Structural Organization of the Spinach Endoplasmic Reticulum-Luminal 70-Kilodalton Heat-Shock Cognate Gene and Expression of 70-Kilodalton Heat-Shock Genes during Cold Acclimation

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The 70-kD heat-shock proteins (HSP70s) are encoded by a multigene family in eukaryotes. In plants, the 70-kD heat-shock cognate (HSC70) proteins are located in organellar and cytosolic compartments of cells in most tissues. Previous work has indicated that HSC70 proteins of spinach (Spinacia oleracea) are adively compartmented in cells in most tissues. Previous work has indicated a multigene family in eukaryotes. In plants, the 70-kD heat-shock cognate protein (HSC70) is constitutively expressed in the cytosol (Winter et al., 1988; Lin et al., 1991; Roberts and Key, 1991; Miernyk et al., 1992), ER (Denecke et al., 1991; Fontes et al., 1991), mitochondrion (Watts et al., 1992; Vidal et al., 1993), and chloroplast (Ko et al., 1992; Marshall and Keegstra, 1992). The fact that HSC70s are distributed in a variety of locations throughout the cell and are expressed during normal growth has been interpreted to suggest that they play an important role in cellular metabolism (Craig and Gross, 1991).

HSP70s also belong to a group of proteins known as molecular chaperones (Ellis and van der Vies, 1991) that transiently interact with a wide variety of other cellular proteins. The exact mechanism of interaction by which HSP70s bind and release proteins is still unclear, as are many of their functions. They do bind ATP tightly and exhibit weak ATPase activity (for review, see Gething and Sambrook, 1992). HSP70s also contain a C-terminal peptide-binding domain (Milaraki and Morimoto, 1989) that enhances the hydrolysis of ATP when associated with peptides. In turn, hydrolysis of ATP favors the release of the bound peptides (Flynn et al., 1989; McCarty and Walker, 1991). Yeast cytosolic HSC70s have been implicated in the transport of proteins to the ER and mitochondria (Craig and Gross, 1991). Miernyk et al. (1992) have also shown that plant cytosolic HSC70 can facilitate the in vitro translocation of secretory protein precursors into microsomes. BiP, the HSC70 of the ER lumen, also known as kar2 in yeast (Normington et al., 1989; Rose et al., 1989) and GRP78 in animals (Munro and Pelham, 1986; Lee, 1987), is essential for the proper translocation and processing of secretory proteins passing through the ER (Hendershot, 1990; Vogel et al., 1990; Nguyen et al., 1991). Mitochondrial HSC70s have also been associated with the translocational import of mitochondrial precursor proteins (Sheffield et al., 1990; Vidal et al., 1993). Under un-
stressed conditions HSP70s are thought to associate transiently with precursor or nascent polypeptides to facilitate their transport to and across cellular organelles by retaining them in an unfolded and transport-competent conformation until targeted for final protein folding or assembly (Deshaies et al., 1988; Rothman, 1989). HSP70s may also function to prevent or disrupt protein aggregates under conditions that prevent proper protein biogenesis or cause native proteins to denature. That HSP70s have been reported to disassemble protein complexes (Schmid et al., 1985; Pelham, 1986) and degrade mutant proteins (Sherman and Goldberg, 1992) and are induced by misfolded or unassembled proteins (Bole et al., 1986; Kozutsumi et al., 1988; Denecke et al., 1991; Fontes et al., 1991) provides further support for a role in maintaining proper protein biogenesis.

Many plants when subjected to low, nonfreezing temperatures undergo a process known as cold acclimation (Levitt, 1980). In hardy plants, cold acclimation has two major functions. The first involves the adjustment of intermediary metabolism and the basic biochemical functions of the cell in an effort to maintain overall homeostasis under the suboptimal conditions of reduced temperature; the second is the induction of increased tolerance to freezing stress (Guy, 1990). In spinach and many other plants, alterations in gene expression have been associated with the cold-acclimation process (reviewed by Guy, 1990). Accordingly, increased expression of several proteins has been linked with the cold-acclimation process in spinach (Guy and Haskell, 1987). One of these proteins has been identified as belonging to the HSC70 family (Neven et al., 1992). Although the pattern of expression of the HS70s in response to exposure to low temperature is quantitatively different than during heat shock, the increased synthesis of HSC70s during cold acclimation suggests a potential role in metabolic adjustments necessary for maintaining homeostasis or in relation to enhanced freezing tolerance (Neven et al., 1992). Here we report the expression of the ER-luminal HSC70 and that of two other HS70 genes during cold acclimation of spinach.

MATERIALS AND METHODS

Plant Material

Spinach seedlings (Spinacia oleracea L. cv Bloomsdale) were grown from seeds under controlled environmental conditions as previously described (Guy and Haskell, 1987). Cold-acclimation and deacclimation treatments were conducted with a 12-h photoperiod as previously described (Guy and Haskell, 1987).

cDNA Library Construction and Screening

Preparation of total RNA and isolation of poly(A+) RNA from 2-d cold-acclimated leaf tissue and the construction of a cDNA library were done as previously described (Neven et al., 1993). The cDNA library was double screened with the PCR-generated clone Spin350 (Neven et al., 1992) and with the partial spinach Bip cDNA clone p73 (1387 bp) random-primed with [α-32P]dCTP. Positive clones were identified by autoradiography. Only those clones that hybridized to both probes were further characterized. The largest clone isolated was approximately 2447 bp and was designated p522. The open reading frame from this clone was designated SOBIP.

Genomic Library Construction and Screening

DNA was extracted from 20 g of leaf tissue as described by Ausubel et al. (1989). A genomic library was constructed by ligating EcoRI-digested, size-selected genomic DNA (3–10 kb) into Uni-ZapII (Stratagene). Approximately 350,000 plaques were screened using randomly primed [α-32P]dCTP-labeled p522 cDNA.

PCR Amplification of Genomic DNA

Synthetic primers were constructed to conserved regions of HSP70s (Neumann et al., 1989). Genomic DNA was amplified using Taq polymerase according to the directions of Perkin-Elmer Cetus. The 5′ and 3′ primers were TTC CAG CAC GGG (CT) AAA GT(A) GAA and CAG AAG CTG GGA CCA CCA CCT, respectively. The two synthetic oligonucleotides were used to amplify genomic DNA in a cycling reaction, which included an initial denaturation of 3 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 55°C, 3 min at 72°C, and a final 10-min extension at 72°C. A 1287-bp fragment (4L) was purified and blunt-end cloned into EcoRV-digested Bluescript (Stratagene).

DNA Sequencing and Analysis

DNA sequencing was accomplished by the Taq DiDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) on an automated sequencer (Applied Biosystems). DNA sequences were further analyzed using DNASTAR (DNASTAR, Inc., Madison, WI). Homology searches of the gene data base (GenBank, EMBL, National Biomedical Research Foundation) and protein data bases (Protein Information Resource and SwissProt) were done using a Genetics Computer Group program.

GST-Bip Fusion Protein

To amplify the coding sequence for the mature protein of p522, two additional synthetic primers were constructed. The 5′ and 3′ primers GGC GGA TCC AAA GT(A) GAA and CAG AAG CTG GGA CCA CCA CCT, respectively, were used to amplify p522 under the same conditions as described above. The 5′ oligonucleotide was designed with a BamHI restriction site immediately 5′ to the codon for amino acid 29 (Lys), the first residue of the processed mature protein. The 3′ oligonucleotide was designed to match a sequence in the 3′ untranslated region 3′ to the EcoRI restriction site at position 2362. Amplified p522 cDNA was digested with BamHI and EcoRI and unidirectionally cloned into pGEX-2T (Pharmacia) digested with the same restriction enzymes. pGEX-2T with the ligated coding region for the mature Bip peptide was transformed into Escherichia coli strain XL1-Blue. Fusion protein synthesis was induced with IPTG (1 mM final concentration) for 4 h at 37°C. Fusion protein was extracted from cells that were pelleted at 1600g for 10 min and resuspended in PBS buffer (pH 8.0) containing 1% Triton X-100 and 1 mM PMSF. After a 10-min incubation...
on ice the cells were ruptured using a French press and centrifuged to remove insoluble material, and the resulting supernatant was applied to an affinity column of GSH-agarose equilibrated with 10 bed volumes of PBS (pH 8.0). The column was washed with 5 column volumes of PBS (pH 8.0) containing 1% Triton X-100, followed by 3 column volumes of PBS. The affinity-bound fusion protein was eluted with 3 column volumes of 5 mM GSH. Fractions containing GST-BiP fusion protein were digested with 1% (w/w) thrombin, and then the sample was reapplied to the GSH-agarose column. Unbound samples were collected and analyzed for BiP.

**Antibody Production**

Purified leaf tissue HSC70 was used to make antibodies (Anderson et al., 1994). Approximately 15 μg of purified monomeric HSC70 in Ribi monophosphoryl lipid A plus trehalose dicymocylolate adjuvant were initially injected into BALB/c mice with booster injections of 15, 8.5, and 8.5 μg of monomeric HSC70 in adjuvant. At 10 weeks the mice were bled, and the spleens rescued for the production of monoclonal antibodies. Cell lines 5B7 and 1D9, producing antibodies monospecific for cytosolic and the ER-luminal HSC70s, were obtained.

**Protein Extraction, Gel Electrophoresis, and Blotting**

Protein extraction buffer (62.5 mM Tris-HCl [pH 6.8], 5% glycerol, 2.5% 2-mercaptoethanol, 1% SDS) was used for homogenization at a weight to volume ratio for root, hypocotyl, cotyledon, and leaf of 1:2 and for dry seed and seed soaked for 4 h of 1:5. Homogenates were denatured by boiling for 2 min, centrifuged for 10 min, transferred, and stored at -20°C. Before loading, samples were diluted in 10 μL of extraction buffer to minimize distortion from salts. For immunoprecipitations, 0.5 g of spinach leaf tissue were ground in 2.5 mL of ice-cold grinding buffer (50 mM Pipes [pH 6.8], 1 mM EDTA, 1 mM DTT, 0.3% Triton X-100, 1.0% [w/v] PVP40) using a 7-mL tissue grinder. The extract was centrifuged at 20,000g for 10 min. Supernatant (50 μL) was placed in a 500-μL microtube along with 1.0 μL of purified ascites 5B7; 100 μL of supplemental grinding buffer was added to ensure that mixing would occur during the 2-h incubation on a rocker table in a 4°C cooler. The contents of the tube then were transferred to a tube containing Protein G (400 μL of Protein G had been microfuged for 30 s, the supernatant was removed, and the pellet was washed twice with 1 mL of grinding buffer) and incubated for 1 h. Next the mixture was microfuged for 30 s and the supernatant discarded. The pellet was washed with 1 mL of grinding buffer five times and washed twice with 1 mL of 50 mM sodium phosphate dibasic buffer (pH 7.0). Protein was extracted by adding SDS buffer to the pellet, heating for 2 min in boiling water, and microfuging for 10 min. This extract (20 μL) was loaded for SDS-PAGE.

SDS-PAGE (10% gels) and native PAGE (7% gels) were done as described by Ausubel et al. (1989). Following electrophoresis, either the gels were stained with Coomassie brilliant blue R 250 or the proteins were transferred to poly-vinylidene difluoride membrane filters using a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) at 20 V for 20 to 30 min. Transfers were done using 48 mL Tris, 39 mM Gly, 0.13 mM SDS, and 20% methanol (pH 9.2) as a transfer buffer. Immunoblots were incubated with various mouse and rabbit antisera and then visualized by alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit (Sigma). Color was developed using 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium. Protein blots were conducted under conditions of excess antibody. Uniformity of protein loading in the protein blot analyses was verified by Coo massie blue staining of duplicate gels.

**RNA and DNA Blotting**

Total RNA and DNA was extracted from leaf tissue as previously described. RNA blots were performed as described by Sambrook et al. (1989). DNA blots were performed using total DNA digested with a 10-fold excess of restriction endonuclease. Digested DNA was electrophoresed in 0.8% agarose gels in Tris/borate/EDTA buffer. The gels were then pressure blotted onto Hybond-N nylon membranes (Amersham) and UV cross-linked (1.2 × 10^6 μJ/cm^2) using a UV Stratallinker 1800 (Stratagene). Prehybridization of RNA and DNA blots was accomplished using 50% formamide, 5XSSPE, 5X Denhardt’s solution, 0.2% SDS, and 10 μg/mL salmon sperm DNA at 42°C for 4 h. [α-32P]dCTP randomly labeled DNA probe p522, p21 (HS701, a full-length cDNA for a constitutively expressed cytosolic HSC70 in spinach), or the PCR-amplified coding portion of p4L was added to the hybridization buffer and allowed to hybridize to the filters for 14 to 16 h at 42°C. After hybridization the DNA blots were washed one time each in 2X, 1X, and 0.5X SSC plus 0.2% SDS for 15 min at 42°C. RNA blots were washed two times each with 6X, 2X, 1X, and 0.5X SSC plus 0.2% SDS for 15 min at 42°C, followed by a 10-min wash in 0.1X SSC plus 0.2% SDS at 50, 55, 60, and 65°C.

**Cell Fractionations**

Cell fractionations and organellar isolations were done as previously described by Neven et al. (1993).

**RESULTS**

**Isolation of a Genomic and cDNA Clone to Spinach BiP**

We isolated two genomic clones (G792 and G793) from a spinach genomic library for analysis. Hybridization analyses of both of these clones indicated that the full-length BiP gene had been cloned in a 5.1-kb DNA fragment as shown in Figure 1. Both Figures 1 and 2 show that spinach BiP contains seven introns, which separate the eight exons encompassing the coding region for BiP. The open reading frame of the BiP gene was determined by computer alignment of the genomic sequence with that of a full-length BiP cDNA (p522) that had been isolated from a spinach cDNA library. Restriction-digested spinach genomic DNA, shown in Figure 3A, probed with the BiP cDNA produced only a single major band. A minor DNA band was observed below 3 kb (approximately 295 bp) in the HindIII-digested lane on DNA blots that were
Figure 1. The complete nucleotide sequence of the spinach BiP gene encoded by pC792 and the corresponding amino acid sequence for the translated regions. Numbers to left of rows refer to the first nucleotide of each row. The nucleotide sequence encoding the open reading frame of BiP is in uppercase letters. The deduced amino acid sequence for the protein is boxed, and the conserveicl sequence (Stevenson and Calderwood, 1990) is designated as a single letter under the nucleotide sequence. Noncoding sequence is designated by lowercase letters. Conserved BiP and CRP78 promoter regions (Liu and Lee, 1991) are indicated by boxes at positions 2221, 2341, 2581, 2761, 3001, 3241, 3481, 3721, 3961, and 4201.

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Expression of a BiP Fusion Protein

Figure 6 shows the in-frame ligation of the mature BiP-coding sequence to the GST sequence encoded in the expression vector pGEX-2T. Expression of pGEX-2T-BiP produced a GST-BiP fusion protein of approximately 97 kD (Fig. 6A). Expression of pGEX-2T alone resulted in accumulation of the 26-kD GST peptide seen as the band at the bottom of lanes 1 and 2 of Figure 6A. The 97-kD fusion protein bound to GSH-agarose and was eluted with GSH. Thrombin cleavage of the 97-kD affinity-purified GST-BiP fusion protein produced two peptides of 79 and 26 kD (Fig. 6A). Protein blots indicate that the 97-kD fusion protein and the 79-kD thrombin-cleaved peptide differentially reacted with antibodies raised against purified native spinach HSC70. The 1D9 an-

binding domain (Figs. 1 and 5), which exhibits 62% homology to the calmodulin-binding domain consensus sequence (Stevenson and Calderwood, 1990).

Figure 3. cDNA restriction map and spinach genomic DNA analysis. A, Total spinach genomic DNA was digested with either EcoRI, BamHI, HindIII, or PstI and separated on a 0.8% agarose gel, transferred onto a nylon membrane, and hybridized with α-32P-labeled cDNA probe to the open reading frame of spinach BiP clone p522. B, Schematic drawing of the restriction sites on clone p522. Numbering on the diagram indicates the nucleotide position of unique restriction sites. Also indicated is the position of the first ATG start site (85), the AAA sequence (169), which codes for the first amino acid of the mature BiP peptide, and the stop codon TAA (2099). Arrows indicate the EcoRI- and KpnI-cloning sites of pBluescript.

Figure 2. Schematic drawing of spinach BiP gene structure. Exons are designated by open boxes, and introns and noncoding regions are designated by solid lines. Numbering at the top and bottom of each open box indicates the nucleotide position at which each exon starts and ends, respectively. Arrows indicate the restriction-cloning sites of pBluescript.

As indicated in Figures 4 and 5, the BiP sequence includes both the β- and γ-phosphate-binding domains as well as the adenosine-binding domain found in HSC70s and other ATPases (Flaherty et al., 1991; Bork et al., 1992). Thr236 (Fig. 4) of the γ-phosphate-binding domain is equivalent to Thr199 of E. coli dnaK (McCarty and Walker, 1991), Thr204 of bovine HSC70 (Flaherty et al., 1991), and Thr229 of mammalian BiP (Gaut and Hendershot, 1992), all of which have been identified as the site of autophosphorylation. We also have identified a second putative adenosine-binding site in the spinach BiP sequence (Figs. 4 and 5) as well as a putative calmodulin-
spinach HSC70 produced a cross-reaction with either E. coli dnaK (Epitenter Technologies) or with recombinant hamster liver BiP (Carlino et al., 1992). These data demonstrate that 1D9 is highly specific for spinach BiP, whereas 5B7 does not recognize recombinant BiP but appears to be specific for spinach cytosolic HSC70s. The slight reactivity observed in the ER fractions (Fig. 6C) probed with 5B7 was attributed to contamination even though the ER fractions were treated with proteinase K.

Isolation of a Partial Genomic Clone to a Spinach HSC70

The sequence of p4L (PCR-generated DNA from spinach genomic DNA) had the highest homology to HS70 genes. The first 870 bp of p4L make up an intron (DNA sequence not shown), and the remaining 417 bp make up a translated sequence for an HS70. The amino acid sequence of the open reading frame of p4L DNA is shown in Figure 4 (HS704). At the amino acid level, the open reading frame for spinach clone p4L (HS704) shows 65.7% homology to the HSC70 of the spinach chloroplast envelope (Fig. 4, SCE70). A comparison between p522 (SOBIP) and p4L (HS704) shows a 59% homology match.

mRNA and Protein Levels of HS70s and HSC70s during Cold Acclimation

RNA blots prepared with total RNA extracted from spinach leaf tissue during a cold-acclimation/deacclimation treatment were hybridized with either labeled p522 (BiP), p21 (HSC701), or p4L (HS704) as shown in Figure 7A. p522 hybridized with a 2.5-kb mRNA (Fig. 7A) expressed during nonacclimation conditions. This mRNA appeared to be up-regulated following 1 d of cold acclimation and remained at an elevated level through 1 d of deacclimation. The BiP mRNA returned to nonacclimated levels by d 7 of deacclimation. RNA blots probed with actin indicate that the reduced hybridization observed for sample CA7 may be due to a slight underload (Neven et al., 1992). p522 cDNA also showed a slight hybridization to a second, smaller mRNA at

Figure 4. Amino acid sequence comparison of spinach BiP with other HS70 proteins SOBIP, BLP4, SCE70, and HS704. The deduced amino acid sequence from spinach p522 (SOBIP), tobacco BLP4 (BLP4; Denecke et al., 1991), spinach chloroplast envelope HSP70 (SCE70; Ko et al., 1992), and spinach p4L (HS704). Numbering to the right of boldfaced lettering indicates the last amino acid for that line. The N-terminal sequence for the mature spinach BiP peptide is underlined. Amino acids that are identical with the spinach BiP sequence (bold letters) are indicated by dots. Areas with no lettering or dots indicate gaps within the sequence homology. Sequences that are highlighted with a shadow box represent the β-phosphate-binding (also underlined) and the γ-phosphate-binding domains (Flaherty et al., 1991). Sequence with double under- and overlines indicates an adenosine-binding domain (1) and a putative second adenosine-binding domain (2). Alignments were accomplished using the ALIGN program of the University of Wisconsin Genetics Computer Group.
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Figure 6. Sequence, SDS-PAGE, and protein blot analysis of BiP fusion protein. DNA and amino acid sequence analysis of the junction between GST (underlined) and the coding region of the mature spinach BiP protein (bold letters). The thrombin cleavage site of the fusion protein is marked by the arrow. A, SDS-PAGE (10%) showing the purification scheme of the recombinant BiP fusion protein. B, Protein blots probed with antibody to spinach BiP (1D9). C, Protein blots probed with antibody to spinach cytosolic HSC70 (5B7). D, Protein blots probed with antibody to recombinant tobacco BiP (BLP4; Denecke et al., 1991). Samples in lanes are as follows: 1, soluble IPTG-induced protein from E. coli harboring pGEX-2T without a DNA insert; 2, GSH-agarose affinity-bound IPTG-induced soluble protein from E. coli harboring pGEX-2T-BiP containing the mature spinach BiP DNA; 3, GSH-agarose unbound protein from thrombin-cleaved GST-BiP fusion protein; 4, ion-exchange chromatography-purified recombinant spinach BiP; 5, purified spinach leaf HSC70 monomer (Anderson et al., 1994); 6, purified oligomeric spinach leaf HSC70 (Anderson et al., 1994); 7, 5B7 immunoprecipitated spinach protein; 8, recombinant hamster BiP (Carlino et al., 1992); 9, E. coli dnaK; 10, fractionated spinach cytosolic protein (Neven et al., 1993); 11, fractionated spinach ER protein. The band at the bottom of lanes 1 and 2 in A is the 26-kD GST peptide.

Figure 7B. Conversely, a heat shock at 37°C for 2 h caused a dramatic reduction in the level of spinach BiP mRNA. Drought-stressed tissue also showed a reduction in the level of BiP mRNA. However, BiP protein levels were unaffected by any of the treatments described (Fig. 7B). In contrast to the BiP mRNA, a second constitutively expressed spinach HSC70 (HSC701) exhibited no loss in mRNA levels during either drought or a heat-shock treatment. In fact, the mRNA for HSC701 increased during both a cold-acclimation and heat-shock treatment.

Figure 8A shows that all tissues of spinach, with the exception of dry seeds, contain the BiP protein. In spinach leaf tissue grown under normal conditions, the level of BiP was always found to be most abundant in the youngest tissue (based on growth) and progressively less as the leaves mature (Fig. 8B). However, it should be noted that spinach leaf tissue contains progressively less protein overall (per gram tissue fresh weight) as it ages. Based on quantity per microgram of total protein, spinach leaf and root tissue contain approximately equivalent concentrations of BiP (Fig. 8C). It is interesting that hypocotyl and petiole contained a proportionately higher concentration of BiP per microgram of total protein.

Tissue-Specific and Environmentally Regulated Expression of BiP

Cold-acclimated leaf tissue shows a moderate accumulation of BiP mRNA after a 7- to 9°C treatment, as indicated in Figure 7B. Conversely, a heat shock at 37°C for 2 h caused a dramatic reduction in the level of spinach BiP mRNA. Drought-stressed tissue also showed a reduction in the level of BiP mRNA. However, BiP protein levels were unaffected by any of the treatments described. In contrast to the BiP mRNA, a second constitutively expressed spinach HSC70 (HSC701) exhibited no loss in mRNA levels during either drought or a heat-shock treatment. In fact, the mRNA for HSC701 increased during both a cold-acclimation and heat-shock treatment.

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DISCUSSION

At least three spinach HS70 genes show increased expression during cold acclimation. BiP and HSC701 are constitutively expressed and appear to be moderately up-regulated during cold-acclimating conditions. Another HSC70 family member, HS704, is not constitutively expressed but is induced in response to cold-acclimating conditions. The finding that BiP is constitutively expressed under normal growing conditions is not novel; however, the fact that mRNA for HSC70s are differentially up-regulated or induced in response to a low-temperature treatment in spinach suggests that they may be important in maintaining proper protein biogenesis in plants at low temperature. This theory is further supported by reports that show that HSP70s are more abundant in maize (Yacoob and Filion, 1987), mung bean (Wu et al.,...
1993), and *Sarcophaga crassipalpis* (Joplin and Denlinger, 1990) in response to cold shock. A prior mild heat-shock treatment of *Drosophila melanogaster* also increases the survival of larvae during subsequent cold treatment (Burton et al., 1988). However, these results are complicated by the fact that protein levels for spinach HSC70s, unlike that of mung bean (Wu et al., 1993), do not change with the corresponding accumulation of its mRNA but instead maintain a steady-state level throughout a cold-acclimation treatment. An explanation for this observation is that up-regulated spinach HSC70 mRNA is either not translated or that translation efficiency is reduced by low temperature. However, this is highly unlikely since Neven et al. (1992) have shown that at least five forms of spinach HSP70 are radiolabeled during a cold-acclimation treatment. Using an antibody that is more reactive to cytosolic HSC70s of spinach, Neven et al. (1992) also observed that a global increase of HSC70 mRNA did not, initially, give a corresponding increase in detectable HSC70.

A more likely explanation for the result reported here is that a low-temperature-induced up-regulation of HSC70 mRNA in spinach is required to maintain a steady-state level of BiP and some cytosolic HSC70s. Perhaps the turnover rate of HSC70 may be affected during a cold-acclimation treatment. Also, the translational contribution from the modest increase in mRNA levels of BiP and a cytosolic HSC70 during cold acclimation may contribute only a small increase to the total pool of spinach HSC70. Another explanation could be that HSC70 mRNA accumulation increases during cold acclimation due to a reduction in the turnover rate of HSC70 mRNA. However, in the case of BiP, the latter explanation is
unlikely, since the expression of mutant proteins that cannot assemble properly in the ER leads to the induction of BiP mRNA without any other stress factor (Kozutsumi et al., 1988). Based on this information, we propose that exposure of spinach leaf tissue to low temperature may result in the altered biogenesis of proteins passing through the ER, which may lead to the formation of misfolded proteins or entrapment of intermediate proteins in the secretory pathway. This theory is supported by evidence that suggests low temperatures can cause the accumulation of such proteins within the secretory pathway (for review, see Rothman and Orci, 1992). If this were to occur, it suggests that the accumulation of secretory proteins could tie up the existing pool of free BiP within the ER and thus signal the up-regulation of BiP mRNA.

In contrast to the multigene BiP family reported in tobacco (Denecke et al., 1991), our data indicate that spinach BiP is likely to be encoded by a single gene with the open reading frame residing on eight separate exons. Other than the multigene family of BiPs reported for tobacco, we have found no other evidence to indicate that BiP should be encoded by a multigene family in other eukaryotes. Based on previous classifications of heat-shock genes, both spinach BiP and p4L (HS704) should be classified as HSC70s, since they both contain introns. However, the fact that HS704 is not constitutively expressed during normal growth or up-regulated during heat shock suggests that it should not be classified as an HSC70 but instead should be called a cold-inducible gene. As for the spinach BiP gene, it is also known that BiP genes of both plants (D. Meyer and A. Bennett, personal communication) and other eukaryotes have introns. Rat and human BiP genes have seven introns (Ting and Lee, 1988; Wooden et al., 1988). In an evolutionary context, this is interesting. Figure 9 shows that yeast BiP genes contain no introns (Pidoux and Armstrong, 1992) and the nematode BiP gene contains three introns (Heschl and Baillie, 1989).

Recently the promoter region of the yeast kar2 (BiP) gene has been shown to contain a regulatory domain that responds to the presence of unfolded proteins in the ER (Kohno et al., 1993). In Figure 1 we have indicated a putative unfolded protein-binding element in the promoter that shows similarity to the consensus sequence of unfolded protein-binding elements among BiPs (Kohno et al., 1993). In addition, we have indicated two other regions of the spinach BiP promoter containing domains that are conserved among BiPs (Liu and Lee, 1991). The putative unfolded protein-binding element and conserved domains observed in the promoter region on the spinach BiP gene are upstream of a domain that contains a conserved CCAAT motif. It has been proposed that these upstream domains of the promoter region are regulatory elements that interact with transactivating factors bound to the proximal CCAAT element upstream of the TATA element (Wooden et al., 1991). This scenario is believed to activate the transcriptional machinery at the TATA element, initiating transcription. It is interesting to note that the CCAAT sequence is also a conserved element of cold-shock promoters (Qoronfleh et al., 1992) and is specifically recognized by the E. coli DNA-binding protein CS7.4 (Wistow, 1990). The CS7.4 protein is known to mediate the activation of some cold-shock genes of E. coli (La Teana et al., 1991) and has been proposed as a possible universal transcriptional regulator of cold-shock genes of bacteria (Qoronfleh et al., 1992).

The fact that the spinach BiP gene was not responsive to a heat-shock treatment is not surprising, since the 5’ untranslated promoter region does not appear to contain the characteristic CnnGAAnnTTCnnG HSE consensus sequence (Pelham, 1985). It is interesting that a heat-shock response of 2 h was sufficient to remove the majority of constitutively expressed BiP mRNA in spinach. This result is not consistent with that observed by Kohno et al. (1993), who demonstrated an up-regulation of the yeast kar2 mRNA level during a 37°C heat treatment. However, they did demonstrate that the up-regulation of kar2 mRNA in yeast peaked after just 10 to 20 min at 37°C and then declined throughout the remainder of their 90-min treatment. The data suggest that the BiP mRNA probably continues to decline after a 90-min treatment at 37°C.

These observations are consistent with the report by Brodl

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**Figure 9.** Diagram of ER-luminal HSC70 gene structure from different eukaryotes. Hatched area indicates the signal peptide, Arabic numerals designate the amino acid residue at exon junctions, Roman numerals denote the exon number. Gene structures were obtained from Schizosaccharomyces pombe (Pidoux and Armstrong, 1992), Saccharomyces cerevisiae (Normington et al., 1989), Caenorhabditis elegans (Heschl and Baillie, 1989), Homo sapiens (Ting and Lee, 1988), Mus norvegicus (Wooden et al., 1988).
et al. (1990), who showed that a heat-shock treatment of barley aleurone cells caused the degradation of ER. Since a drought treatment also was sufficient to reduce the BiP mRNA level in spinach, we suspect that heat-shock and drought treatments may reduce protein export through the secretory pathway and thus may signal a down-regulating event for BiP mRNA. However, the reduced levels of BiP mRNA observed during a heat-shock treatment did not appear to affect the level of BiP protein in spinach leaf tissue. Taken together, these results suggest that the constitutively expressed pool of BiP in the ER is sufficient to maintain whatever level of protein biogenesis of secretory proteins that is occurring during heat and drought stress. We also have preliminary evidence to suggest that not all constitutively expressed spinach HSC70 family members are down-regulated during a heat-shock or drought treatment. The data clearly show that a constitutively expressed cytosolic HSC70 in spinach leaf tissue is up-regulated during heat shock and remains constant during water-stress conditions. Genomic Southern data suggest that there are at least 10 H570 genes (J.V. Anderson, G.-B. Li, D.W. Haskell, C.L. Guy, unpublished data) of which at least 5 HSC70s are expressed in spinach during low temperature (Neven et al., 1992). With three members of this family cloned, we are only beginning to understand the complexity that is involved in the regulation of different HS70s genes and HSC70s during suboptimal conditions.

We have observed a putative calmodulin-binding site in the spinach BiP sequence. Based on a Kyte and Doolittle (1982) hydropathy profile for the amino acid sequence of spinach BiP, a putative calmodulin-binding domain is located within a hydrophilic region. This is in contrast to the finding of Ko et al. (1992), who showed that the calmodulin-binding domain for the HSC70 of spinach chloroplast envelopes (SCE70) is located within a hydrophilic region of the sequence. Such differences in structural identity could affect the calmodulin-binding capacity of HSC70s. Since HSP70s may bind calmodulin (Clark and Brown, 1986; Stevenson and Calderwood, 1990), it has been suggested that intracellular increases in calcium concentrations during heat shock could enhance the binding of calmodulin to HSP70s and thus inhibit the interaction of HSP70s with other proteins (Stevenson and Calderwood, 1990). At this point it is unclear what purpose a calmodulin-binding site on BiP would serve, since the ER is currently not recognized as an organelle that contains detectable levels of calmodulin.

HSC70s have previously been postulated to contain both a catalytic and hydrolytic ATP-binding site (Schmid et al., 1985). Based on the spinach BiP sequence data, we have identified what we believe could be a potential second adenosine-binding site. This putative adenosine-binding site is located within the C-terminal portion of the BiP, which is a region of HS70s that still has not been analyzed by x-ray crystallography. The ATP hydrolytic site is contained within the 44-kD N-terminal fragment, which has been analyzed by x-ray crystallography (Flaherty et al., 1991). This hydrolytic site has been associated with the release of peptides from BiP (Flynn et al., 1989) and is located within the ψ-phosphate-binding domain, which contains the Thr residue that undergoes autophosphorylation (Flaherty et al., 1991; Gaut and Hendershot, 1992). The postulated catalytic ATP-binding site of HSC70s is believed to regulate the binding of proteins and the dissociation of oligomeric and dimeric forms of HSC70s and does not require the hydrolysis of ATP to fulfill this task (Schmid et al., 1985). Based on preliminary data, we believe spinach BiP may contain a second ATP-binding site that could participate in determining both its oligomeric and conformation state (J.V. Anderson, G.-B. Li, D.W. Haskell, C.L. Guy, unpublished data).

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