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A heat shock gene from *Saccharomyces cerevisiae* encoding a secretory glycoprotein

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ABSTRACT **We report the finding of a secretory heat shock protein, HSP150, of *Saccharomyces cerevisiae*, and the characterization of the gene coding for it. HSP150 is constitutively expressed, extensively O-glycosylated, and secreted efficiently to the growth medium. When cells grown at 25°C were shifted to 37°C, a 7-fold increase in the level of HSP150 was observed within 1 hr. The *HSP150* gene encodes a primary translation product of 412 amino acids. Direct amino acid sequencing of the mature secreted protein showed that an N-terminal sequence of 18 amino acids is removed, and a KEX2 protease-specific site is cleaved to yield two subunits of 53 and 341 amino acids, which remain noncovalently associated during secretion. The larger subunit is highly repetitive, containing 11 tandem repeats of a 19-amino acid sequence. Northern blot hybridization analysis showed a substantial increase in *HSP150* mRNA level after heat shock. The upstream flanking region of the gene contains several heat shock element-like sequences. Disruption of *HSP150* did not lead to inviability or significant effects on growth rate, mating, or thermotolerance. However, heat-regulated antigenic homologs of HSP150 were found in divergent yeasts such as *Schizosaccharomyces pombe*.**

About 15 secretory glycoproteins have been identified from *Saccharomyces cerevisiae*. The expression of most of them is regulated by nutritional conditions or the mating type of the cells. Most of them remain intercalated in the cell wall, and only a few are secreted across the cell wall to the growth medium (1). No secretory protein has been reported to be regulated by heat stress. Heat-regulated proteins are found in all organisms and fall into two groups: the conserved stress proteins, which include the 70-kDa, 90-kDa, and low molecular weight heat shock proteins, and unclassified ones such as "household" enzymes and ubiquitin. All stress proteins characterized so far are cell-associated (2). In this report we characterize *HSP150*, a gene coding for a protein that is regulated by heat stress, O-glycosylated, and secreted to the growth medium.†

MATERIALS AND METHODS

Strains and Media. The *S. cerevisiae* strains SEY2101a (*MATa*$_1$ *ade2-101 ura3-52 leu2-3*,112 *suc2-Δ9 gal2*), S288C (*MATα*), SF821-8A (*MATa*$_1$ *sec7-1 his4-580 ura3-52 leu2-3,112 trp1-289*), W303D (*MATa/MATα ura3-1/ura3-1 his3-11/his3-11 his3-15/his3-15 leu2-3/leu2-3 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ade2-1/ade2-1 can1-100/can1-100*) and the *Schizosaccharomyces pombe* (*leu1-32, h*$^-$), *Kluyveromyces marxianus* (NCYC587), and *Torulaspora delbrueckii* strains were grown at 25°C in YPD medium containing 1% yeast extract (Oxoid, Basingstoke, U.K.), 2% bacto peptone (Difco), and 2% glucose (BDH), to midlogarithmic phase. *Escherichia coli* strain XL1-Blue was from Stratagene. Synthetic complete (SC), presporulation, and sporulation media were made as in ref. 3.

Metabolic Labeling and Immunoprecipitation of HSP150. Cells were labeled for 1 hr in YPD medium with [^{35}S]methionine (>1000 Ci/mmol; 1 Ci = 37 GBq) at 50–100 μCi/ml or [2-^{3}H]mannose (11.5 Ci/mmol; Amersham), at 300 μCi/ml, washed, and lysed mechanically with glass beads. Lysates and media were immunoprecipitated, as described before for α-factor (4), with anti-HSP150 antiserum (1:100) raised in rabbits against purified HSP150. The antiserum specifically recognized mature and nonglycosylated HSP150, and the immunoprecipitations were quantitative.

Direct Amino Acid Sequencing of HSP150. SF821-8A cells were incubated for 1 hr at 37°C; under these conditions there is a preferential secretion of HSP150. The HSP150 protein (subunits I and II) was purified from the growth medium by concentration on an ion exchanger, followed by dialysis, gel filtration, and anion exchange chromatography. The purified protein was cleaved with trypsin, and the peptides were purified by reversed-phase chromatography. N-terminal amino acid sequencing was carried out on the native mature protein, and on subunits I and II after separation by reversed-phase chromatography. Amino acid sequence analysis was performed with a gas-phase sequencer equipped with an on-line phenylthiohydantoin amino acid analyzer (5).

Cloning and Sequencing. Peptide 5 (see Fig. 1) was used to synthesize a 32-mer oligonucleotide probe with inosines at certain ambiguous codon positions (6). The probe was radiolabeled with ^{32}P by T4 polynucleotide kinase (7) and used to screen >300,000 plaques of a yeast cDNA library made in λgt11 (7, 8). Putative clones were subcloned into Bluescript II vectors (Stratagene) and subjected to double-stranded DNA sequencing with the chain-termination method (9). A yeast genomic library (10) was screened with the *HSP150* cDNA clone, which was radiolabeled with [α-^{32}P]dCTP by the random primer technique (11). Two putative genomic clones were isolated, subcloned, and subjected to sequencing. Both strands of the entire *HSP150* coding region and the flanking regions were sequenced.

Northern Blot Analysis. Total RNA was isolated from SEY2101a cells, electrophoresed, and subjected to Northern blot analysis as described (12), except that hybridizations were for 12–18 hr at 42°C. The probe used for Northern (and Southern) blots was the same as used for screening the yeast genomic library.

Gene Disruption. The *HSP150* chromosomal locus was disrupted by the one-step gene disruption method (13). The genomic *HSP150* clone, with ≈2-kilobase (kb) and 1-kb flanking sequences at the 5′ and 3′ ends, respectively, was subcloned into the *Sal* I site of the Bluescript II SK(±) vector (Stratagene), which had its *Pst* I site mutated. A *Pst* I fragment coding for amino acids 61–301 in the *HSP150* gene was replaced by a *Hin*dIII fragment containing a yeast *URA3*

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M88698).

gene (supplied by Fred Sherman, University of Rochester). The *Pst* I and *Hin*dIII 5′ cohesive ends were filled in by the combined 3′-to-5′ exonuclease and 5′-to-3′ polymerase activities of T4 DNA polymerase in the presence of deoxynucleoside triphosphates, to create blunt ends for ligation (14). The diploid yeast strain W303D was then transformed with this integrating plasmid, *URA3*$^+$ transformants were sporulated, asci were dissected (15), and *URA3*$^+$ spores were analyzed for the disruption of the *HSP150* gene.

Immunoblotting. Cells grown overnight were washed and resuspended in fresh YPD medium (2×10^8 cells in 400 μl) and then incubated for 1 hr at either 25°C or 37°C. The cells were pelleted and lysed, and the medium was precipitated with 14% (wt/vol) trichloroacetic acid. Five percent of each cell lysate and growth-medium sample was subjected to SDS/PAGE, except in the case of *Sch. pombe*, where 10% of the sample volume was electrophoresed. The proteins were transferred to nitrocellulose filters, which then were probed with anti-HSP150 antiserum (1:1500) and developed by using alkaline phosphatase-conjugated anti-rabbit antibody according to the supplier's instructions (Amersham). Immunostaining was blocked when a 1:10 dilution of growth medium in which *S. cerevisiae* cells (5×10^8 per ml) had been incubated for 1 hr at 37°C was added to the above antiserum preparation.

Other Methods. To determine thermotolerance, cells that had been grown overnight at 25°C were incubated for up to 20 min at 50°C, with or without a preincubation of 30 min at 37°C. Aliquots were plated on solid YPD media and scored for colony formation at 25°C. SDS/PAGE was done in 7.5–15% polyacrylamide Laemmli gels. Gels were processed for autoradiography and exposed to Kodak X-Omat AR film (Eastman Kodak). Radioactivity in gel slices was quantitated by scintillation counting after solubilization with NCS tissue solubilizer (Amersham). HF treatment was performed to release O-glycans (16). Trichloroacetic acid (14% TCA) precipitation was for 30 min on ice. Tunicamycin (Sigma; preincubation for 30 min) was used at a concentration of 10 μg/ml.

RESULTS AND DISCUSSION

Heat Shock Increases the Level of a 150-kDa Secretory Glycoprotein. Cells (SEY2101a unless otherwise stated) were labeled with [^{35}S]methionine at various temperatures, and the proteins of the growth media were analyzed in SDS/PAGE. After labeling at 25°C, a minor band of 150 kDa could be detected among other bands. When the labeling was performed at 37°C, the intensity of the 150-kDa band was much higher than at 25°C. Quantitations showed that there was 7 times more radioactivity in the 150-kDa band at 37°C ($n = 6$; range 5.5–9.3), 6.3 times more at 39°C, and very little at 41°C as compared with 25°C (Fig. 1*A*). Similar results were obtained with an α mating-type strain, S288C. The 150-kDa protein was designated HSP150. The protein was determined to be a glycosylated secretory protein, since it could be labeled with [^{3}H]mannose. However, tunicamycin had no effect on its [^{3}H]mannosylation or electrophoretic migration (data not shown), suggesting that the protein was not N-glycosylated. Instead, HF treatment, which removes O-glycans, reduced its apparent molecular mass to 47 kDa (Fig. 1*B*). Immunoprecipitations of [^{35}S]methionine-labeled growth medium and cell lysate with anti-HSP150 antiserum confirmed that heat stress increased the level of HSP150, and further showed that >90% of the newly synthesized HSP150 was secreted to the medium (Fig. 1*C*). Thus, HSP150 appears to be an extensively O-glycosylated protein that is secreted efficiently to the growth medium and regulated by heat stress. To confirm this, we cloned and characterized the *HSP150* gene.

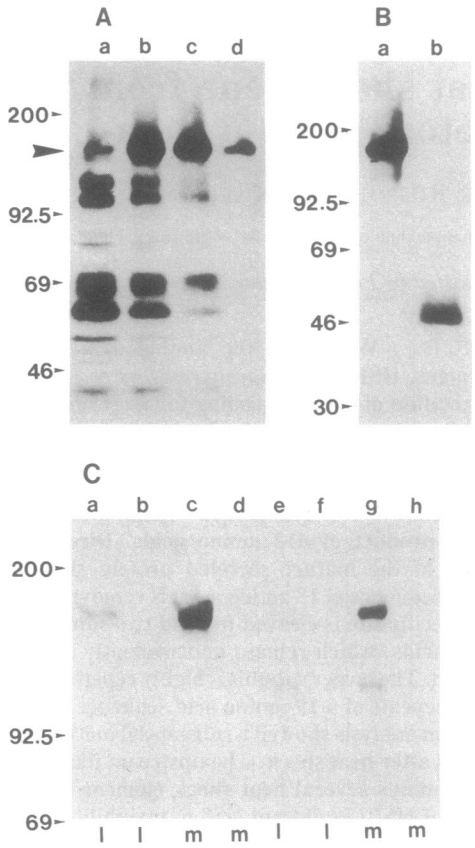


FIG. 1. Heat stress increases the level of a 150-kDa secretory glycoprotein. (*A*) Cells were labeled for 1 hr with [^{35}S]methionine at 25°C (lane a), 37°C (lane b), 39°C (lane c), or 41°C (lane d). Proteins were precipitated from the growth media with trichloroacetic acid and analyzed by SDS/PAGE followed by autoradiography. Molecular size markers (kDa) are at left. The arrowhead indicates HSP150. (*B*) [^{35}S]Methionine-containing HSP150 before (lane a) or after (lane b) HF treatment to remove O-glycans. (*C*) Cells were labeled as in *A* at 37°C (lanes a–d) or 25°C (lanes e–h), and the cell lysates (l) and growth media (m) were subjected to immunoprecipitation with anti-HSP150 antiserum (lanes a, c, e, and g) or preimmune serum (lanes b, d, f, and h).

Cloning and Primary Sequence Analysis. A *S. cerevisiae* cDNA library made in λgt11 (7) was screened with an oligonucleotide probe, which was synthesized according to the sequence of a peptide obtained from trypsin-digested purified HSP150 protein. Six putative clones were isolated and found to have the same restriction endonuclease digestion fragments. Two of them were sequenced, and both clones had an open reading frame of 1236 nucleotides that included the amino acid sequence of the peptide used for design of the oligonucleotide probe, as well as the sequences of six other peptides determined from the purified protein (Fig. 2).

The *HSP150* gene encodes a primary translation product of 412 amino acids with a calculated molecular mass of 41,633 Da (Fig. 2). The polypeptide starts with a putative signal sequence of 18 amino acids. It has the following features common to yeast signal sequences: two lysine residues in the N-terminal region, a hydrophobic core, and small uncharged amino acids at positions −1 and −3 relative to the cleavage site (17).

Direct N-terminal sequence analysis of the native mature protein harvested from the growth medium gave a double sequence corresponding to peptides 1 and 2, in approximately equimolar ratio. Reversed-phase chromatography resolved the native protein into two components, subunits I and II, which in N-terminal sequence analysis gave single sequences

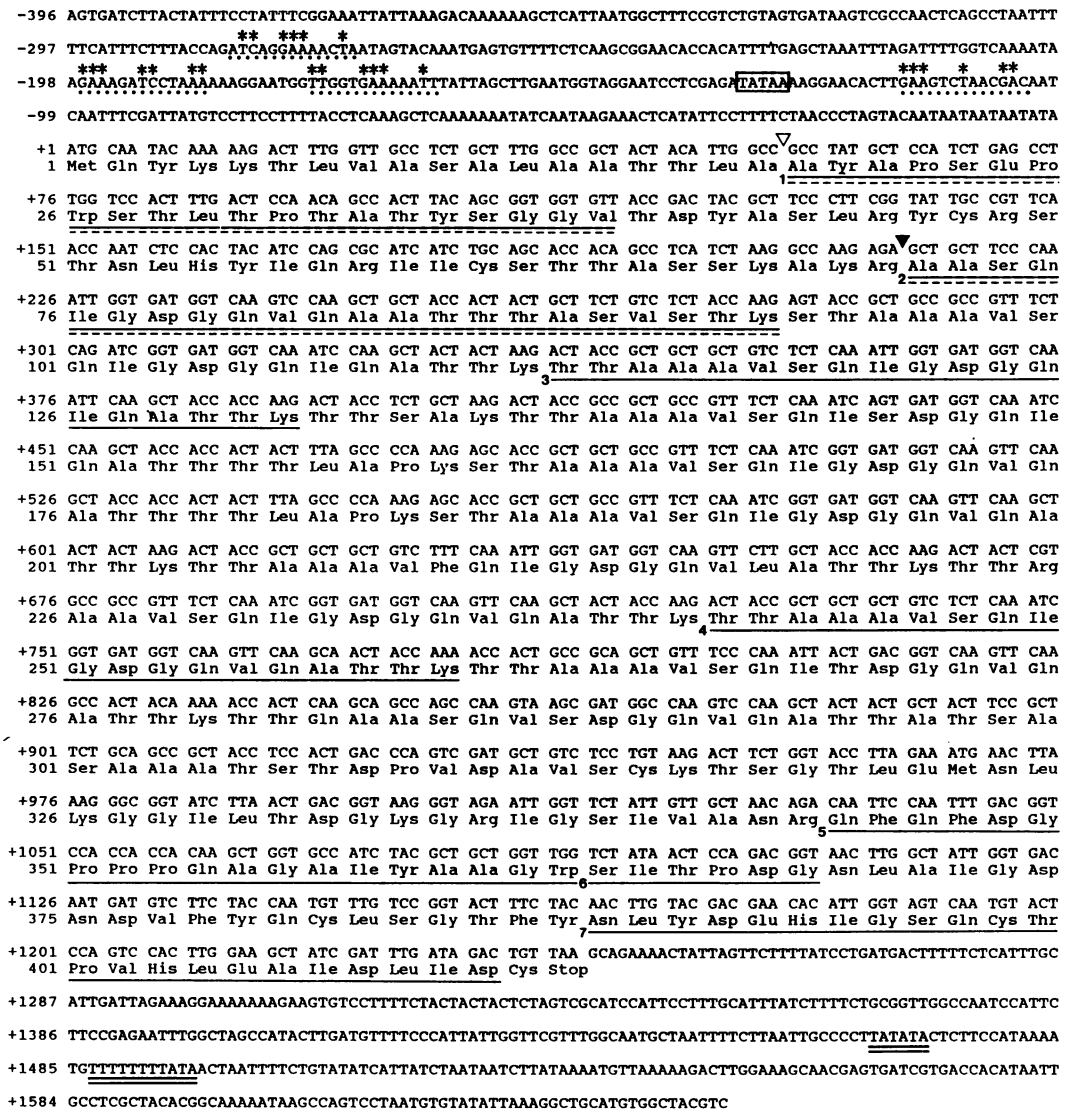


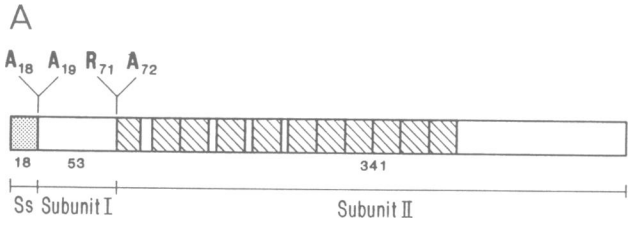
FIG. 2. Nucleotide sequence of *HSP150* and predicted amino acid sequence of the HSP150 protein. Three putative regulatory elements are indicated in the flanking sequences of the coding region: the TATA element (box), heat shock element (HSE)-like sequences (dotted underline), and two transcription termination signals (double underline). Nucleotides matching the HSE consensus are indicated by asterisks. The open and full arrowheads show the signal sequence and the KEX2 protease-specific cleavage sites, respectively. The underlined and numbered amino acid sequences were confirmed by direct amino acid sequencing of tryptic peptides of mature secreted HSP150. The sequences of peptides 1 and 2 (dashed underline) were obtained by direct N-terminal sequence analysis and thus represent the N termini of subunits I and II.

starting at amino acids 19 (peptide 1) and 72 (peptide 2), respectively (Fig. 2). This suggests that the signal sequence was cleaved and that a second cleavage took place directly after Lys^{70}-Arg^{71}, a putative KEX2 protease-recognition site, to yield subunits I and II, which remained noncovalently associated in the mature secreted protein. Whether the C terminus of subunit II remained intact was not studied. Thus, the 150-kDa band in SDS/PAGE (Fig. 1) represents glycosylated subunit II, whose apparent molecular mass is highly exaggerated, since the calculated molecular mass of the apoprotein is ≈34 kD. Subunits I and II lack potential N-glycosylation sites (Asn-Xaa-Ser/Thr), whereas 25% of their amino acids are serines or threonines, which are potential O-glycosylation sites.

The cleavage of subunits I and II may have been carried out by the serine protease KEX2, which cleaves the precursors of the mating pheromone α-factor and the M_1 killer toxin at pairs of basic amino acids (18, 19). The KEX2 protease is thought to be located in the late Golgi compartment (20). In the case of α-factor and killer toxin, the released effector molecule is secreted to the growth medium, whereas the rest of the precursor remains cell-associated.

The N-terminal portion of subunit II, 227 out of 341 amino acids, is highly repetitive. It consists of 11 tandem repeats of a 19-amino acid sequence (Fig. 3). Some of the repeats are separated by 5 or 7 amino acids. Although the nucleotide sequences coding for the repeats vary, the amino acid sequences are conserved (Fig. 3*B*).

Regulation of the *HSP150* Gene. Sequence analysis of the genomic clones revealed the same open reading frame found in the cDNA clones with no introns, plus putative regulatory signals in the sequences flanking the coding region (Fig. 2). The flanking region downstream of *HSP150* contains two putative yeast mRNA 3′ end-forming signals, TATATA (21) and TTTTTTTTATA (22), starting at nucleotides +1466 and +1487, respectively. The region upstream from the translation initiation codon revealed a putative TATA box (TATAA) starting at position −131. The 396 nucleotides 5′ to the translation initiation codon contain four nucleotide sequences that resemble the consensus heat shock element,



B

^{72}--AA-SQIGDGQVQAATTTASVSTKS^{94}
^{95}TAAAVSQIGDGQIQATTKT^{113}
^{114}TAAAVSQIGDGQIQATTKTTSAKT^{137}
^{138}TAAAVSQISDGQIQATTTTLAPKS^{161}
^{162}TAAAVSQIGDGQVQATTTTLAPKS^{185}
^{186}TAAAVSQIGDGQVQATTKT^{204}
^{205}TAAAVFQIGDGQVLATTKT^{223}
^{224}TRAAVSQIGDGQVQATTKT^{242}
^{243}TAAAVSQIGDGQVQATTKT^{261}
^{262}TAAAVSQITDGQVQATTKT^{280}
^{281}TQAA-SQVSDGQVQATTAT^{298}

FIG. 3. (*A*) Primary translation product of the *HSP150* gene. The putative signal sequence (Ss) is removed by a cleavage between Ala^{18} and Ala^{19}. Subunit I is cleaved directly after a KEX2 protease-specific site (Lys^{70}-Arg^{71}), between Arg^{71} and Ala^{72}. Subunit II contains 11 repeats of a 19-amino acid peptide (hatched boxes). The numbers 18, 53, and 341 denote the number of amino acids in the signal sequence and the subunits. (*B*) Amino acid sequence of the repetitive area in subunit II. Dots above the amino acids indicate nonconservative changes, and dashes denote gaps. The sequences between the repeats are underlined.

three or more contiguous repeats of the element NGAAN in alternating orientations (23, 24). Northern analysis of total RNA revealed a constitutively expressed *HSP150* transcript of ≈1.6 kb that which showed a substantial increase in steady-state level after heat shock (Fig. 4). This, along with the presence of heat shock elements, suggests that heat regulation of *HSP150* occurs at the transcriptional level.

Disruption of the *HSP150* Gene. A single-step gene disruption experiment (13) was carried out to assess whether *HSP150* is essential. The genomic copy of *HSP150* was disrupted with a *URA3* gene (Fig. 5*A*), and the *hsp150*::*URA3* construction was transformed into the diploid strain W303D. Integrants were isolated on SC plates lacking uracil. Four *URA3*$^+$ integrants were sporulated and tetrad analysis was performed on 20 asci from each integrant. Greater than 90% spore viability for each integrant suggests that *HSP150* is not essential. The disruption of *HSP150* was confirmed by Southern and Northern analyses (data not shown), and immuno-

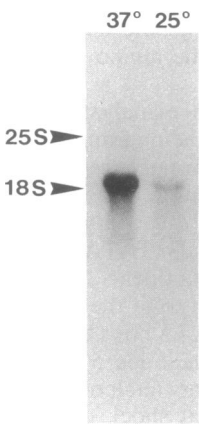


FIG. 4. Northern blot analysis of *HSP150* mRNA levels under normal and heat shock conditions. Cells were grown to midlogarithmic phase, and then the culture was divided in two and incubated for 1 hr at 37°C or 25°C. Total RNA was isolated and subjected to Northern blot analysis. The ^{32}P-labeled *HSP150* cDNA clone was used as the probe. The positions of the 25S and 18S ribosomal RNAs are indicated at left.

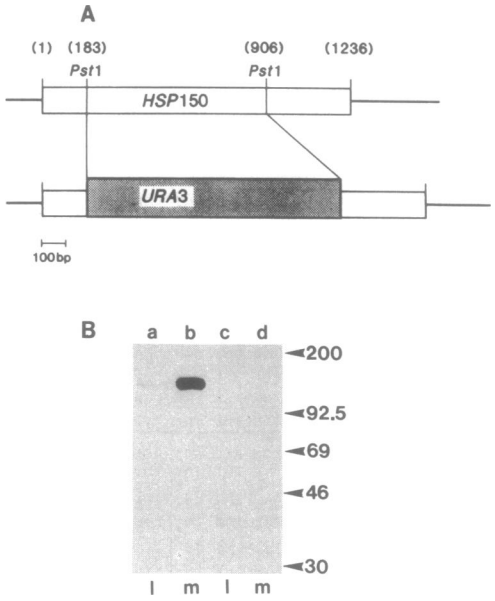


FIG. 5. Disruption of the *HSP150* gene. (*A*) The nucleotides coding for amino acids 61–302 in the genomic copy of *HSP150* were replaced by a *URA3* gene. (*B*) A *HSP150* strain (lanes a and b) and a *hsp150*::*URA3* strain (lanes c and d) were labeled with [^{35}S]methionine for 1 hr at 37°C, and the cell lysates (l; lanes a and c) and growth media (m; lanes b and d) were subjected to immunoprecipitation with anti-HSP150 antiserum. The precipitates were subjected to SDS/PAGE analysis. Molecular size markers (kDa) are at right.

precipitation experiments demonstrated the lack of HSP150 protein in both cell lysates and media (Fig. 5*B*). The disruption of *HSP150* gene had no effect on growth at 25°C or 37°C, mating, and thermotolerance (data not shown). In comparison, two members of the 70-kDa heat shock gene family, *SSC1* and *KAR2*, have been found to be essential (25–27).

Conservation of the HSP150 protein. Computer-aided searches of nucleic acid and protein files in the EMBL data base showed no significant homologies between HSP150 and any known genes or proteins, thus providing no hypothesis for the function of the HSP150 protein. Thus, we studied whether HSP150 is conserved, by searching for antigenic homologs in other yeasts. *T. delbrueckii*, *K. marxianus* and *Sch. pombe* cells were incubated at 25°C and 37°C, and the growth media and cell lysates were subjected to immunoblot analysis using anti-HSP150 antiserum. Antigenic homologs were discovered in all three yeasts. *T. delbueckii* (Fig. 6*A*) and *K. marxianus* (Fig. 6*B*) synthesized secretory homologs with apparent molecular masses of 54 kDa and 54–150 kDa, respectively. In both cases, similar homologs were found in the cell lysates, together with putative precursors of smaller molecular size. The heterogeneous electrophoretic migration of the homolog of *K. marxianus* may be due to heterogeneous N-glycosylation. A homolog of 120 kDa was found in *Sch. pombe*; however, it was not secreted but remained cell-associated (Fig. 6*C*). Excess HSP150 protein of *S. cerevisiae* inhibited the immunostaining of the *Sch. pombe* protein. The levels of the antigenic homologs of each organism were increased by heat shock.

Several heat shock proteins have previously been found to serve important physiological functions as chaperonins, which are involved in the maintenance or change of the conformation of other proteins (28, 29). Most stress proteins reside in the cytoplasm, nucleus, or mitochondria (2), and some are known to be segregated to the secretory pathway; however, they are not secreted. The BiP protein and grp94 are resident proteins of the endoplasmic reticulum (30, 31) and a 118-kDa glycoprotein of yeast has an unknown intra-

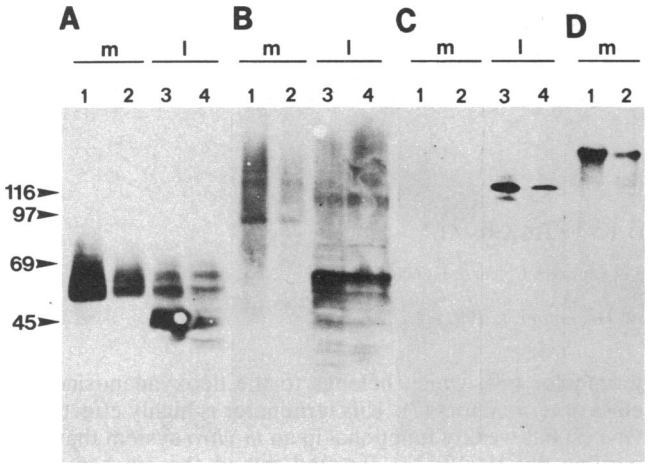


FIG. 6. Antigenic homologs of HSP150 in other yeasts. *T. delbrueckii* (*A*), *K. marxianus* (*B*), and *Sch. pombe* (*C*) cells were incubated for 1 hr at 25°C (lanes 2 and 4) or 37°C (lanes 1 and 3). The growth media (m) and lysed cells (l) were subjected to SDS/PAGE followed by immunoblot analysis with anti-HSP150 antiserum. HSP150 (subunit II) secreted to the medium by *S. cerevisiae* cells served as a control (*D*). Preimmune serum gave negative results for all samples (data not shown). Molecular size markers (kDa) are at right.

cellular location (32). Thus, HSP150 appears to be the first secretory stress protein reported. Though we have no data to suggest a possible function for HSP150, the apparent conservation of HSP150 in divergent yeasts suggests that HSP150-like proteins may constitute a class of secretory stress proteins.

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