a higher plant and bovine brain differ in only 13 residues. Most, but not all, of the amino acid substitutions are conservative in nature.

Given the high degree of amino acid sequence homology

among calmodulins isolated from different sources, and the availability of a cloned cDNA probe encoding a vertebrate calmodulin (13), experiments were performed to identify calmodulin mRNAs in plant cells using a heterologous DNA probe. Several recent reports have documented changes in the level of calmodulin protein in developing plant systems (1, 11, 15); calmodulin protein levels are typically elevated in plant meristematic zones. This study addresses the question of whether similar increases in calmodulin mRNA can be detected in a plant meristematic zone.

Although Ca^{2+} is clearly involved in mediating a number of developmental processes in plants (reviewed in Refs. 9 and 12), calmodulin is the only well-characterized Ca-modulated plant protein. Proteins possessing chemical properties similar to calmodulin, however, have been reported as minor contaminants in calmodulin preparations isolated from plant sources (*e.g.* Schleicher *et al.* [17]). The possibility was explored that a heterologous eel calmodulin DNA probe might be used to detect mRNAs encoding other Ca-modulated proteins in plants. A portion of this work has appeared previously in abstract form (28).

MATERIALS AND METHODS

Plant material. Barley (*Hordeum vulgare* L., var. Glen) was grown in a greenhouse and harvested at 5 to 7 d after planting. Dark-grown plants were raised in an incubator held at 16 to 18°C. Greening was induced by exposing the plants to fluorescent lighting (10–25 $\mu E\ m^{-2}\ s^{-1}$).

Isolation and Fractionation of RNA. Total cellular RNA was prepared by grinding plant material to a powder under liquid N_2 in a mortar and extracting nucleic acids in buffer containing 50 mM Tris-HCl (pH 9), 10 mM Na_2EDTA, 200 mM NaCl, 0.1% (w/v) SDS, 100 $\mu g/ml$ proteinase K for 30 min at 37°C. The extract was centrifuged for 15 min at 10,000g, and the remaining protein removed by extraction with phenol:chloroform:isoamyl alcohol (50:50:1, by vol), followed by chloroform:isoamyl alcohol (50:1, by vol). After precipitations with isopropanol, ethanol, and LiCl, fractions of poly(A^+)-enriched RNA were prepared by affinity chromatography on Poly(U)-agarose (type 6, PL-Pharmacia, Milwaukee, WI). After two or three precipitations with ethanol, the RNAs were dissolved in sterile water and stored at −70°C. In some experiments, poly(A^+) RNA was further fractionated by sedimentation in gradients of 10 to 30% (w/w) sucrose containing 10 mM CH_3HgOH, 10 mM Tris/HCl (pH 7.5), 0.1 mM Na_2EDTA. Centrifugation was performed at 30,000 rpm for 16 h at 4°C in a Sorvall TH 641 rotor.

Electrophoresis of RNA in Formaldehyde Gels. Electrophoresis of poly(A^+) RNA was performed in formaldehyde gels composed of 2% (w/v) agarose, 40 mM triethanolamine/H_3PO_4 (pH 7.5), 2 mM Na_2EDTA, and 3% formaldehyde. Fractionated RNA was transferred to nitrocellulose, and hybridized with ^{32}P-labeled anti-sense eel calmodulin mRNA (described below). Hy-

bridizations were carried out in 50% (v/v) formamide, 5× SSPE (1× SSPE is 0.135 M NaCl, 0.01 M NaH₂PO₄/Na₂HPO₄ [pH 7.4], 1 mM Na₂EDTA), 1× Denhardt's solution, 0.1% (w/v) SDS, and ^{32}P-labeled probe at 1 to 2 × 10⁶ cpm (Cerenkov)/ml for 24 h with constant shaking. Hybridization temperatures are indicated in the figure legends. Prehybridization was for 16 h in a similar solution without labeled probe, except that the Denhardt's solution was increased to 5× and 250 μg/ml denatured calf thymus DNA was included in the mixture. Post-hybridization washes in all experiments were performed in 2× SSPE, 0.1% (w/v) SDS for 30 min at room temperature, followed by 0.1× SSPE, 0.1% (w/v) SDS for at least 2 h at 60°C.

Calmodulin cDNA probe. The eel calmodulin cDNA probe subclone, pCaM-1 (27), that represents the coding region corresponding to amino acids 93 through 134 was transferred into the phage T7 promoter-containing plasmid pT7.1 (US Biochemicals, Cleveland, OH). This recombinant molecule was termed pT7.1-CaM, and it contains calmodulin coding sequences in an inverted orientation with respect to the T7 promoter. The plasmid was linearized with HindIII, and used to synthesize anti-sense calmodulin mRNA transcripts in vitro (diagrammed in Fig. 1). The reaction mixture contained 40 mM Tris-HCl (pH 8), 20 mM MgCl₂, 10 mM DTT, 600 units/ml RNasin (Promega Biotec, Madison, WI), 100 μg/ml BSA, 20 μg/ml linearized DNA template, 12 μM UTP (400 Ci/mmol, SP6-grade, Amersham Corp., Arlington Heights, IL), and 500 units/ml T7 RNA polymerase; incubation was for 90 min at 37°C. Template DNA was removed by digestion with RNase-free DNase (25 μg/mL) for 10 min at 37°C. The in vitro-synthesized RNA was recovered by ethanol precipitation, and it typically had a specific activity of about 5 × 10⁸ Cerenkov cpm/μg.

In Vitro Translation of Poly(A⁺) RNA. Extracts of wheat germ (obtained from Arrowhead Mills, Hereford, TX) and in vitro translation reaction mixtures were prepared as described by Erickson and Blobel (10). Labeling was with [^{35}S]methionine (Amersham, 1000–1300 Ci/mmol). To assay incorporation of [^{35}S]methionine into calmodulin, translation products were processed as described (19, 27), with the exception that no unlabeled calmodulin carrier was added. Briefly, translation mixtures were adjusted to 10 mM EDTA, 50% (v/v) ethanol, and incubated 1 h at −20°C. The precipitated proteins were removed at 12,000g for 15 min, and the supernatant adjusted to 80% (v/v) ethanol. After 1 to 4 h at −20°C, the 50 to 80% ethanol-precipitated proteins were collected by centrifugation as described above, dried, suspended in 50 mM Tris-HCl (pH 8), 1 mM EDTA, 10 mM 2-mercaptoethanol, and subjected to non-denaturing PAGE in a gel system in which calmodulin migrates with a unique mobility (23, 27). The labeled polypeptides were detected by fluorography.

RESULTS

Detection of Putative Calmodulin mRNAs by Blot Hybridization. The amino acid sequence of calmodulin is highly conserved in a wide variety of eukaryotes. At the nucleotide sequence level, however, it is known that the coding regions of calmodulin mRNAs from different vertebrates can be diverged by 25% (16), even though the amino acid sequences they encode are nearly identical (99⁺% homology). In a first series of experiments, poly(A⁺) RNAs isolated from barley leaves were fractionated by gel electrophoresis, transferred to nitrocellulose filters, and hybridized with a nick-translated eel calmodulin cDNA probe. The Northern blot profiles observed using this method, however, were subject to tremendous variation, both with respect to the number of hybridizing RNA species and their relative hybridization intensities. To overcome this reproducibility problem, blot-transferred mRNAs were probed in a second set of experiments using an eel anti-sense calmodulin mRNA probe at increasingly stringent criteria. This probe encodes the region of eel calmodulin mRNA corresponding to amino acids 93 through 134. There are three amino acid sequence changes in this 42-residue region between spinach and vertebrate calmodulin (14). The nucleotide sequence homology between eel and chicken calmodulin mRNA in this region is about 77% (16). Thus, hybridization conditions were chosen assuming a minimum nucleotide divergence of 25% between plant calmodulin mRNAs and the eel anti-sense probe. Figure 2 depicts three such hybridization experiments. Four size classes of mRNA molecules were found to hybridize with the calmodulin probe under the least stringent conditions. In separate experiments, the sizes of these molecules were estimated using a series of calibrated RNA markers, which were produced by in vitro transcription (data not shown). The hybridizing RNAs range in size from about 650 to 4000 nucleotides (calmodulin would require a minimum of 444
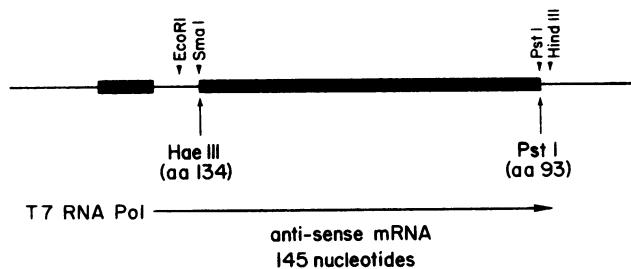

FIG. 1. Physical and transcriptional map of pT7.1-CaM. The region encoding amino acids 93 through 134 of an eel calmodulin cDNA probe was transferred from pCaM-1 (27), to the phage T7 promoter-containing plasmid pT7.1 with the usual mRNA 5'-3' orientation reversed with respect to the T7 promoter. This DNA was cleaved with HindIII and used as a template for transcription by phage T7 RNA polymerase to produce a 145-nucleotide anti-sense RNA probe.
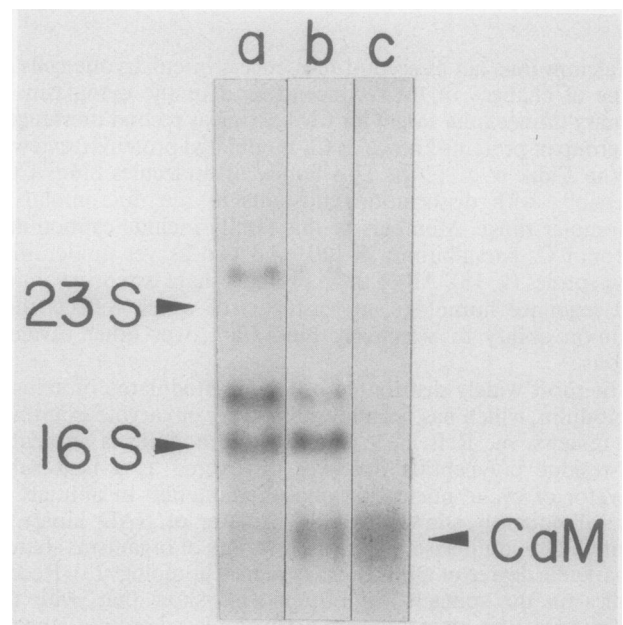

FIG. 2. Detection of putative calmodulin mRNA by blot hybridization. Poly(A⁺) mRNA (approximately 1 μg) from barley leaves was fractionated by gel electrophoresis in a 2% agarose gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled anti-sense eel calmodulin mRNA. Hybridization was performed with ^{32}P-labeled probe for 24 h at (a) 42°C, (b) 47°C, or (c) 52°C. Post-hybridization washes were done under identical conditions (final washes in 0.1 × SSPE, 0.1% SDS at 60°C). The position of the strongest hybrid (putative calmodulin mRNA) is indicated in the figure (CaM)

nucleotides of coding region). The 650-nucleotide molecule, however, shows the highest thermal stability, as can be seen in lane C of Figure 2. In a parallel experiment, similar results were observed using gel-fractionated pea poly(A^+) RNAs (data not shown).

In order to establish whether all the RNA species detected in Figure 2 that share homology with the eel calmodulin probe represent authentic calmodulin messengers, poly(A^+) RNAs from both barley and pea were fractionated by sedimentation in CH_3HgOH-containing sucrose density gradients. After ethanol-precipitation, an aliquot of each gradient fraction was separated by gel electrophoresis, transferred to nitrocellulose, and hybridized with the eel calmodulin probe. The results of such a fractionation of barley mRNAs are shown in Figure 3A. Gradient fractions were obtained that were highly enriched for the 650-, the 1600-, and 2100-, and the 4000-nucleotide hybridizing RNA species. A second aliquot from each gradient fraction was then translated in vitro and the translation products fractionated by ethanol precipitation. The newly synthesized polypeptides were then analyzed for the presence of calmodulin by PAGE in a non-

FIG. 3. Identification of calmodulin mRNA by sucrose density gradient fractionation and in vitro translation. Poly(A^+) mRNA (approximately 200 μg) from barley leaves was fractionated by sedimentation in 10 to 30% (w/w) sucrose density gradients containing 10 mM CH_3HgOH. The fractionated RNA was collected, ethanol-precipitated, and aliquots of each fraction analyzed by (A) Northern blot hybridization (as described in "Materials and Methods" and the legend to Fig. 2), or (B) in vitro translation, ethanol fractionation, non-denaturing PAGE and fluorography. The mobility of authentic calmodulin polypeptide (CaM), detected by staining, is indicated in the figure.

denaturing gel in which calmodulin migrates with a unique mobility (23, 27). The identity of the putative calmodulin zone and the unique mobility of barley calmodulin synthesized by in vitro translation in this gel electrophoretic system were verified by the following criteria: homogeneity of the protein zone after elution from the gel and fractionation by SDS-PAGE in the presence of 0.1 mM $CaCl_2$ or EDTA; immunoreactivity of protein eluted from the gel with anti-calmodulin antiserum; and comigration in HPLC analysis of limited proteolytic digestion products of the in vitro-synthesized protein with those obtained from authentic barley calmodulin (data not shown). As shown in Figure 3B, only the gradient fractions containing the 650-nucleotide hybridizing mRNA are capable of directing the synthesis of calmodulin.

The 1600- and 2100-nucleotide RNAs detected in the lower stringency hybridizations probably represent spurious cross hybridization of the eel anti-sense probe with rRNAs. In support of this interpretation, it should be noted that the poly(A^+)-enriched mRNA fractions employed in these experiments contain significant amounts of 16S and 18S rRNA. In addition, probes made by reverse transcribing barley 18S rRNA hybridize with a 2100-nucleotide RNA species in the poly(A^+) RNA preparation used in these experiments. The identity of the 4000-nucleotide species, on the other hand, is not yet known. It does not appear to comigrate with stainable rRNAs, but attempts to rescue the putative mRNA and identify its polypeptide product by in vitro translation have not been successful.

Quantitation of Calmodulin mRNA Levels. To quantitate the level of calmodulin mRNA in barley leaf poly(A^+) RNA fractions, the RNA was translated in vitro and, after estimating the total incorporated radioactivity, the translation mix was fractionated with ethanol. The 50 to 80% ethanol fraction was then subjected to electrophoresis by non-denaturing PAGE as described above. Newly translated calmodulin was located, after Coomassie blue staining, by observing the migration of unlabeled calmodulin derived from the wheat germ extract. The calmodulin protein zone was excised and counted in a liquid scintillation counter. This method has been used previously (27) to estimate the calmodulin mRNA levels in chick embryo fibroblast mRNA fractions. Control experiments, described in the previous section, indicated this approach also to be a valid one for estimating the level of translatable calmodulin mRNA in barley, if not for higher plants in general. In barley leaf poly(A^+) RNA preparations, the level of calmodulin mRNA accounts for between 0.01 and 0.06% of the total translatable mRNA, depending upon the age of the tissue. In general, the younger the tissue, the higher the apparent level of calmodulin mRNA (data not shown). Similar results have also been observed for poly(A^+) RNA fractions isolated from a variety of higher plants, including pea, maize, spinach and Arabidopsis thaliana, and the green alga, Chlamydomonas reinhardtii. In every case tested, calmodulin mRNA represented 0.01 to 0.1 % of the translatable mRNA.

Poly(A^+) RNA fractions from the above-mentioned plants were assayed by blot hybridization for the presence of calmodulin mRNA. As is shown in Figure 4, however, a more complex picture is seen in all species tested compared with barley and pea. Multiple mRNAs are observed that cross-hybridize with the heterologous calmodulin probe. Just as in the well-characterized barley mRNA fraction, the two hybridizing RNA species migrating at about 16S and 18S probably represent spurious hybridization with rRNA. Because of the technical difficulty in separating the lower mol wt (<16S) hybridizing RNA species, however, it is not yet clear whether each of these molecules represents an authentic calmodulin mRNA.

Modulation of Calmodulin mRNA Levels during Leaf Cell Differentiation. The leaves of monocotyledonous plants contain, from the base to the tip, a continuous transition from meriste-
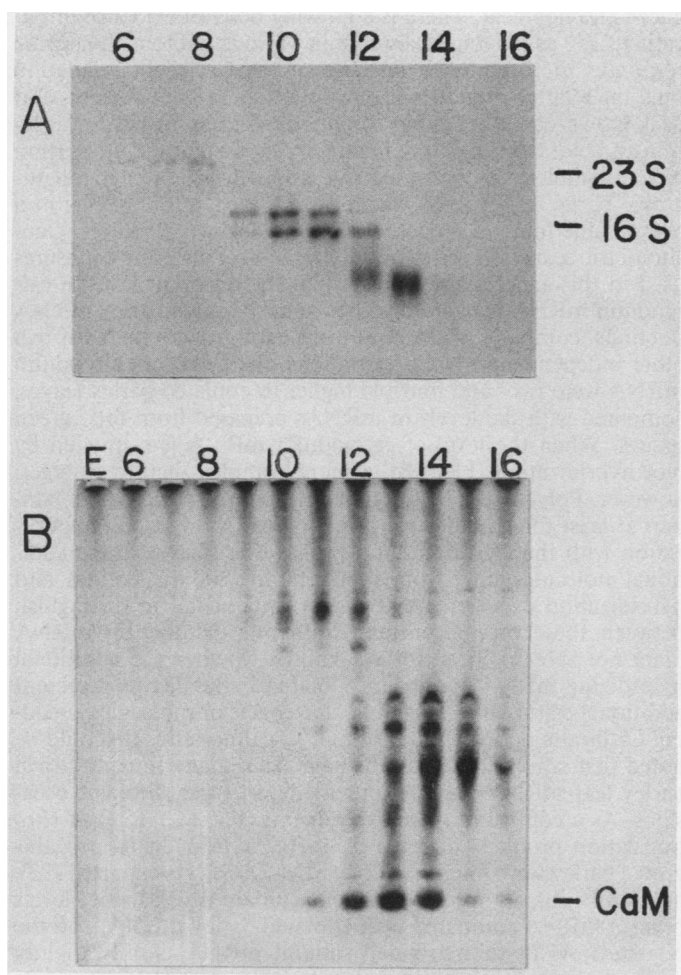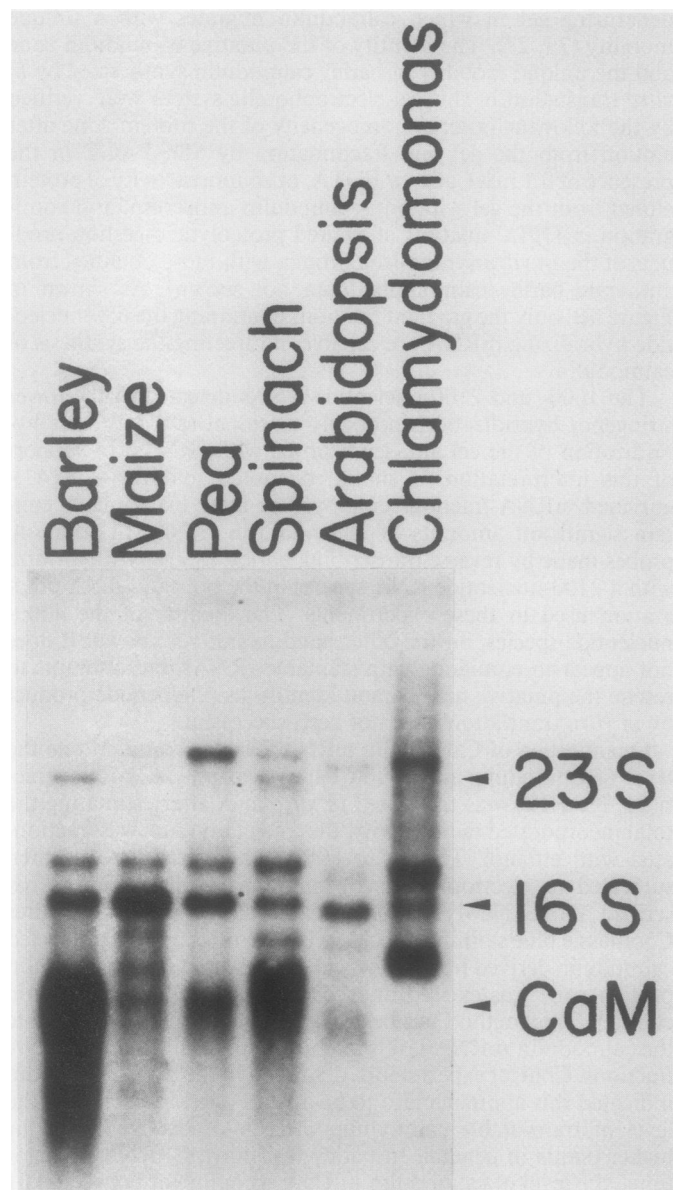
FIG. 4. Northern blot detection of calmodulin-like mRNAs in higher plants and a green alga. Poly(A$^+$) RNAs (approximately 1 μg) were fractionated in formaldehyde-agarose gels, transferred to nitrocellulose, and probed with ^{32}P-labeled eel anti-sense calmodulin mRNA. Hybridization was performed at 50°C for 24 h. The position of authentic calmodulin mRNA (CaM), as demonstrated for barley mRNA is indicated in the figure.

matic to terminally differentiated cells. This developmental gradient was utilized to examine the levels of calmodulin mRNA during the course of leaf cell differentiation. Leaves of 7-d-old barley seedlings, grown under natural light conditions, were harvested by cutting at the base. The coleoptiles and any second leaf material were removed, and the leaves dissected into six segments of equal length. Poly(A$^+$) RNA fractions were prepared from each leaf segment (derived from about 500 plants), and analyzed by blot hybridization and translation in vitro for their relative calmodulin mRNA contents. The results of one such experiment are summarized in Figure 5A. In this, and in two other separate experiments, the basal leaf segment (which contains the meristematic region of the leaf) was found to contain, proportionally, about twice as much calmodulin mRNA, as

detected by hybridization or by in vitro translation, as do the remaining segments. As a control in these experiments, samples of gel-fractionated poly(A$^+$) RNA from each leaf segment were tested for their content of mRNA for the light-harvesting Chl a/b-binding protein precursor (pa/b) by hybridization with the cloned pAB96 cDNA sequence from pea (2). As was originally shown by Viro and Kloppstech (21), maximal pa/b mRNA accumulation occurs in the second leaf segment; and the mRNA level declines dramatically in barley leaf segments containing progressively older cell populations.

To further enrich for dividing leaf cells, the basal 0.5 cm portion was dissected from leaves of 7-d-old barley seedlings, and its relative calmodulin mRNA content was compared with that of the remainder of the first leaf. Figure 5B shows that the basal section of the leaf clearly contains a higher proportion of calmodulin mRNA than does the remainder of the leaf (about 3.5-fold, in this experiment as measured by densitometry of the autoradiograph shown in the figure). Similar results were observed (3.2-fold higher levels in basal cell mRNA fractions) when this assay was performed by in vitro translation (data not shown).

Modulation of Calmodulin mRNA Levels during Light-Induced Development. There is a growing body of evidence implicating Ca^{2+} as playing a key role in various photomorphogenic responses in plants (12). Because of the Ca^{2+}-light regulation link, the relative proportions of calmodulin mRNA in light- and dark-grown barley seedlings were compared by in vitro translation and blot hybridization. Figure 6A shows that the proportion of translatable calmodulin mRNA is somewhat lower in a light-grown barley leaf mRNA fraction compared with the level in a comparable fraction prepared from dark-grown seedlings. Densitometric scans of a series of fluorographs of differing exposures used in this experiment indicate that the apparent level of calmodulin mRNA is twofold higher in dark-grown barley mRNA fractions, compared with those from light-grown plants. In two other independent RNA preparations, the levels of calmodulin mRNA were two- and fourfold higher in etiolated barley leaves, compared with the levels in mRNAs prepared from fully green tissues. When the level of calmodulin mRNA is estimated by blot hybridization (Fig. 6B), a more complex picture emerges, however. Poly(A$^+$) RNA fractions from dark-grown barley contain at least three additional mRNAs that show strong hybridization with the eel calmodulin probe. The sizes of these additional molecules range from about 500 to 1200 nucleotides; and hybridization experiments at higher criteria fail to distinguish between these messengers and authentic calmodulin mRNA (data not shown). It is not yet known whether the additional hybridizing mRNA species in etiolated barley leaves represent additional calmodulin-encoding messengers or molecules encoding Ca-binding polypeptides similar to calmodulin. It should be noted that similar observations have been made in dark-grown barley leaf mRNA preparations made on three different occasions. As a control in these experiments (Fig. 6C), total in vitro translation products directed by mRNAs from light- or dark-grown barley were fractionated by SDS-PAGE. As expected (22), light-grown barley mRNA fractions contain much higher levels of pa/b mRNA compared with etiolated barley mRNA; and the relative level of rubisco small subunit mRNA is only slightly higher in light-grown barley mRNA. RNAs isolated from green and etiolated tissues for these assays had equal translational efficiencies.

DISCUSSION

It has been shown that calmodulin polypeptides are encoded by a mRNA population having a mean size of 650 nucleotides in barley. This conclusion is based upon two lines of evidence: higher thermal stability of the 650-nucleotide mRNA/eel calmodulin probe hybrids detected in northern blot experiments
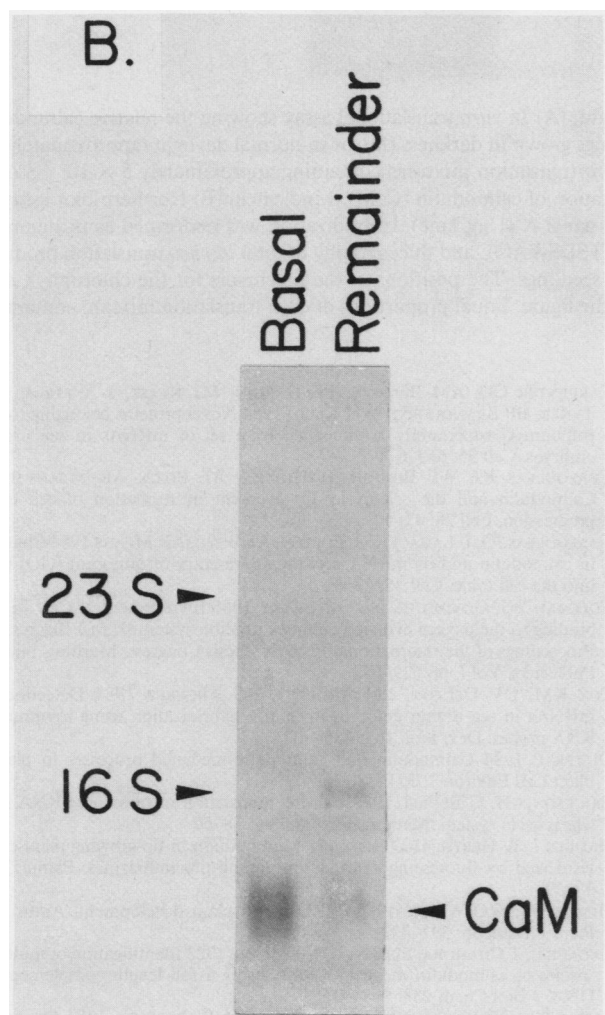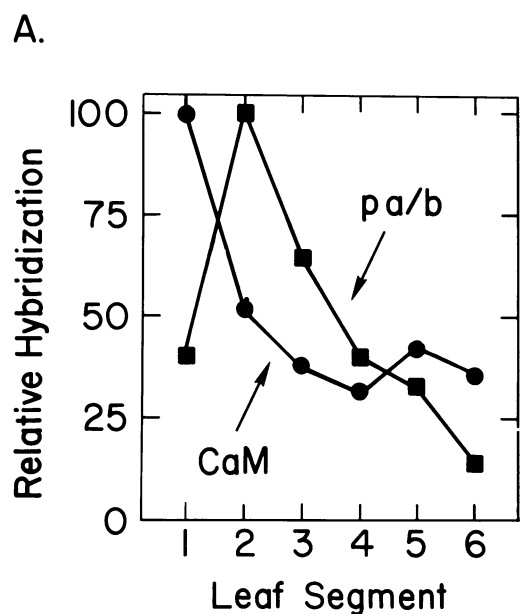
(Fig. 2); and, more directly, that synthesis of calmodulin polypeptide is directed *in vitro* only by sucrose gradient fractions enriched in the 650-nucleotide mRNA (Fig. 3). This mRNA population may be composed of at least two similar-sized mRNA species in barley, as judged by northern blot hybridizations of de-adenylated RNA fractions or of poly(A$^+$) RNAs separated in high resolution agarose gels (data not shown). Multiple mRNAs encoding calmodulin polypeptides have been described previously in mRNA fractions isolated from the electroplax of electric eel (13).

The molecular probe used in the experiments in this report consisted of ^{32}P-labeled anti-sense eel calmodulin mRNA produced by *in vitro* transcription of cloned cDNA by T7 RNA polymerase. It was found that this method of probe labeling gave more consistent and reproducible blot hybridization results than did nick translation. This observation is likely to be a consequence of the higher affinity of RNA-RNA compared with DNA-RNA hybrids, and the fact that the length of the RNA probe is very near the size estimated to be optimal for hybridization rate and specificity (8).

Ca has been implicated as an important regulatory component for cell proliferation (24). Calmodulin involvement in mediating this Ca-regulation is inferred from the observations that the intracellular concentration of the receptor is acutely regulated at the G1/S transition of the cell cycle (5). The change in calmodulin protein level is preceded by an increase in the calmodulin mRNA level (6). In addition, calmodulin has been localized in the mitotic spindles of animal (26) and plant (25) cells. Actively growing or dividing plant cells contain higher concentrations of calmodulin than do nongrowing cells (1, 11, 15). The data presented in this study (Fig. 5) demonstrate that calmodulin mRNA levels are elevated in barley leaf meristematic zones, and support the hypothesis that the content of calmodulin is regulated at the level of mRNA production in dividing plant cells.

The steady-state proportion of calmodulin mRNA in nondividing barley leaf cells appears to be affected to a small degree by light. RNA preparations made from dark-grown barley leaves contain about twice as much translatable calmodulin mRNA as do corresponding preparations made from plants grown under normal illumination. This change, albeit a small one, is quite reproducible, and has been observed in three independent sets of experiments. Little, if any, difference was observed in the yields of RNA from etiolated *versus* green barley leaves in these experiments. A portion of the decrease in the relative calmodulin mRNA level observed, however, may be accountable to a dilution effect resulting from light-induced RNA synthesis. Nevertheless, the multiple *in vitro* regulatory activities of calmodulin (9), and its role as a primary intracellular Ca^{2+} receptor (3, 20), suggest that even small alterations in the intracellular level of calmodulin protein or mRNA could profoundly affect a number of cellular activities.

In animal cells, calmodulin is a member of a family of structurally related polypeptides, the Ca-modulated proteins (20). Are there Ca-binding polypeptides in barley, or other higher plants, that are structurally related to calmodulin? The blot hybridization data shown in Figure 6B suggests that such proteins exist, particularly in etiolated tissues. mRNAs were consistently observed that show weak to relatively strong hybridization with the heterologous calmodulin probe. These RNAs cannot be distin-

FIG. 5. Relative calmodulin mRNA levels are elevated in barley leaf meristematic zones. First leaves of 7-d-old barley seedlings were dissected into 6 segments of equal length (1 = basal, 6 = tip). Poly(A$^+$) mRNA (1 μg) was assayed by northern blot hybridization or *in vitro* translation for calmodulin mRNA. Northern blots were hybridized at 50°C for 24 h with 10^6 cpm/ml of anti-sense RNA probe. (A) Summary of densito-metric quantitation of calmodulin mRNA (CaM) detected by northern blot hybridization in leaf gradient mRNA fractions compared with the Chl *a/b* binding protein mRNA (*pa/b*). (B) Northern blot analysis for calmodulin mRNA in poly(A$^+$) mRNA fractions (1 μg/lane) prepared from the basal 0.5-cm or the remainder of approximately 500 7-d-old barley leaves. The position of calmodulin mRNA (CaM) is indicated in the figure.
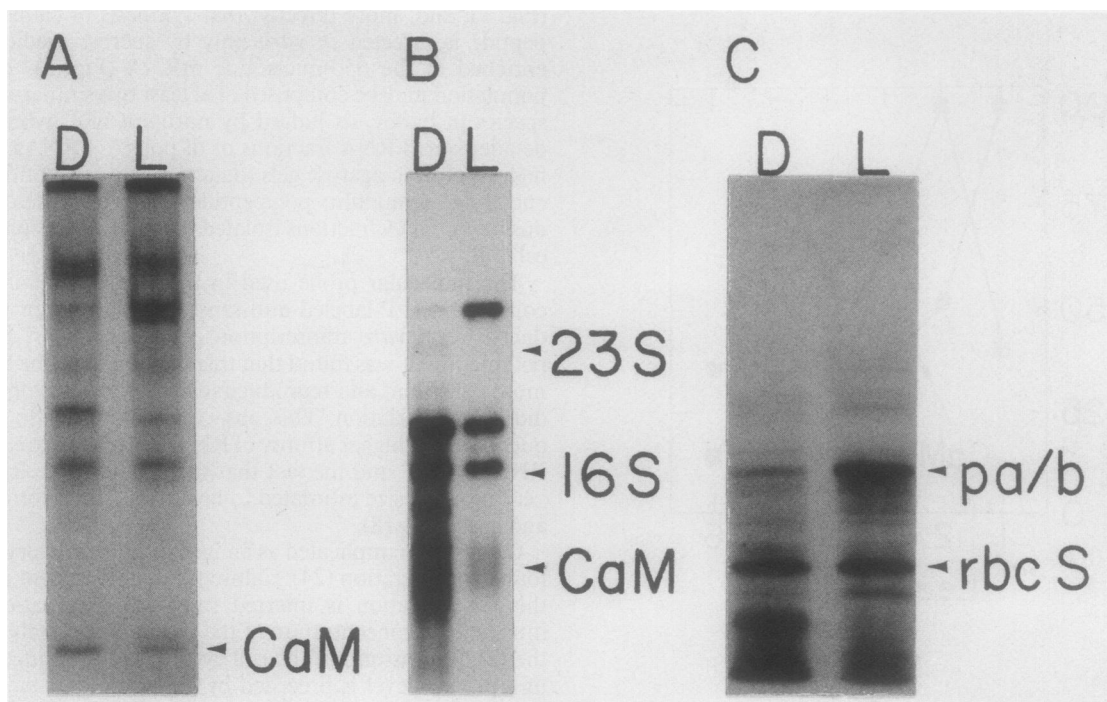
FIG. 6. Apparent down regulation of relative calmodulin mRNA levels by light. (A) *In vitro* translational assay showing the relative calmodulin mRNA content in poly A^+ mRNA fractions prepared from 5-d-old barley seedlings grown in darkness (D), or in normal daylight (approximately 12 h light, 12 h dark) conditions (L). The 50 to 80% ethanol fractions from *in vitro* translation mixtures containing approximately 5×10^6 ^{35}S cpm were applied to the non-denaturing gel, and detected by fluorography. The migration of calmodulin (CaM) is indicated. (B) Northern blot assay of calmodulin mRNA content in the same poly(A^+) mRNA fractions analyzed in panel A (1 µg/lane). Hybridization was performed as in Figure 5, but at 42°C. The position of authentic calmodulin mRNA (CaM) is indicated. (C) SDS-PAGE and fluorography of total *in vitro* translation products directed by poly A^+ mRNAs (0.5 µg) from dark- or light-grown 5-d-old barley seedlings. The positions of the precursors for the chlorophyll *a/b*-binding protein (*pa/b*) and the small subunit of rubisco (*rbc* S) are indicated in the figure. Equal proportions of each translation mixture containing approximately 10^6 cpm were loaded onto the gel.

guished from authentic calmodulin mRNA by thermal discrimination; they do not comigrate with rRNAs, nor do they translate polypeptides that comigrate with authentic calmodulin in non-denaturing PAGE. In animal cells, there is ample precedent for the existence of mRNAs encoding polypeptides related, but not identical, to known members of the Ca-modulated protein family. These include mRNAs that are either restricted in tissue distribution (18) or modulated in one particular tissue during development (4). The identities of putative calmodulin-like polypeptides potentially encoded by cross-hybridizing mRNAs in barley are unknown, but two likely candidates include the 33 kD polypeptide of PSII (7), and a polypeptide that cross-reacts with antibodies to the 35 kD vertebrate calcimedin (S Roux, personal communication). Future experiments using homologous plant calmodulin cDNA probes will be particularly directed toward resolving the question of whether Ca-binding protein mRNAs related to calmodulin mRNA exist in plant cells.

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