Cloning of cDNA Sequences Encoding the Calcium-Binding Protein, Calmodulin, from Barley (Hordeum vulgare L.)

Vincent Ling and Raymond E. Zielinski

Department of Plant Biology, University of Illinois, Urbana, Illinois 61801

ABSTRACT

Full- and partial-length cDNAs encoding calmodulin mRNA have been cloned and sequenced from barley (Hordeum vulgare L.). Barley leaf mRNA, size-fractionated in sucrose density gradients, was used to synthesize double-stranded cDNA. The cDNA was cloned in λgt10 and screened with a synthetic, 14-nucleotide oligonucleotide probe, which was designed using the predicted coding sequences of the carboxy termini of spinach and wheat calmodulin proteins. The primary structure of barley calmodulin, predicted from DNA sequencing experiments, consists of 148 amino acids and differs from that of wheat calmodulin in only three positions. In two of the three positions, the amino acid changes are conservative, while the third change consists of an apparent deletion/insertion. The overall nucleotide sequence similarity between the amino acid coding regions of barley and vertebrate calmodulin mRNAs is approximately 77%. However, a region encoding 11 amino acids of the second Ca²⁺-binding domain is very highly conserved at the nucleotide level compared with the rest of the coding sequences (94% sequence identity between barley and chicken calmodulin mRNAs). Genomic Southern blots reveal that barley calmodulin is encoded by a single copy gene. This gene is expressed as a single size class of mRNA in all tissues of 7-day-old barley seedlings. In addition, these analyses indicate that a barley calmodulin cDNA coding region subclone is suitable as a probe for isolating calmodulin genes from other plants.

All CaM proteins isolated and characterized thus far possess four Ca²⁺-binding domains, remarkably similar amino acid sequences, and most consist of approximately 148 amino acid residues (7, 22). Increases in intracellular free Ca²⁺ cause the Ca²⁺-binding sites of CaM to become occupied, which alters the conformation of the protein and facilitates its interaction with other cellular enzymes. The association of CaM with its target enzymes can profoundly alter cellular metabolism. Through this system, Ca²⁺ acts as a second messenger for stimulus response coupling in all eukaryotes. Although a number of cellular processes in plants are known to be regulated by the Ca²⁺-CaM complex, few of the specific target enzymes of this second message complex have been identified (5, 15, 17). To dissect the responses of plants to increases in intracellular Ca²⁺, we have cloned cDNA sequences encoding plant CaM.

Genomic DNA and cDNA sequences encoding cam have been isolated and cloned from vertebrates (1, 9, 14, 16), invertebrates (4), fungi (2), and protozoa (21). None of the sequences has been cloned from any plant source, however. We report in this paper the cloning of barley cam cDNAs using a plant-specific synthetic cam oligonucleotide as probe. We demonstrate that cam mRNAs are expressed in all tissues of vegetatively growing barley seedlings, and that barley cam cDNAs are useful probes for cloning cam from other plants. A preliminary report of this work has been presented previously in abstract form (10).

MATERIALS AND METHODS

Size Fractionation and cDNA Cloning of Barley Leaf mRNAs

Fractions of barley (Hordeum vulgare L.) leaf poly(A⁺) RNA enriched in cam mRNA sequences were isolated by CH₃HgOH-sucrose density gradient centrifugation (25). These mRNAs were used to prepare cDNA (24), which was cloned in λgt10 (6).

Preparation of Synthetic cam Oligonucleotides

Synthetic, cam-specific oligonucleotides (composition described below) were synthesized by the phosphoramidite method at the University of Illinois Biotechnology Center. The oligonucleotide mixture was purified on a Varian Model 5000 HPLC fitted with C4 reverse phase column (5-μm particle size, 330A pore size, Vyead). Following detritylation in 80% acetic acid, the oligonucleotide probe was concentrated by ethanol precipitation, resuspended in sterile water, and stored at -20°C.

Calmodulin (CaM²) is a small, acidic protein that binds Ca²⁺ with high affinity (Kd = 1 μM), which has been identified in all eukaryotic species examined (7). It is a pleiotropic modulator of physiological function in animal cells (22), and a growing body of evidence suggests that CaM plays a similar role in plants (5, 15, 17). The high degree of amino acid sequence homology between CaM proteins isolated from organisms of widely divergent phylogeny lends credence to the idea that CaM is an essential protein for normal growth and development performing similar functions in all eukaryotes. More recently, mutational analyses of the cam locus in yeast demonstrated that this protein plays an indispensable function in cell growth. In these experiments, progeny from mutants heterozygous at the cam locus were nonviable when they contained a cam null allele (2).

1 Supported by grants from U.S. Department of Agriculture Competitive Research Grants Office (86-CRCR-11932) and the Graduate Research Board of the University of Illinois (to R. E. Z.)
2 Abbreviations: CaM, calmodulin protein; cam, calmodulin gene(s); bp, base pair; kb, kilobase.

Received for publication November 28, 1988
and in revised form February 7, 1989

Downloaded on December 29, 2020. Published by https://plantphysiol.org
Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.
Plaque Hybridization

The cam oligonucleotides were end-labeled with $[^\gamma-32]$P ATP using T4 polynucleotide kinase (12). Plaque lifts onto nitrocellulose discs were performed according to the Schleicher & Schuell (Keene, NH) product manual. Prehybridizations and hybridizations were performed using 5× SSPE (1× SSPE is: 0.135 M NaCl, 10 mM NaH$_2$PO$_4$/NaOH pH 7.4, 1 mM Na$_2$EDTA), 1× Denhardt’s solution, 0.1% (w/v) SDS, and 100 μg/mL denatured calf thymus DNA. Hybridizations were carried out at 37°C overnight with 10$^4$ cpm/mL of solution (approximately 10 ng oligonucleotide/mL), followed by two washes with 2× SSPE, 0.2% (w/v) SDS, and two washes with 1× SSPE, 0.1% (w/v) SDS at hybridization temperature.

Northern Blots

RNA samples were fractionated in agarose-formaldehyde gels (25). The RNAs were transferred onto either nitrocellulose or nylon membranes overnight. The filters were then baked and prehybridized overnight in a solution of 50% (v/v) formamide, 5× SSPE, 2× Denhardt’s solution, 0.1% (w/v) SDS, and 100 μg/mL carrier DNA at 42°C. Hybridization was carried out in fresh solution containing the same components, except that Denhardt’s solution was reduced to 1× and labeled probe was included at 5 to 10 ng/mL or 25 ng/mL for RNA and DNA probes, respectively. The filters were then washed as described previously (25). Radiolabeled RNA probes were made by T7 RNA polymerase transcription in vitro (25) using pcbam-l (the partial-length cDNA described below) linearized with HindIII as template. DNA probes were prepared by randomly priming (3) cam cDNA inserts.

Genomic Southern Blots

Ten μg of restriction enzyme-digested barley DNA were fractionated on 0.8% agarose gel and transferred to nitrocellulose filters. Prehybridization and hybridization were carried out in 6× SSPE, 0.5% (w/v) SDS, 1× Denhardt’s solution, and 100 μg/mL carrier DNA at 65°C. Radiolabeled probes were prepared and included in the hybridization mixture as described above for Northern blots.

Plasmid DNA Sequencing

The putative barley cam cDNA inserts from positively hybridizing recombinant phage were subcloned into the sequencing/transcription vector pGEM-3Z (Promega Biotec, Madison, WI). Double-stranded plasmid sequencing was performed using T7 DNA polymerase (Sequenase, US Biochemicals, Cleveland, OH) and a modified sequencing method. Two μg of DNA were denatured in 0.4 N NaOH in a final volume of 10 μL for 10 min at room temperature. Two hundred ng of sequencing primer and 3 μL of 3 M Na-acetate (pH 4.5) were added, and the final volume adjusted to 17 μL. The mixture was precipitated with 75 μL of ethanol for 5 min at −70°C, and collected by centrifugation. Following a rinse in 80% (v/v) ethanol, the pellet was resuspended and subjected to sequencing using $[\alpha-35]$PdATP according to the Sequenase product manual. Samples were heated to 90°C for 1 min prior to loading onto 40-cm, 8% (w/v) acrylamide sequencing gels (12). Three hundred to four hundred nucleotides of sequence information were routinely obtained from a standard sequencing gel, when two successive loadings of each set of reaction products were fractionated.

RESULTS

Earlier studies in this laboratory (25) identified barley cam mRNA using antisense RNA probes produced from a heterologous eel cam cDNA, which primarily contained sequences from the third Ca$^{2+}$-binding loop domain. These results suggested that such a probe might be suitable for screening cDNA libraries for inserts encoding cam. In practice, however, this heterologous probe generated numerous false positives under a variety of hybridization stringencies. In addition, although a number of specific anti-CaM antisera are available, the affinities of those antisera were insufficient to be useful for immunological plaque screening of cDNA expression libraries. To circumvent these problems, a synthetic oligonucleotide was constructed for screening λgt10 cDNA libraries. We compared the published amino acid sequences of CaM from wheat (20) and spinach (11), and identified the region of amino acid sequence with the least possible codon degeneracy, taking into account the estimation that the lower limit of effective oligonucleotide probe length is 14 to 17 nucleotides (19). The most suitable region for designing such a probe lies between residues 144 and 148 of wheat CaM (residues 143–147 of the spinach protein), which contains two contiguous methionines in the sequence: K-V-M-A. Eight DNA sequences could potentially encode such a peptide (i.e. 5'-AG/A/ATG/ATG-GC-3', where N = any nucleotide), if the third position of the alanine codon is omitted. To facilitate hybridization of the oligonucleotide to cam mRNA as well as cam DNA, complementary oligonucleotides were constructed with the sequence: 5'-GC-CAT-CAT-=-NAC-YTT-3', where Y = a pyrimidine, and N = any nucleotide. This mixed oligonucleotide was also used as a primer for double-stranded DNA sequencing experiments.

To estimate the optimal temperature conditions for plaque screening, the cam oligonucleotide mixture was end-labeled with $^{32}$P using T4 polynucleotide kinase and hybridized to Southern blots of barley genomic DNA. Two EcoRI restriction fragments and four HindIII fragments hybridized under the most stringent conditions (37°C) (data not shown).

Plaque hybridizations were then performed with the cam oligonucleotide, using the conditions empirically determined from genomic Southern blots, and a number of putative positive clones were picked. Upon rescreening, one of the positives continued to hybridize with a very strong signal. Southern blots of EcoRI-cleaved DNA isolated from small-scale liquid lysates of this recombinant phage clone indicated that a 500-bp insert was responsible for the signal generated. The hybridizing phage was amplified and the insert transferred to the EcoRI site of the plasmid vector, pGEM-3Z. The resulting plasmid subclone was termed pcbam-1; and the DNA sequence of the plasmid insert was determined using a modified double-stranded DNA sequencing protocol (described in “Materials and Methods”). The resulting sequence (Fig. 1) was 489 bp in length, and translation of the nucleotide sequence revealed that 108 bp of the cDNA encodes a polypeptide identical in amino acid sequence with the carboxy-
Figure 1. Nucleotide and derived amino acid sequences of full-length (pBcam-3), and 489-bp, partial-length (pBcam-1) barley leaf cam cDNA clones. Amino acid residues are numbered in parentheses beginning with the presumptive amino-terminal alanine residue, and are given in the single letter IUPAC nomenclature. These residues are aligned to illustrate the sequence relationships of the four Ca\(^{2+}\)-binding domains of CaM. Residues marked with asterisks are those which act as Ca\(^{2+}\)-binding ligands. The region corresponding to the sequence of one of the specific oligonucleotide probes is marked by a bar.

terminal region of plant CaM. The encoded sequence includes the entire fourth Ca\(^{2+}\)-binding domain of the protein (amino acid residues 113 through 148), followed by a termination codon, and 381 bp of 3'-untranslated region.

Once the partial-length cam cDNA had been characterized, we initiated a series of plaque screening experiments to isolate a clone that was full-length with respect to the CaM-coding region. In these experiments, 32P-labeled partial-length cam cDNA insert (pBcam-1), or antisense RNA transcripts of the pBcam-1 insert region were used as hybridization probes to screen a second, cam-enriched cDNA library. This new library was constructed in Agt10 using poly(A)\(^{+}\) RNA obtained from barley leaf basal meristematic zones, which is about 3.5-fold enriched in cam mRNA compared with total leaf poly(A)\(^{+}\) RNA (25). From these experiments, a single full-length cam cDNA was isolated and sequenced (pBcam-3, Figure 1). A second, partial-length cam cDNA (pBcam-2) was also obtained from further screening of the original cDNA library, and was sequenced only sufficiently to establish its identity unambiguously. Both of these longer clones share complete DNA sequence identity with the overlapping region of the original, partial-length cam cDNA, and with each other. The amino acid sequence derived from the three cam cDNA clones (Fig. 1) yields a putative polypeptide whose primary structure is very similar, but not identical, to that of spinach and wheat CaM, which were previously determined by direct amino acid sequencing (11, 20). Two of the amino acid sequence changes are conservative in nature. Position 6 in barley is changed from glutamate to aspartate compared with either spinach or wheat CaM; position 97 in barley represents an amidation change (Asn) compared with wheat CaM (Asp). This residue is asparagine in the spinach protein sequence. Compared with the wheat CaM sequence, barley and spinach CaM are smaller by one amino acid residue, due to an apparent insertion/deletion event between residues 8 and 9 in the derived barley CaM sequence (Fig. 1). These results are consistent with observations that CaM isolated from barley leaves possesses biochemical and immunological properties similar to those of the spinach protein (18). Barley CaM also shares approximately 92% amino acid sequence identity with vertebrate CaM. But, as expected, the nucleotide sequences encoding the plant and vertebrate proteins have diverged considerably over the course of evolution. Barley cam cDNA sequences share about 75% nucleotide sequence similarity with the corresponding cDNA sequences from a variety of other organisms. Typically, the sequences encoding the second Ca\(^{2+}\)-binding domain of cam are somewhat more conserved among the organisms surveyed. Interestingly, the coding sequence of the second Ca\(^{2+}\)-binding subdomain (encoding amino acid residues 56–67) is as conserved between barley and chicken cam mRNAs (94% sequence identity) as it is between chicken and any other animal cam mRNA (Table I) (16).

The Ca\(^{2+}\)-binding domains of CaM are hypothesized to have arisen from two gene duplication events that transformed an ancestral single-domain molecule into a protein capable of binding four Ca\(^{2+}\) (7, 8). By comparing the nucleotide sequence similarities of the four Ca\(^{2+}\)-binding domains of CaM, we find that domain pairs I/III and II/IV share somewhat higher degrees of sequence identity than do other pair-wise combinations, with the exception of the domain I/IV pair (Table II). These results are consistent with the gene duplication model described above, but are less persuasive than the results of identical analyses performed with vertebrate cam sequences (16).

Because CaM is generally considered to be a constitutively expressed protein (7, 22), we utilized the cloned cam cDNA probe to ask whether cam mRNA sequences accumulate to measurable levels in all vegetative tissues of young barley seedlings. Total RNA was isolated from root, seed, coleoptile, and leaf tissues dissected from etiolated, 7-d-old seedlings and...
Table I. Intradomain Nucleotide Similarities between Ca\textsuperscript{2+}-Binding Domains and Subdomains in Barley and Animal cam cDNAs

The assignment of domains is based on the amino acid sequence of bovine brain cam (23). These are: I, amino acids 8–40; II, amino acids 44–76; III, amino acids 81–113; IV, amino acids 117–148. The Ca\textsuperscript{2+}-binding subdomains are defined as: I*, amino acids 21–30; II*, amino acids 56–67; III*, amino acids 93–104; IV*, amino acids 129–140.

<table>
<thead>
<tr>
<th>Domain</th>
<th>Percent Nucleotide Similarity with Barley cam*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chicken</td>
</tr>
<tr>
<td>I</td>
<td>71</td>
</tr>
<tr>
<td>I*</td>
<td>78</td>
</tr>
<tr>
<td>II</td>
<td>82</td>
</tr>
<tr>
<td>II*</td>
<td>94</td>
</tr>
<tr>
<td>III</td>
<td>66</td>
</tr>
<tr>
<td>III*</td>
<td>67</td>
</tr>
<tr>
<td>IV</td>
<td>71</td>
</tr>
<tr>
<td>IV*</td>
<td>78</td>
</tr>
</tbody>
</table>

* Data for the coding region nucleotide sequence comparisons were taken from the following: chicken (16), eel (9), rat (14), Xenopus (1), trypanosome (21), and urchin (4).

Table II. Nucleotide Sequence Similarities within the Four cam Ca\textsuperscript{2+}-Binding Loop Regions

<table>
<thead>
<tr>
<th>Ca\textsuperscript{2+}-Binding Loop (amino acid residues)</th>
<th>Percent Nucleotide Sequence Similarity with Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (20–31)</td>
<td>100</td>
</tr>
<tr>
<td>II (56–67)</td>
<td>50 100</td>
</tr>
<tr>
<td>III (93–104)</td>
<td>64 47 100</td>
</tr>
<tr>
<td>IV (129–140)</td>
<td>67 58 47 100</td>
</tr>
</tbody>
</table>

Figure 2. cam mRNA sequences expressed in all tissues of etiolated, 7-d-old barley seedlings. Duplicate, 5-µl samples of total high molecular weight RNA isolated from root (R), seed (S), coleoptile (C), and leaf (L) tissues were fractionated in formaldehyde-agarose gels, and either transferred to a nitrocellulose filter (panel a) or stained with ethidium bromide (panel b). The filter-bound RNA was hybridized with randomly primed, \textsuperscript{32}P-labeled pBcam-1 cDNA insert as described in "Materials and Methods" and cam mRNA sequences detected by autoradiography (Fig. 3). Three 3-d at –80°C with one intensifying screen). The positions of 25S and 18S rRNA are noted in the figure.

equal mass amounts subjected to Northern blot analysis. Figure 2 shows that cam mRNA sequences were readily detected in each tissue examined, and that seed and coleoptile tissues are enriched in cam mRNA compared with root and leaf tissues. The single cam mRNA band detected in each RNA sample used in this experiment is approximately 0.85 kb in length, a size that is slightly longer than anticipated from previous studies (25). The slightly larger apparent size of coleoptile cam mRNA shown in Figure 2 is a consequence of slightly anomalous migration of the coleoptile RNA sample in this particular blot experiment. (Note the slightly larger apparent size of 18S rRNA in this sample.) The mRNA size we have determined in these experiments is about 0.2 kb shorter than the longest cam cDNA we isolated (1.086 kb), however. The reasons for this discrepancy are not yet clear, but we suspect that cDNA synthesis reactions added sequences artifically to the 5'-end of our longest clone (pBcam-3). The length of this untranscribed sequence (255 bp) is unusually large compared with most eukaryotic mRNAs (13). In addition, it contains three potential ATG initiation codons, which are not normally found upstream from translational initiation sites in eukaryotic mRNAs (13). Similar, artificial 5' additions (unrelated in sequence with those observed in this instance) have been noted in some other cDNAs constructed in our laboratory (SJ Rundle, RE Zielinski, unpublished experiments). In the future, the nature and extent of cam mRNA 5'-untranslated sequences will be resolved by structural analyses of barley cam genomic DNA sequences.

Northern blot analyses were also performed with poly(A\textsuperscript{+}) RNAs isolated from different plant sources (Fig. 3). In these analyses, we wanted to determine the level of hybridization between the barley cam cDNAs and other plant cam-encoding sequences. Either randomly primed cam coding region cDNA inserts, or antisense cam mRNA transcript probes generated by \textit{in vitro} transcription with T7 RNA polymerase of a linearized cam coding region plasmid derived from pBcam-1 were employed in the hybridizations. The coding region probe was constructed by subcloning a 108-bp EcoRI-HinPI fragment, which encoded the cam mRNA region corresponding to amino acids 115 through 148 (i.e. the fourth Ca\textsuperscript{2+}-binding domain region), into EcoRI/AccI-cleaved pGEM-4Z. This probe (pBcam1Δ1) hybridized to a single mRNA size class
Figure 3. Detection of cam mRNA sequences in different plant species using a barley cam cDNA probe. Poly(A') RNAs (~1 μg) were denatured and fractionated in a formaldehyde-agarose gel, transferred to nitrocellulose and hybridized with a randomly primed, 32P-labeled cam coding region cDNA insert (pBcam1.2). Samples used in the blot were: 1, barley; 2, pea; 3, soybean; 4, maize; and 5, Arabidopsis. Autoradiographic exposure was for 1 d at −80°C with one intensifying screen.

Figure 4. Southern blot analysis of cam sequences in barley genomic DNA. Ten-μg aliquots of high mol wt barley DNA were digested to completion with either EcoRI (lane 1) or HindIII (lane 2), fractionated in an agarose gelf, and transferred to a nylon membrane. The filter replica was probed with randomly primed, 32P-labeled pBcam-1 insert DNA. Autoradiography was for 7 d at −80°C with one intensifying screen. The small arrow heads indicate the positions of restriction fragments hybridizing with intensities of less than one copy equivalent per haploid genome, which consistently appear in addition to the major cam-encoding fragments.

in poly(A') RNA fractions from pea, soybean, maize, and Arabidopsis thaliana, albeit with lower apparent efficiency than with barley mRNA. Figure 3 also shows that cam mRNAs from these other plant sources, with the possible exception of Arabidopsis, are approximately the same size as barley cam mRNA. Other studies in this laboratory indicate that the cam coding region probe can also specifically detect carrot and Chlamydomonas reinhardtii cam mRNA sequences (V. Ling, I. Perera, and R. E. Zielinski, unpublished results). Chlamydomonas cam mRNA, however, appears to be significantly larger than cam mRNAs from higher plants (data not shown). These results are consistent with previous Northern blot assays of higher plant mRNA, in which antisense RNA produced from a heterologous eel cam cDNA was employed as a hybridization probe (25).

To gain some information on the potential number of cam-encoding sequences there are in the barley genome, we probed Southern blots of barley total DNA with 32P-labeled cam cDNA inserts. These analyses yielded a single hybridizing band of 2.8 kb for EcoRI, and two, less strongly hybridizing bands of 3.2 and 2.2 kb for HindIII-digested DNA, respectively (Fig. 4). Inclusion of EcoRI-cleaved pBcam-1 in similar analyses at levels equivalent to 1 and 10 gene copies per haploid barley genome revealed a hybridization intensity consistent with the idea that the 2.8-kb EcoRI band bears a single cam copy (data not shown). In addition, several bands of much lesser intensity can also be seen in this assay (~4.6 kb in the EcoRI lane, and ~6.5, and 1.3 kb in the HindIII lane of Fig. 4). It is not yet clear whether these additional hybridizing restriction fragments represent additional cam sequences, or closely related sequences encoding a CalM-like protein.

**DISCUSSION**

Three cDNA clones, including one that spans the entire protein coding region, encoding barley cam have been isolated and two of them completely sequenced. A 489-bp, partial-length cDNA corresponding to amino acids 115 through 149, encoding the fourth Ca2+ binding loop and the 3' untranslated region of cam mRNA, was initially isolated using a mixed oligonucleotide probe. This partial-length molecule was then utilized to isolate a full-length (with respect to the protein coding sequences) cDNA clone and a second partial-length cam cDNA. The overlapping regions of the three clones are identical in their nucleotide sequence, including the 3' untranslated region. This result indicates that all three clones represent copies of mRNA transcribed from the same structural gene. These results are consistent with our Southern blot (Fig. 4) and genomic reconstruction experiments, which indicated that cam is encoded by a single-copy gene in barley. We cannot yet exclude the possibility that the single, 2.8-kb EcoRI fragment that hybridizes with the barley cam cDNA contains tandemly repeated cam genes, as was previously reported for cam genes in trypanosomes (21).

As expected from the conserved nature of CaM, barley cam coding sequences share a relatively high degree of sequence similarity with vertebrate cam coding sequences. However, our attempts to use an eel cam cDNA as a probe for barley cam sequences, which relied on a subclone of the eel cDNA that encoded the third Ca2+ binding domain (25), were not successful (i.e. numerous false positives were obtained in plaque screening experiments). Although the amino acid sequences in this region of the vertebrate and plant proteins are reasonably well conserved, Table I shows that the third Ca2+ binding domain is the least conserved at the nucleotide sequence level between plant and eel cDNA sequences. In general, the third Ca2+ binding domain is the least conserved among cam coding sequences between barley and other organisms. In retrospect, therefore, it is not surprising that we encountered numerous false positives in our original screenings. We suspect that the sequences encoding Ca2+ binding domains II or IV will serve as better heterologous probes for cam genes. In support of this idea, we note that we have isolated Arabidopsis and carrot cam cDNA sequences using
the Ca²⁺-binding domain IV subclone (pBcam1Δ1) described above (V Ling, RE Zielinski, unpublished experiments).

Northern blots of barley RNA using homologous cam cDNA probes indicate that barley cam mRNA is approximately 850 bases in length, a size that is somewhat larger than our previous estimates of cam mRNA length in plants (25). RNA from other plant sources, with the exception of Arabidopsis, were also shown to contain cam mRNA sequences of a size similar to that of barley. In no case did we observe any indication of multiple cam mRNA size classes as were originally observed in eel (9) and chicken (16). Although cam mRNA was detected in all tissues of 7-day-old barley seedlings (Fig. 2), steady state levels of the messenger were significantly greater in seeds and coleoptiles relative to leaves and roots. We do not yet know what mechanisms are responsible for these differences, but note that they could be accounted for by different rates of cam mRNA transcription or turnover, or by the expression of additional cam structural sequences in seeds and coleoptiles. In addition, we do not know whether the increased steady state mRNA levels of coleoptiles and seed results in greater accumulation of CaM in these tissues.

Ca²⁺ has been implicated to play an important role in regulating a variety of physiological responses in plants to signals as diverse as phytohormones, light and gravity (5, 15, 17). In most instances, it is tacitly assumed that the physiological effects of Ca²⁺ are mediated by CaM. In plant cells, however, Ca²⁺ regulation mediated via CaM, has been demonstrated for only a few enzymes, including NAD kinase, a microsomal Ca²⁺ transport ATPase, quinata:NAD⁺ oxidoreductase, and various protein kinases (15). In animal cells, it is clearly established that many Ca²⁺-regulated processes are mediated by Ca²⁺-receptor proteins sharing extensive structural homology with CaM (reviewed in ref. 22). Expression of these receptor proteins is typically restricted, temporally or spatially, while CaM is expressed constitutively (7, 22). We have preliminary evidence that plant soluble protein extracts contain a number of Ca²⁺-binding polypeptides that may share structural similarities with CaM (I Perera, RE Zielinski, unpublished experiments). The success we reported here in isolating cDNAs encoding CaM using specific, degenerate oligonucleotide probes suggests that a similar approach, using oligonucleotides designed on the basis of amino acid sequences encoding Ca²⁺-binding domains, may be fruitful to isolate plant genes encoding proteins sharing structural similarity with CaM.

ACKNOWLEDGMENTS

We thank Mary Schuler for critically reading the manuscript.

LITERATURE CITED