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The Independent Stage-Specific Expression of the 18-kDa Heat Shock Protein Genes During Microsporogenesis in *Zea mays* L.

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ABSTRACT The small (18-kDa) heat shock proteins (hsps) of maize are encoded by a complex multigene family. In a previous report, we described the genetic information from cDNAs encoding two different members of the family. In this communication, we report the isolation and characterization of cDNA and genomic clones encoding information for a third member of this hsp family (c/gMHSP18–1). DNA fragments containing nucleotide sequences common to, or specific for, each of these characterized 18-kDa genes were prepared and used as probes to assess the expression of these genes during microsporogenesis and development of the gametophyte in an inbred line of maize (Oh43). Our results demonstrate (1) that mRNA transcripts encoding the 18-kDa hsps are expressed and/or accumulate during microsporogenesis, and (2) that genes encoding two of the characterized 18-kDa hsps are expressed and/or accumulate independently, in a stage-specific manner during microsporogenesis. These observations imply that the stage-specific expression of particular 18-kDa hsp genes results from gene-specific regulation during microsporogenesis and gametophyte development rather than from an overall activation of the heat shock or stress response.

Key words: Heat shock protein, maize, microsporogenesis, gametogenesis

INTRODUCTION

All organisms possess genes whose expression is up-regulated by heat shock/stress and code for a group of proteins referred to as the heat shock proteins (hsps) [Atkinson and Walden, 1985; Lindquist and Craig, 1988; Vierling, 1991]. In plants as well as other organisms, these proteins range in size from 15 to >100 kDa and are thought to serve protective functions against damages imposed by various types of environmental stress, including heat [Berger and Woodward, 1983; Key *et al.*, 1985; Landry *et al.*, 1989; Lin *et al.*, 1984; Nover *et al.*, 1989; Schlessinger *et al.*, 1991]. Unlike animal hsps, the majority of hsps synthesized in plants appear to consist of a complex group of low molecular weight proteins with M_r of 15,000–30,000 [Key *et al.*, 1985; Raschke *et al.*, 1988]. Although the size and complexity of the low molecular weight hsps vary among plant species, proteins of approximately 18 kDa comprise the most prominent hsp class in maize [Atkinson *et al.*, 1989; Baszczynski *et al.*, 1982; Goping *et al.*, 1991] and, much like their counterparts in other organisms [Lindquist, 1986; Nagao, 1985; Southgate, 1985], are considered to be encoded from a multigene family.

An increasing amount of evidence suggests that in addition to serving a putative protective function during heat stress, specific hsps and/or specific members of particular hsp families may also serve important and, perhaps, common functions during normal development in a variety of different organisms and tissues [see reviews in Bond and Schlessinger, 1987; Nover and Hightower, 1991]. Although the expression of genes encoding the high molecular weight hsps has been reported in a variety of different developmental processes, it is becoming apparent from studies in a number of evolutionarily divergent eukaryotes that the expression and/or accumulation of mRNAs encoding specific members of the small hsp families quite often accompanies gametogenesis [Kurtz et al., 1986; Pauli *et al.*, 1990; Frappier *et al.*, 1991; Dietrich *et al.*, 1991].

In previous reports, we characterized the genetic information encoding two members of the small (18-kDa) hsp family in an inbred line of maize (cv. Oh43) and, as a result of some preliminary observations, suggested

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that the expression of these 18-kDa hsp genes changes during microsporogenesis [Goping *et al.*, 1991; Frappier *et al.*, 1991]. In this communication, we characterize the genetic information encoding a third member of the 18-kDa hsp family from the same maize inbred and demonstrate differences in the expression of the 18-kDa hsp genes in maize microsporogenic tissue. Our results indicate that each of the 18 kDa genes we characterized is expressed independently during microsporogenesis, and that their expression in the male gametophyte results from gene-specific regulation rather than from an overall activation of the heat shock or stress response.

MATERIALS AND METHODS

Growth and Treatment of Maize Seedling and Preparation of Premeiotic Tassels, Staged Anthers, and Pollen From Nursery-Grown Maize (Oh43)

Dry seeds of *Zea mays* L. (Oh43) were treated with a fungicide (Vitaflo; Uniroyal Chemical, Aylmer, Ontario, Canada) and were germinated in the dark on moistened filter paper at 27°C for 5–6 days. The intact, etiolated seedlings were maintained at 27°C or subjected to a 1-h incubation at 42.5°C (heat shock). After 1 h, radicles and plumules were excised from the seedlings, and total RNA [Puissant and Houdebine, 1990] was isolated from them. In other cases, the seedlings were maintained at 27°C or 42.5°C and labeled for an additional 2 h with 100 μCi/ml (1 Ci = 37 GBq) of L-[^{35}S]methionine (Dupont Canada, Mississauga, Ont., Canada; specific activity 1042 Ci/mmol); subsequently, the radicles and plumules were excised and their proteins extracted [Baszczynski *et al.*, 1982].

Premeiotic tassels and tassels for another isolation were harvested early in the morning (before 9:30 AM) from nursery grown maize (Oh43). Premeiotic tassels (~3–4 cm) and shed pollen were collected and frozen immediately in liquid nitrogen. Tassels for another isolation were dissected out in the field and kept on ice until they could be staged; "stage" of anthers being defined in terms of microspore development and/or stage of meiosis [Chang and Neuffer, 1989] within the anther. In most cases, anther stage was first assessed by the allometric relationship [Bouchard and Walden, 1990] between anther length and established developmental stage; for every tassel used, a cytological check [Chang and Neuffer, 1989] of anther stage from acetoorcein squashes was also done. The range of microsporocyte stages found within the anthers used for a particular preparation are designated in the Results section. Sorting of anthers was done under a dissecting microscope, and, in order to ensure that the tissues were not stressed, the base of the microscope was kept in a bed of crushed ice. The stage-sorted anthers were then placed in 1.5-ml microcentrifuge tubes, frozen in liquid nitrogen, and stored at −70°C until used.

Construction and Screening of a cDNA Library Prepared From Poly(A)$^+$ RNAs Isolated From Premeiotic Tassels, Stages Anthers and Fresh Pollen of Maize (Oh43)

To produce a cDNA library with a high probability of including clones representing transcripts made during all stages of microsporogenesis and gametophyte development, large-scale total RNA preparations were made [Davis *et al.*, 1986] from pooled staged material of inbred Oh43. All the RNA preparations were pooled and a single batch of poly(A)$^+$ RNA was isolated, using PolyATract mRNA isolation system (Promega Corp., Madison, WI) for use in construction of the library. This maize cDNA library was constructed in the *Eco*RI/*Xho*I sites of a Uni-Zap XR vector (Stratagene, LaJolla, CA).

The maize cDNA library was screened [Maniatis *et al.*, 1982] with a 0.342-kb *Pst*I/*Spe*I DNA fragment excised from the open reading frame of a maize (Oh43) cDNA encoding an hsp 18 mRNA [cMHSP18–9; Goping *et al.*, 1991]. This DNA fragment is shown, through hybrid selection (see Fig. 4), to share homology with at least nine different members of the 18 kDa hsp family in this inbred, and, hereafter is referred to as scMHSP18-9-2. The purified restriction fragment, used as a probe, was radioactively labeled with [α-^{32}P]-dCTP (Dupont Canada; sp. act. 3,00 Ci/mmol) to a specific activity of 1×10^9 cpm/μg using a T_7 Polymerase Quickprime Kit (Pharmacia Fine Chemical, Piscataway, NJ), and the labelled DNA was separated from unicorporated nucleotides using Pharmacia Nick-Columns developed in 1× NTE (0.1 M NaCl, 10 mM Tris, and 1 mM EDTA, pH 8.0). The library was screened in a solution containing 50% formamide, 5× SSPE (1× = 0.18 M NaCl, 0.01 M Na_2 HPO_4, 1 mM EDTA), and 0.1% sodium dodecyl sulfate (SDS) at 42°C. The final stringency wash was done in 1× SSC (0.15 M NaCl, 0.015 M Na citrate) and 0.1% SDS at 55°C. Selected clones were purified by two or more rounds of screening, and the Bluescript II plasmid was subcloned into XL1-Blue cells (Stratagene).

Construction and Screening of a Maize (Oh43) Genomic Library

A maize (cv. OH43) genomic DNA library, consisting of 4.0×10^6 independent recombinant phage, was constructed in the *Xho*I site of a λFIX II vector (Stratagene). The genomic library was screened [Maniatis *et al.*, 1982] with scMHSP18-9-2, the same DNA fragment used for screening the cDNA library. Hybridizations and washes were performed as described for the cDNA library. A number of positive clones were detected, and two of them, with inserts of approximately 12.2 and 24.5 kb, were selected for further study. The Not I cassette in λFIX II, containing insert and flanking T3 and T7 promoter vector sequences, was excised, cut with various restriction enzymes, and the resultant

fragments were separated on a 1.0% agarose gel and vacuum blotted, using a LKB 2016 Vacugene vacuum blotter (LKB Instruments, Rockville, MD), onto a Zetaprobe membrane (BioRad, Richmond, CA). The DNA on the blots was UV-crosslinked to the membranes, using a Stratagene 1800 UV-Stratalinker (Stratagene), and hybridized with the same [α-^{32}P]-dCTP-labeled DNA fragment used for screening the library, and sequentially with [α-^{32}P]-dCTP-labeled DNA fragments *specific* for each of three different 18-kDa hsps from this inbred; namely, a 0.235-kb *SalI*/*Eco*RI DNA fragment from the 3′ untranslated region (UTR) of cMHSP18-9 [Goping *et al.*, 1991], a 0.139-kb *Hind*III/*Eco*RI DNA fragment from the 3′ UTR of cMHSP18-3 [Goping *et al.*, 1991], and a 0.242-kb *Bst*XI/*Xho*I DNA fragment from the 3′ UTR of cMHSP18-1 (described in this report). Two genomic DNA fragments were selected and subcloned into a pBluescript II SK$^-$ cloning vector (Strategene). One of the genomic DNA fragments, a 6.6-kb *Xba*I/*Xba*I fragment from the 12.2-kb genomic clone, hybridized with the probe specific for cMHSP18-1 and was designated as gMHSP18-1, and the other fragment, a 5.5-kb *Xba*I/*Xba*I fragment from the 24.5-kb genomic clone, hybridized with the probe specific for cMHSP18-3 and was designated as gMHSP18-3.

Sequencing of Maize cDNA and Genomic DNA Clones

The inserts in the cDNA and genomic clones were sequenced by a modification of the dideoxy chain termination method [Chan and Seeberg, 1985] using [^{35}S]-dATP (Dupont Canada; specific activity 600 Ci/mmol) and a Sequenase Version 2 Kit (United States Biochemical Corporation, Cleveland, OH). Universal T_3 and T_7 and/or SK and KS primers were initially used to sequence from the Bluescript vector into either end of the cloned DNA inserts. Once the nucleotide sequences in the external regions of the DNA inserts were established, specific, 17-oligomer primers were prepared for the established sequences (VetroGen, London, Ontario, Canada) and used to sequence the internal regions of the DNA inserts. For complete sequencing of the DNA inserts in both directions, DNA fragments were generated by restriction endonuclease digestion of the cloned DNA, and the excised fragments were subcloned into the appropriate cloning sites in a pBluescript II SK$^-$ vector (Strategene). Universal SK and KS primers, as well as specific internal primers were prepared (VetroGen) and used for full sequencing of the DNA fragments in both directions.

Southern Analysis

DNA was isolated from maize plumules [Dellaporta *et al.*, 1983] and digested with *Bam*HI, *Hind*III, and *Eco*RI. The fragmented DNA was separated on a 1% agarose gel, transferred onto a Zetaprobe membrane by capillary transfer [Southern, 1975], and UV-crosslinked using a UV Stratalinker (Stratagene). The membrane, containing the transferred DNA, was hybridized with scMHSP18-9-2, washed, and sequentially hybridized with the [α-^{32}P]-dCTP-labeled DNA fragments (as described above) specific for each of the sequenced maize 18-kDa hsps (i.e., cMHSP18-9, cMHSP18-3 and cMHSP18-1). In each case, the membrane was prehybridized in a solution containing 10% formamide, 3× SSPE, 1.0% SDS, 0.5% Blotto [Carnation nonfat dry milk in diethyl pyrocarbonate (DEPC)-treated water] and 0.5 mg/ml of sheared salmon sperm DNA for 4 h at 55°C. Hybridization was performed in a similar solution containing 10% dextran sulfate and the [α-^{32}P]-dCTP-labeled probe for 24 h at 55°C. The DNA blots were washed four times at room temperature in 2, 2, 0.5, and 0.1× SSC containing 0.1% SDS (15 min per wash) and subsequently, washed for 30 min at 55°C in 0.1× SSC containing 1% SDS. The washed blots were exposed to preflashed Kodak XR-OMAT film (Eastman Kodak Co., Rochester, NY) with an intensifying screen at −70°C.

Northern and Dot-Blot Analyses

Aliquots of total RNA and/or poly(A)$^+$ RNA extracted from tissues of nursery-grown corn and from radicles and plumules of control and heat-shocked maize seedlings, were size-fractionated by electrophoresis on 1% agarose gels and transferred to Zetaprobe GT membranes (BioRad). Dot-blots were prepared [Sambrook *et al.*, 1989] from the same samples with a dot-blotting apparatus (BioRad); autoradiograms of the dot-blots were quantitated by laser densitometry using a LKB Ultrascan XL Laser Densitometer (LKB Instruments). The blots were hybridized with a [α-^{32}P]-dCTP-labeled DNA fragment, scMHSP18-9-2, which is common to cMHSP18-1, cMHSP18-3, and cMHSP18-9, as well as with DNA fragments, which, as described above, are specific for each of these maize 18-kDa hsp cDNAs. Hybridizations were performed in a Robbins model 310 Incubator (Robbins Scientific Corp., Sunnyvale, CA) using a prehybridization solution containing 50% deionized formamide, 1.5× SSPE, 1% SDS, 0.5% Blotto, and 500 μg/ml herring sperm DNA, and a hybridization solution made up of the prehybridization solution, 10% dextran sulfate, and the heat-denatured probe at 55°C. Following hybridization, the blots were washed as described for Southern hybridizations.

Hybrid Selection and In Vitro Translation

mRNAs were hybrid-selected [Atkinson *et al.*, 1989; Goping *et al.*, 1991] from poly(A)$^+$ RNAs isolated from total RNA of radicles from heat-shocked maize seedlings and used to direct the synthesis of polypeptides in a rabbit reticulocyte lysate cell-free translation system (Dupont Canada) in the presence of L-[^{35}S]methionine (1042 Ci/mmol) according to the directions of the supplier. The translation reaction was terminated after 1 h

at 37°C, and the radioactivity incorporated was measured as described previously [Baszczynski *et al.*, 1982].

In Vitro Transcription and Translation of the Inserts in the hsp 18 cDNAs

The T_3 and T_7 promoter binding sites in the pBluescript vector were used to synthesize RNA transcripts from the cDNAs in cMHSP18-1, cMHSP18-3 and cMHSP18-9. In each case, the plasmids were linearized with restriction enzymes whose sites are confined to the region of the pBluescript vector attached to the 3′ end of the inserted DNA. The linearized plasmids were transcribed with T_3 or T_7 polymerases (depending on the orientations of the insert) according to the directions from the supplier (Stratagene). Each transcription reaction was translated in a cell-free rabbit reticulocyte lysate system in the presence of L-[^{35}S]methionine. The radioactivity incorporated was determined, translation products were electrophoretically separated on two-dimensional (IEF/SDS) polyacrylamide gels, and the radiolabeled proteins were visualized by fluorography.

Gel Electrophoresis and Fluorography of Maize Proteins

Proteins synthesized in vivo as well as those synthesized in a cell-free system were separated by both one- and two-dimensional (IEF/SDS) polyacrylamide gel electrophoresis and visualized by fluorography [Atkinson *et al.*, 1989; Baszczynski *et al.*, 1982]. The isoelectric focusing (IEF) gels and dilution buffer contained 2% ampholines (LKB Instruments) made up of 2 parts pH 3.5–10 and 3 parts pH 5–8 ampholines. The SDS-separating gels were composed of a 7.5–17.5% linear gradient of polyacrylamide. Marker proteins (Pharmacia) of known molecular mass (M_r) were electrophoretically separated on the same gels as the polypeptides.

Computer Analysis

Characterization of the cloned DNA inserts was accomplished using DNA Strider Version 1.0 (Marck, C., Service de Biochimie-Département de Biologie. Institut de Recherche Fondamentale, Commissariat a l'Energie Atomique-France), and DNA Inspector II$^+$ (Textco, West Lebanon, NH) software. The EMBL and GenBank databases were searched using the Wisconsin Genetics Computer Group sequence analysis software package Version 6.1 [Devereux *et al.*, 1984]

RESULTS

Isolation and Characterization of a cDNA From a Maize Microsporogenic cDNA Library Which Shows Sequence Homology With Sequences Encoding the 18-kDa hsps in Maize

A maize cDNA library, prepared from poly(A)$^+$ RNAs isolated from maize (Oh43) microsporogenic tissue, was screened with a nucleotide fragment from the open reading frame (ORF) of a cDNA (cMHSP18-9) previously shown to encode an 18-kDa hsp in this same maize inbred [Goping *et al.*, 1991]. The DNA fragment used for screening this library contains a nucleotide sequence that shares homology with a sequence in the ORF of another characterized maize 18-kDa hsp (cMHSP18-3; Goping *et al.*, 1991) and is common, as shown by hybrid selection (see Fig. 4), to a sequence found in the mRNAs of at least nine different maize 18-kDa hsps. Screening the library with this nucleotide fragment resulted in the selection and isolation of a cDNA clone designated as cMHSP18-1. This cDNA was sequenced at least twice from both strands and found to consist of 780 nucleotides (Fig. 1). The longest ORF contains 465 nucleotides, translates into a protein of 154 amino acid residues, and is terminated by a TGA codon. The ORF is flanked by a 5′ untranslated region (UTR) of 58 nucleotides, and a 3′ UTR of 257 nucleotides. The 3′ UTR of cMHSP18-1 terminates in a string of adenine residues (>40) and contains a complete animal-like polyadenylation signal, AATAAA, which begins 21 nucleotides upstream from the polyadenylated site.

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   1 cgaattaatccccaatcaacacagcaagtcagcaacaagcaaagcagcga
  51 cccgagagATGGACGCGAGGATGTTCGGGCTGGAGACCCCCCGGGTGGCG
 101 GCGCTGCATCACCTGCTGGACGTGCCCGACGGCGACAAGGCGGGCGGCGG
 151 CGCCACGCGCACCTACGTCCGCGACGCGCGCGCCATGGCGGCCACCCCGG
 201 CCGACGTCAAGGAGCTCGCGGGAGCGTACGCGTTCGTGGTGGACATGCCG
 251 GGGCTGAGCACGGGCGACATCAGGGTGCAGGTGGAGGACGAGCGGGTGCT
 301 GGTGATCAGCGGCGAGCGCCGCCGGGAGGAGCGCGAGGACGCCAAGTACC
 351 TGCGCATGGAGCGGCGGATGGGCAAGTTCATGCGCAAGTTCGTGCTGCCG
 401 GACAACGCCGACGTGGACAAGGTCGCCGCCGTGTGCAGGGACGGCGTGCT
 451 CACGGTGACCGTCGAGAAGCTGCCCCCGCCGGAGCCCAAGAAGCCCAAGA
 501 CCATCGAGATTAAGGTCGCCTGAggggagaccgttgtcggagcagtgcca
          BstXI
 551 gtgagctgggtggcaacctgcgggactctggtgaggaagagcaaatggta
 601 gaaagcagtagctgtgcttctgaacattgtgtgtgtttgtttcccttgtt
 651 accgccaatcatcctacctgatgtgtcgtctgggggtttcgtttgaaccat
 701 gctgggtttggagctttgtgatgcaaagcagaatgtcctgatgcttttctt
                                                  XhoI
 751 tctccatagaataaatctctcctgttacctaaaaaaaaaa...
```

Fig. 1. Nucleotide sequence for the cDNA in cMHSP18-1. Numbers to the left refer to the nucleotide locations with respect to the 5′-end of the cDNA insert. The 5′- and 3′-untranslated regions (UTRs) are indicated in lower case letters and the ORF is shown in uppercase letters. The translational start (ATG) and stop (TGA) codons are shown in boldface type, and an internal, in-frame ATG codon is underlined. A putative polyadenylation signal sequence is underlined in the 3′ UTR. The enzyme restriction sites used for preparing a 3′ UTR nucleotide fragment specific for cMHSP18-1 are indicated.

A comparison of the nucleotide sequence in cMHSP18-1 with the nucleotide sequences reported [Goping *et al.*, 1991] to encode two 18-kDa hsps in this same maize inbred reveals that the 3′ and 5′ UTRs of cMHSP18-1 share little identity (34–54%) with the nucle-

otide sequences found in the 3′ and 5′ UTRs of the cDNAs (cMHSP18-3 and cMHSP18-9) encoding the previously characterized maize 18-kDa hsps. However, the nucleotide sequence in the putative ORF of cMHSP18-1 appears to share 94.6% identity with the ORF sequence found in both maize 18 kDa hsp cDNAs (Fig. 2A). Analyses of the amino acid sequence derived from the putative ORF of cMHSP18-1 (shown in Fig. 2B) predict that the protein product has a relative molecular mass (M_r) of 17,027 and an isoelectric point (pI) of 8.4. Although the M_r and pI predicted for the protein derived from the ORF of cMHSP18-1 results in a protein slightly smaller and much more basic than the 18-kDa hsps derived from cMHSP18-3 (M_r = 17,779 and pI = 5.19) or from cMHSP18-9 (M_r = 17,491 and pI = 5.47), the derived amino acid sequence of cMHSP18-1 shares 93% identity with the one derived from cMHSP18-3 and 89% identity with the one derived from cMHSP18-9 (Fig. 2B).

Northern Hybridization Analyses Reveal That mRNA Transcripts With Sequences Corresponding to cMHSP18-1 Accumulate in Tissues From Heat-Shocked Maize Seedlings

An assessment of the size and relative amounts of the mRNAs with sequences corresponding to cMHSP18-1 in radicles and plumules from control (25°C) and heat-shocked (42.5°C) maize (Oh43) seedlings is shown in Figure 3. These Northern blot hybridizations demonstrate that radicles and plumules from heat-shocked seedlings accumulate enhanced levels of mRNA transcripts with sequences which hybridize with a nucleotide fragment specific for cMHSP18-1 (a 0.242-kb *Bst*XI/*Xho*I DNA fragment from the 3′ UTR of cMHSP18-1), as well as with a subcloned nucleotide fragment from the ORF of cMHSP18-9 (cMHSP 18-9-2), which shares identity with sequences in the ORF of cMHSP18-1 and cMHSP18-3, and a 4.0-kb *Eco*RI/*Bam*HI nucleotide fragment encoding the maize hsp 70 gene [pMON9502; provided by D.E. Rochester, as described by Shah *et al.*, 1985]. For comparison, and as a means of establishing a quantitative control for the relative amounts of mRNAs available to hybridize with the ^{32}P-labeled cDNA probes, the same blots were hybridized with a 9.0-kb *Eco*RI nucleotide fragment encoding the 26S, 17S, and 5.8S transcribed regions of maize ribosomal RNA [McMullen *et al.*, 1986]. These Northern blot hybridization results demonstrate that cMHSP18-1 encodes an RNA which, like the maize hsp 70 and the previously reported maize 18-kDa hsps accumulates during heat shock.

Transcription/Translation and Hybrid Selection Analyses Identify the 18-kDa hsp Encoded by c/g MHSP18-1

In order to establish which member, if any, of the maize 18-kDa hsp family is encoded by cMHSP18-1, the cloned DNA was transcribed in vitro, using T_3 polymerase and its product translated in a cell-free system containing [^{35}S]methionine. The ^{35}S-labeled protein, resulting from the transcription and translation of cMHSP18-1, was separated by two-dimensional (IEF/SDS) gel electrophoresis, and its mobility (Fig. 4D) was compared with the two-dimensional gel electrophoretic mobility of proteins synthesized by poly(A)$^+$ RNAs isolated from plumules of heat-shocked maize seedlings (Fig. 4A), as well as with the proteins translated from the transcripts of cMHSP18-3 and cMHSP18-9 (not shown but refer to Goping *et al.*, 1991). The results from these comparisons demonstrate that the electrophoretic mobility of the protein encoded from cMHSP18-1 is different from the proteins synthesized from either cMHSP18-3 or cMHSP18-9, but its mobility corresponds to the electrophoretic mobility of one of the other maize 18-kDa hsps (denoted with a double arrow in Fig. 4A). To substantiate that cMHSP18-1 encodes this particular 18-kDa hsp, poly(A)$^+$ RNAs were isolated from the plumules of control and heat-shocked maize seedlings and hybrid-selected with the nucleotide fragment specific for the 3′ UTR of cMHSP18-1 as well as with the ORF nucleotide fragment common to a sequence in cMHSP18-1, cMHSP18-3, and cMHSP18-9. The hybrid-selected mRNAs were translated in a cell-free system containing [^{35}S]methionine, and the proteins were separated by two-dimensional (IEF/SDS) gel electrophoresis. Hybrid selection of the mRNAs from plumules of *control* seedlings did not result in the detectable synthesis of any proteins (not shown). However, when mRNAs from plumules of *heat-shocked* seedlings were hybrid-selected with the common ORF nucleotide fragment, at least nine members of the 18-kDa hsp family were synthesized (Fig. 4B), and when they were hybrid-selected with a nucleotide fragment specific for cMHSP18-1, a single protein was synthesized and its mobility (Fig. 4C) corresponded with the one synthesized by cMHSP18-1. These results support the contention that cMHSP18-1 encodes information for an 18-kDa hsp protein, and that the two-dimensional electrophoretic mobility of that protein corresponds to the electrophoretic mobility of one of the 18-kDa hsps synthesized by the mRNAs in plumules from heat-shocked maize seedlings.

Isolation and Characterization of the Genes Encoding 18-kDa hsps Corresponding to the Sequences in cMHSP18-1 and in cMHSP18-3

To further characterize the genetic information encoding the 18-kDa hsp synthesized from cMHSP18-1, we screened (see Materials and Methods) our genomic library for clones with sequences corresponding to the highly conserved nucleotide sequence from the ORF of cMHSP18-9, and, subsequently, with nucleotide fragments specific for cMHSP18-1, −3, or −9. Although we were unable to obtain a genomic sequence corresponding to that found in a cMHSP18-9, we successfully obtained genomic clones with sequences corresponding to

A

```
+1    ATGGACGCGA  GGATGTTCGG  GCTGGAGACC  CCCCGGGTGG  CGGCGCTGCA       cMHSP18-1
+1    -------G--  ----------  T--C------  ----T-A---  T---------       cMHSP18-9
+1    ---------G  T---------  ---------T  ----T-A---  ----------      cMHSP18-3

+51   TCACCTGCTG  GACGTGCCCG  ACGGCGAC**  **********  *********A
+51   G-----T---  ----------  --------GC  CGGCGCGGGC  GGCGAC***-
+51   G---------  ----------  --------GC  CGGCGCGGGT  GGCGACAAC-

+80   AGGCGGGCGG  CGGCG*****  *CCACGCGCA  CCTACGTCCG  CGACGCGCGC
+98   ----------  -----GGC**  *---------  --------GC  ----------
+101  --A-----A-  -----GCAGC  G---------  ----------  ----------

+124  GCCATGGCGG  CCACCCCGGC  CGACGTCAAG  GAGCTCGCGG  GAGCGTACGC
+145  ----------  T---------  ----------  ------C---  -C--------
+151  ----------  ----------  ------G---  -----GC-C-  -C--------

+174  GTTCGTGGTG  GACATGCCGG  GGCTGAGCAC  GGGCGACATC  AGGGTGCAGG
+195  ----------  ----------  -----G----  ----------  -A--------
+201  ----------  ----------  -----G----  ----------  C---------

+224  TGGAGGACGA  GCGGGTGCTG  GTGATCAGCG  GCGAGCGCCG  CCGGGAGGAG
+245  ----------  ----------  ----------  -------G--  ----------
+251  ----------  ----------  ---G------  -------G--  ----------

+274  CGCGAG***G  ACGCCAAGTA  CCTGCGCATG  GAGCGGCGGA  TGGGCAAGTT
+295  ------***-  ----------  ----------  ----------  ----------
+301  ------GAC-  ----------  ----------  ----------  ----------

+321  CATGCGCAAG  TTCGTGCTGC  CGGACAACGC  CGACGTGGAC  AAGGTCGCCG
+342  ----------  ----------  ----------  ----A-----  ---A--T-G-
+351  ----------  ----------  ----------  ------C---  ----------

+371  CCGTGTGCAG  GGACGGCGTG  CTCACGGTGA  CCGTCGAGAA  GCTGCCCCCG
+392  -G--------  ----------  -----C----  ----------  ----------
+401  ----------  ----------  ----------  ----T-----  ----------

+421  CCGGAGCCCA  AGAAGCCCAA  GACCATCGAG  ATTAAGGTCG   CCTGA +465nt; cMHSP18-1
+442  --C-------  ----------  ----------  G-C-------   ----- +486nt; cMHSP18-9
+451  ----------  ----------  ----------  --C-------   ----- +495nt; cMHSP18-3
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B

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MDARMFGLET  PRVAALHHLL  DVPDGD****  ***KAGGG**  ATRTYVRDAR
--G-------  -LMY--Q---  ------AGAG  GD*-----G*  P-----A---
---V------  -LM---Q---  ------AGAG  GDN-T-S-GS  ----------

AMAATPADVK  ELAGAYAFVV  DMPGLSTGDI  RVQVEDERVL  VISGERRREE
---Y-R----  --P-------  -----G----  K---------  ----------
----------  --P-------  -----G----  ----------  -Y--------

RED*AKYLRM  ERRMGKFMRK  FVLPDNADVD  KVAAVCRDGV  LTVTVEKLPP
---*------  ----------  --------M-  -IS-------  ----------
---D------  ----------  ----------  ----------  ----------

PEPKKPKTIE  IKVA  154 amino acids ;  cMHSP18-1
----------  V---  161 amino acids ;  cMHSP18-9
----------  V---  164 amino acids ;  cMHSP18-3
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Fig. 2. Nucleotide (**A**) and deduced amino acid (**B**) sequences in the open reading frame of cMHSP18-1 compared with those reported for two other 18 kDa hsp mRNAs in this maize inbred; cMHSP 18-9 and cMHSP 18-3 [Goping *et al.*, 1991]. In the nucleotide and amino acid sequences, dashes indicate identity, and the asterisks, between adjacent nucleotides or amino acids, indicate insertions or deletions to allow for maximal alignment.

cMHSP18-1 and cMHSP18-3 and designated them as gMHSP18-1 and gMHSP18-3, respectively.

Figure 5 shows only the sequence in gMHSP18-1 (Fig. 5A) and gMHSP18-3 (Fig. 5B), which is 300 nucleotides 5′ to the ATG start codon in each genomic clone (the sequences which are 3′ to the ATG start codon are longer than, but contain sequences exactly the same as those reported for the 3′ region of cMHSP18-1 (see Fig. 1) and for the 3′ region of cMHSP18-3 [Goping *et al.*, 1991]). The 5′ sequence of gMHSP18-1 (Fig. 5A) contains three paired (decanucleotide), exact heat shock element (HSE) consensus sequences (all within an area of 62 nucleotides) and two putative TATA boxes. One TATA box is located 44 nucleotides upstream from the most 5′ HSE and the other is located 18 nucleotides downstream from the most 3′ HSE. The 5′ sequence of gMHSP18-3 (Fig. 5B) also contains three paired, exact HSEs (all within an area of 129 nucleotides), but only

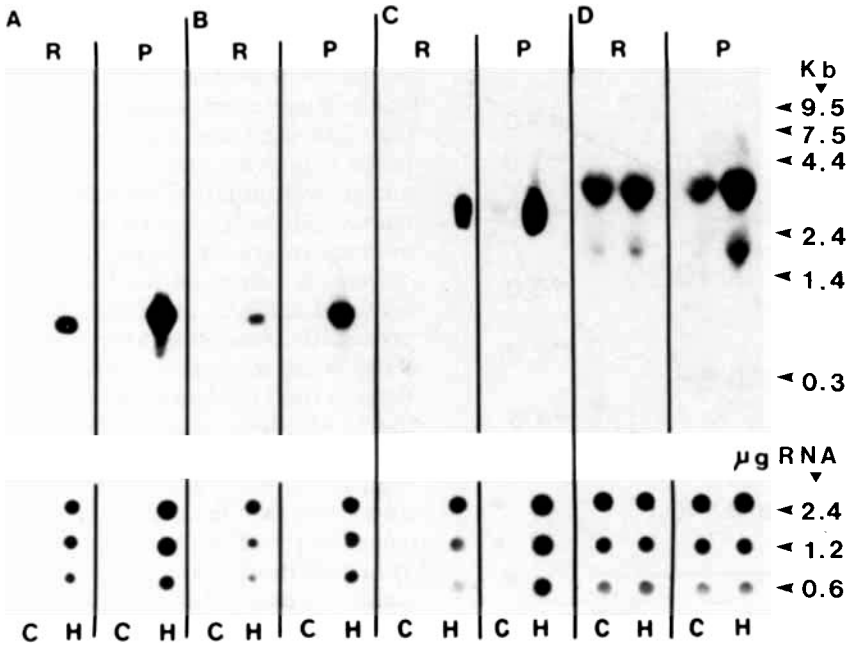

Fig. 3. Northern and dot-blot hybridization analyses of RNAs isolated from radicles (R) and plumules (P) of control (C) and heat-shocked (H) maize seedlings. The same blots (as well as duplicates) were hybridized with DNA fragments containing an ORF sequence shared by cMHSP18-1, −3, and −9 (**A**), a 3′ UTR sequence specific for cMHSP18-1 (**B**), a sequence encoding a maize hsp70 gene (**C**), and a sequence encoding the 26S, 17S, and 5.8S transcribed regions of maize rRNA (**D**). The size of coelectrophoresed standards and the amount of RNA used for the dot-blots are indicated.

one putative TATA box, located 27 nucleotides downstream from the most 3′ HSE.. A comparison of the genomic sequences for gMHSP18-1 and gMHSP18-3 with their corresponding cDNA sequences establishes that no introns are present in the genes encoding these two 18-kDa hsps. Characterization of the 5′ genomic sequence of gMHSP18-3 also clarifies a question regarding the first 194 nucleotides we reported in the 5′ end of cMHSP18-3. In our previous report [Goping *et al.*, 1991], we raised the possibility that the first 194 nucleotides in cMHSP18-3 might be the result of a cloning artifact. The genomic sequence of gMHSP18-3 establishes that the 5′-most 194 nucleotides detected in the cDNA are not constituents of gMHSP18-3 but, instead, are the result of a cDNA cloning artifact.

Southern blot hybridization analyses of maize (Oh43) genomic DNA digested with various restriction enzymes (Fig. 6) demonstrate the specificity of the 3′ UTR nucleotide fragments from cMHSP18-1, −3, and −9, and validate them for use as probes to assess the expression of specific 18-kDa hsp gene sequences. Aliquots of maize DNA were digested separately with *Hind*III, *Bam*HI, and *Eco*RI (enzymes for which no recognition sites were found in cMHSP18-1, -3, or -9), electrophoretically separated on a 1% agarose gel, blotted, and initially hybridized with a ^{32}P-labeled nucleotide fragment subcloned from the ORF region of cMHSP18-9 (cMHSP 18-9-2). Duplicate blots, as well as the same blot, were subsequently hybridized with ^{32}P-labeled nucleotide fragments from the 3′ UTRs of cMHSP18-1, −3, and −9 (see Materials and Methods)l The nucleotide fragment from the ORF recognized a number of nucleotide fragments from each enzyme digestion (Fig. 6A), and each of the 3′ UTR probes recognized nucleotide fragments specific for the enzyme used but different for each of the 3′ UTR sequences (Fig. 6B–D). These observations support the contention that the 3′ UTR nucleotide fragments excised from cDNAs encoding these 18-kDa hsps are unique enough to detect and assess the expression of specific 18-kDa hsp gene sequences.

Differential Expression of Maize 18-kDa hsp Genes During Microsporogenesis

The constitutive expression of the maize 18-kDa hsp genes was assessed in various tissues from nursery-grown maize by Northern blot hybridization analyses. In these pilot investigations (not shown), we used the ORF nucleotide fragment, which appears to recognize a number of members of the 18-kDa hsp gene family as a probe and found that microsporogenic tissue appeared to exhibit enhanced expression and/or an accumulation of the mRNAs encoding members of this hsp family. To assess and quantify our initial observations adequately, we prepared Northern (not shown) and dot blots (Fig. 7) of the RNAs isolated from premeiotic tas-

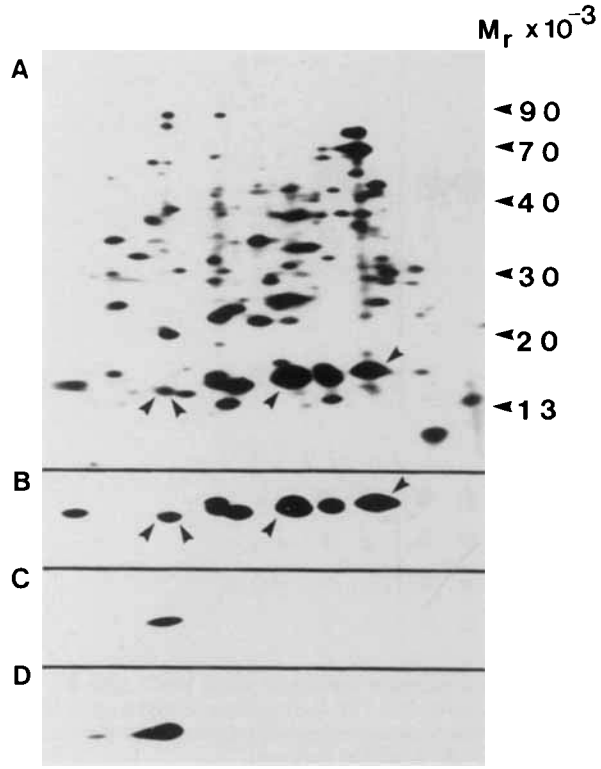

Fig. 4. Identification of the 18-kDa hsp synthesized from cMHSP18-1. **A:** Fluorogram from a two-dimensional gel (IEF/SDS) electrophoretic separation of the proteins synthesized from poly(A)$^+$ RNAs isolated from plumules of heat-shocked maize seedlings. **B,C:** Fluorograms from similar electrophoretic separations for the proteins synthesized by mRNAs hybrid-selected from the poly(A)$^+$ RNAs used in panel A. The common ORF DNA sequence and a 3′ UTR fragment specific for cMHSP18-1 were used for the hybrid selections shown in B and C, respectively. **D:** The two-dimensional gel electrophoretic mobility of a protein synthesized from cMHSP18-1 corresponds with one synthesized by both the total poly(A)$^+$ RNAs and the hybrid-selected mRNAs from the heat-shocked plumules. A,B: the 18-kDa hsp protein synthesized by cMHSP18-1 is identified by a double arrow, and the other characterized 18-kDa hsps are denoted by single arrows. Although the pictures for B–D have been trimmed to show only the relevant portions of the fluorograms, the M_r of the proteins were established in each case by coelectrophoresed M_r standards.

sels (lane 3), staged anthers [containing microsporocytes from mid-prophase to metaphase I or II (lane 4) and binucleate microspores (lane 5)] and shed pollen (lane 6), and hybridized them with a ^{32}P-labeled nucleotide fragment containing the common ORF sequence (Fig. 7A) and with ^{32}P-labeled 3′ UTR nucleotide fragments specific for cMHSP18-1 (Fig. 7B), cMHSP18-9 (Fig. 7C) and cMHSP18-3 (Fig. 7D). Preparations of RNAs isolated from plumules of control (lane 2) and heat-shocked (lane 1) maize seedlings were assessed and used as somatic controls, on the same blots. The autoradiograms from the dot blot hybridization analyses (shown in the upper portions of Fig. 7A–D) were scanned with a laser densitometer and the relative intensity (see figure legend for details) of the radioactivity in the dot-blots was determined (shown graphically in the lower portions of Fig. 7A–D). Visual inspection and relative quantification of the results from the dot blot hybridization analyses with the common ORF probe (Fig. 7A) reveal that an enhanced expression and/or accumulation of mRNA transcripts from the maize 18-kDa hsp genes occurs in anthers containing microsporocytes in stages ranging from mid-prophase of meiosis I through the binucleate gametophyte; the levels of mRNAs encoding the 18-kDa hsp genes in premeiotic tassels and shed pollen are no greater than those found in plumules from control (25°C) seedlings. Results from analyses using probes specific for each of the 18-kDa hsps (Fig. 7C–E) disclose that the cumulative expression of the 18-kDa hsp genes, as shown in Figure 7A, represents a stage-specific expression distinct for each of these three members of the 18-kDa hsp gene family. For example, while mRNA transcripts from g/cMHSP18-3 (Fig. 7E) do not accumulate significantly at any stage of microsporogenesis, the mRNA transcripts from g/cMHSP18-1 (Fig. 7C) are predominant early in microsporogenesis (mid-prophase through meiosis) and those from cMHSP18-9 (panel D) appear most abundant in the binucleate stage of the gametophyte. These observations, using gene-specific hsp probes, suggest that the expression of hsp gene transcripts throughout microsporogenesis, as detected with the common ORF probe, reflects the cumulative expression of specific 18-kDa hsp family members whose developmental timing and peak levels of accumulation are regulated independently.

DISCUSSION

When maize seedlings are subjected to a brief heat shock, most of their cells synthesize a spectrum of hsps [Atkinson *et al.*, 1989; Baszczynski *et al.*, 1982, 1983]. The most prominent hsps synthesized in maize tissues appear to consist of a complex group of low-molecular-weight proteins with M_r of approximately 18,000 [Atkinson *et al.*, 1989]. In an earlier study, using the inbred Oh43 of maize, we reported the nucleotide sequence in mRNAs encoding two different members of this 18-kDa hsp family [Goping *et al.*, 1991]. In the present study, we report the isolation and characterization of a cDNA, cMHSP18-1, encoding another member of this 18-kDa hsp family. In this case, cMHSP18-1 was obtained from a cDNA library prepared from poly(A)$^+$ RNAs isolated from a mixture of maize microsporogenic tissues. This cDNA is shown to encode a protein which has an M_r (17,027) and a pI (8.4) quite distinct from the other characterized members of this 18-kDa hsp family. A comparison of the nucleotide sequence in cMHSP18-1, with the nucleotide sequences encoding the two other 18-kDa hsps characterized in this same maize inbred [see Goping *et al.*, 1991] reveals

A

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-300 TTTATATTTATACTAACTTTAAACCTCGTGGCAACACACGGGCATATACC
-250 TAATCTATATAAAAAGACCCTACAAAGTAGTACAAAGACTCTAGTTTATT
-200 CTGGATTCTTCCGGAATCCCGACCATCCCACGGAGCCACCTTTCAGAACC
-150 TTCCTTCGCCTTCCAGAAGCCCACCCGTCACCACCATATAAACCGCCCCT
                                                  *
-100 GTTCGCGTCGGCATTCCCCACACCGAGAATCACAATACGAAACGAATTAA
 -50 TCCCCAATCAACACAGCAAGTCAGCAACAAGCAAAGCAGCGACCCGAGAG
   1 ATG
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B

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-300 AAGCACAGAGCCTTCTATTTATTCGGGACTCTTCCGGAATCCCGACCATC
-250 CCACACAGAGCCACGGACACGGCCGGGAGTCCGGGACGCCTACGCCTCGC
-200 CGCGCCCGGGGCCCCGCACACTCCACAGCCTTTCAGAACCTTCCGTGGCC
-150 TTCCAGAAGAACAGAAGCCCACCCGTCACCACCAATATAAACTGCCCCTC
-100 CGGATCGGCACTCCGCACACCAAGAATCACATCACAATTCACACAGCGAA
                  *
 -50 CCGAGAAACCAACACAGCAACAAGCAAAGCAGCGATCCGACATCCGAGAG
   1 ATG
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Fig. 5. Comparison of the distribution of heat shock elements in two different maize genes encoding distinctly different 18-kDa hsps. Since consensus sequences for HSEs were only detected relatively close to the putative transcriptional start site in the genes encoding these 18-kDa hsps (>800 nucleotides upstream from the putative transcriptional start site have been sequenced in both genes), only the sequence of the 300 nucleotides 5′ to the ATG start codon are shown for g/cMHSP18-1 (**A**) and g/cMHSP18-3 (**B**). In both gene sequences, the HSE consensus sequences are double underlined, the putative TATA boxes are underlined once, the nucleotide corresponding to the 5′-terminal nucleotide in the homologous cDNA clone has an asterisk over it, and the ATG codon is in bold type.

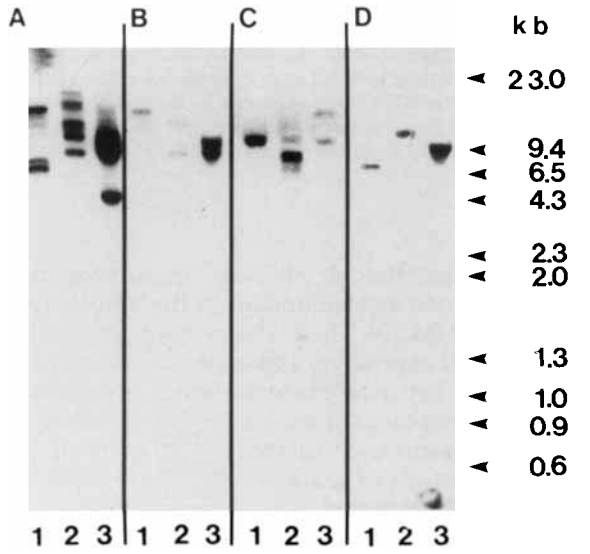

Fig. 6. Southern blot hybridization analyses of restriction enzyme-cleaved maize (Oh43) DNA. DNA, isolated from the plumules of maize seedlings, was digested with *Eco*RI (lane 1), *Bam*HI (lane 2), and *Hind*-III (lane 3), separated on a 1% agarose gel and blotted onto a Zetaprobe membrane. The DNA in the membrane was hybridized with a ^{32}P-labeled DNA sequence common to at least nine of the 18-kDa maize hsps (**A**) and, sequentially, with sequences specific for c/gMHSP-1 (**B**), c/gMHSP-3 (**C**), and cMHSP-9 (**D**) (see Materials and Methods for details). The relative sizes of the DNA fragments (kb; kilobases) were estimated from the mobility of coelectrophoresed DNA standards.

that the nucleotide sequence in the ORF of cMHSP18-1 shares 94.6% identity with the ORF sequences in the other two hsps, while the 3′ and 5′-UTR nucleotide sequences are quite different. The similarity of the ORF nucleotide sequences in these cDNAs enabled us to prepare a DNA fragment from the ORF sequence, for use as a probe, which shares high identity with, and, as shown by hybrid selection, is recognized by each of the mRNAs encoding these and other members of the 18-kDa hsp family. In addition, the dissimilarity of the 3′ UTR nucleotide sequences in these cDNAs enabled us to prepare DNA fragments from the 3′ UTRs of these cDNAs, for use as probes specific for each of the mRNAs encoding these particular 18-kDa hsps. The specificity of these 3′ UTR DNA fragments was confirmed by their ability to hybrid-select mRNAs encoding specific members of the 18-kDa hsp family, and by their ability to hybridize with different size fragments on Southern blots of maize genomic DNA which had been cut with the same restriction enzymes.

Through the use of these 3′ UTR DNA fragments, we were able to isolate clones from our maize genomic library, which contained sequences corresponding to those in cDNAs, cMHSP18-1 and cMHSP18-3, encoding two different members of the 18-kDa hsp family. A comparison of the genomic sequences with the cDNA sequences found for each of these 18-kDa hsps established that no intervening sequences (introns) are present in the genes encoding these two different 18-kDa hsps. Inspection of the genomic sequences reveals that the 5′ UTRs of both genes contain a putative TATA box preceded by three paired (decanucleotide) complete HSE consensus sequences and that only gMHSP18-1 contains a TATA box motif located upstream from the most 5′ HSE. The presence of these HSEs is consistent with the view that the expression of these sequences may be enhanced by heat shock [Xiano and Lis, 1988]. However, the presence of a putative TATA box motif upstream from the HSEs in gMHSP18-1 raises the possibility that the expression of this particular gene may also be influenced by other upstream regulatory sites that may rely on the upstream TATA

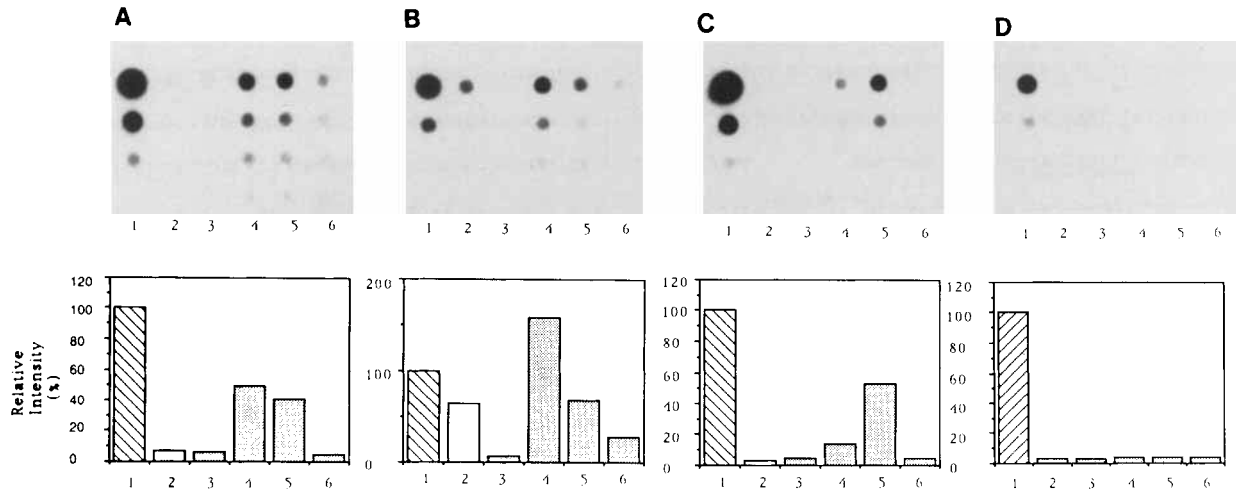

Fig. 7. Transcripts encoding different 18-kDa hsps in maize (Oh43) were expressed, and accumulated, in a gene-specific manner, at specific stages during microsporogenesis. A–D: upper portions show autoradiograms of RNA which was isolated from maize plumules of control and heat-shocked seedlings and from tassels at various stages of microsporogenesis, dot-blotted to Zetaprobe membranes, and hybridized with ^{32}P-labeled nucleotide sequences common to all of the 18-kDa hsp genes (**A**) and specific for cMHSP18-1 (**B**), cMHSP18-9 (**C**), and cMHSP18-3 (**D**). The RNAs used in the dot blots include RNA from heat-shocked plumules (1), control plumules (2), premeiotic tassel (3), midprophase I to microspore-containing anthers (4), binucleate microspore anthers (5), and fresh pollen (6). The amount of RNA used for the top row of dots was 5 μg; subsequent rows of dots represent dilutions by full-logs (1/10, 1/100, and 1/1000) for the plumule RNAs and half-logs (1/3, 1/10, and 1/30) for each of the microspore developmental stages. Lower portions of A–D are graphs, derived from laser densitometry scans, of the relative intensity of the radioactivity in the dot-blots shown in the upper portions. The signal intensities (relative intensity percent) in the graphs in A–C are quantitated relative to the 1/10 dilution of heat-shocked plumules as 100%; those in D are relative to the full-strength signal from heat-shocked plumules as 100%. Standard deviations for these reference heat-shock intensities were calculated from five separate determinations, and the coefficients of variation, expressed as percentages, are 1.5% (A), 0.9% (B), 1.7% (C), and 1.0% (D).

box [Kloeckener-Gruissem *et al.*, 1992] as an alternative or tissue-specific transcriptional start site.

Northern and dot blot hybridization analyses, using the 3′ UTR probes specific for the 18-kDa hsps encoded from cMHSP18-1, cMHSP18-3, and cMHSP18-9 demonstrate that varous somatic tissues from heat-shocked maize seedling accumulate and/or exhibit an enhanced expression of the mRNA transcripts recognized by these specific DNA fragments. Studies in our laboratory [Frappier *et al.*, 1991] and in other laboratories [Dietrich *et al.*, 1991] with maize, as well as with *Lilium* [Bouchard, 1990], have reported enhanced expression and/or an accumulation of 18-kDa hsp mRNAs in the male gametophyte in the absence of heat stress. In this communication, we substantiate our previous observations, and, through use of the 18-kDa hsp gene-specific probes, report that a stage-specific expression of mRNAs encoding distinct members of the 18-kDa hsp family occurs in the maize microsporocyte and gametophyte. Although mRNA transcripts encoded from one of the 18-kDa hsp genes (g/cMHSP18-3) do not appear to accumulate significantly at any stage of microsporogenesis, mRNA transcripts recognized by both g/cMHSP18-1 and cMHSP18-9 accumulate independently, in a stage-specific manner during microsporogenesis; mRNA transcripts recognized by g/cMHSP18-1 are predominant early in microsporogenesis (mid-prophase through meiosis); those recognized by cMHSP18-9 are most abundant in the binucleate stage of the gametophyte. These observations imply that the stage-specific expression and/or accumulation of particular 18-kDa hsp gene products, which is apparent during the development of male gametes in maize, results from gene-specific regulation rather than from an overall activation of the heat shock or stress response.

The studies reported in this communication clearly direct attention to the possibility that certain members of the 18-kDa hsp family in maize may have specific roles to play during the development of the male gametophyte and possibly male gametes in maize, as well as in other plants, such as *Lilium* [Bouchard, 1990]. Indeed, it is becoming apparent from studies in a number of evolutionarily divergent eukaryotes that gametogenesis is accompanied quite often by gene-specific expression and/or accumulation of mRNAs encoding specific members of the hsps, particularly from those in the small (16–30 kDa) hsp families. For example, mRNAs encoded by the sole known member of the small hsp gene family in *Saccharomyces* accumulate during sporulation and meiosis [Kurtz *et al.*, 1986], and mRNAs for specific members of the small hsp-gene family in *Drosophila* accumulate in spermatocytes (hsp 26) [Pauli *et al.*, 1990] as well as in the ovaries (hsp 27) during the meiotic interval [Zimmerman *et al.*, 1983].

While studies with the *Drosophila* hsp 26 and hsp 27 promoters have identified ovarian-, spermatocyte-, ecdysone-, and heat shock-specific regulatory sites [Cohen and Messelson, 1985; Glaser and Lis, 1990; Hoffman *et al.*, 1987], the study reported herein identifies only the heat shock regulatory sites in genes encoding the small, 18-kDa hsps of maize. Presumably, the promoter regions of the 18-kDa hsp family in maize contain, in addition to their HSE sites, regulatory sites controlling their stage-specific and/or tissue-specific induction during microsporogenesis. The identification of such regulatory sites in the maize 18-kDa hsp genes may help identify regulatory sequences shared by other genes active in meiosis and during the development of the gametophyte. Whatever the case, the finding that transcription and/or accumulation of specific small hsp mRNAs can be correlated with meiosis and gametogenesis in organisms from three eukaryotic kingdoms, implies that some small hsps may serve a specific and, perhaps, common function in the normal development of meiotic cells and/or in the gametes or gametophytes these produce.

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