Gene Sequence and Analysis of hsp30, a Small Heat Shock Protein of *Neurospora crassa* Which Associates with Mitochondria*

(Norah Plesofsky-Vig§$ and Robert Brambl§‖)

From the §Department of Plant Biology, the ‖Department of Genetics and Cell Biology, and the §Plant Molecular Genetics Institute, the University of Minnesota, Saint Paul, Minnesota 55108

hsp30 is a small heat shock protein of *Neurospora crassa* which earlier studies suggested may associate with mitochondria during cellular heat shock. We show here that the association of hsp30 with mitochondria is reversible and that hsp30 dissociates after cells are returned to normal temperature. We sequenced the gene for hsp30 and defined its transcript by S1 nuclease analysis and cDNA sequencing. The gene apparently is present in the genome as a single copy, and it contains no introns. The encoded 25.3-kDa peptide is related to other small heat shock proteins, especially those from green plants. According to its deduced sequence, hsp30 can form two strongly amphiphilic α-helices, including one at its amino terminus. In binding assays, *in vitro* synthesized hsp30 bound strongly to mitochondria isolated from heat-shocked cells but not to mitochondria prepared from cells incubated at normal temperature. A mutant hsp30 peptide, deleted in the amino-terminal amphiphilic helix, bound more avidly than the full-length hsp30 to mitochondria isolated from heat-shocked cells and exhibited less stringent requirements for binding. The mutant peptide also showed strong affinity for mitochondria isolated from unstressed cells.

The cells of every organism examined respond to sublethal high temperature by synthesizing a characteristic group of heat shock proteins (hsp)1 (Lindquist, 1986). These proteins have been shown to protect cells against damage from higher temperatures, but the specific functions of most heat shock proteins have not been identified. One proposed function of some of these proteins is to disrupt complexes of denatured proteins that form at high temperature, thereby allowing restoration of proper higher order structure to the proteins (Pelham, 1988). This suggestion that heat shock proteins affect the conformation of other cellular proteins is strengthened by the finding that certain normal proteins that mediate peptide transport and assembly are either closely related to heat shock proteins or are themselves synthesized at increased levels during heat shock. hsp70, a ubiquitously and highly conserved heat shock protein, is closely related to various normal cellular proteins that facilitate translocation of peptides into the endoplasmic reticulum and mitochondria (Deshayes et al., 1988) or participate in protein assembly in the endoplasmic reticulum (Munro and Pelham, 1986). In mitochondria and chloroplasts the assembly of enzyme complexes requires proteins homologous to the heat shock-induced groEL protein of *Escherichia coli* (Cheng et al., 1989; Hemmingsen et al., 1988), and the organellar proteins are themselves synthesized at increased levels during heat shock (McMullin and Hallberg, 1988).

Some of the heat shock-induced proteins are located, like the groEL-related proteins, in specific cellular organelles, including the small heat shock proteins in chloroplasts of green plants (Kloppstech et al., 1985; Vierling et al., 1988) and a mitochondrial hsp70-related protein (Craig et al., 1989). However, the subcellular location of other heat shock proteins is more variable and problematic. hsp70 itself appears to be distributed between the cytoplasm and the nucleus, and in mammalian cells it becomes concentrated in nucleoli as a result of heat shock (Pelham, 1984). Diverse intracellular locations have been reported for a group of small heat shock proteins that share homology with lens α-crystallin. An α-crystallin-related protein of mammalian cells is apparently intranuclear during heat shock (Arrigo et al., 1988), but related proteins of *Drosophila* have been reported to be either nuclear (Arrigo et al., 1980) or associated with perinuclear cytoskeletal elements (Leicht et al., 1986). Related heat shock proteins in green plants are found in cytoplasmic granules (Nover et al., 1983), in addition to those specifically translocated into chloroplasts. In several of these cases, a change in the temperature of cell exposure redirected the heat shock proteins to a different cellular location (Arrigo et al., 1980, 1988; Nover et al., 1983; Pelham, 1984), but in yeast, nutrient status determined the nuclear or cytoplasmic location of a small heat shock protein (Rossi and Lindquist, 1989).

*Neurospora crassa* synthesizes six major heat shock proteins at 45 °C, of approximately 98, 83, 67, 38, 34, and 30 kDa, and we earlier found that the three smaller peptides cofractionated with mitochondria (Plesofsky-Vig and Brambl, 1986). Although induction of the 38- and 34-kDa proteins only occurred after conidiospores had begun to germinate, hsp30 and the high molecular weight heat shock proteins were strongly produced by cells at all stages of development (Plesofsky-Vig and Brambl, 1988). In this report we have explored further the association of hsp30 with mitochondria, using *in vitro* binding assays. We determined the sequence of the hsp30 gene, including 5′ sequences that conform to the regulatory elements found upstream of other heat shock genes. Its pre-

*This research was supported by United States Department of Agriculture Competitive Research Grants Office Grant 85-CRCR-1-1655 and by National Institute of General Medical Sciences Grant GM-19398. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05601.

$To whom correspondence should be sent: Dept. of Plant Biology, 220 BioSciences Center, the University of Minnesota, Saint Paul, MN 55108.

1The abbreviations used are: hsp, heat shock protein; MOPS, 3-(N-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; HSE, heat shock element.
dicted amino acid sequence places hsp30 in the class of small heat shock proteins that have a region of homology to vertebrate α-crystallin.

A striking structural feature of the predicted hsp30 peptide is its potential to form two amphiphilic helices of high hydrophobic moment. We tested the importance of the aminoterminus helix for hsp30 localization by performing binding assays with mitochondrial fractions from heat-shocked and non–heat-shocked cells. Comparison of the binding properties of full-length hsp30 and a mutant peptide that lacked the amino-terminal helix suggests that the amphiphilic helix, rather than being solely responsible for the binding capability, is likely involved in regulating the membrane binding of hsp30, possibly in concert with a resident mitochondrial protein.

MATERIALS AND METHODS

Mitochondrial Isolation—Mycelial cells or germinating spores (1 g) were ground in a mortar and pestle at 4 °C with acid-washed sand (1.5 g) in a buffer (pH 7.2) containing 10 mM MOPS, 0.6 M sucrose, and 1 mM EDTA (Plesofsky-Vig and Brambl, 1987). The cells were subjected to two low speed centrifugations for 5 min each, at 1,500 × g and at 2,300 × g, respectively. The mitochondrial fraction was collected from the low speed supernatant by centrifugation at 13,000 × g for 12 min, and the pellet was washed with extraction buffer. Proteins in an aliquot of the postmitochondrial supernatant were precipitated with methanolic 0.1 M ammonium acetate and suspended in sample buffer for electrophoresis.

For gradient purification of mitochondria, cells were disrupted in buffered 0.25 M sucrose in a Braun MSK homogenizer. Unbroken cells and nuclei were removed by a 10-min centrifugation at 1,500 × g, and the mitochondrial fraction sedimenting at 20,800 × g was resuspended in a small volume of extraction buffer and brought to 40% Percoll (Pharmacia LKB Biotechnology Inc.) and 0.25 M sucrose. A gradient of Percoll was established by centrifugation at 58,400 × g in a Ti-70.1 rotor for 50 min; and a discrete band of mitochondria was collected, diluted with 20 volumes of extraction buffer, resuspended, and washed by the same procedure (Nicholson et al., 1987).

Isolation of Nuclei—The isolation procedure of Talbot and Russell (1982) was used with modifications. Cells were disrupted for 30 s at 4 °C in a Braun MSK homogenizer, and nuclei were extracted by Dounce homogenization in 1 M sorbitol, 7% Ficoll 400, 20% glycerol, 5 mM MgCl2, 10 mM CaCl2, adjusted to pH 7.5, and 0.5% Triton X-100. The nuclei were collected by centrifugation at 2,300 × g for 10 min, the nuclei were sedimented at 9,200 × g for 35 min and were resuspended in a small volume of 1 M sucrose, 50 mM Tris (pH 7.5), 5 mM MgCl2, 10 mM CaCl2, and 0.5% Triton X-100. The nuclei were mixed with a final concentration of 37% Percoll (Pharmacia) and 0.25 M sucrose. A gradient of Percoll was established after a 40-min centrifugation at 58,400 × g in a Ti 70.1 rotor, and the nuclei formed a band near the center of the gradient (Pelham and Russell, 1982). The nuclei were diluted with 10 volumes of resuspension buffer containing Triton X-100, collected by centrifugation at 3,900 × g for 10 min, and washed with buffer alone.

Labeling of Cellular Proteins and Gel Fluorography—Incubation conditions for Neurospora and preparation of whole cell extracts for electrophoresis have been described (Plesofsky-Vig and Brambl, 1985). Neurospora cells were grown at 30 °C for 3 or 5 h before transfer to 45 °C. Cellular proteins were radiolabeled for 15 min, between 15 and 30 min of heat shock, with 1 μCi/ml [3H]leucine (128 Ci/mmol) followed by 10 μM unlabeled leucine as a chase. The postmitochondrial fraction was treated with the fluorography agent Amplify (Amersham Corp.).

Gene Identification and Mapping—DNA from mycelial cells of Neurospora was partially digested with Sau3A1 and ligated into the BamHI site of the λ-cloning vector EMBL4 (Promega). The constructed λ-library was screened successively at high and low density with [32P]labeled insert cDNA for Neurospora hsp30, pH30, whose isolation we described earlier (Plesofsky-Vig and Brambl, 1987). Complementary λ-DNA was purified and cut with EcoRI, XhoI, PstI, HindIII, BamHI, and SalI, and a blot of the electrophoretically separated fragments was probed with radiolabeled hsp30 insert cDNA. XhoI fragments were subcloned into pUC18 (Viazzi and Manning, 1982), and a partial restriction map was derived for the genomic clone by cutting each XhoI subclone with EcoRI and SalI. The cloned cDNA was analyzed with the same restriction enzymes. High molecular weight genomic DNA of Neurospora was restricted with XbaI, EcoRI, and SalI; the fragments were separated in a 0.7% agarose gel for 14 h at 30 V, blotted to nitrocellulose, and probed with [32P]labeled inserts. Replicate blots were washed at either 65 °C (stringent) or at 42 °C (nonstringent).

Gene Sequencing—Cloned genomic XbaI fragments of 2.17 and 0.58 kilobases and the hsp30 cDNA were inserted into M13mp19 (Messing, 1983). Deletions were made for both strands of each DNA with T4 DNA polymerase (Dale et al., 1985), and overlapping clones were sequenced by the dideoxy chain termination method (Sanger et al., 1977), defining restriction fragments that were sequenced where necessary. The analog of 7-deaza-dGTP (Boehringer Mannheim) was used in several reactions to minimize band compression (Mizusawa et al., 1986), and radioabelling was with [32P]dATP or thio-[32P]dATP. Reaction products were separated in 5% polyacrylamide gel urea sequencing gels.

Nucleic Acid Analysis—An end-labeled (Maxam and Gilbert, 1980) XbaI-XhoI segment of the genomic fragment was denatured and hybridized in 80% formamide at 52 °C to 5 μg of poly(A)+ RNA or 100 μg of total RNA isolated from mycelial cells incubated at 45 °C (Plesofsky-Vig and Brambl, 1987). The hybridized nucleic acid was digested with 200 units/ml S1 nuclease for 30 min at 37 °C (Berk and Sharp, 1977) and the precipitated reaction products were separated in a sequencing gel.

Computer Analysis of Gene and Protein Sequence and Structure—DNA sequence analysis, peptide structural analysis, sequence alignment, and Protein Identification Resource data bank searches were performed with the aid of Intelligenetics programs SEQ, PFP, and IFIND. The algorithm for analyzing amphiphilic α-helical structure was written by Stephen Gantt, University of Minnesota.

Mutant Construction and in Vitro Transcription and Translation—The transcription vector pGEM4Z (Promega) was used for cloning an EcoRI fragment of a 5'-deleted hsp30 gene that includes the mRNA leader and coding sequences. To delete the helix-coding region, the ends of the hsp30 gene were marked with NruI (at base 99), filled in the AuaI site of the gel-purified DNA with the polymerase Klenow fragment, and introduced an 10-base MuiI linker (Lahe et al., 1994). This procedure eliminated 17 encoded amino acids (positions 17–33) and introduced 4 residues (Thr-Thr-Arg-Arg), all of which were included within the preexisting sequence and 2 of which were identical in position. The DNA templates in pGEM4Z were linearized with BamHI, and they were transcribed with SP6 RNA polymerase (Galili et al., 1986) in the presence of mG(5)ppp(5)'G (Pharmacia) for capping; the ATPase subunit 9 cDNA in pGEM3 (Pfanner et al., 1987) was linearized with EcoRI. The precipitated mRNAs were translated in a rabbit reticulocyte (Amersham Corp.) or T7 RNA-labeled peptide.

In Vitro Mitochondrial Binding Assays—Mitochondria, isolated from 16-h cultures, were resuspended at 8 mg of protein per ml in MOPS-sucrose buffer (pH 7.2), containing 1 mM dithiothreitol, 10 mM succinate, 0.15 mM ADP, and 2.5 mM potassium phosphate (Argun et al., 1988). The mitochondria (15 μl) were mixed with the radiolabeled assay peptide in reticulocyte lysate (5 μl), and the mixture was incubated at 29 °C for 45 min in the standard assay. o-Methylglucolulose was added to 0.33% and aprotinin to 0.02% where included in the assay, and phenylmethylsulfonyl fluoride was added to 1 mM from a 200 mM (in ethanol) stock solution. At the conclusion of the reaction MOPS-sucrose buffer (27 μl) was added, and the mixture was digested with 200 units/ml Sl nuclease for 30 min at 37 °C (Berk and Sharp, 1977), and the precipitated reaction products were separated in a sequencing gel.

RESULTS

Subcellular Localization of hsp30—We compared the amount of hsp30 that concentrated in the mitochondrial fraction of heat-shocked cells with the amount found associated with nuclei, an organelle to which several heat shock proteins have been found to localize (Arrigo et al., 1980, 1998; Pelham, 1984). Nuclei were isolated from heat-shocked cells...
by procedures of differential centrifugation and Percoll density fractionation that would maximize their separation from both unbroken cells and mitochondria. Mitochondrial fractions were separately purified on Percoll density gradients, and the integrity of the mitochondria was confirmed by electron microscopy (data not shown). Comparing equivalent amounts of protein in an isolated nuclear fraction and a purified mitochondrial fraction, we found that hsp30 was enriched severalfold in the mitochondrial fraction (Fig. 1).

The association of hsp30 with mitochondria depends on continued cellular exposure to high temperature and is apparently reversible. We labeled Neurospora cells with [3H]leucine at 15 min after their transfer to a 45 °C heat shock, added unlabeled leucine at 30 min, and after 45 min of heat shock the cells were either collected or transferred back to the normal growth temperature of 30 °C for another 30 min. Fig. 2B shows that there was a significant, approximately 50%, loss of hsp30 from the mitochondrial fraction after cells were returned to normal temperature although the peptide was stable in whole cell extracts (Fig. 2A). Analysis of the postmitochondrial supernatants indicated that hsp30 had moved into the soluble fraction of these down-shifted cells (compare lanes 3 and 4 of Fig. 2B). In contrast, when cells were maintained at 45 °C, hsp30 appeared to remain associated with the mitochondrial fraction (Fig. 2C). The dissociation of hsp30 from the organelle after cells are shifted to normal growth temperature suggests that the peptide is located at the periphery of mitochondria.

Nucleotide Sequence—We screened a λ-library of Neurospora genomic DNA with the cDNA insert of pNC-Hsp-30 (Plesofsky-Vig and Brambl, 1987) and purified complementary bacteriophage. Restriction mapping of the λ-insert showed that the complementary sequences were contained within a 2.17-kilobase XbaI fragment and that adjacent to this coding sequence was a 0.58-kilobase XbaI fragment (Fig. 3). High molecular weight DNA of Neurospora was cut with SalI, EcoRI, and XbaI, restriction enzymes used to map the cloned hsp30 gene. Southern analysis with the hsp30 cDNA probe revealed the same pattern of hybridization in the genomic DNA as in the λ-cloned gene, and no additional hybridizing bands of similar intensity were evident (Fig. 4). The faintly hybridizing larger bands may be due to incomplete cutting of the genomic DNA; these bands did not become more prominent, nor were additional ones revealed under less stringent hybridization conditions (data not shown). hsp30 cDNA also was found to have the same internal restriction sites as the genomic clone.

We sequenced both strands of the two XbaI genomic fragments and of the hsp30 cDNA (dashed arrows). 89% of the genomic sequence was determined from both strands, but with the cDNA sequences included 94% of the final sequence was determined from both DNA strands. Restriction sites used in analysis and gene cloning are indicated for the sequenced DNA (below) and its 5' extension (above, drawn to smaller scale). The transcribed sequences and protein coding region are also marked. b, bases.

stringent hybridization conditions (data not shown). hsp30 cDNA also was found to have the same internal restriction sites as the genomic clone.

We sequenced both strands of the two XbaI genomic fragments and of the hsp30 cDNA. We also sequenced a SalI fragment of the λ-insert where it crossed the internal XbaI site. The sequencing strategy is shown in Fig. 3. The sequenced gene contains one long open reading frame encoding a protein of 228 amino acids with a predicted molecular weight of 25,267. The hsp30 cDNA sequence begins 49 bases upstream of the initiating ATG and is identical to the gene sequence until the poly(A) tract at 1061 (Fig. 5).

The 5' end of the transcript was determined by S1 nuclease digestion of a genomic XbaI-AvaI restriction fragment (Fig. 3), annealed to RNA from heat-shocked cells. Both total RNA and poly(A)+ RNA protected 170 bases of the end-labeled probe, which was subjected to fluorography. The positions of the six heat shock proteins of Neurospora are indicated on the left by an arrow for hsp30 and by lanes for hsp8, 38, 34, and 34. Equal amounts of protein (38 μg) were applied to lanes N and M; 53 μg of protein was applied to lane C. Radiolabeled proteins were separated in a 10% polyacrylamide-SDS gel, which was subjected to fluorography.

Fig. 1. Comparison of radiolabeled proteins extracted from purified nuclei or purified mitochondria. Nuclei and mitochondria were isolated separately by Percoll gradient sedimentation. Lane C, unfractionated extracts of heat-shocked cells; lane N, purified nuclei from heat-shocked cells; lane M, purified mitochondria from heat-shocked cells. The positions of the six heat shock proteins of Neurospora are indicated on the left by an arrow for hsp30 and by lanes for hsp8, 38, 34, and 34. Equal amounts of protein (38 μg) were applied to lanes N and M; 53 μg of protein was applied to lane C. Radiolabeled proteins were separated in a 10% polyacrylamide-SDS gel, which was subjected to fluorography.

Fig. 2. Comparison of cellular fractions, showing temperature-dependent localization of hsp30 in vivo. The cells were pulse labeled and chased at 45 °C, and after 45 min they were either collected (45 °C) or incubated at 30 °C for an additional 30 min (45 °C → 30 °C). Proteins were separated in a 10% polyacrylamide-SDS gel, which was subjected to fluorography. The position of hsp30 in all lanes is marked by an arrow. Lines indicate the other five heat shock proteins of Neurospora, hsp98, 83, 67, 38, and 34, in the unfractionated extract. A, unfractinated cell extracts. Lane I, 45 °C; lane 2, 45 °C → 30 °C, B, fractionated cell extracts. Lane I, mitochondrial fraction, 45 °C; lane 2, mitochondrial fraction, 45 °C → 30 °C; lane 3, postmitochondrial supernatant, 45 °C; lane 4, postmitochondrial supernatant, 45 °C → 30 °C. C, mitochondrial fractions. Lane 1, 3.25 h at 45 °C; lane 2, 45 °C → 30 °C. Equal amounts of protein were applied to panel B, lanes 1 and 2 (150 μg) and to panel B, lanes 3 and 4 (350 μg). Equal portions of the cell extracts were applied to panel A, lanes 1 and 2, and equal portions of the mitochondrial extracts were applied to panel C, lanes 1 and 2.

Fig. 3. Map of cloned hsp30 gene, showing the sequencing strategy for genomic DNA (solid arrows) and for the cloned cDNA (dashed arrows). 89% of the genomic sequence was determined from both strands, but with the cDNA sequences included 94% of the final sequence was determined from both DNA strands. Restriction sites used in analysis and gene cloning are indicated for the sequenced DNA (below) and its 5' extension (above, drawn to smaller scale). The transcribed sequences and protein coding region are also marked. b, bases.

Downloaded from http://www.jbc.org/ by guest on November 6, 2017
most abundant transcript for hsp30 begins 121 bases upstream of the initiating methionine and excludes the upstream ATG at -156. A likely TATA box (Breathnach and Chambon, 1981) -TATAAA- appears 46 bases upstream of the transcriptional start site (Fig. 5), a spacing similar to that found in other Neurospora genes (Munger et al., 1985; Roberts et al., 1988). The transcript includes an open reading frame of 687 nucleotides and a 374-base untranslated region at the 3' end. An adenine residue appears in the -3 position, a context that is essential for translation initiation (Kozak, 1986). The sequence -AATAAA- appearing 23 bases upstream of the cDNA poly(A) tract resembles the polyadenylation signal -AATAAA- (Proudfoot and Brownlee, 1976). Without the poly(A) tail, hsp30 mRNA would be 1182 bases long, which is consistent with our earlier estimation of the polyadenylated mRNA as 1400 bases (Plesofsky-Vig and Brambl, 1987).

The promoter regions of heat shock genes contain a heat shock consensus element (HSE), CtnGAAAnnTTTcAGN, with 10 out of the 14 nucleotides conserved. It has been demonstrated that this HSE is essential for gene transcription (Pelham, 1982) and that it binds a heat shock transcription factor (Parker and Topol, 1984; Wu, 1985). In the Neurospora hsp30 gene we found a sequence at -461 which matches 9 of the 10 consensus nucleotides, and there are three elements with 7 consensus nucleotides at -280, -600, and -725, respectively. Each of the HSEs that are closest to the TATA box, at -280 and -461, has overlapping elements with 6 matching bases; there is one overlapping element for the distal HSE and two for the TATA-proximal HSE (Fig. 5). At -536, upstream from these two HSEs, is a homopolymeric tract of 53 almost uninterrupted adenines, and downstream of the gene at 1366 is a tract of 67 purines which ends with 26 consecutive guanines.

**FIG. 4.** Autoradiogram of a Southern blot of restriction fragments from the λ-insert (on the left) and Neurospora high molecular weight DNA (on the right), hybridized to 32P-labeled hsp30 cDNA. Both DNAs were digested with Sall (S), EcoRI (R), and XbaI (X), and fragments were separated in a 1% (left) or a 0.7% (right) agarose gel. The restriction map of the hsp30 gene and flanking sequences is shown in Fig. 3. Ab, kilobases.

**FIG. 5.** Nucleotide and predicted amino acid sequence of the hsp30 gene and cDNA. Likely promoter elements are boxed, including sequences that match the heat shock consensus element CtnGAAAnnTTTcAGN and the TATA box. The beginning of transcription and the polyadenylation site are marked with arrows, and a possible polyadenylation signal is underlined with a dashed line. Tandem repeated sequences within the mRNA leader are denoted by a double line. An open arrowhead indicates the 5' end of the cDNA. The region of the peptide that is conserved in small heat shock proteins and n-crystallin is denoted by a double line.
Peptide Sequence—The encoded hsp30 (Fig. 5) is a hydrophilic protein with short hydrophobic stretches, especially near the carboxyl terminus. It is slightly acidic, having a pI of 6.08. The peptide has no internal methionine, in agreement with its inability to incorporate [35S]methionine in vivo (Plesofsky-Vig and Brambl, 1985), and it has no cysteine, but more than 10% of its amino acids are threonine residues. Like several small heat shock proteins of other organisms, Neurospora hsp30 has sequence homology to mammalian α-crystallins. Comparison with the α-crystallin A chain (de Jong et al., 1975) and Drosophila hsp23 (Ingolia and Craig, 1982) in Fig. 7B indicates that similarity begins at amino acid 185 of hsp30 and continues through amino acid 216. When the sequence of hsp30 is aligned with two heat shock proteins from plants, hsp17.5E of soybean (Czarnecka et al., 1985) and the chloroplast-associated hsp22 of Chlamydomonas reinhardtii (Grimm et al., 1989), similarity extends from amino acid 175 of hsp30 through the next 53 residues to the end of the protein (Fig. 7A). In addition, within its first 28 amino acids and in residues 55–100, there is similarity between Neurospora hsp30 and the plant proteins. The mitochondrial association of hsp30 which we observed earlier (Plesosfky-Vig and Brambl, 1985) and in this study led us to analyze its peptide sequence for the ability to form strongly amphiphilic helices, a property described first for surface-seeking peptides (Kaiser and Kezdy, 1984) and also characteristic of mitochondrial targeting sequences (von Heijne, 1986). Structure analysis revealed that hsp30 has the potential to form two extensive amphiphilic α-helices of high hydrophobic moment (Fig. 8), according to von Heijne's modification (von Heijne, 1986) of the criteria of Eisenberg et al. (1984). An 18-residue helix near the amino terminus, within amino acids 18–35, would have a high hydrophobic moment of 10.2 and a strong maximum hydrophobicity of 6.6. A second helix from amino acids 179–196 would have a hydrophobic moment of 10.1 and a maximum hydrophobicity of 4.0, slightly below the 4.5 characteristic of surface-seeking peptides (von Heijne, 1986). Both helices contain acidic as well as basic residues, unlike the positively charged targeting sequences of most imported mitochondrial proteins (Hurt and van Loon, 1986).
Neurospora HSP30:18-35, \( \mu_g^{10.2}, H_{max} = 6.6 \)

Pho Thr Thr Leu Phe Arg Leu Arg Leu Asp Asp Phe Asp Thr Tyr Thr Arg Glu Val

Neurospora HSP30:179-196, \( \mu_g^{10.1}, H_{max} = 6.0 \)

Ser Glu Ser Ser Ile Gly Phe Ser Arg Thr Phe Asn Phe Pro Gly Arg Val

Chlamydomonas HSP22:14-31, \( \mu_g^{11.1}, H_{max} = 6.7 \)

\( \mu_g \) Ser Pro Phe Thr Thr Gly Ser Met Asp Arg Met Ala Ala Ala Ala Ala Asp

Drosophila HSP92:2-19, \( \mu_g^{12.1}, H_{max} = 5.1 \)

Arg Ser Val Phe Gly Tyr Leu Ala Asp Met Gly Glu Arg Cln Phe

Soybean HSP17:93-112, \( \mu_g^{13.3}, H_{max} = 5.0 \)

Asp Asp Thr Thr His Arg Val Glu Arg Ser Gly Lys Leu Val Arg Arg Phe

FIG. 8. Strongly amphiphilic \( \alpha \)-helical regions predicted for hsp30 and related heat shock proteins. \( \mu_g \), hydrophobic moment; \( H_{max} \), maximal summed hydrophobicity of 7 neighboring residues along the helical face (von Heijne, 1986). The 7 amino acids that contribute to the maximal summed hydrophobicity of each sequence are underlined. Presented are the 18-residue linear sequences of Neurospora hsp30, Chlamydomonas hsp22 (Grimm et al., 1988), Drosophila hsp22 (Ingolia and Craig, 1982), Caenorhabditis hspl6-1 (Russnak and Candido, 1985), and soybean hsp6871 (Nagao et al., 1985).

Responsible for its association with mitochondria, as well as other factors that may promote binding, we employed hsp30 that was synthesized by \textit{in vitro} transcription/translation in binding assays with isolated mitochondrial fractions. The hsp30 that we synthesized in \textit{vitro} had the same mobility in a one-dimensional gel as extracted hsp30 (data not shown). By comparing the amount of the peptide that became mitochondrial associated with the amount left unbound in postmitochondrial supernatants, we could estimate relative efficiencies of \textit{in vitro} binding. We found that hsp30 bound strongly to mitochondria isolated from cells that were heat shocked, with 35-50% of the available peptide binding to these mitochondria (Figs. 9 and 10).

Since hsp30 appears to associate reversibly with mitochondria \textit{in vitro}, dissociating after the temperature of cell incubation is lowered, it seemed necessary to determine whether \textit{in vitro} binding of hsp30 displayed a similar preference for heat-shocked mitochondria over mitochondria isolated from non-heat-shocked cells. To minimize the active proteolysis of hsp30 in normal mitochondrial preparations, we included the protease inhibitors \( \alpha \)-macroglobulin and aprotinin in the binding assays. We found that hsp30 did not bind or bound only weakly to mitochondria from unstressed cells (Fig. 9A) despite the availability of unbound hsp30 in the postmitochondrial supernatant (Fig. 9B). There was an 80% decrease in the amount of hsp30 that bound to these mitochondria from unstressed cells, relative to stressed cells, and a two-thirds decrease in binding efficiency. This result indicates that hsp30 has a much greater affinity \textit{in vitro} for mitochondria derived from heat-shocked cells than for mitochondria from normal cells. The mitochondria from both types of cells bound equivalent amounts of an identified mitochondrial protein, the nucleus-encoded subunit 9 of \textit{Neurospora} mitochondrial ATPase, but only the mitochondria isolated from normal cells processed the precursor peptide to its mature size (Fig. 9C) (Pfanner et al., 1987).

The binding of hsp30 to mitochondria from heat-shocked cells, although strong, was not complete, and we found that the extent of binding could be increased or decreased by changes in the assay conditions. Binding efficiency was decreased by two-thirds if the reaction was conducted at 4°C rather than at 29°C (Fig. 10), and shorter reaction times at 29°C also decreased binding (data not shown). On the other hand, inclusion of the protease inhibitors \( \alpha \)-macroglobulin and aprotinin in the binding assay produced a 50% increase in the amount of hsp30 that bound to mitochondria as well as a 7% increase in the proportion of available hsp30 that bound (Fig. 9), suggesting that the inhibitors also protected an additional protein in the mitochondrial membranes or the reticulocyte lysate that was required for hsp30 binding.

The strong preference of hsp30 for binding to mitochondria from heat-shocked cells raised the possibility that in \textit{vitro} synthesized hsp30 might be associating with other heat shock proteins, including endogenous hsp30, in the mitochondrial fractions. To test this possibility we blocked heat shock protein synthesis by including cycloheximide (100 \( \mu \)g/ml) in the cell cultures throughout heat shock. Binding was as strong, however, to mitochondria isolated from these cells as to...
mitochondria from uninhibited cells (Fig. 10). The same results were obtained with 5-h cells heat shocked early in growth (data not shown) as with the older 16-h cultures used in these binding assays, indicating that the inducible heat shock proteins themselves are not the factors promoting hsp30 binding.

One requirement for translocation of a protein into or across mitochondrial membranes is an amino-terminal domain that has the potential to form an amphiphilic helix (von Heijne, 1986). We were especially interested, therefore, in determining the importance for mitochondrial binding of the amphiphilic helix predicted near the amino terminus of hsp30. We constructed a mutant gene lacking the amino-terminal 14 of the 18 residues in the predicted helix and containing linkers designating 2 new residues (see "Materials and Methods"). A mutant hsp30 peptide, with a predicted size of 23.7 kDa, was synthesized in vitro by transcription/translation, and its binding to mitochondria was assayed.

Surprisingly, we found that rather than exhibiting weaker binding, the mutant peptide bound in vitro more strongly to heat shock mitochondria than did the full-length protein (Figs. 9A and 10A), with very little of the mutant peptide remaining unbound in the postmitochondrial supernatant (Figs. 9B and 10B). Also in contrast to the native peptide, the mutant hsp30 bound almost as avidly to mitochondria isolated from unstressed cells as it did to heat shock mitochondria. Approximately 60–75% of the available mutant peptide bound to normal mitochondria (Fig. 9), whereas binding was less than 20% for the full-length peptide.

In several other characteristics of its binding the mutant hsp30 also differed from the native protein. Its binding to heat shock mitochondria appeared to be independent of assay conditions. Mitochondrial binding of the truncated hsp30 was as strong (or stronger) at 5 min of the reaction as at 45 min (data not shown), and its binding was not diminished when the reaction was performed at 4 °C rather than at 29 °C (Fig. 10), unlike binding of the native protein. Furthermore, binding of the mutant protein did not appear to require the additional protein component(s) from the mitochondrial membranes or lysate required by binding of native hsp30 since mutant protein binding did not increase when the protease inhibitors a2-macroglobulin and aprotinin were included in the reaction (Fig. 9). Under all these conditions the mutant hsp30 appeared to bind quantitatively to mitochondria whereas the binding of full-length hsp30 was strongly influenced by assay conditions.

We attempted to increase the binding affinity of full-length hsp30 by introducing other variations in the assay conditions. Preincubation of precursor peptides in urea has been reported to accelerate their import into mitochondria dramatically, possibly by unfolding the peptides (Eilers et al., 1988). However, preincubating hsp30 in 5 M urea, prior to dilution during the binding assay, did not increase its binding to mitochondria, and pretreatment with urea depressed binding of the truncated peptide noticeably (data not shown). Supplementation of the reaction with reticulocyte lysate, included elsewhere to facilitate translocation (Argan et al., 1983) of the ATPase subunit 9 into mitochondria, also inhibited binding of the truncated hsp30, without affecting binding of the full-length protein. Other reagents that were included in the binding assay, such as ADP, phosphate, or succinate, could be eliminated without affecting the extent of binding for either peptide. However, excluding dithiothreitol from the reaction reduced binding of both the full-length and mutant peptides.

**DISCUSSION**

hsp30 is one of three small heat shock proteins synthesized by *Neurospora* which we find associated with the mitochondrial fraction. Earlier experiments showed that purified mitochondrial hsp30 had a 7–8-fold greater concentration of radiolabeled hsp30 than did whole cell extracts (Plesofsky-Vig and Brambl, 1985). Unlike the other two small heat shock proteins whose synthesis depends on age of the cells (Plesofsky-Vig and Brambl, 1985), hsp30 is synthesized by all heat-shocked cells of *Neurospora*, and its RNA is accumulated to high levels (Plesofsky-Vig and Brambl, 1987). But under normal growth conditions, there is no hsp30 RNA detectable in dormant spores, germinating spores, or mycelial cells (Plesofsky-Vig and Brambl, 1987).

We have found that hsp30 is enriched in the mitochondrial fraction of heat-shocked cells compared with a purified nuclear fraction. Our studies reveal that hsp30 dissociates from mitochondria when the temperature of cell incubation is lowered and that continued association with the mitochondrial fraction requires sustained high temperature. The reversible nature of this association makes it more likely that hsp30 is on the periphery of mitochondria rather than integrated within them. Although hsp30 is likely peripheral to the membranes, it is closely associated with them. We were unable to extract hsp30 by washing mitochondria with high concentrations of KCl (up to 1 M) or by treating them with 25 mM EDTA. Only a minor fraction of hsp30 dissociated when mitochondria were washed with 50 or 125 mM EDTA or when they were incubated in 1% Triton X-100.²

*Neurospora* appears to have a single copy of the gene for hsp30. If there are related genes within its genome, they differ too much in sequence to cross-hybridize readily. This contrasts with the four hsp30 genes of soybean, whose encoded peptides are more than 90% homologous (Nagao et al., 1985), and with the multiple genes for human hsp27 (Hickey et al., 1986). However, sequence variability is found among the hsp16 genes of *Caenorhabditis* (Rusnak and Candido, 1985) and among the several small heat shock proteins of *Drosophila* (Ingolia and Craig, 1982) some of whose genes do not cross-hybridize (Ayne and Tissières, 1985).

The 5' region of the hsp30 gene has heat shock elements

² N. Plesofsky-Vig and R. Brambl, unpublished results.
that match the consensus sequence found upstream of other heat shock genes (Pelham, 1982). Maximum induction of the Drosophila hsp70 gene requires two distinct TATA-proximal HSEs, to which a heat shock transcription factor binds cooperatively (Amin et al., 1987). Two elements that seem likely to be active in hsp30 begin 108 and 289 bases upstream of the TATA sequence. Each has flanking HSEs with 4 overlapping bases, and such overlapping elements have been shown to be more effective than a single HSE in binding the transcription factor and in promoting transcription (Sorger and Pelham, 1997). The promoter elements of the hsp30 gene may be strengthened further by periodic blocks of -GAA- (Amin et al., 1988) that extend beyond the strict HSEs.

The hsp30 gene is flanked by two long homopolymeric sequences, an adenine-rich sequence 363 bases upstream from the presumed TATA box and a guanine-rich tract a few hundred bases downstream of the gene. Both types of homopolymeric tracts are reported to affect the structure of adjoining DNA. Tracts of dG . dC can unwind adjacent chromosomal DNA (Kohwi and Kohwi-Shigematsu, 1988), and homopolymeric tracts of dA . dT appear to produce bends where they adjoin other DNA sequences (Koo et al., 1986). Although the significance of these tracts for the hsp30 gene is unknown, a tract of 14 contiguous adenes appears about 230 bases upstream of a soybean heat shock gene TATA box in a region that lacks HSEs but is required for full gene expression in a heterologous system (Baumann et al., 1987).

Studies with Drosophila indicate that the leader regions of heat shock mRNAs confer a translational advantage in heat-shocked cells (Klenz et al., 1985; McGarry and Lindquist, 1985), an advantage also displayed in vivo by heat shock mRNAs of Neurospora (Plesofsky-Vig and Brambl, 1987). The 121-base leader of hsp30 mRNA is enriched in adenine (36%) and cytosine (32%) residues and, like the leaders of other heat shock mRNAs (McGarry and Lindquist, 1985), has little obvious secondary structure, an attribute that may enhance mRNA translatability during stress (Kozak, 1988). Two tandem repeated sequences appear in the leader, AGATCA/AGATCA at -119 near the cap site and CACTCTgAA/CACTCTcAA/CA at -89 (Fig. 5), but their contribution to translation is unknown. Splicing of mRNA precursors has been shown to be inhibited in Drosophila by a severe heat shock (Yost and Lindquist, 1986) and like the related genes of Drosophila (Ingolia and Craig, 1982) and soybean (Czarnecka et al., 1985; Nagao et al., 1985), the Neurospora hsp30 gene has no introns. Introns are present in the homologous genes of nematodes (Russnak and Candido, 1985) and humans (Hickey et al., 1986).

hsp30 belongs to the group of eukaryotic heat shock proteins that have sequence homology to vertebrate α-crystallin of the eye lens. It has been pointed out that members of this group form complex higher order structures similar to those formed by α-crystallin (Arrigo et al., 1988; Nover et al., 1983). Another characteristic they may share is the extreme structural stability of α-crystallin, even when heated to 100 °C (Maiti et al., 1988). The most conserved portion of these proteins is a 33-amino acid region near their carboxyl termini, the latter half of which is hydrophobic. Neurospora hsp30 has greater similarity to small heat shock proteins of soybean (Nagao et al., 1985) and Chlamydomonas (Grimm et al., 1989) than to the homologous proteins of Drosophila (Ingolia and Craig, 1982), nematodes (Russnak and Candido, 1985), and humans (Hickey et al., 1986). This similarity to the plant proteins extends beyond their carboxyl regions, also encompassing 38 of the 100 amino-terminal residues of hsp30. The small heat shock proteins of animals, in turn, show more extensive homology to one another and to α-crystallin (Hickey et al., 1986), with an additional 45-amino acid region of homology immediately preceding the tightly conserved carboxyl domain (Fig. 7B) (Ingolia and Craig, 1982).

Apart from this relationship to other heat shock proteins, Neurospora hsp30 shows no sequence similarity to any characterized peptides that might otherwise suggest a function. However, within its unique central region, the sequences surrounding histidine 126 (Fig. 7C) resemble the sequences flanking histidine 215 in the chloroplast D1 and D2 proteins; this conserved histidine in the chloroplast photosystem II proteins appears to bind non-heme iron (Sayre et al., 1986). There is also some similarity to a histidine-containing sequence in yeast adenylate kinase (Schulz et al., 1986), in which the analogous histidine 45 is reported to bind magnesium of MgATP/MgADP (Egner et al., 1987). This suggests the possibility that hsp30 binds a divalent cation.

hsp30 does not possess the targeting structure characteristic of internalized mitochondrial proteins, typically an amino-terminal sequence with basic and hydroxylated residues, which is cleaved after peptide import (Hurt and van Loon, 1986). However, its sequence predicts that hsp30 can form two extensive, amphiphilic α-helices of high hydrophobic moment, one at the amino terminus and the other near the carboxyl end, overlapping the region of α-crystallin homology. Formation of such helices is known to guide some proteins to insert into membranes or, in other cases, to promote specific interactions among proteins. An analysis of related heat shock proteins indicates that some of these can also form amphiphilic α-helices with hydrophobic moments near 10 or above. These are at the amino termini of Chlamydomonas hsp22, Drosophila hsp22, and hsp16-1 of Caenorhabditis and at the carboxyl terminus of soybean hsp17 (Fig. 8). However, this is not a consistent feature of the α-crystallin-related heat shock proteins.

We found that deletion of the sequences comprising most of the amino-terminal helix of hsp30, rather than reducing, strikingly increased the peptide’s in vitro binding to mitochondria. Typically, 35-50% of the available hsp30 bound to mitochondrial membranes in in vitro assays whereas binding approached 100% for the peptide that was deleted in the helical domain. This result indicates that the potential amphiphilic helix is not required for hsp30 to interact with mitochondrial membranes. Instead, the amino-terminal helix may be a structure through which hsp30 binding can be regulated. More stringent requirements for binding were exhibited by the native hsp30, whose binding depended upon physiological temperatures and was slower than binding of the mutant peptide. An important finding of this study is that hsp30 binds selectively in vitro to mitochondria from heat-shocked cells, but deletion of the helical domain abolished this specificity. The truncated peptide showed a high affinity for mitochondria from both stressed and unstressed cells.

We believe that the amino-terminal helix of hsp30 may interact with a protein resident in mitochondrial membranes. Peptide regions characterized by amphiphilic helical structure have been described in several proteins as the sites at which specific interactions between proteins occur. An amphiphilic helix is not required for hsp30 to interact with mitochondrial membranes in in vitro assays. A shortened proteolytic product of the large subunit, lacking the amino-terminal helical domain, was found to be dramatically more effective than the full-length subunit at aggregating granules at low calcium concentrations, although not as effective as the assembled calpactin I complex. The amino terminus was suggested to...
function as a regulatory region that modulates the calcium sensitivity of binding sites elsewhere in the protein (Drut and Creutz, 1988). The amino-terminal helix of hsp30 may play an analogous role, affecting the availability or affinity of other binding sites within the peptide.

The presence of an accessory protein in the mitochondrial membranes of Neurospora gains experimental support from the enhanced binding of full-length hsp30 that was produced by including protease inhibitors in the reaction. Since cycloheximide did not interfere with the development of binding capibility in heat shocked mitochondria, the hypothetical accessory protein would likely be a normal cellular constituent that becomes modified by heat shock to interact effectively with hsp30. In preliminary experiments, we found that antibodies prepared against purified outer mitochondrial membranes of Neurospora partially blocked the in vitro binding of hsp30 to heat-shocked mitochondria whereas preimmune serum did not diminish binding.1

The actual biological function of the helical domain may be determined by transforming Neurospora with the mutant gene and comparing in vivo localization of the truncated peptide with that of native hsp30. The mutant peptide in vivo might bind irreversibly to mitochondria and be unable to dissociate from the organelles, as the native protein does, after cells are returned to normal temperatures and are no longer under stress. Alternatively, the truncated hsp30 in vivo might have a different intracellular distribution from the full-length protein, either a more general membrane distribution or, possibly, no membrane association at all if only regulated binding occurs in vivo.

Acknowledgments--We thank Daniel Geraghty for invaluable help with sequencing techniques and strategy, Bernhard Grimm and Klaus Kopp for communicating the Chlamydomonas hsp22 sequence before publication, and Walter Neupert for the ATPase subunit 9 cDNA cloned into pGEM3. We are also grateful to Stephen Gantt for advice on peptide structure analysis, Rodney Kuehn for electron microscopy, and Ross Readstrom for technical assistance.

REFERENCES

1 N. Plesofsky-Vig, C. Mannella, and R. Brambl, unpublished results.
Gene sequence and analysis of hsp30, a small heat shock protein of Neurospora crassa which associates with mitochondria.
N Plesofsky-Vig and R Brambl


Access the most updated version of this article at http://www.jbc.org/content/265/26/15432

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/26/15432.full.html#ref-list-1