The EMBO Journal vol.8 no.7 pp.1927 - 1934, 1989

Mutation in a heat-regulated hsp70 gene of *Ustilago maydis*

David W.Holden[1], James W.Kronstad[2] and Sally A.Leong

Department of Plant Pathology and Plant Disease Resistance Unit USDA-ARS, University of Wisconsin, Madison, WI 53706, USA

[1]Present address and to whom correspondence should be addressed: Genetics Division, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK
[2]Present address: Biotechnology Laboratory, The University of British Columbia, Vancouver, British Columbia V6T 1W5, Canada

Communicated by J.Fincham

Four genes (*ums1, ums2, ums3* and *ums4*) representing an hsp70-related gene family were isolated from a genomic library of *Ustilago maydis*. All four genes are transcriptionally active during normal growth. Following a heat shock, the mRNA levels of *ums1* and *ums2* increase by ~5-fold, whereas the *ums3* transcript becomes less abundant. The amount of *ums4* mRNA remains relatively unchanged after heat treatment. The nucleotide sequence of the 5′ non-coding and a portion of the *ums2* coding region was determined. The sequence encoding the first 90 amino acids is 73% identical to corresponding regions of the *Drosophila* and yeast (*SSA1*) hsp70 genes. To investigate the effect of a mutation in *ums2*, a plasmid was constructed in which most of the transcriptional unit of *ums2* was deleted and substituted with the *Escherichia coli* hygromycin B (hygB) phosphotransferase gene. Transcription of this gene is controlled by the *ums2* promoter, allowing the expression of hygB resistance in *Ustilago*. The marker was introduced into diploid cells as a linear sequence with termini homologous to the 5′ and 3′ flanking regions of *ums2*. In ~50% of transformants examined, one of the two wild-type *ums2* alleles had been replaced by the mutated sequence, demonstrating the feasibility of using one-step gene disruption to create heterozygous diploids in *Ustilago*. The *ums2/ums2*::hygB^r heterozygote produced teliospores after injection into corn plants, but only cells carrying functional *ums2* were found among their meiotic progeny. Therefore *ums2*::hygB^r segregates as a recessive lethal, which strongly suggests that *ums2* is essential for growth in *Ustilago*.

Key words: gene disruption/gene family/hsp70 mutation/ *Ustilago*

Introduction

The major heat-inducible protein of most eukaryotic cells, hsp70, is encoded by one or a small number of genes that form part of a larger multigene family. In *Drosophila*, a total of 13 hsp70-related genes have been identified (Craig *et al.*, 1987), and mammalian genomes contain, in addition to functional copies, numerous pseudogenes (Sorger and Pelham, 1987). Since some genes are active during normal growth and development (Milarski and Morimoto, 1986; Palter *et al.*, 1986) and are not necessarily heat inducible (Palter *et al.*, 1986; Sorger and Pelham, 1987), it has been assumed that they have functions unrelated to the stress response. Evidence for complex relationships between members of the *Saccharomyces cerevisiae* hsp70 gene family has come from detailed studies on their nucleotide sequences, regulation and mutant phenotypes. As a result of these studies, the eight genes isolated to date have been put into functionally distinct subgroups (Craig *et al.*, 1987). Strains with single mutations in any of the four genes of one subgroup appear wild-type, but one double mutant has reduced growth rates. Triple mutants are able to survive through compensation and, in one case, overproduction from the remaining functional gene (Craig and Jacobsen, 1984; Werner-Washburne *et al.*, 1987). Considered together, the four genes encode an essential subfamily of hsp70s. Recently it has been shown that these hsp70s are involved in the translocation of secreted and mitochondrial proteins across the endoplasmic reticulum and mitochondrial membranes (Chirico *et al.*, 1988; Deshaies *et al.*, 1988). A second group comprises two genes. Mutations in either one have no detectable phenotype, but cells carrying mutations in both genes are cold-sensitive (Craig and Jacobsen, 1985). A third group consists of one essential gene. No phenotypic effects of a mutation in the remaining gene have been observed (Craig *et al.*, 1987).

Analysis of defined mutations in hsp70 genes from other eukaryotes might help to establish (i) whether such genetically distinct subfamilies exist in other organisms; (ii) what relationships exist between these genes and thermotolerance; and (iii) the functions of those genes that are developmentally regulated. Attempts to make mutations in eukaryotic hsp70 genes by classical means have not been successful (Gausz *et al.*, 1981). To date, the use of *in vitro* mutagenesis and gene replacement to make preselected mutations in hsp70 genes has been confined to yeast.

We used an hsp70 gene promoter from *Ustilago maydis* to construct a selectable marker for transformation of this fungus (Wang *et al.*, 1988). We have now extended our analysis of the hsp70-related gene family, and describe here the cloning and expression of four of its members. *Ustilago* acquires thermotolerance following a heat shock, a response that is dependent on protein synthesis (Taylor and Holliday, 1984). It was therefore of interest to know whether the *Ustilago* hsp70 genes are required for thermotolerance. Recently it was shown that it is possible to apply gene replacement methods to *Ustilago* (Banks and Taylor, 1988; Kronstad and Leong, 1989). In this study, we obtained diploid transformants in which one chromosomal allele of a heat-regulated gene was replaced by a mutated sequence. Our failure to recover mutant basidiospore segregants from

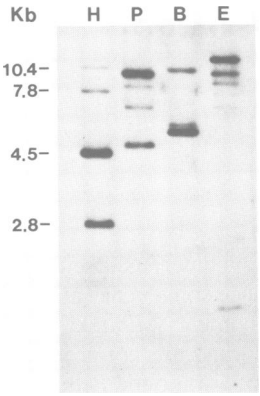

Fig. 1. Southern hybridization between *Ustilago* nuclear DNA and a yeast hsp70 gene. DNA samples (5 μg) were digested with the restriction enzymes *Hin*dIII, *Pst*I, *Bam*HI or *Eco*RI (**lanes H, P, B** and **E** respectively), electrophoresed through a 0.6% agarose gel and transferred to a nylon filter. The blot was hybridized under non-stringent conditions with a portion of the yeast hsp70 gene *SSA2*. The sizes in kb refer to the bands of hybridization in lane H only.

their progeny suggests that this hsp70 gene is essential for cellular growth.

Results

Cloning

To identify sequences in the *Ustilago* genome that are related to yeast and *Drosophila* hsp70 genes, *Ustilago* nuclear DNA was digested with a variety of restriction endonucleases, and analysed by Southern hybridization under non-stringent conditions with portions of the protein-coding regions of *Drosophila* and yeast hsp70 genes. The *Drosophila* probe gave an identical pattern of hybridization to that obtained with the yeast gene. Results of hybridization with the yeast gene are given in Figure 1. Depending on the restriction enzyme used, between four and six bands of hybridization were obtained.

A partial genomic library of *Ustilago* was created by cloning *Hin*dIII-generated DNA fragments of 4−5 kb into pUC12. Of 2000 clones that were screened by hybridization under non-stringent conditions to the yeast probe, one positive recombinant was detected. A restriction map of this clone [designated pUMS2H, referred to previously (Wang *et al.*, 1988) as pSL7] is shown in Figure 2. Regions of homology between the cloned DNA and the yeast gene were determined by digesting the cloned DNA with restriction enzymes, and probing Southern blots made from size-fractionated fragments (unpublished data). We then mapped the approximate locations of the putative 5′ and 3′ termini of the mRNA for this gene by S1 nuclease protection experiments (for details, see Materials and methods and Figure 2). A 0.8 kb *Bgl*II fragment from within the transcriptional unit was isolated from pUMS2H, and used as a probe to identify related sequences in a nuclear genomic cosmid library of *Ustilago*. Seven positive clones were obtained from a total of 3180 that were screened. DNA from the positive clones was digested with *Hin*dIII and analysed by Southern hybridization to the 0.8 kb *Bgl*II fragment of pUMS2H. In each of the seven cosmids, hybridization to a single *Hin*dIII fragment was observed. The sizes of these fragments were 4.5 kb for three clones, 10.4 kb for two

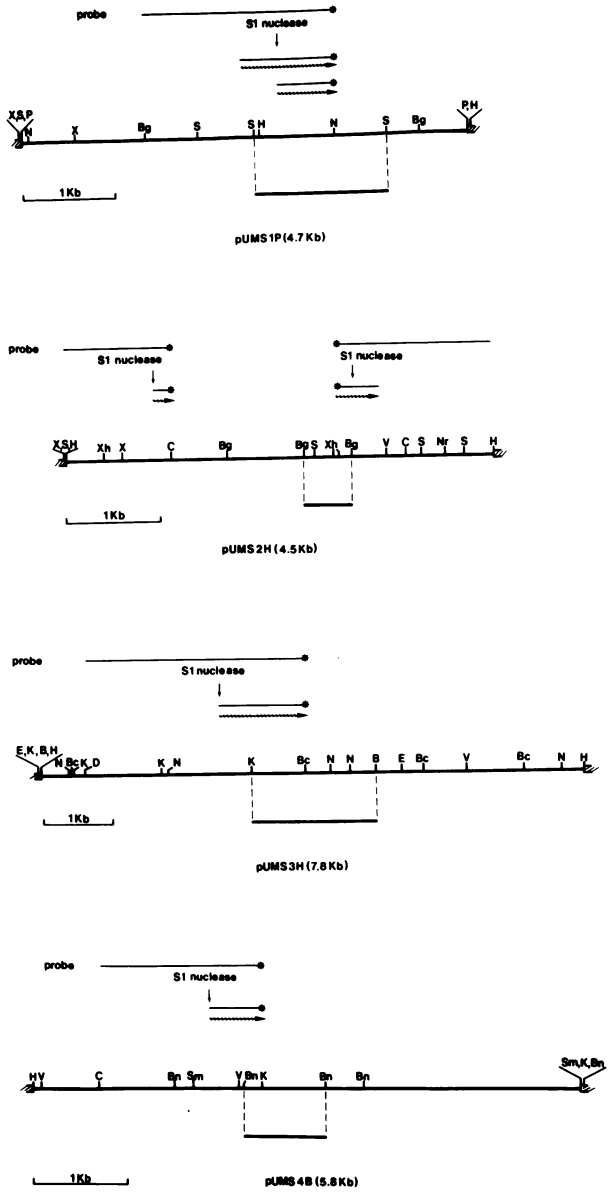

Fig. 2. Restriction maps of four clones (pUMSIP, 2H, 3H and 4B) that contain *Ustilago* hsp70-related genes. Restriction sites shown are: *Bam*HI (B), *Ban*II (Bn), *Bcl*I (Bc), *Bgl*II (Bg), *Cla*I (C), *Dra*I (D), *Eco*RI (E), *Eco*RV (V), *Hin*dIII (H), *Kpn*I (K), *Nco*I (N), *Nru*I (Nr), *Pst*I (P), *Sal*I (S), *Sma*I (Sm), *Xba*I (X), *Xho*I (Xh). Probes for transcript mapping (above each restriction map) were 5′ or 3′ end-labelled at the restriction sites shown (asterisks). The lengths of RNA−DNA hybrids surviving S1 nuclease treatment and the direction of transcription (wavy lines) are indicated beneath each probe. Bars beneath the restriction maps represent fragments that were used for Northern analysis (see Figure 5).

clones, 7.8 kb for one clone and 2.8 kb for one clone. These sizes correspond to those observed on Southern blots of *Hin*dIII-digested nuclear DNA (Figure 1). The 4.5 kb fragments were found, on the basis of restriction digestion patterns, to be identical to pUMS2H and were not investigated further. *Hin*dIII fragments representing each of the other size classes of hybridizing fragments were subcloned into pUC18 and physical maps obtained by single and double digests with restriction endonucleases. The clone harbouring the 7.8 kb *Hin*dIII fragment was designated

Fig. 3. Nucleotide sequence for the 5′ coding and non-coding region of *ums2*. A putative heat-shock element in the promoter is underlined. The transcriptional initiation site (see Figure 4A) is indicated by an asterisk. The extent of a *ums2* deletion (Δ10) from the coding region of *ums2* to 21 bp from the transcriptional start site is shown. After attachment of *Bgl*II linkers, this deletion was fused to the *E.coli* hygB phosphotransferase gene to construct pHL1 (Wang *et al.*, 1988). The deduced sequence for the first 90 amino acids of *ums2* is shown above the nucleotide sequence. Mismatches with the corresponding region of the yeast *SSA1* gene are aligned below. The *Cla*I site at codons 7 and 8 (underlined) corresponds to that shown in the restriction map of pUMS2H in Figure 2.

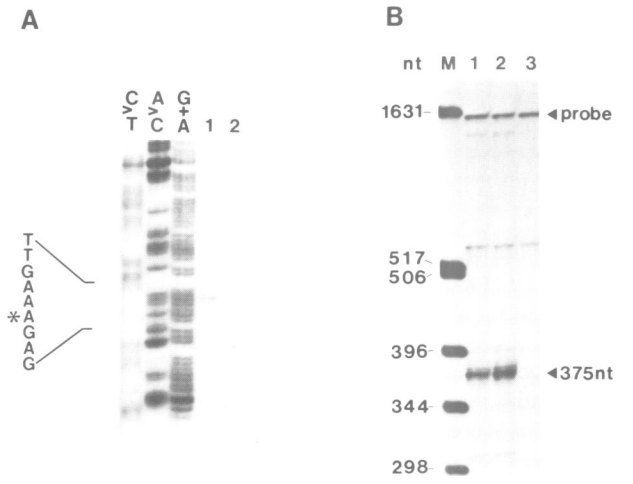

Fig. 4. S1 mapping of the *ums2* transcript. (**A**) High-resolution mapping of the 5′ terminus. The 1.1 kb *Cla*I−*Hin*dIII fragment of pUMS2 (Figure 2) was 5′ end-labelled with ^{32}P at the *Cla*I site and either subjected to chemical sequencing reactions (first three lanes) or incubated with 20 μg RNA from heat-shocked cells (**lane 1**) or 20 μg yeast tRNA (**lane 2**). RNA−DNA hybrids were digested with S1 nuclease, and reaction products analysed by 6% urea−acrylamide gel electrophoresis and autoradiography. The sequence around the major initiation site is shown on the left. The asterisk corresponds to the major initiation site after compensating for the slower migration of S1 nuclease products (Sollner-Webb and Reeder, 1979). (**B**) Low-resolution mapping of the 3′ terminus. The *Xho*I−*Hin*dIII fragment of pUMS2 (Figure 2) was 3′-end labelled at the *Xho*I site and used as a template for hybridization with 30 μg (**lane 1**) or 60 μg (**lane 2**) of RNA from heat-shocked cells, or 30 μg yeast tRNA (**lane 3**). RNA−DNA hybrids were digested with S1 nuclease and analysed as above. Size markers (**lane M**) were end-labelled DNA fragments of *Hin*fI-digested pBR322.

pUMS3H (Figure 2). Southern analysis and S1 nuclease protection experiments with the 2.8 kb insert indicated that one of the transcriptional boundaries lay outside the cloned fragment (unpublished data). Therefore this gene was subsequently reisolated on a 4.7 kb *Pst*I fragment from the same cosmid, cloned into *Pst*I-digested pUC18, and designated pUMS1P. The region within the 10.4 kb *Hin*dIII fragment that hybridized to the probe was localized to a 5.8 kb *Bgl*II fragment. This was subcloned into the *Bam*HI site of pUC18 and constitutes pUMS4B. Restriction maps for pUMS1P, pUMS2H, pUMS3H and pUMS4B are shown in Figure 2.

The presence and orientation of transcriptional units within clones pUMS1P, pUMS3H and pUMS4B were established by S1 nuclease protection experiments. The approximate lengths of the S1 nuclease-resistant fragments are shown in Figure 2. Sequences from within the transcriptional units of each of the four genes (Figure 2) were isolated and hybridized to *Ustilago* genomic DNA on Southern blots. Each probe cross-hybridized only to the other three genes, to give hybridization patterns similar to that shown in Figure 1, although the intensity of each band varied depending on the probe used (unpublished data).

Nucleotide sequence of the 5′ region of pUMS2

To obtain the nucleotide sequence of the 5′ coding and non-coding region of *ums2*, and to construct fusions between the *ums2* promoter and the *Escherichia coli* hygromycin B (hygB) phosphotransferase gene, we made a series of deletions from the coding region into the 5′-non-transcribed sequence of *ums2*. The nucleotide sequence extending upstream from various deletion endpoints was determined (Figure 3). Analysis of this region revealed one contiguous open reading frame (ORF) within the first 270 nucleotides that were sequenced, ending at the presumed initiation codon AUG. This sequence is 73% identical to the corresponding regions of the yeast hsp70 gene *SSA1* and the *Drosophila* hsp70 gene (Ingolia *et al.*, 1982). The derived sequence for the first 90 amino acid residues is 82% identical to *SSA1* (Figure 3). The *ums2* transcriptional start site was positioned by high-resolution S1 nuclease mapping to a thymine residue 126 nucleotides upstream from the initiation codon (Figure 4A). A sequence with a 6 out of 8 match to the consensus heat shock element (HSE) (Pelham, 1985) is found between −80 and −94 with respect to the transcriptional start site (Figure 3). The putative 3′ terminus of *ums2* was located by low-resolution S1 mapping (Figure 4B) to a position ~370 nt downstream of the 3′ *Xho*I site in *ums2* (Figure 2). From the sizes of the 5′ and 3′ protected fragments, and from the size of the *ums2* RNA detected on Northern blots (Figure 5A), it appears that *ums2* is transcribed, uninterrupted, as an mRNA of ~2.2 kb.

Regulation of mRNA levels of ums1, 2, 3 and 4 during heat shock and recovery

A potential problem in studying RNA levels of closely related genes arises from the possibility of cross-hybridization of different mRNAs to the same DNA probe. By probing Northern blots both independently (Figure 5A) and sequentially (unpublished data) with labelled DNA fragments from each of the four genes under stringent conditions, we were able to distinguish between hybridizing RNAs on the basis of their size, and thereby show that with these probes (shown as bars beneath restriction maps in Figure 2), cross-hybridization was negligible. The sizes of RNAs were

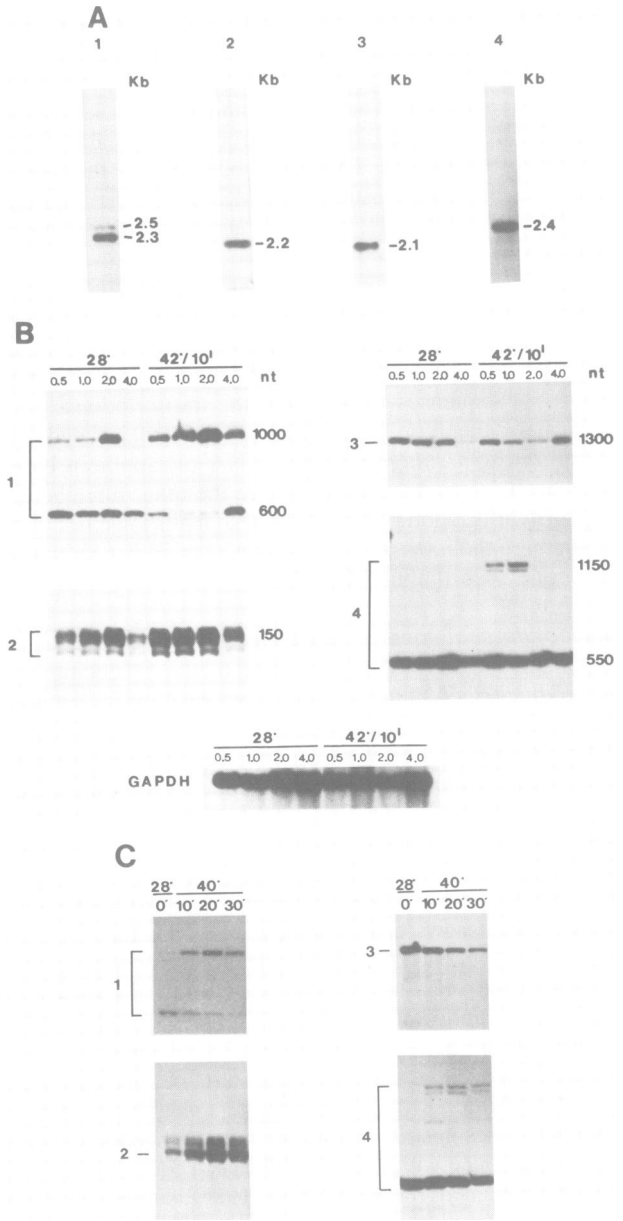

Fig. 5. RNA levels of *ums1, 2, 3* and *4* during heat shock and recovery. (**A**) Northern analysis of RNA samples (30 μg) from heat-shocked cells. **Lanes 1, 2, 3** and **4** were probed under stringent conditions with the portions of pUMS1, 2, 3 and 4 shown as bars beneath restriction maps in Figure 2. (**B**) Quantitative S1 nuclease analysis of RNA levels of *ums1, 2, 3* and *4* (**panels 1, 2, 3** and **4** respectively) after a 10 min, 42°C heat shock. Cells at 28°C were harvested at 0.5, 1, 2 and 4 h after a 10 min, 42°C heat shock (last four lanes of each panel) and at the same time points from a control culture that was not heat-treated (first four lanes of each panel). RNA samples (30 μg) representing each time point were hybridized to the end-labelled DNA templates from pUMS1, 2, 3 and 4 shown in Figure 2. Following S1 nuclease treatment, the resistant fragments were analysed by urea–acrylamide gel electrophoresis and autoradiography. Approximate lengths in nucleotides (nt) of S1 nuclease-resistant fragments are given at the right of each panel. To show that the amounts of RNA analysed at each time point were equivalent, samples were also subjected to Northern hybridization using a probe consisting of a portion corresponding to the 3′ coding and non-coding region of the *Ustilago* glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) (T.Smith and S.Leong, unpublished data). (**C**) RNA levels of *ums1, 2, 3* and *4* during a 40°C heat shock. RNA levels were measured by S1 nuclease protection as above. Signals from control RNA samples isolated from cells grown at 28°C at the beginning (shown) and end (not shown) of the 30-min experiment were indistinguishable.

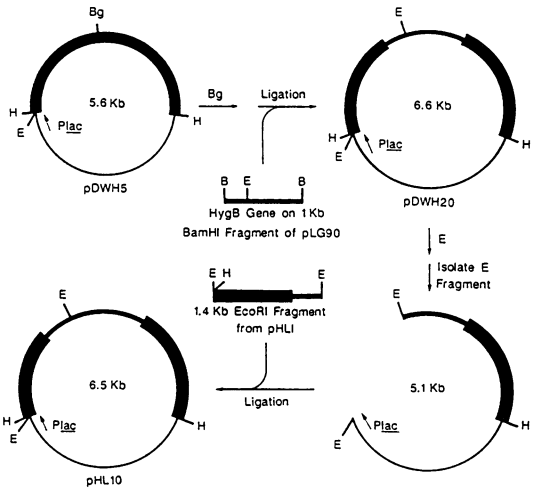

Fig. 6. Construction of plasmid pHL10. Restriction sites shown are: *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H). *Ustilago* DNA is denoted by the thickest line, the *E.coli* hygB phosphotransferase gene by the thinner line, and pUC12 DNA by the thinnest line. The *Hin*dIII insert of pHL10 constitutes the replacement fragment HL10H (Figure 7). For details of plasmid construction, see Materials and methods.

~2.5 kb and ~2.3 kb for *ums1*, ~2.2 kb for *ums2*, ~2.1 kb for *ums3* and ~2.4 kb for *ums4*. RNA samples used for quantitative S1 nuclease protection experiments (Figure 5B and C) were also analysed by hybridizing Northern blots with the probes shown as bars beneath restriction maps in Figure 2. Because the patterns of RNA accumulation shown in Figure 5B were essentially the same as those obtained by Northern analysis (unpublished data), we conclude that these patterns reflect the hybridization of each probe to its own transcript.

In one set of experiments (Figure 5B), mRNA levels were assayed over a 4 h period after cells had been shifted from 28 to 42°C for 10 min; this treatment was chosen because it is a strong inducer of thermotolerance in *Ustilago* (Taylor and Holliday, 1984). To provide a more prolonged heat stimulus, a second set of experiments was performed, in which cells were maintained at 40°C for 30 min after transfer from the normal growing temperature of 28°C (Figure 5C). DNA probes used for these S1 nuclease experiments are indicated above the restriction maps in Figure 2. Detection of RNAs by S1 nuclease protection of a template from pUMS1 showed that two transcripts are present during logarithmic growth of *Ustilago*. The sizes of the protected fragments differed by ~400 nt, in contrast to the estimated 200 nt difference between the mRNA species that was detected on Northern blots (Figure 5A). This apparent discrepancy could be due to the relatively poor size resolution obtained on Northern blots, or reflect a difference caused by sequences in *ums1* that affect processing of the mRNA downstream of the S1 nuclease-protected region. It is apparent from Figure 5 that changes in the mRNA levels for *ums1, 2* and *3* occur during normal growth, although the higher levels of the larger transcript of *ums1* at 2 h was not consistently observed in replicate experiments. These changes may result from the increase in cell titre, which quadruples over the course of the experiment, as the culture shifts from log to late-log phase.

For *ums1*, a pronounced shift in the relative levels of each transcript was consistently observed 2 h after a 10 min, 42°C

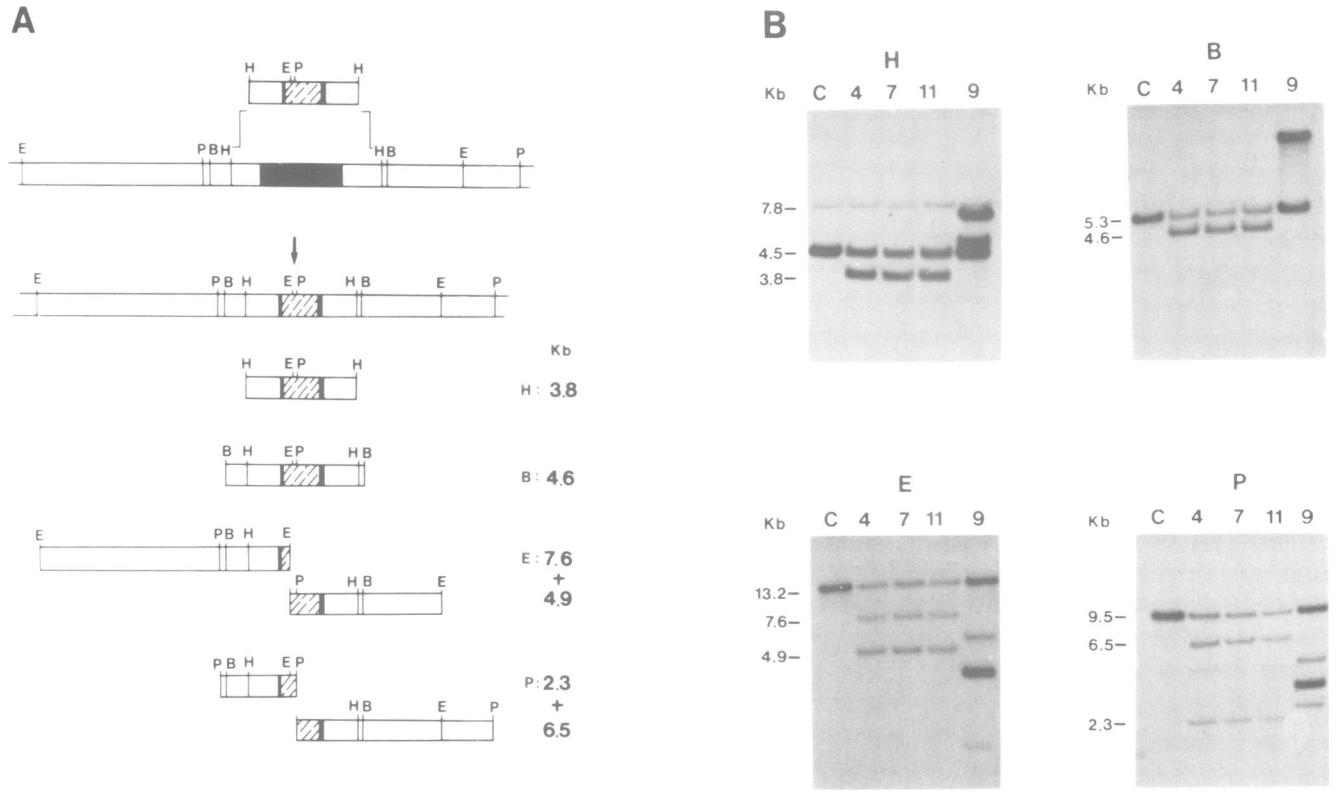

Fig. 7. Replacement of *ums2* by HL10H. (**A**) Predicted structure of the *ums2* locus after replacement of *ums2* by HL10H. Restriction enzymes are: *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Pst*I (P). Prior to replacement, digestion with these enzymes produces fragments bearing *ums2* of 5.3, 13.2, 4.5 and 9.5 kb respectively. These can be seen in **lanes C** in (**B**), and as the most strongly hybridizing bands in each lane of Figure 1. The predicted sizes of DNA fragments after replacement of *ums2* are given in kb. Open regions, *Ustilago* DNA sequences flanking *ums2*; black regions, the *ums2* transcriptional unit; cross-hatched regions, the hygB phosphotransferase gene. (**B**) Southern hybridization analysis of DNAs from diploid cells following transformation by HL10H. DNA samples from four transformants, d132t4, t7, t11 and t9, and an untransformed control (C) were digested with *Hin*dIII (H), *Bam*HI (B), *Eco*RI (E) or *Pst*I (P). DNA fragments were electrophoresed through 0.6% agarose gels and transferred to nylon filters. The filter shown in **panel H** was hybridized sequentially (not shown) and then simultaneously (shown) to three probes: (i) the 1.8 kb *Kpn*I–*Bam*HI fragment of pUMS3H, (ii) the 0.5 kb *Bgl*II fragment of pUMS2, and (iii) the 1 kb *Bam*HI fragment of pLG90, which carries the coding sequence of the hygB phosphotransferase gene (Gritz and Davies, 1983). The band at 7.8 kb represents hybridization to pUMS3; the band at 4.5 kb results from hybridization of the 0.5 kb *Bgl*II fragment of pUMS2 to the intact chromosomal copy of *ums2*; the band at 3.8 kb is due to hybridization to the hygB phosphotransferase gene in the disrupted copy of *ums2*. Filters shown in **panels B, E** and **P** were probed with HL10H.

heat shock, with a decline in the levels of the smaller transcript and an ~5-fold increase in the abundance of the larger. Normal levels of the smaller transcript were detected 4 h after heat shock. A similar response was observed during the 40°C heat shock (Figure 5C), maximal levels of the larger transcript being detected at the 20 min time point. An increase in the abundance of the *ums2* transcript was also observed in response to both heat treatments. In the case of the 40°C heat shock, this was of similar magnitude and kinetics to the larger transcript of *ums1*. A decline in the *ums3* mRNA level occurred over the 4 h time course at 28°C (Figure 5B). This decline was accentuated when cells were exposed to either heat treatment. The cellular levels of the major mRNA of *ums4* appeared to be largely unaffected by heat shock. However, RNA from heat-treated cells protected small quantities of at least two additional fragments, the most prominent of these being ~600 nt longer than the major protected fragment.

Inactivation of ums2 by gene replacement

To begin a genetic analysis of the *Ustilago* hsp70 gene family, we attempted to construct a mutant strain lacking *ums2* by replacing one chromosomal allele with a cloned sequence in which most of the transcriptional unit had been deleted. The construction of pHL10 from which the replacement fragment was derived is shown in Figure 6 and described in Materials and methods. This plasmid consists of a deletion of *ums2* that extends from the coding region to 22 bp downstream of the transcriptional start site, and to ~300 bp upstream of the transcriptional terminus. The *ums2* coding region has been substituted with the *E.coli* hygB phosphotransferase gene; the fusion site with the *ums2* promoter (Figure 3) is the same in pHL10 as in pHL1 (Wang *et al.*, 1988) and allows the expression of hygB resistance in *Ustilago*. The replacement fragment could not be isolated directly from pHL1, because in this plasmid the deletion extends beyond the 3′ transcriptional terminus of *ums2*. For gene replacement experiments, pHL10 was digested with *Hin*dIII, and the 3.8 kb insert was purified from pUC12 DNA. This linearized sequence (HL10H) has ~1 kb of homology at the 5′ end, and 1.4 kb at the 3′ end, to the corresponding flanking regions of *ums2* in the chromosome (Figure 7A). Since it was not known whether *ums2* is an essential gene, a diploid strain was chosen as a recipient for transformation experiments in the expectation that some of the transformants would be heterozygous for the *ums2* disruption. The strain d132 was chosen because it is solopathogenic; that is, it is capable (by virtue of heterozygosity

Table I. Segregation of auxotrophic markers and hygB resistance in progeny of d132 and two transformants

Strain	Segregation[a]		
	ad^-	met^-	$hygB^r$
d132	98	89	0
d132t9	82	83	87
d132t11	84	91	18

[a]Among 200 random basidiospore segregants.

at the *b* locus) of infecting corn directly, resulting in the production of teliospores that undergo meiosis during germination. If *ums2* is not an essential gene, then haploid cells bearing disrupted copies should be present among the meiotic progeny of the diploid transformant.

As shown in Figure 7A, direct replacement of the genomic sequence via homologous recombination with HL10H should result in changes in the digestion patterns produced by the restriction enzymes *Hin*dIII, *Bam*HI, *Eco*RI and *Pst*I. The sizes of fragments bearing *ums2* after digestion with these enzymes were the same in d132 and the haploid strain 518. These were 4.5 kb for *Hin*dIII, 9.5 kb for *Pst*I, 5.3 kb for *Bam*HI and 13.2 kb for *Eco*RI (the most strongly hybridizing bands in each lane in Figure 1). For a diploid strain in which one allele has been replaced with HL10H, digestion with *Hin*dIII should result in one unaltered 4.5 kb fragment and an additional fragment of 3.8 kb, representing the integrated disrupted hsp70 sequence. Digestion with *Bam*HI should produce a new 4.6 kb fragment in addition to the unaltered 5.3 kb sequence. As the HygB gene contains single sites for *Pst*I and *Eco*RI, digestion with these enzymes should yield two additional fragments in each case, of 7.6 and 4.9 kb for *Eco*RI, and 2.3 and 6.5 kb for *Pst*I.

HygB-resistant transformants of d132 were obtained by standard procedures (Wang *et al.*, 1988), using HL10H. Hybridization patterns obtained by Southern analysis of DNA from four independent diploid transformants and an untransformed control are shown in Figure 7B. Southern blots containing *Hin*dIII-digested DNA were hybridized separately (not shown) then simultaneously (Figure 7B) with three probes: (i) pUMS3, as a control to show that approximately equal quantities of DNA from each transformant were analysed; (ii) the 0.5 kb *Bgl*II fragment of pUMS2, which is not present in the replacement fragment, and will therefore only hybridize to intact *ums2*; (iii) the hygB gene itself, which should hybridize to sequences of 3.8 kb from transformants where replacement has occurred. Transformant DNAs that had been digested with *Bam*HI, *Eco*RI or *Pst*I were probed with HL10H, which also hybridizes to the unaltered *ums2* allele, as a result of the homologous flanking sequences at the ends of HL10H. Three of the four transformants shown in Figure 7B (d132t4, d132t7 and d132t11), gave hybridization patterns expected for direct replacement of one allele. In all cases the hybridization signals to *ums2* from untransformed cells (Figure 7B, lanes c) were approximately twice the intensity of those from transformants d132t4, 7 and 11, indicating that in untransformed cells, these bands represent unresolved doublets. The patterns obtained for d132t9 indicate that HL10H had integrated by non-homologous recombination. In other experiments a total of >50 independent diploid transformants have been analysed: ~50% of these gave Southern hybridization patterns indicative of direct replacement of one of the two alleles (unpublished data).

Teliospores were obtained from galls on corn plants that had been injected with d132, and transformants d132t11 and d132t9. Tetrad analysis in *U.maydis* is complicated by the absence of complete tetrads produced from the majority of teliospores (Holliday, 1974; and unpublished data from this study). For this reason, random basidiospore segregants were analysed. Teliospores were germinated (Holliday, 1974) and progeny pooled from colonies containing up to 100 cells. The segregation of two auxotrophic markers (*ad*1-1 and *met*1-1), and hygB resistance is shown in Table I. Close to a 1:1 segregation ratio for the auxotrophic markers was obtained in the untransformed control and both transformants. The slight excess of wild-types may be due to the presence of a small percentage of unreduced diploids among the haploid progeny (Holliday, 1974). In d132t9, resistance to hygB also segregated normally. However, in d132t11 only 18 out of 200 segregants were resistant to HygB. In nine of these, resistance to HygB segregated with one or both of the auxotrophic markers, suggesting that these cells were haploid. DNA from these 18 HygB-resistant cells was analysed by probing Southern transfers of *Hin*dIII-digested DNA with the 0.5 kb *Bgl*II fragment of *ums2*. Hybridization to a 4.5 kb fragment was detected in every case, indicating the presence of an intact copy of *ums2*, in addition to the disrupted sequence. These cells are, therefore, not true haploids but the fact that some were auxotrophic suggests they are aneuploid rather than diploid. If a mutation in *ums2* resulted in hypersensitivity to hygB, such mutants might not be recovered among the hygB-resistant progeny. To examine this possibility, DNAs from 20 HygB-sensitive progeny of d132t11 were subjected to Southern analysis as described above: hybridization to a 4.5 kb fragment was again found in every case (unpublished data).

Discussion

We have cloned four genes, (*ums1, ums2, ums3* and *ums4*) from a genomic library of *U.maydis* that are related to the yeast and *Drosophila* hsp70 genes. Their identity was confirmed by (i) the high degree of sequence similarity between the 5′ coding region of *ums2* and the yeast and *Drosophila* hsp70 genes; (ii) demonstrating that *ums1, 2* and *3* are heat responsive; and (iii) establishing that all four RNAs are of sizes expected for proteins of ~70 kd. No additional, strongly hybridizing sequences were detected by the genomic library screen or when Southern blots were probed under non-stringent conditions with portions of the yeast, *Drosophila* or the four *Ustilago* genes. The *Ustilago* family therefore appears to be unusually small. However, very faint hybridization signals did become visible with some probes after prolonged autoradiographic exposures of Southern blots; these sequences could represent distantly related members of the same gene family. If there are only four genes, then this may reflect less redundancy in *Ustilago*, as mutations in all eight of the cloned hsp70 genes of yeast show them to be associated with only three distinct functions (Craig *et al.*, 1987) The sizes of mammalian hsp70 gene families are also smaller than at first thought, since the majority of genes that have been cloned are non-functional (Mues *et al.*, 1986; Sorger and Pelham, 1987).

All four *Ustilago* genes are transcribed during normal

growth of haploid cells. The mRNA levels of *ums1* and *2* increase by ~5-fold in response to heat shock, the *ums3* mRNA declines and that of *ums4* remains relatively unaltered. A similar range of responses is found in the mRNA levels of members of the yeast hsp70 gene family (Ellwood and Craig, 1984; Craig and Jacobsen, 1985; Craig *et al.*, 1987). It is possible that similarly regulated genes of the two organisms are functionally related. These relationships should become clearer when the remaining nucleotide sequences and mutant phenotypes of the *Ustilago* genes have been determined. The nucleotide sequence of *ums1* would also provide a basis for experiments to resolve the mechanism by which this gene produces two transcripts under normal growing conditions. The transcripts could arise from the presence of two transcriptional start sites separated by ~400 bp; alternatively the smaller transcript could result from processing of the larger. The increased abundance of the larger transcript after heat shock could be accounted for by a heat-responsive distal promoter, a greater stability of the larger RNA at elevated temperatures or an interruption of processing.

Despite the wide range of studies that have been carried out on many eukaryotic hsp70 genes, mutational analyses have been confined to yeast, where strains carrying hsp70 disruptions were constructed by *in vitro* mutagenesis and gene replacement. Previous work has shown that targeted gene replacement, equivalent to one-step gene replacement in yeast (Rothstein, 1983), will occur with high frequency in *Ustilago*, when haploid cells are transformed with linear DNA molecules containing sequences homologous to chromosomal DNA (Banks and Taylor, 1988; Kronstad and Leong, 1989). This finding led us to ask if, by analogy to yeast (Shortle *et al.*, 1982), it would be possible to use diploid cells to obtain transformants that are heterozygous for a *ums2* disruption. Southern analysis of DNA from diploid cells that had been transformed with the mutated sequence showed that this event occurred frequently. Cells heterozygous for the *ums2* disruption produced normal galls following their injection into corn plants. However, we were unable to recover haploid progeny that had inherited the disrupted gene from teliospores of these diploids, indicating that the *ums2*::hygB^r null mutation is a recessive lethal. To establish firmly that *ums2* is essential for vegetative growth, and not merely required for sporulation or germination, additional tests must be carried out. Tests based on the loss of a plasmid-borne copy of the gene or the repression of its expression are not possible in *Ustilago*, because centromeric plasmids have yet to be developed, and the sexual stage of its life-cycle can only be completed *in planta*. However, other approaches, such as the construction of conditional mutants, may prove feasible. Our failure to recover haploid *ums2*::hygB^r cells is consistent with the lack of type III integration (gene replacement) in haploid cells transformed with the related vector pHL1—a result that also suggested that *ums2* is indispensable (Wang *et al.*, 1988).

An important metabolic function of hsp70 proteins in unstressed cells is the translocation of secretory and mitochondrial polypeptides across organellar membranes (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Zimmermann *et al.*, 1988). Part of the evidence for this is that yeast cells that carry disruptions in three of the four member *SSA* subfamily accumulate unprocessed precursors of organelle-targeted polypeptides in the cytosol (Deshaies *et al.*, 1988). The finding that these proteins fulfil such a basic metabolic requirement provides an explanation for their collective indispensability, and the structural conservation of hsp70s in general. Rescue of yeast mutant(s) by *ums2* might be used to establish the function of *ums2* in unstressed *Ustilago* cells. Whether *ums2* is required for thermotolerance in *Ustilago* remains an open question.

Materials and methods

Fungal strains, culture and heat shock treatment

The two strains of *U.maydis* used in this study were 518 (*a2b2*) a wild-type haploid, and d132 (*ad1-1, me1-2, nar1-6, a2b1/pan1-1, nar1-1, a1b2*), an artificially synthesized diploid. Both strains were obtained from Dr R.Holliday (National Institute for Medical Research, Mill Hill, London). Media for routine culture, auxotrophic tests and transformation of *U.maydis* have been described previously (Holliday, 1974; Wang *et al.*, 1988). For heat-shock experiments, cells were grown overnight in 500 ml of complete medium in Fernbach flasks at 28°C, until the cell density reached ~2 × 10^7/ml (log phase). The culture was then split into 100 ml aliquots and transferred to 500 ml Erlenmeyer flasks. For heat treatment, flasks were immersed in a shaking water bath at 42 or 40°C for varying periods of time then returned to 28°C or rapidly chilled for RNA extraction (see below).

Nucleic acid isolation

Nuclear DNA was prepared by the method of Specht *et al.* (1982). DNA from fungal transformants was isolated by the rapid procedure for yeast described by Elder *et al.* (1983), except that the aqueous phase was extracted twice with phenol–chloroform and once with chloroform. To isolate total RNA, cells were transferred from Erlenmeyer flasks to 40 ml Oakridge tubes, and immediately immersed and shaken in a dry ice–ethanol bath until the temperature reached ~2°C (within 1 min). Tubes were centrifuged at 3000 *g* for 5 min at 0°C and RNA extracted from cell pellets as described by Fonzi and Sypherd (1985).

Library screening and restriction mapping

To prepare a partial genomic library, nuclear DNA was digested with *Hin*dIII and electrophoresed through an agarose gel in Tris–acetate EDTA buffer. Fragments of 4–5 kb were collected by electrophoresis onto Whatman DE81 paper and recovered by the method of Dretzen *et al.* (1981). These fragments were ligated into *Hin*dIII-digested pUC12 and recombinant clones propagated in *E.coli* strain TB1. The library was screened by colony hybridization on Whatman 541 paper (Taub and Thompson, 1982) with a 1.5 kb *Kpn*I–*Hin*dIII fragment from the yeast gene *SSA2*, which spans codons for amino acids 161–660 (E.Craig, personal communication). The fragment was labelled with ^{32}P by nick translation (Rigby *et al.*, 1977). The nuclear genomic library was constructed in this laboratory by T.Smith and consisted of partially digested *Sau*3AI fragments of 30–40 kb cloned in the cosmid vector pKBY2 (Yelton *et al.*, 1985). The library was screened after transfer to Whatman 541 filters by non-stringent hybridization with the 0.8 kb *Bgl*II fragment of pUMS2, using conditions for Southern hybridizations (see below).

Restriction maps of subcloned fragments from cosmids were determined by single and double digests, carried out according to the supplier's recommendations (New England Biolabs, Bethesda Research Laboratories). Digested DNA samples were fractionated through 2 and 0.5% agarose gels using *Hae*III-digested φX174 RF DNA and *Hin*dIII-digested phage λ as size markers.

Nucleic acid hybridizations and S1 nuclease protection experiments

Southern blots were prepared by transferring DNA fragments from agarose gels to Zetabind (AMF-Cuno) filters. DNA probes were labelled with ^{32}P by nick translation and were hybridized to filters under stringent (50% formamide, washing at 65°C) or non-stringent (30% formamide, washing at 42°C) conditions, as described by Amasino (1986), omitting PEG and NaCl from the hybridization buffer. Probes used for the detection of *Ustilago* hsp70-related sequences consisted of the portion of the yeast *SSA2* gene described above, and a 1.1 kb *Pst*I fragment from clone B8, which includes 155 bp of 5′ untranslated sequence, and the coding region for amino acids 1–312 of a *Drosophila* hsp70 gene (Ingolia *et al.*, 1980).

For Northern analysis, RNA samples were glyoxylated (Maniatis *et al.*, 1982) and resolved in 1.2% agarose gels containing Tris–borate EDTA buffer (pH 7.5). Brome mosaic virus RNAs served as size standards and

were visualized by staining the gel with acridine orange. RNA was transferred to Zetabind filters, then hybridized and washed under stringent conditions as for Southern blots.

DNA templates for S1 nuclease protection experiments were end-labelled with [^{32}P]ATP by T4 polynucleotide kinase or the Klenow fragment of *E.coli* DNA polymerase I. For pUMS1P, pUMS3H and pUMS4B, the selection of fragments for end-labelling was based on preliminary experiments in which restriction endonuclease-cleaved fragments of each of these clones were hybridized to probes representing either 5′ or 3′ regions of the *ums2* transcriptional unit. This gave the probable orientations of the three genes within clones pUMS1P, pUMS3H and pUMS4B (unpublished data). The labelled templates described in Figure 2 were hybridized with 30 μg aliquots of RNA essentially as described by Favaloro *et al.* (1980). Temperatures that favoured RNA−DNA hybridizations were determined empirically for each probe, and for quantitative analyses, the DNA probes were shown to be in excess. Fragments were analysed by autoradiography after electrophoresis through 6% acrylamide−urea gels (Maniatis *et al.*, 1982) using ^{32}P-labelled *Hin*fI fragments of pBR322 as size markers. Protected fragments were cut out from the gel, immersed in Aquasol and quantified by scintillation counting after 24 h.

Deletions, sequencing and plasmid construction

Plasmid pSL11, derived from pUMS2H by the removal of two internal *Bgl*II fragments, has been described previously (Wang *et al.*, 1988). To create deletions for sequencing and plasmid construction, *Bal*31 nuclease digestion was initiated from the *Bgl*II site of pSL11, and terminated at various time points. The digested fragments were blunt-ended with the Klenow fragment of *E.coli* DNA polymerase I and synthetic *Bgl*II linkers were attached using T4 DNA ligase (Maniatis *et al.*, 1982). Linkered fragments were subsequently digested with *Bgl*II and religated prior to transformation of *E.coli* strain HB101 (Maniatis *et al.*, 1982). The nucleotide sequence of both strands extending upstream from deletion end-points was determined by a modification of the chemical degradation method (Bencini *et al.*, 1984). A transcriptional fusion between the *E.coli* hygB phosphotransferase gene (Gritz and Davies, 1983) and the *ums2* promoter was constructed using the deletion plasmid pDWH10 (Wang *et al.*, 1988). The resultant plasmid pHL1 could not be used for gene replacement because the deleted region extended beyond the 3′ transcriptional terminus of *ums2*. To construct the replacement plasmid pHL10, pDWH5, which carries a deletion that terminated ~300 bp from the 3′ transcriptional boundary, was used. pDWH5 was linearized with *Bgl*II, and ligated with the *Bam*HI fragment of pLG90, which carries the coding region of the hygB phosphotransferase gene (Gritz and Davies, 1983), to make pDWH20. The 5.1 kb *Eco*RI fragment of pDWH20 was then ligated with the 1.3 kb *Eco*RI fragment of pHL1 (Wang *et al.*, 1988) to reconstitute the hygB phosphotransferase gene, in transcriptional fusion with the *ums2* promoter. The resulting plasmid pHL10 was cleaved with *Hin*dIII, and the 3.8 kb insert purified by electroelution from an agarose gel, for transformation of *Ustilago*. The construction of pHL10 is summarized in Figure 6.

Other procedures

Transformation of *U.maydis* spheroplasts was as described (Wang *et al.*, 1988). Genetic tests were performed by standard methods (Holliday, 1974), after injecting diploid cells of *U.maydis* into seedlings of the corn cultivar Golden Bantam. Briefly, teliospores were harvested by grinding gall tissue in a mortar and suspended overnight in 1.5% $CuSO_4$ solution, then washed with water before plating on complete medium. Colonies of up to 100 haploid segregants were collected from plates, mixed thoroughly, then replated at low density to fresh medium. Resulting single colonies were picked to complete medium containing hygB (350 μg/ml), to minimal medium supplemented with adenine, methionine and pantothenic acid, and to media in which one of the three supplements was omitted.

Acknowledgements

We thank Elizabeth Craig and members of her laboratory for many useful discussions and for providing us with plasmids. We are especially grateful to Geoff Banks, in whose laboratory some of the genetic studies were carried out. We also thank Jun Wang and Al Budde for their help at various times, Tim Smith for providing us with the cosmid library, Steve Vicen for photographic work, and Diane Moss and Lucille Strassman for preparing the manuscript. This work was supported by the USDA, Public Health Service grant 1 RO1 GM33716 from the National Institutes of Health (USA), and later by the Medical Research Council (UK).

References

Amasino,R.M. (1986) *Anal. Biochem.*, **152**, 304−307.
Banks,G.R. and Taylor,S.Y. (1988) *Mol. Cell. Biol.*, **8**, 5417−5424.
Bencini,D.A., O'Donovan,G.A. and Wild,J.R. (1984) *Biotechniques*, **2**, 4−5.
Chirico,W.J., Waters,G.M. and Blobel,G. (1988) *Nature*, **332**, 805−810.
Craig,E.A. and Jacobsen,K. (1984) *Cell*, **38**, 841−849.
Craig,E.A. and Jacobsen,K. (1985) *Mol. Cell. Biol.*, **5**, 3517−3524.
Craig,E.A., Kramer,J. and Kosic-Smithers,J. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4156−4160.
Deshaies,R.J., Koch,B.D., Werner-Washburne,M., Craig,E.A. and Schekman,R. (1988) *Nature*, **332**, 800−805.
Dretzen,G., Bellard,M., Sassone-Corsi,P. and Chambon,P. (1981) *Anal. Biochem.*, **112**, 295−298.
Elder,R.T., Loh,E.Y. and Davies,R.W. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 2432−2436.
Ellwood,M. and Craig,E.A. (1984) *Mol. Cell. Biol.*, **4**, 1454−1459.
Favaloro,J., Treisman,R. and Kamen,R. (1980) *Methods Enzymol.*, **65**, 718−749.
Fonzi,W.A. and Sypherd,P.S. (1985) *Mol. Cell. Biol.*, **5**, 161−166.
Gausz,J., Gyurkovics,H., Bencze,G., Awad,A.A.M., Holden,J.J. and Ish-Horowicz,D. (1981) *Genetics*, **98**, 775−789.
Gritz,L. and Davies,J. (1983) *Gene*, **25**, 179−188.
Holliday,R. (1974) In King,R.C. (ed.), *Handbook of Genetics*. Plenum Press, New York, Vol. 1, pp. 575−595.
Ingolia,T.D., Craig,E.A. and McCarthy,B.J. (1980) *Cell*, **21**, 669−679.
Ingolia,T.D., Slater,M.R. and Craig,E.A. (1982) *Mol. Cell. Biol.*, **2**, 1388−1398.
Kronstad,J.W. and Leong,S.A. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 978−982.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Milarski,K.L. and Morimoto,R.I. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9517−9521.
Mues,G.I., Munn,T.Z. and Raese,J.D. (1986) *J. Biol. Chem.*, **261**, 874−877.
Palter,K.B., Watanabe,M., Stinson,L., Mahowald,A.P. and Craig,E.A. (1986) *Mol. Cell. Biol.*, **6**, 1187−1203.
Pelham,H. (1985) *Trends Genet.*, **1**, 31−35.
Rigby,P.W.J., Diekmann,M., Rhodes,C. and Berg,P. (1977) *J. Mol. Biol.*, **113**, 237−251.
Rothstein,R. (1983) *Methods Enzymol.*, **101**, 202−211.
Shortle,D., Haber,J.E. and Botstein,D. (1982) *Science*, **217**, 371−373.
Sollner-Webb,B. and Reeder,R.H. (1979) *Cell*, **18**, 485−499.
Sorger,P.K. and Pelham,H.R.B. (1987) *EMBO J.*, **6**, 993−998.
Specht,C.A., DiRusso,C.C., Novotny,C.P. and Ullrich,R.C. (1982) *Anal. Biochem.*, **119**, 158−163.
Taub,F. and Thompson,E.B. (1982) *Anal. Biochem.*, **126**, 222−230.
Taylor,S.Y. and Holliday,R. (1984) *Curr. Genet.*, **9**, 59−63.
Wang,J., Holden,D.W. and Leong,S.A. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 865−869.
Werner-Washburne,M., Stone,D.E. and Craig,E.A. (1987) *Mol. Cell. Biol.*, **7**, 2568−2577.
Yelton,M.M., Timberlake,W.E. and van den Hondel,C.A.M.J.J. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 834−838.
Zimmermann,R., Sagstetter,M., Lewis,M.J. and Pelham,H.R.B. (1988) *EMBO J.*, **7**, 2875−2880.

Received on January 27, 1989; revised on April 13, 1989