

Mutational Analysis of the Human HSP70 Protein: Distinct Domains for Nucleolar Localization and Adenosine Triphosphate Binding

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Abstract. The human HSP70 gene was modified *in vitro* using oligonucleotide-directed mutagenesis to add sequences encoding a peptide from the testis-specific form of human lactate dehydrogenase (LDH) to the carboxy terminus of HSP70. The peptide-tagged HSP70 can be distinguished from the endogenous HSP70 protein using an LDH peptide-specific antiserum in indirect immunofluorescence assays of cells transiently transfected with an expression vector containing the tagged HSP70 gene regulated by the human HSP70 promoter. A series of deletion mutants within the HSP70 protein coding region were generated. Using double-label indirect immunofluorescence with the LDH peptide-specific antiserum and HSP70-specific mAbs, we compared the intracellular distribu-

tion of the deletion mutants to that of endogenous HSP70. We have determined that sequences in the carboxy terminus of HSP70 are necessary for proper nucleolar localization after heat shock. In contrast, sequences in the amino terminus of HSP70 are responsible for the ATP-binding ability of the protein. Mutants that were unable to bind ATP, however, still displayed nucleolar association, indicating that ATP binding is apparently not required for interaction with substrate. Additional support that HSP70 appears to be composed of at least two domains follows from the results of trypsin digestions of wild type and mutant HSP70. Protease digestion of the mutant HSP70 proteins identified a region of HSP70 that, when deleted, affected HSP70 conformation.

ALL cells respond in a similar manner to abrupt changes in their environment. Exposure to temperatures a few degrees above normal, and to a variety of other chemicals (amino acid analogues, ethanol, arsenite, various heavy metals, and certain inhibitors of mitochondrial function) results in rapid changes in gene expression. The synthesis of a small set of proteins is induced, whereas synthesis of the normal complement of proteins present before the stress treatment is diminished (reviewed in references 10 and 18). The heat- or stress-induced proteins are referred to by their molecular mass, and their collective induction is thought to confer upon the cells the ability to recover from the stress treatment, and to provide a transient degree of protection from subsequent stress. Characterization of the induction of the various stress proteins by a multitude of agents in many different cell types, as well as the apparent protective effects of the stress proteins have been well documented (see reference 19). However, the biochemical functions of the individual stress proteins have yet to be determined.

Because of their abundance and high degree of induction in stressed cells and their striking conservation among species (1, 14), the 70-kD class of stress proteins has attracted

attention. In every species examined thus far, a stress-induced protein of ~70 kD has been found. In mammalian cells, at least four different HSP70-related proteins have been characterized. HSP70, the most highly stress-induced protein, and P72, which is constitutively expressed and only slightly inducible, are both found in the cytoplasm and nucleus, and become associated with nucleoli after heat shock (40, 42). GRP78, related to HSP70 both by sequence homology and antigenically, resides in the endoplasmic reticulum, and has been demonstrated to be the heavy-chain immunoglobulin binding protein, BiP (26, 39). Finally, a 75-kD protein antigenically related to HSP70 and P72 appears to reside within mitochondria (Mizzen, L. A., C. Chang, J. Garrels, and W. J. Welch, 1989, manuscript submitted for publication). The identification of HSP70-related proteins in distinct subcellular compartments has led to the suggestion that each protein performs similar tasks, but within its own intracellular locale.

With the exception of HSP70, these proteins are all expressed at considerable levels and are quite abundant in unstressed cells, suggesting that they participate in normal biochemical processes in the absence of stress. HSP70 is strictly stress-inducible in most species, the notable exception being primate cells (43). In human cells, HSP70 is cell cycle regulated (22). HSP70 thus also appears to function in the ab-

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sence of stress in human cells. All four proteins also share the common feature of binding to ATP-agarose, and this has facilitated their purification and biochemical characterization (41).

We have been interested in the function of HSP70 in both unstressed and heat-shocked cells, with the aim of identifying cellular processes in which HSP70 participates. Clues to the identity of these processes can be obtained by analyzing the cellular components that HSP70 interacts with. After heat shock, HSP70 becomes associated with the preribosomal-containing granular region of the nucleolus, and during recovery a fraction of HSP70 colocalizes with polyribosomes suggesting that HSP70 may participate in maintaining ribosome assembly, and/or translation (43). Recent biochemical evidence suggests that HSP70 and P72 are involved in the translocation of nascent polypeptides across membranes of the endoplasmic reticulum and mitochondria (7, 12, 28, 47). HSP70 and P72 have also been shown to interact with clathrin, the cellular protooncogene p53, and the viral transactivating proteins of adenovirus (E1A) and SV40 (T antigen) (4, 5, 13, 34, 37, 38, 44). Finally, we have demonstrated that HSP70 displays a number of cell cycle-specific interactions with other proteins (23).

Given the number and variety of reported interactions, we became interested in identifying the domains of HSP70 responsible for its interactions with other molecules. To this end, we have constructed a series of deletion mutants within the HSP70 coding region. To distinguish the mutant proteins from the endogenous cellular HSP70 protein, we first marked the human HSP70 gene *in vitro* by adding sequences that encode a peptide from the testis-specific form of lactate dehydrogenase (LDH)¹ to the extreme carboxy terminus of the HSP70 coding region (24). This marked HSP70 gene was then used to construct the deletion mutants. Using an LDH peptide-specific antiserum we were able to detect the products of the marked genes after transient transfection into HeLa and CV1 cells. We present results indicating that sequences in the carboxy terminus of HSP70 are necessary for nucleolar localization after heat shock, whereas sequences in the amino terminus are necessary for the ATP-binding ability of the HSP70 protein. In addition, we analyzed the ability of each deletion mutant to accelerate the recovery of cells after heat-shock treatment.

Materials and Methods

Construction of Tagged HSP70 Expression Vector and Deletion Mutants

The human HSP70 gene was tagged at the carboxy terminus of the coding region with sequences coding for amino acids 5–15 of human testis-specific LDH (24) by oligonucleotide-directed *in vitro* insertional mutagenesis using the Amersham system (Amersham Corp., Arlington Heights, IL) based on a method described previously (29). The Bam HI-Hind III fragment from pH 2.3 (45) containing the HSP70 coding region and flanking sequences was inserted into the polylinker region of the replicative form of M13mp18 and single-stranded phage DNA was prepared (21). A 67-base oligonucleo-

tide (5' GAA AGG CCC CTA CTC GTC GTC CTC GAT CAG CTT CTC GAT CAG CTG CTC ACA GAA TTC TAC CTC CTC A 3') was synthesized on a DNA synthesizer (model 380B; Applied Biosystems, Foster City, CA) and was gel purified before use. 12 bases at the 3' end of the oligonucleotide hybridized to the last few codons of the HSP70 coding sequence, whereas 12 bases at the 5' end of the oligonucleotide hybridized to the termination codon and the 3' untranslated region. The intervening 43 bases of non-hybridizing sequence contained codons specifying amino acids 5–15 of LDH. Mutagenesis resulted in the positioning of the LDH peptide tag at the extreme carboxy terminus of the HSP70 coding region and creation of an Eco RI restriction endonuclease cleavage site at the HSP70-tag junction. Replicative form DNA was prepared from the mutated construct, and the Bam HI-Hind III fragment was shuttled into an expression vector containing 188 bp of the human HSP70 promoter (46) and the remaining 5' untranslated sequences to generate the wild type, tagged HSP70 expression construct shown in Fig. 1 A.

To construct the deletion mutants, the mutated Bam HI-Hind III fragment was inserted into the polylinker region of pGEM1 (Promega Biotec, Madison, WI), and the HSP70 sequences between the indicated restriction sites were deleted. The proper reading frame was restored by modifying the restriction endonuclease-generated ends with either Mung bean nuclease, T4 DNA polymerase, or the Klenow fragment of DNA polymerase I before religation. SN was generated by deleting sequences between the Sac II and Nco I sites, NF by removing sequences between the Nco I and Fsp I sites, PB by deleting sequences from Pst I to Bcl I, BC by removing the Bc II-Cla I fragment and SMA by deleting sequences between two internal Sma I sites. The NH₂-terminal deletion, NSC, was generated by deleting sequences between the Sac II and Cla I sites, and the COOH-termination deletion, CRI, was generated by fusing two Eco RI sites that were generated by *in vitro* mutagenesis. The location of restriction sites is shown in Fig. 1 A and a diagram of the deleted regions of the HSP70 protein is shown in Fig. 1 B.

After construction of the deletion mutants, the Bam HI-Hind III fragment from each clone was shuttled into the expression vector as described for the wild-type construct. The expression plasmids were used for transfection studies, while the pGEM constructs were used for *in vitro* transcription/translation.

Transcription, Translation, and Immunoprecipitation of Mutants

The pGEM constructs containing the wild-type, tagged HSP70 gene and the deletion mutants were linearized with Hind III and transcribed *in vitro* using T7 RNA polymerase. The resulting RNAs were translated in a rabbit reticulocyte lysate in the presence of [³⁵S]methionine as described (33). After translation, the reactions were diluted 20-fold with RIPA buffer (150 mM NaCl, 10 mM Tris-HCl [pH 7.4], 1.0% deoxycholate, 1.0% Triton X-100, 0.1% SDS) and 3 μ l of the LDH peptide-specific rabbit antiserum was added. The reactions were rocked at 4°C for 2 h and the immune complexes were isolated by addition of protein A-agarose. After being washed five times with RIPA buffer, the immunoprecipitated proteins were released by boiling in SDS sample buffer and analyzed by SDS-PAGE and fluorography.

Transfections

HeLa and CV1 (monkey kidney) cells were transiently transfected by a modification, of existing protocols (6 and 8). Briefly, plasmids to be transfected were brought to a final concentration of 20 μ g DNA/ml in 125 mM CaCl₂, 25 mM BES (*N,N*-bis[2-hydroxyethyl]-2-aminoethane sulfonate), 140 mM NaCl, and 0.75 mM Na₂HPO₄. Cells growing in 10-cm² dishes were trypsinized, pelleted by centrifugation, and resuspended in the DNA solution. After 15 min at room temperature the cells were diluted with DME plus 10% calf serum, 0.025 vol of 0.25 M CaCl₂, and 0.025 vol of 2 \times BBS (50 mM BES [pH 6.95], 280 mM NaCl, 1.5 mM Na₂HPO₄), and plated. The plates were placed at 37°C in a humidified atmosphere equilibrated with 3% CO₂. After 16–18 h the plates were washed twice with DME and allowed to recover in DME plus 10% calf serum for 12–24 h at 37°C in 7–10% CO₂.

Immunofluorescence of Transfected Cells

CV1 cells transfected as described above were plated directly onto glass coverslips after exposure to the DNA solution. After 24 h of recovery at 37°C in 7–10% CO₂, the cells were incubated at 43°C for 2 h and then fixed in absolute methanol at –20°C for 2 min. The distribution of the endogenous HSP70 protein and of the transfected, tagged protein was determined by double-label immunofluorescence using a mixture of HSP70-specific mAbs

1. Abbreviations used in this paper: BBS, 50 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethane sulfonate (BES), pH 6.95, 280 mM NaCl, 1.5 mM Na₂HPO₄; E1A, adenovirus; FI-GAM, fluorescein isothiocyanate-conjugated goat anti-mouse antibodies; LDH, lactate dehydrogenase; Rh-GAR, rhodamine-conjugated goat anti-rabbit antibodies; RIPA buffers, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1.0% deoxycholate, 1.0% Triton X-100, 0.1% SDS.

(C92, N6, N15, N21, and N33, generously provided by Dr. W. Welch, University of California, San Francisco) diluted 1:250, and the LDH-specific rabbit antiserum (generously provided by Dr. E. Goldberg, Northwestern University, Chicago, IL) diluted 1:300. The primary antibodies were detected after incubation with fluorescein-conjugated goat anti-mouse (F1-GAM) and rhodamine-conjugated goat anti-rabbit antibodies (Rh-GAR), both diluted 1:70. All antibodies were diluted in PBS containing 0.5% BSA. The cells were photographed on a photomicroscope III (Carl Zeiss, Inc., Thornwood, NY).

Immunoblots

Cell lysates prepared from transfected cells were resolved by SDS-PAGE and the proteins were transferred to nitrocellulose (3). The filters were incubated with the LDH-specific antiserum diluted 1:500 or with a 1:300 dilution of an HSP70-specific rabbit polyclonal antiserum (generously provided by Dr. W. Welch, reference 39). The primary antibodies were detected after incubation with ¹²⁵I-labeled goat anti-rabbit antibodies and autoradiography.

Cell Fractionation

Cytoplasmic and nuclear fractions were prepared by hypotonic lysis. Cells incubated in cold hypotonic buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 0.1 mM EDTA) were lysed by Dounce homogenization. The nuclei were pelleted by centrifugation at 2,000 g for 10 min, and the supernatant and pellet were added to SDS sample buffer such that the final volumes were the same. Equal amounts of cytoplasmic and nuclear fractions were used for immunoblots as described above.

ATP-binding Assays

ATP-agarose (Sigma Chemical Co., St. Louis, MO, linked through C8) was washed and stored at 4°C as a 1:1 solution in buffer B (20 mM Tris-HCl [pH 7.4], 20 mM NaCl, 0.1 mM EDTA, 2 mM DTT). Each mutant was synthesized *in vitro* by T7 RNA polymerase transcription followed by translation in the presence of [³⁵S]methionine as described above. The translation reactions were diluted 40-fold with buffer B containing 0.5% BSA, 200 μl of ATP-agarose was added, and the samples were rocked at 4°C for 6–8 h. The ATP-agarose beads were pelleted by centrifugation and the unbound material was removed and immunoprecipitated with the LDH-specific antiserum. The ATP-agarose beads were washed twice with buffer D (B supplemented with 3 mM MgCl₂) containing 500 mM NaCl, twice with buffer D alone and twice with buffer D containing 5 mM GTP. The material bound to the ATP-agarose beads was eluted by rocking the beads in buffer D plus 20 mM ATP at 4°C for 2 h. The beads were pelleted and the ATP-eluted material was removed and immunoprecipitated with the LDH antiserum. The immunoprecipitated proteins from the unbound and ATP-eluted fractions were analyzed by SDS-PAGE and fluorography.

Trypsin Digestions

After *in vitro* translation of the wild type or mutant proteins the reaction mixtures were divided into three aliquots, and either 0, 0.5, or 1.0 μg of trypsin was added. The reactions were incubated at 30°C for 10 min and quenched by addition of protease inhibitors and boiling in SDS sample buffer. The digestion products were analyzed by SDS-PAGE and fluorography.

Results

Construction of Tagged Mutants

To distinguish proteins encoded by the transfected genes from endogenous cellular stress proteins, we constructed a human HSP70 gene tagged at the carboxy terminus with sequences encoding amino acids 5–15 of the human testis-specific LDH protein. The choice of this peptide tag was based on the availability of a high-titer, peptide-specific rabbit antiserum to these amino acids, which showed no cross-reactivity with somatic cell proteins, and because of the

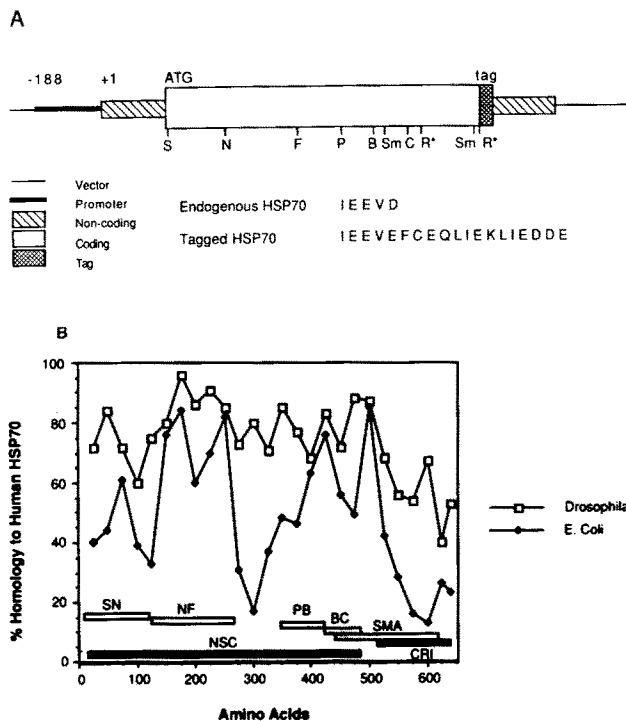


Figure 1. Peptide-tagged HSP70 expression vector and HSP70 deletion mutants. (A) The human HSP70 gene was marked at the carboxy terminus of the protein coding region by addition of sequences encoding amino acids 5–15 of human LDH, and inserted into the expression vector diagrammed above. The sequence of the last five amino acids of HSP70 is shown, as is the sequence at the HSP70-peptide tag junction. The restriction sites used to create the peptide-tagged deletion mutants are indicated beneath the coding region by the following letters: Sac II, S; Nco I, N; Fsp I, F; Pst I, P; Bcl I, B; Sma I, Sm; Cla I, C. R* denotes two Eco RI restriction sites that were created by *in vitro* mutagenesis. (B) The graph shows the homology of the human HSP70 protein to *Drosophila* HSP70 (*open squares*) or *E. coli* dnaK (*closed squares*) along the length of the protein. At the bottom of the graph the region deleted from each of the mutants is shown. Internal deletions are represented by shaded boxes, whereas terminal deletions are shown as black boxes. The nomenclature of the mutants refers to the restriction sites used to create the deletions. The precise amino acids deleted from each mutant are as follows: NSC, 5–479; SN, 5–122; NF, 122–264; PB, 351–414; BC, 415–479; SMA, 437–617; CRI, 504–641.

hydrophilic nature of the peptide (24). The human HSP70 gene was tagged *in vitro* by oligonucleotide-directed insertional mutagenesis as described in Materials and Methods. The marked gene was introduced into an expression vector containing 188 bp of the human HSP70 promoter, sufficient for both basal and stress-induced transcription of linked genes (46), and the complete 5' and 3' untranslated regions of the HSP70 gene. The peptide tag is the only feature that distinguishes this construct from the endogenous HSP70 gene (Fig. 1 A).

Once the wild-type gene was tagged, we constructed a series of deletion mutants within the coding sequences of HSP70 as described in Materials and Methods. Fig. 1 B shows the region of the HSP70 protein deleted from each mutant. Also shown is the level of sequence identity between human HSP70 and *Drosophila* HSP70 or *E. coli* dnaK, to indicate the regions of HSP70 that are most highly con-

Table I. Summary of the Phenotypes and Biochemical Properties of HSP70 Mutants

Mutant	Deleted amino acids	Localization		Association with		
		Cytoplasmic	Nuclear	Nucleoli	Other nuclear structures	ATP binding
CRI	504-641	+	+	+/-	-	+
NSC	5-479	+	+	+	+	-
SN	5-122	+	+	+	+	+
NF	122-264	+	+	+	+	-
PB	351-414	+	-	-	-	-
BC	415-479	+	+	+	+	+
SMA	437-617	+	+	-	-	+

served. The precise amino acids deleted from each mutant are given in the legend and in Table I.

To insure that the cloning procedures restored the proper reading frame, we took advantage of the carboxy-terminal location of the peptide tag in each of the proteins. The ability to immunoprecipitate a mutant polypeptide of the predicted molecular weight with antibodies to the peptide tag indicated that the translational reading frame remained intact throughout the length of the protein. Plasmid DNAs for each of the mutant constructs were linearized and transcribed *in vitro* using T7 RNA polymerase, and the resulting RNAs were translated *in vitro* in the presence of [³⁵S]methionine using an mRNA-dependent rabbit reticulocyte lysate (33). The translation reactions were boiled in SDS sample buffer, used

in denaturing immunoprecipitations using the LDH-specific antiserum followed by protein A-agarose and analyzed by SDS-PAGE. Fig. 2 shows the proteins immunoprecipitated following transcription and translation of the wild-type tagged gene (WT) and each of the mutant constructs. The different signal intensities reflected the translational efficiencies of the individual *in vitro*-synthesized RNAs. For example, the SN and SMA RNAs were repeatedly less efficiently translated than the others. In each case, though, the apparent mobility of the immunoprecipitated polypeptide corresponded well with the predicted molecular weight of each deletion mutant. The predicted sizes are as follows: WT, 72 kD; CRI, 57 kD; NSC, 20 kD; SN, 59 kD; NF, 56 kD; PB, 66 kD; BC, 65 kD; SMA, 52 kD.

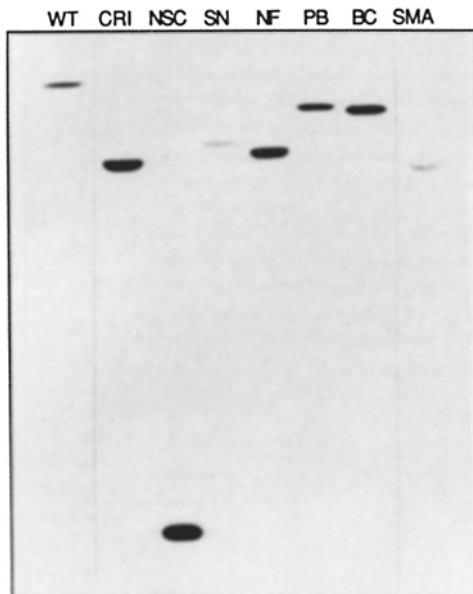


Figure 2. *In vitro* transcription, translation, and immunoprecipitation of the mutants. pGEM constructs containing the deletions were linearized and transcribed *in vitro*. The resulting RNAs were translated *in vitro* in rabbit reticulocyte lysate in the presence of [³⁵S]methionine, and the translation reactions were immunoprecipitated with antibodies to the peptide tag. The immunoprecipitated proteins were analyzed by SDS-PAGE. A fluorograph of the gel is shown. WT refers to the wild-type, tagged protein. The predicted molecular weights of the tagged proteins are as follows: WT, 72-kD; CRI, 57 kD; NSC, 20 kD; SN, 59 kD; NF, 56 kD; PB, 66 kD; BC, 65 kD; SMA, 52 kD.

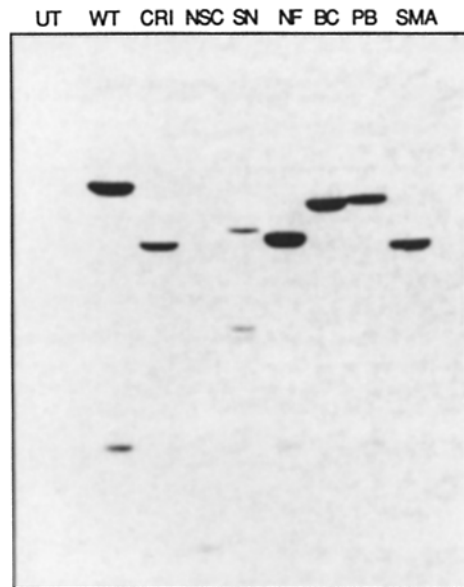


Figure 3. Expression of mutant proteins in transiently transfected cells. HeLa cells were transfected with 10 μg of each DNA construct. After transfection, the cells were incubated at 43°C for 2 h and lysed in SDS sample buffer. The transfected cell lysates were resolved by SDS-PAGE and the proteins were transferred to nitrocellulose. The filter was probed with the LDH peptide-specific antiserum followed by ¹²⁵I-labeled goat anti-rabbit antibodies. Shown is an autoradiograph of the filter. UT, untransfected cell lysate.

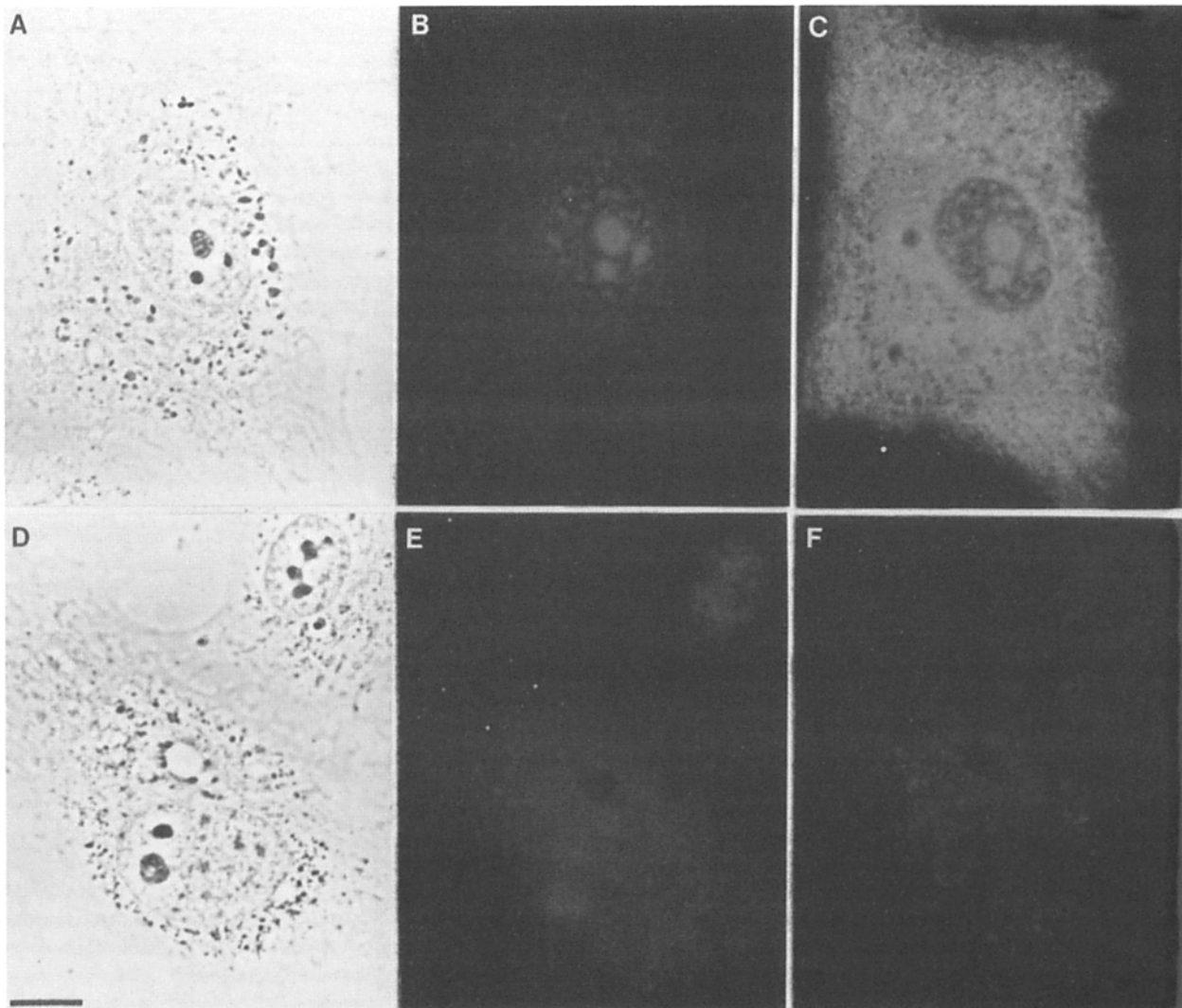


Figure 4. Intracellular distribution of peptide-tagged and untagged HSP70 in transiently transfected cells, as determined by double-label indirect immunofluorescence. CV1 cells were transfected with the wild-type, tagged HSP70 construct (*A*, *B*, and *C*) or the parental expression vector containing an unmarked HSP70 gene (*D*, *E*, and *F*). After transfection, the cells were incubated at 43°C for 2 h and fixed for double-label indirect immunofluorescence as described in Materials and Methods. The distribution of the tagged protein was determined using the LDH peptide-specific antiserum followed by Rh-GAR, and the localization of the endogenous HSP70 protein was analyzed using a mixture of HSP70-specific mAbs followed by FI-GAM. (*A*, *B*, and *C*) Cells transfected with the WT-tag construct. (*D*, *E*, and *F*) Cells transfected with the parental, untagged HSP70 construct. (*A* and *D*) Phase-contrast micrographs. (*B* and *E*) endogenous HSP70 distribution. (*C* and *F*) Distribution of tagged protein.

Expression in Transfected Cells

After confirming that the translational reading frame of each construct was intact, the coding sequences of the deletion mutants were shuttled into the expression vector described for the wild-type construct. Each of the expression plasmids was transiently transfected into HeLa cells as described in Materials and Methods. For each mutant, a single 10-cm² plate was transfected with 10 μg of DNA. After transfection, the plates were incubated at 43°C for 2 h to induce expression of the transfected genes. The cells were lysed in SDS sample buffer, and the expression of the peptide-tagged mutants was assayed by immunoblot analysis using the LDH-specific antiserum. This antiserum did not cross-react with any proteins in untransfected HeLa cells (Fig. 3, UT).

After transfection with each of the mutant constructs, a

single major polypeptide of the predicted molecular weight was detected by the LDH antibody after heat-shock treatment. In some cases low levels of a single degradation product were also detected, but could be eliminated by preparing the cell lysates in the presence of protease inhibitors. In addition, all of the mutant proteins could be detected before heat-shock treatment (data not shown), consistent with basal expression of the HSP70 promoter in unstressed primate cells, and indicating that the proteins were apparently stable. Differences in the levels of detection of the individual mutants reflected the transfection efficiencies of the DNA constructs rather than the abundance of the proteins. For instance, the transfection efficiency of NSC (determined by immunofluorescence) was very low, but within a single transfected cell the intensity of immunofluorescence was similar to that of the other mutants.

We next determined whether the presence of the peptide tag affected the intracellular localization of the wild-type protein. The distribution of the peptide-tagged, wild-type protein was compared with that of the endogenous HSP70 protein after heat-shock treatment by double-label indirect immunofluorescence using the anti-LDH rabbit antiserum and a mixture of HSP70-specific mAbs. FI-GAM antibodies were used to detect the mAbs, whereas Rh-GAR antibodies were used to detect the peptide-specific antibodies. Thus, the rhodamine staining revealed the distribution of the tagged protein alone, whereas the fluorescein pattern represented both the endogenous protein and the tagged protein.

HeLa cells and monkey kidney (CV1) cells were transiently transfected with the WT construct and plated onto glass coverslips. The transfected cells were incubated at 43°C for 2 h, fixed, and processed for immunofluorescence as described in Materials and Methods. The results obtained with HeLa and CV1 cells were identical in all experiments. Micrographs of CV1 cells are shown because the CV1 cells were more well spread and revealed more structural detail.

After heat-shock treatment, HSP70 migrates to the nucleus and becomes associated with nucleoli (40). Fig. 4 *B* illustrates the nucleolar localization of HSP70 after heat shock. In addition, the association of HSP70 with other nuclear structures was also detected. The distribution of the peptide-tagged HSP70 protein is shown in Fig. 4 *C*. The localization of the tagged, wild-type protein and the endogenous HSP70 protein were similar (Fig. 4, *B* and *C*). Therefore, the addition of the LDH peptide to the carboxy terminus of HSP70 did not appear to alter the distribution of the protein following heat shock. These results were confirmed using the LDH antiserum in single-label indirect immunofluorescence to insure that the nucleolar staining detected by the Rh-GAR actually reflected the distribution of the tagged protein, and was not simply bleedover due to the intensity of the fluorescein staining (data not shown).

The LDH antiserum did not cross-react with any proteins in untransfected cells (Fig. 3), only transfected cells were stained by the LDH antibodies and Rh-GAR. In addition, the transfected cells could be distinguished by observing only the fluorescein immunofluorescence because they displayed increased amounts of cytoplasmic fluorescence compared to untransfected cells (Fig. 4 *E*). To determine if the increased cytoplasmic staining was simply because of the high level of expression of the transfected gene or if the addition of the peptide tag was causing cytoplasmic accumulation of the protein, we transfected cells with a plasmid containing the untagged, wild-type HSP70 gene driven by the same promoter sequences (Fig. 4, *D*, *E*, and *F*). Because this protein was not tagged, staining with the LDH antiserum provided a negative control (Fig. 4 *F*). Indirect immunofluorescence with HSP70-specific mAbs revealed that a small percentage of cells, similar to the frequency of WT tag-transfected cells, displayed increased cytoplasmic fluorescence similar to the levels of cytoplasmic staining seen in cells transfected with the tagged protein (Fig. 4 *E*). Therefore, the high level of expression of the transfected gene may be responsible for the increased cytoplasmic distribution of the protein since transfection with an untagged HSP70 protein gave the same result.

Intracellular Localization of Mutant Proteins

We examined the intracellular distribution of each mutant af-

ter heat-shock treatment of transiently transfected CV1 cells to determine what regions of the HSP70 protein are important for its localization. CV1 cells were transfected with each deletion mutant and plated on glass coverslips. After transfection, the cells were incubated at 43°C for 2 h and fixed for double-label indirect immunofluorescence as described. Fig. 5 shows the results for three mutants, SN (Fig. 5, *A*, *B*, and *C*), NF (Fig. 5, *D*, *E*, and *F*), and BC (Fig. 5, *G*, *H*, and *I*), which all appeared similar to wild-type HSP70. In transfected cells containing SN, NF, or BC the distribution of the mutant proteins mirrored the distribution of the endogenous HSP70 protein. Specifically, both nucleolar deposition of the mutant proteins and association with other nuclear components were evident. Therefore, deletion of amino acids 5–122 (SN), 122–264 (NF), or 415–479 (BC) did not appear to alter the intracellular localization of the protein.

Mutants that revealed information about regions of HSP70 that are important for proper localization are shown in Fig. 6. The NSC mutant, which contains only the last 160 amino acids of HSP70, was similar in intracellular distribution to the endogenous protein (Fig. 6, *B* and *C*), indicating that the carboxy-terminal quarter of HSP70 contains sequences sufficient for association with nucleoli and other nuclear structures. The localization of the SMA mutant supports this conclusion. When amino acids 437–617 were deleted, as in SMA, the resulting protein no longer associated with the nucleolus of heat-shocked cells (Fig. 6 *F*), although the endogenous HSP70 protein displayed nucleolar localization (Fig. 6 *E*). Despite the lack of nucleolar deposition of the SMA mutant, the protein still appeared to be located in the nucleus of the cell.

The PB mutant, which lacks amino acids 351–414, appeared incapable of entering the nucleus, and remained in the cytoplasm of heat-shocked cells (Fig. 6 *I*). However, the endogenous HSP70 protein showed the expected nucleolar distribution in cells transfected with the PB mutant (Fig. 6 *H*). The inability of PB to enter the nucleus was confirmed by biochemically fractionating heat shock-treated cells that were transiently transfected with the PB construct and analyzing the cytoplasmic and nuclear fractions by immunoblotting (data not shown). The PB proteins were found only in the cytoplasm of heat-shocked cells with no detectable PB protein in the nucleus.

The final mutant that was informative with regard to nucleolar localization was the carboxy-terminal deletion, CRI, lacking the last 138 amino acids. This particular mutation yielded somewhat variable results. In some CRI-transfected cells, the mutant protein was detected within the nucleolus as shown in Fig. 6 *L*. However, in some cases the protein was distributed in a ring just outside the nucleolus (data not shown). The nucleolar localization of this mutant varied depending on the conditions of heat shock and the overall level of expression of the mutant protein in an individual cell. Generally, more intense heat-shock treatments were necessary to cause nucleolar localization of the CRI protein, and cells expressing very high levels of the CRI mutant were more likely to display nucleolar deposition of the mutant protein than cells expressing lower levels of CRI. Additionally, the association of the CRI mutant with nonnucleolar structures in other areas of the nucleus also appeared diminished as compared with either the endogenous protein (Fig. 6 *K*) or to other mutants (Figs. 5 *I* or 6 *C*). Again the intracellular distribution of the CRI mutant supports the con-

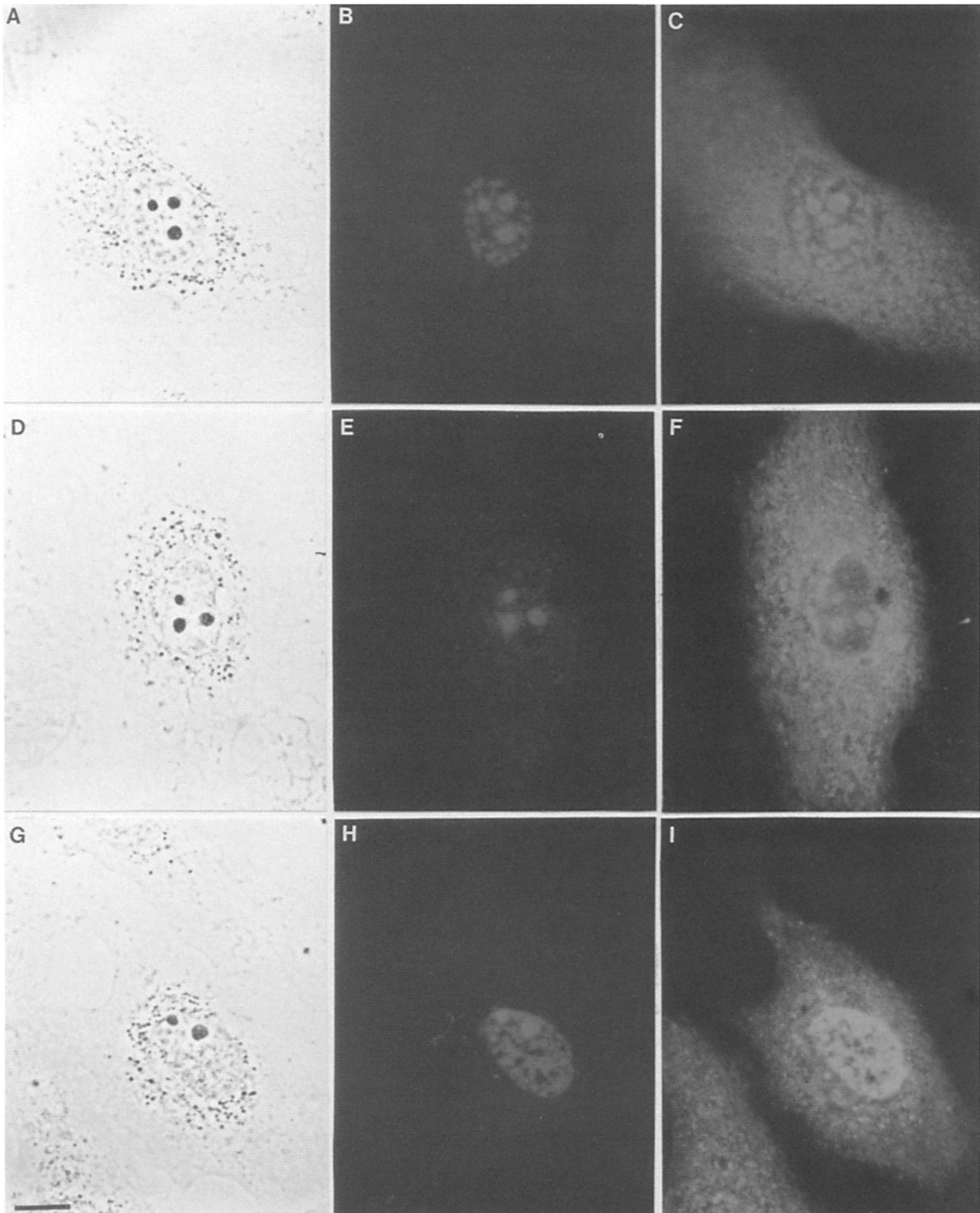
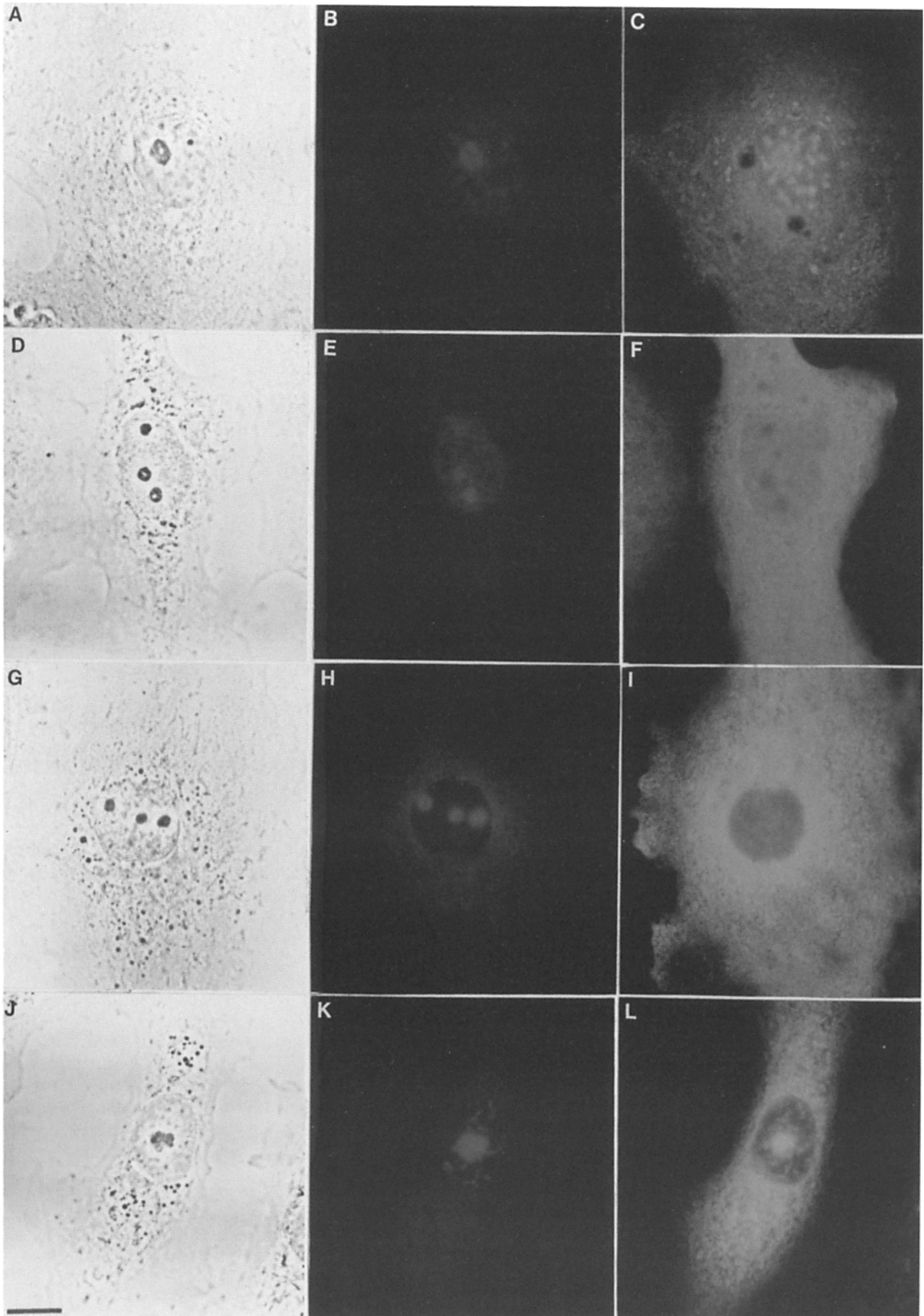


Figure 5. Intracellular localization of deletion mutants SN, NF, and BC. CV1 cells transfected with mutants SN (*A*, *B*, and *C*), NF (*D*, *E*, and *F*) or BC (*G*, *H*, and *I*) were incubated at 43°C for 2 h and fixed for double-label indirect immunofluorescence as described in Fig. 4. (*A*, *B*, and *C*) Cells transfected with SN. (*D*, *E*, and *F*) Cells transfected with NF. (*G*, *H*, and *I*) Cells transfected with BC. (*A*, *D*, and *G*) Phase-contrast micrographs. (*B*, *E*, and *H*) Endogenous HSP70 fluorescence. (*C*, *F*, and *I*) Distribution of transfected deletion mutants.



clusion that sequences in the carboxy terminus of the HSP70 protein are important for nucleolar localization and association with nuclear structures.

ATP-binding Properties

One feature common to all members of the 70-kD family of stress proteins is their affinity for and ability to be purified by ATP-agarose (41). Having mapped the regions of HSP70 necessary for nucleolar localization to the carboxy terminus of the protein, we next used the deletion mutants to identify HSP70 sequences involved in ATP binding. We took advantage of the fact that each of the mutants could be synthesized *in vitro* in the presence of [³⁵S]methionine by transcribing the pGEM constructs with T7 RNA polymerase and then translating the resulting RNAs in a rabbit reticulocyte lysate. Each of the *in vitro*-synthesized mutants was incubated with ATP-agarose beads and the unbound material was removed after pelleting the beads. The ATP-agarose beads were washed extensively and the bound material was eluted with ATP as described in Materials and Methods. The unbound and ATP-eluted fractions were immunoprecipitated with the LDH antiserum and the precipitated proteins were analyzed by SDS-PAGE.

The wild-type (WT), CRI, BC, SMA, and SN proteins were able to bind to ATP-agarose beads and be eluted by ATP-containing buffer (Fig. 7 A). In each case, a significant proportion of the protein remained in the unbound fraction, even if more ATP-agarose was added. This was not entirely unexpected because a fraction of the endogenous cellular HSP70 protein always fails to bind ATP-agarose during purification (unpublished observations). Also, the presence of ATP and an ATP regenerating system in the translation reactions may have affected the ability of the proteins to bind ATP-agarose.

Mutants PB, NF, and NSC were completely unable to bind to ATP-agarose (Fig. 7 B). When each of these mutants was incubated with ATP-agarose beads all of the protein was recovered in the unbound fraction, and none appeared in the ATP-eluted fraction. To be certain that these mutant proteins were not somehow irreversibly bound to ATP-agarose, the beads were boiled in SDS sample buffer. Again no detectable protein was found in this fraction (data not shown).

We repeated the ATP-binding assay with the PB, NF, and NSC mutants including the wild-type protein as an internal control for the integrity of the ATP-agarose beads. Each of the proteins was translated independently, and the translation reactions were mixed before the addition of ATP-agarose. As shown in Fig. 7 C, the wild-type protein was found in both the unbound and ATP-eluted fractions, whereas the PB, NF, and NSC proteins were present only in the unbound fractions.

A summary of the data to this point is presented in Table I. Mutations in the HSP70 coding region that affected the intracellular localization of the protein were exclusively in the

carboxy-terminal portion of the protein. Whereas mutations in two distinct and distant regions abolished ATP-binding activity. These were located in a highly conserved region of the amino terminus (amino acids 122–264) and a less well-conserved region roughly two-thirds of the way through the protein (amino acids 351–414).

Trypsin Digestion of Deletion Mutants

The PB mutant was somewhat peculiar in that deletion of this region of the HSP70 protein (amino acids 351–414) affected both the intracellular localization and ATP-binding properties of the protein, whereas results from other mutants indicated that these functions mapped to distinct regions of the protein. This observation led us to suspect that the PB protein had an altered conformation. To examine this, we used protease digestion to analyze the structure of the mutant proteins. A conserved feature of HSP70 proteins from several species is that cleavage with proteases such as chymotrypsin and trypsin leads to production of a stable fragment of roughly 45 kD (4, Carillo, A., and R. I. Morimoto, unpublished observation). Amino-terminal sequencing of this trypsin-resistant fragment revealed that it is derived from the amino terminus of the HSP70 protein. This fragment contains many potential trypsin cleavage sites that are inaccessible in the native protein, but not after denaturation. Production of the trypsin-stable fragment thus can be used as an indication of proper folding of the amino terminus of the HSP70 protein.

We synthesized each of the mutants by *in vitro* translation in the presence of [³⁵S]methionine, and analyzed the trypsin digestion products of the translation reactions by SDS-PAGE (Fig. 8). When no exogenous RNA was added to the translation reaction an endogenous rabbit reticulocyte protein of ~48 kD was labeled (Fig. 8 E). This polypeptide was not cleaved by addition of 0.5 or 1 μg of trypsin, and was present in all the translation reactions. Trypsin digestion of the wild-type, tagged protein (Fig. 8, WT) resulted in the production of a stable 45 kD fragment as described above.

When a mutant containing a carboxy-terminal deletion (CRI) was digested with trypsin the same stable 45-kD fragment was produced, suggesting that deletion of the COOH terminus did not alter the folding of the amino terminus (Fig. 8, CRI). Trypsin digestion of mutants BC and SMA, which contain internal deletions of carboxy-terminal sequences also resulted in the production of the stable 45-kD fragment (data not shown). When mutants containing internal deletions within the amino terminus of the HSP70 protein (SN and NF) were exposed to trypsin a stable fragment of lower molecular weight was generated (Fig. 8, SN). The size of this fragment (~32 kD) corresponded to the 45-kD fragment minus the deleted sequences. The 32-kD fragment was apparently less stable than the 45-kD fragment, as the amount of the smaller fragment was slightly diminished upon trypsin treatment.

Figure 6. Intracellular distribution of deletion mutants NSC, SMA, PB, and CRI. CV1 cells were transfected with mutants NSC (A, B, and C), SMA (D, E, and F), PB (G, H, and I) and CRI (J, K, and L). After transfection, the cells were incubated at 43°C for 2 h and fixed for double-label indirect immunofluorescence as described previously. (A, B, and C) cells transfected with NSC. (D, E, and F) Cells transfected with SMA. (G, H, and I) Cells transfected with PB. (J, K, and I) Cells transfected with CRI. (A, D, G, and J) Phase-contrast micrographs. (B, E, H, and K) Endogenous HSP70 fluorescence. (C, F, I, and L) Distribution of transfected deletion mutants.

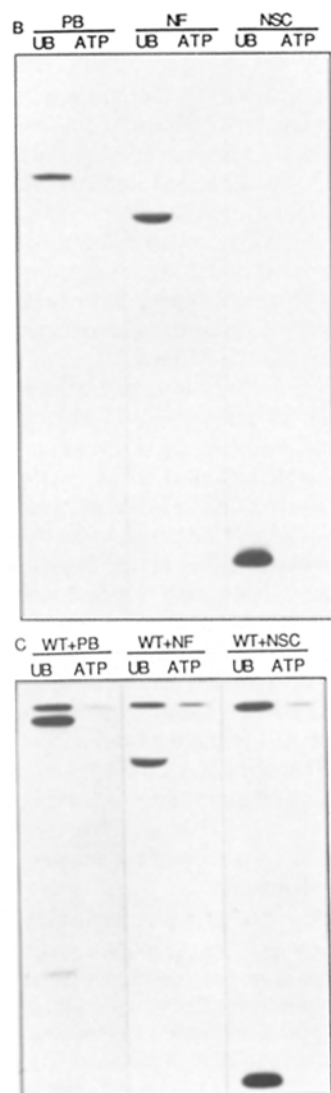
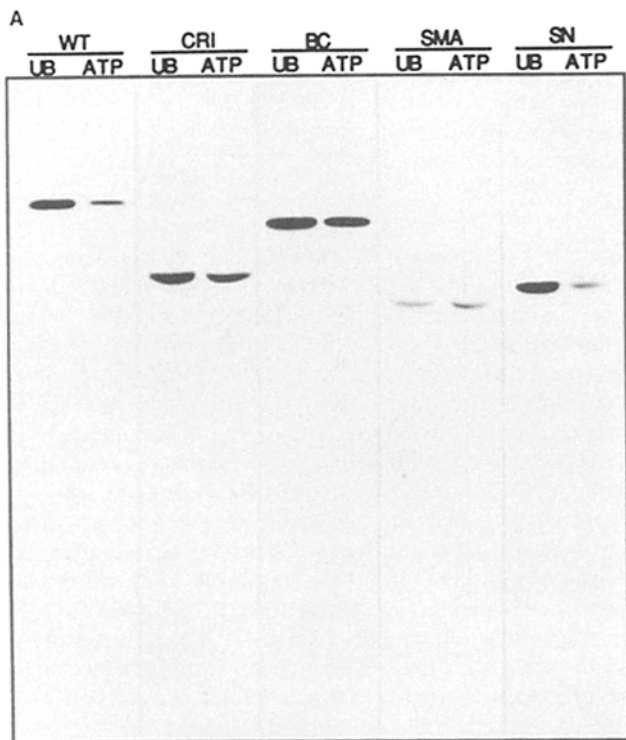


Figure 7. ATP-binding properties of deletion mutants. Each of the mutant proteins was synthesized *in vitro* by translation in a rabbit reticulocyte lysate as described in Materials and Methods. The translation reactions were incubated with ATP-agarose beads and the unbound fraction was removed after pelleting the beads. After washing the beads, the bound material was eluted with ATP. Both the unbound (UB) and the ATP-eluted (ATP) fractions were immunoprecipitated with the LDH antiserum and the precipitated proteins were analyzed by SDS-PAGE. Shown are fluorographs of the gels. (A) Unbound (UB) and ATP-eluted (ATP) fractions of WT, CRI, BC, SMA, and SN. (B) Unbound (UB) and ATP-eluted (ATP) fractions of PB, NF, and NSC. (C) Unbound (UB) and ATP-eluted (ATP) fractions from combined reactions containing WT and PB, WT and NF, or WT and NSC.

Trypsin digestion of the PB protein under identical conditions or even using 10–20-fold less trypsin did not give rise to any stable fragments (Fig. 8, PB). These results indicate that deletion of amino acids 351–414 from the HSP70 protein apparently affects the ability of the mutant protein to fold properly.

Recovery after Heat Shock

One proposed function of the HSP70 protein is to aid in the repair of damage sustained by cellular structures caused by heat-shock treatment. The nucleolar association of HSP70 after heat shock is correlated with visible alterations in nuclear morphology. Upon recovery, as nucleoli regain their structural integrity, HSP70 exits the nucleolus and accumulates in the cytoplasm (43). Constitutive expression of HSP70 from transfected genes in unstressed cells was demonstrated to accelerate the recovery of heat-induced nucleolar damage (31). This has led to the suggestion that HSP70 is involved in the repair of nucleolar damage after stress (17, 32, 40, 42, 43).

Many of the reported interactions of HSP70-related proteins with other cellular components, including nucleoli, have been demonstrated to be ATP dependent. Specifically, addition of ATP results in the release of the 70 kD stress proteins from protein complexes *in vitro* (4, 9, 17, 26, 37, 48). Having identified a mutant HSP70 protein that was incapable of binding to ATP (NF), but still showed proper localization we were interested in determining how this mutant might affect the recovery of cells from stress *in vivo*. CVI cells were transiently transfected with the WT construct or the NF mutant and plated on glass coverslips. After incubation at 43°C for 2 h, one coverslip from each transfection was removed and fixed for double-label indirect immunofluorescence as described, whereas the remaining coverslips were allowed to recover at 37°C for 2, 4, 6, and 8 h (Fig. 9).

Using the exit of HSP70 from nucleoli and its cytoplasmic accumulation as a measure of nucleolar recovery (43), after 4 h at 37°C cells transfected with the WT construct appeared fully recovered (Fig. 9 C). Both the tagged HSP70 protein and the endogenous protein had exited nucleoli (Fig. 9, B and C). By contrast, in the surrounding untransfected cells the distribution of the endogenous HSP70 protein was still predominantly nucleolar (Fig. 9 B). Thus, cells expressing the transfected WT protein showed an accelerated ability to recover after heat-shock treatment. In the case of NF, we found that cells transfected with the mutant protein did not show accelerated recovery when compared to the surrounding untransfected cells. After 4 h at 37°C, both the NF protein and the endogenous HSP70 protein were still associated with nucleoli (Fig. 9, E and F).

The immunofluorescence results were confirmed by immunoblot analysis of fractionated cells after recovery. CVI cells were transfected with WT or NF, incubated at 43°C for 2 h, and allowed to recover at 37°C for 4 h. The cells were fractionated into cytoplasmic and nuclear lysates as described previously, and the lysates were analyzed by immunoblotting. Equal amounts of cytoplasmic and nuclear fractions were used to prepare duplicate blots, which were probed with either the LDH antiserum or the HSP70-specific rabbit polyclonal serum. When the distribution of the transfected proteins was analyzed (Fig. 10, LDH blot), the WT protein was predominantly cytoplasmic, but the NF

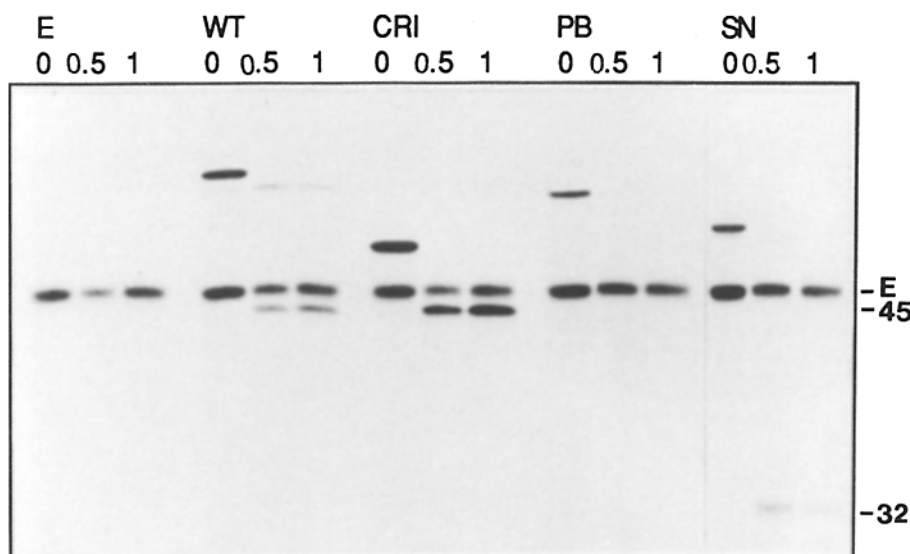


Figure 8. Trypsin digestions of deletion mutants. Each of the mutant proteins was synthesized by in vitro translation as described in Materials and Methods. The translation reactions were divided into three aliquots and 0, 0.5, or 1 μ g of trypsin was added. After 10 min at 30°C, the digestion products were analyzed by SDS-PAGE. Shown is a fluorograph of the gel. E, endogenous translation reaction to which no exogenous RNA was added. WT, trypsin digestion of the wild-type, tagged HSP70. (CRI) Trypsin digestion of the CRI mutant. PB, Trypsin digestion of the PB mutant. SN, trypsin digestion of the SN mutant. Approximate molecular weight markers are indicated at the right, as is an endogenous rabbit reticulocyte protein (E) labeled in the translation reactions in the absence of added exogenous RNA.

protein was present in both the cytoplasmic and nuclear fractions. Since the efficiency of transfection was generally <10%, the 70K blot for the most part represents the distribution of the endogenous HSP70 in untransfected cells. The distribution of the endogenous HSP70 protein was similar to that of the NF protein (Fig. 10, 70K blot), being present in both the cytoplasmic and nuclear fractions. These results indicate that cells transfected with the WT protein, but not NF, recovered faster than the surrounding untransfected cells.

Compared with the WT protein, the NF mutant did not appear to be functional in accelerating the recovery process. Later recovery timepoints revealed that some NF-transfected cells actually recovered more slowly than untransfected cells. Fig. 11 shows two different fields of NF-transfected cells after 8 h at 37°C. The majority of the untransfected cells appeared fully recovered at this time. In contrast, some NF-transfected cells still displayed nucleolar localization of the NF protein (Fig. 11, B and C), whereas others appeared fully recovered (Fig. 11, E and F).

We analyzed the ability of the remaining mutants to accelerate the recovery of cells from heat shock exactly as described for WT and NF (data not shown). SN and BC both displayed normal intracellular localization after heat shock, were capable of binding to ATP, and were able to facilitate accelerated recovery similar to the WT protein. Not surprisingly, the PB mutant, which apparently fails to fold properly, was incapable of aiding the recovery process. PB-transfected cells recovered from heat-shock treatment with the same kinetics as the surrounding untransfected cells.

The SMA mutant, which binds to ATP but fails to associate with nucleoli after heat shock, still allowed the accelerated recovery of transfected cells. The ability of CRI to speed the recovery of transfected cells was intermediate. After 4 h at 37°C, only some of the CRI-transfected cells had recovered. However, by 6 h the recovery of transfected cells was complete, whereas the surrounding untransfected cells were just beginning to recover. CRI thus was capable of accelerating recovery, but not as efficiently as the WT protein. Curiously, the ability of the carboxy-terminal fragment, NSC, to aid in cellular recovery appeared similar to the WT protein.

Discussion

We have used the method of peptide-tagging to mark the human HSP70 gene to distinguish the protein product of a transfected gene from the endogenous HSP70 protein. Sequences encoding amino acids 5–15 of the human testis-specific LDH protein were added to the extreme carboxy terminus of the coding region of the human HSP70 gene, and the peptide-tagged HSP70 gene was then used to construct a series of deletion mutants within the HSP70 coding region. The deletion mutants were reintroduced by transient transfection into HeLa and CV1 cells under the regulation of a fragment of the human HSP70 promoter sufficient for both basal and heat-inducible transcription. These deletion mutants were used to map regions of the HSP70 protein responsible for ATP binding, intracellular localization, and association with nuclear structures (see Fig. 12).

The carboxy terminus of the HSP70 protein appears to be involved in association with nucleoli after heat shock. Three separate deletion mutants support this conclusion. Deletion of amino acids 437–618 (SMA) resulted in a protein that, although still present in the nucleus, did not accumulate in nucleoli after heat shock. The CRI mutant, which lacks amino acids 504–641, displayed variable results with respect to nucleolar localization. Finally, a small carboxy-terminal fragment of HSP70, containing only amino acids 480–641, displayed normal nucleolar localization. Together, these results indicate the importance of sequences between amino acids 480 and 618 for nucleolar association of the HSP70 protein. We cannot, however, rule out the possibility that the nucleolar localization of the deletion mutants may be a consequence of dimerization or multimerization of the mutants with the endogenous HSP70 protein.

We should note that our results contrast a previous study that concluded that sequences between amino acids 114 and 338 of the *Drosophila* HSP70 protein were responsible for nucleolar association (25). The studies differ in several important respects, however. We have marked the human HSP70 gene and examined deletion mutants in human cells and another primate line (CV1 cells), the results being the

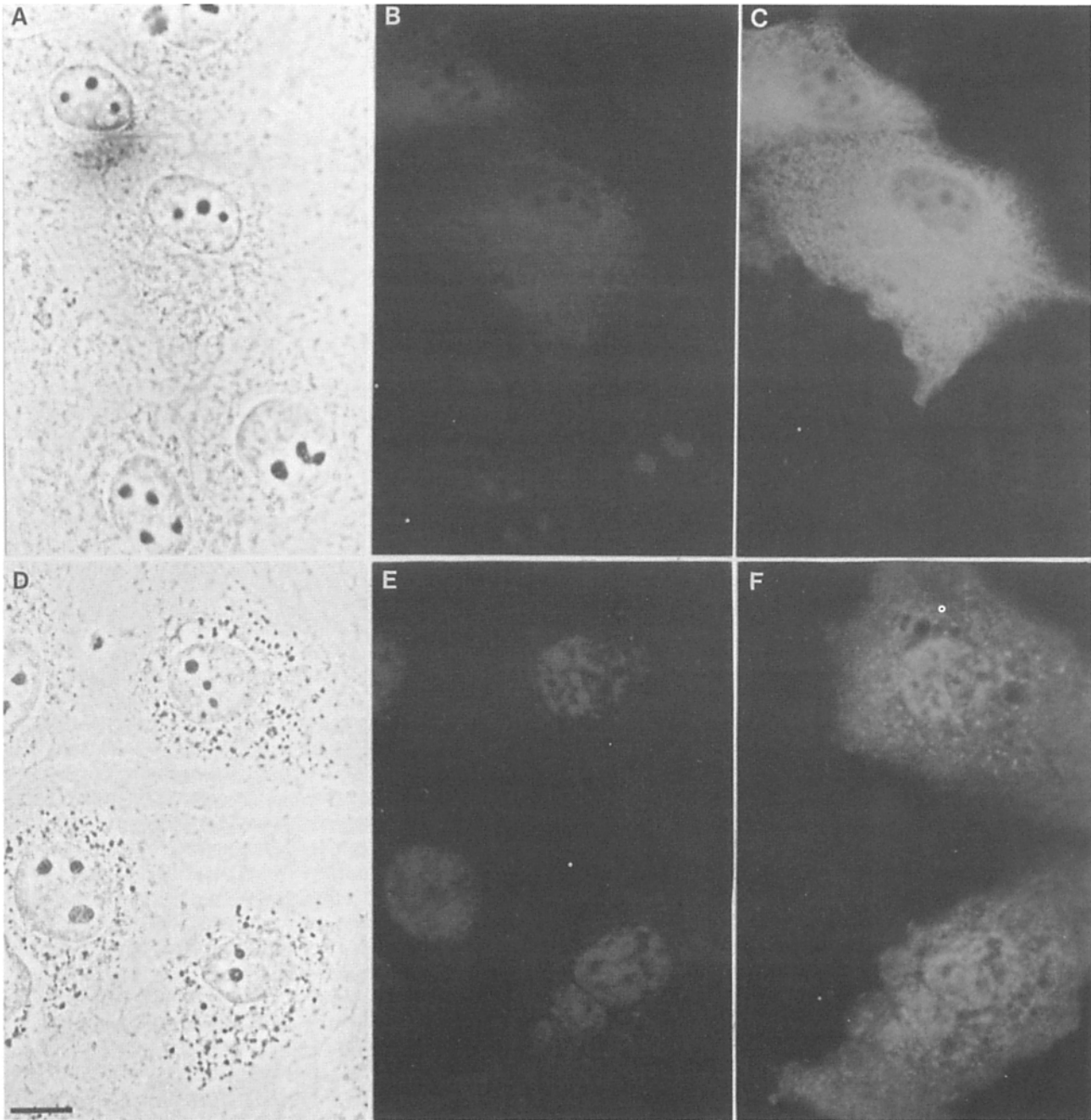


Figure 9. Intracellular distribution of the WT and NF proteins following recovery from heat-shock treatment. CV1 cells transfected with either the WT construct (A, B, C) or the NF deletion (D, E, F) were plated on glass coverslips. After incubation at 43°C for 2 h, the cells were allowed to recover at 37°C for 4 h. After recovery, coverslips were fixed for double-label indirect immunofluorescence as described previously. (A, B, and C) Cells transfected with the WT construct. (D, E, and F) Cells transfected with the NF construct. (A and D) phase contrast micrographs. (B and E) Endogenous HSP70 fluorescence. (C and F) Distribution of transfected proteins.

same in both cell types, whereas the previous study examined deletion mutants of *Drosophila* HSP70 in monkey cells. That *Drosophila* proteins are adapted to a different range of temperatures than are primate proteins may explain why many of their deletion mutants were unstable in monkey cells. Also, the previous deletions were all either amino-terminal or carboxy-terminal deletions, which may cause more drastic structural alterations and instability than the internal deletions we have constructed. Using a computer-generated prediction of HSP70 protein structure, we deter-

mined that the deletion endpoints chosen did not disrupt major regions of the predicted secondary structure. The previous study also used the method of peptide-tagging to distinguish the product of the transfected gene. However, the peptide chosen required modification in vitro by amidation for antibody recognition. Finally, we have designed our system such that the mutant proteins have the same pattern of expression as the endogenous protein; they are both constitutively expressed and induced following heat shock. In the previous study the mutants were linked to the adenovirus major late

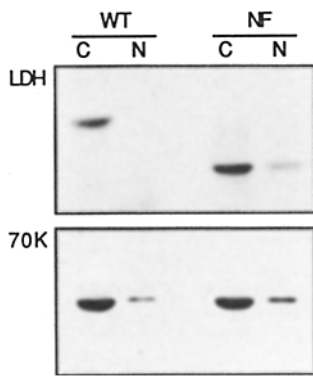


Figure 10. Immunoblot analysis of cytoplasmic and nuclear fractions of transfected cells during recovery from heat shock. HeLa cells transfected with either the WT construct or the NF deletion were incubated at 43°C for 2 h and allowed to recover 4 h at 37°C. After recovery cytoplasmic and nuclear fractions were prepared by hypotonic lysis. Equal amounts of the lysates were resolved by SDS-PAGE and transferred to nitrocellulose.

Duplicate blots were probed with either the LDH antiserum or the HSP70-specific rabbit polyclonal antiserum followed by ^{125}I -labeled goat anti-rabbit antibodies. Shown are autoradiographs of the filters. LDH, immunoblot probed with the LDH antiserum. 70K, immunoblot probed with the HSP70 antiserum. C, cytoplasmic fraction of WT- or NF-transfected cells. N, nuclear fraction of WT- or NF-transfected cells.

promoter and thus not heat inducible. Whether these points can account for the difference in results is not clear.

Many nuclear proteins have a specific targeting signal consisting of a helix-destabilizing amino acid followed by a stretch of basic amino acids (2, 11, 15, 20, 35). Proteins that bear these signals are generally exclusively nuclear. Analysis of the HSP70 sequence does not identify typical nuclear localization sequences. Our results indicate that no single region of the HSP70 protein is responsible for its intracellular localization in unstressed cells. We have demonstrated that the only mutant that was incapable of entering the nucleus, PB, was not properly folded. Thus, the exclusively cytoplasmic distribution of the PB protein was presumably due to its aberrant conformation. In all of the remaining deletion mutants, the tagged HSP70 molecules were distributed throughout the cytoplasm and nucleus, even in cells maintained at physiological temperatures (unpublished observations). Therefore, the nuclear localization of HSP70 appears to be a constitutive function of the protein. Both biochemical and immunological studies have revealed that in cells maintained at 37°C HSP70 is distributed throughout the cytoplasm and nucleus (40, 41). The proportion of nuclear-associated HSP70 increases in early S phase in cells maintained at physiological temperatures (22), or in response to heat shock or other stress treatments (40). Changes in HSP70 distribution appear to be dictated by the stress-induced appearance or accumulation of substrates within specific cellular compartments. Thus the mechanism of intracellular targeting of HSP70 is likely to be very different than that for proteins that are exclusively nuclear in distribution.

HSP70 associates with nucleoli after stress treatments, such as heat shock or exposure to amino acid analogues, that induce visible alterations in nucleolar morphology (40, 43). Treatment with agents that induce the stress response, but do not cause nucleolar damage (arsenite, for example) do not lead to nucleolar accumulation of HSP70 (40, 43). Although we have identified carboxy-terminal sequences important for the nucleolar association of HSP70, this region thus does not function like a constitutive nucleolar targeting signal (36). Rather, the accumulation of HSP70 within nucleoli requires the appearance of damaged substrates within the organelle.

Deletion of sequences in the amino terminus of HSP70 affected the ATP-binding ability of the protein. Removal of the most highly conserved region of HSP70 (amino acids 122–264) abolished ATP binding. This region also corresponds to one of the most highly conserved regions among different members of the 70-kD family of stress proteins. Human HSP70 and rat ATPase (P72) share 90% amino acid identity in this region of the protein, and rat ATPase and rat GRP78 also show 90% homology if conservative amino acid substitutions are included (14, 26, 30). That deletion of this region affected ATP binding is consistent with the fact that the ability to bind ATP is a feature shared by four different members of the 70-kD class of stress proteins. The major heat-inducible HSP70 protein, the related constitutively expressed P72, and GRP78, which resides in the endoplasmic reticulum, can all be isolated by ATP-affinity chromatography (41). In addition, a recently identified mitochondrial HSP70-related protein also shares this ATP-binding capacity (Mizzen, L. A., C. Chang, J. Garrels, and W. J. Welsh, 1989, manuscript submitted for publication). Thus, the emerging hypothesis is that members of the HSP70 family perform a common function in their specific cellular compartments, whether it be ATP-dependent protein unfolding or solubilization of aggregated structures. We suggest that the highly conserved amino terminus may contain the common ATP-binding domain, whereas the carboxy terminus may direct substrate specificity and intracellular localization such as the nucleolar association of HSP70. For GRP78, sequences at the carboxy terminus have been demonstrated to be important for retention of this protein in the endoplasmic reticulum (27).

Results from protease digestion of the HSP70 deletion mutants support the suggestion that HSP70 is composed of two distinct domains. Trypsin digestion of HSP70-related proteins yields a stable fragment of 45 kD derived from the amino terminus of the protein (5, Carillo, A., and R. I. Morimoto, unpublished observations). This trypsin-resistant fragment contains nearly 50 potential trypsin cleavage sites that are inaccessible to protease in the native HSP70 protein, but not after denaturation. Thus, we have used the production of this trypsin-resistant fragment as an indicator of the conformation of the amino terminus of the HSP70 protein. Trypsin digestions of mutants containing internal deletions within the carboxy terminus or of the carboxy-terminal deletion mutant generated the 45-kD stable fragment; indicating that proper folding of the amino-terminal domain is independent of sequences in the carboxy terminus. Deletion of amino acids 351–414 (PB), however, resulted in a protein that was completely degraded by trypsin. Interestingly, based on the size of the amino-terminal protease-resistant fragment, the trypsin cleavage site that generates this 45-kD fragment is probably located within the region deleted from PB. This region of the HSP70 protein may act as a hinge, separating the amino-terminal domain from the carboxy-terminal region. Mutants containing deletions within the amino terminus also yielded trypsin-resistant fragments that were smaller than 45 kD by the size of the deletion. Thus, the amino terminus seems to fold into a structure in which much of the protein is inaccessible to trypsin. The carboxy terminus of HSP70 contains fewer trypsin cleavage sites than the amino terminus, yet is very trypsin sensitive. In support of this suggestion that the carboxy terminus is an accessible re-

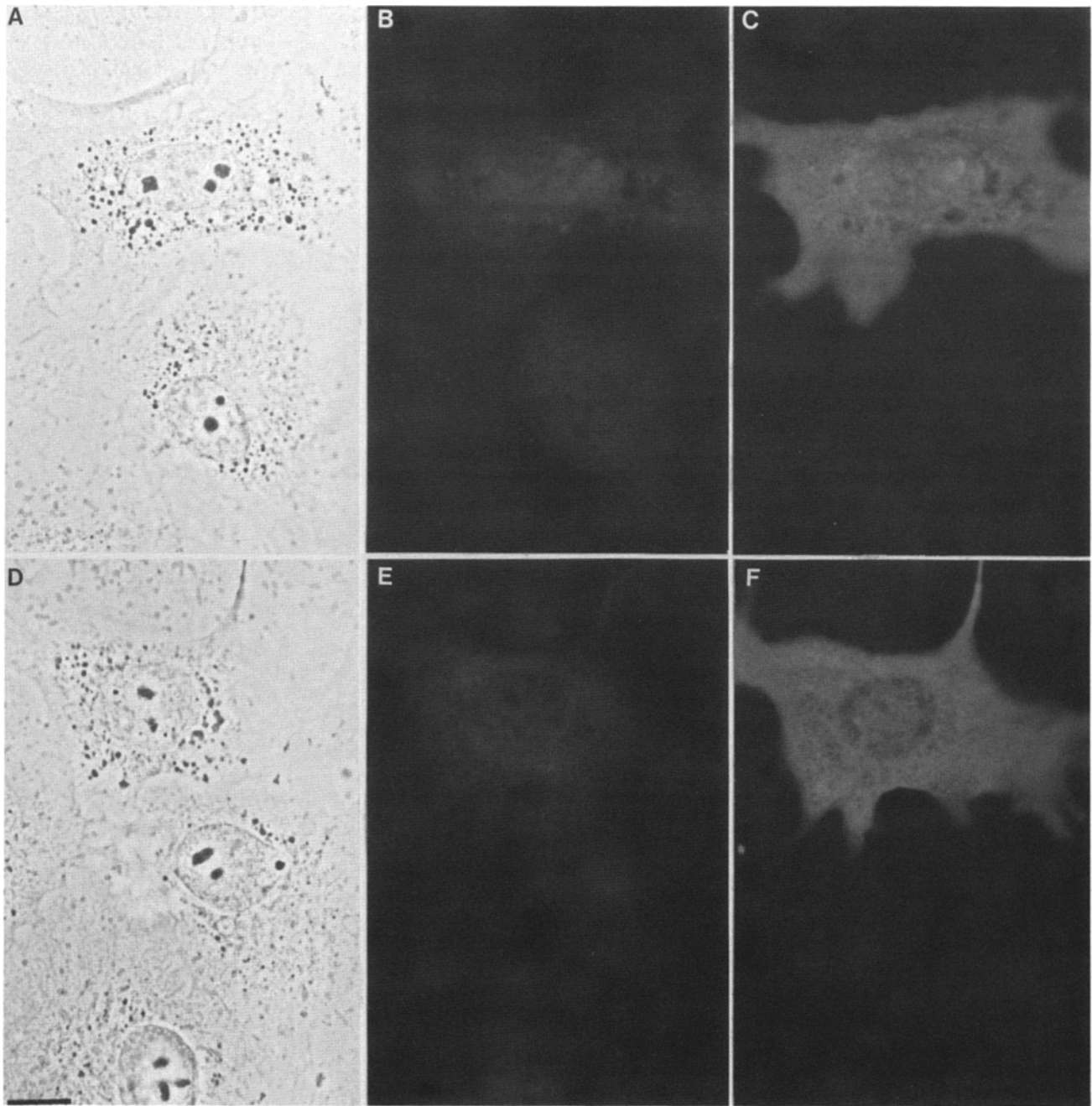


Figure 11. Intracellular distribution of the NF protein after 8 h of recovery from heat-shock treatment. CV1 cells transfected with the NF construct were plated on glass coverslips and incubated at 43°C for 2 h and then allowed to recover at 37°C for 8 h. After recovery the cells were fixed for double-label indirect immunofluorescence as described. (A and D) Phase-contrast micrographs. (B and E) Endogenous HSP70 fluorescence. (C and F) Distribution of the NF protein.

gion of the HSP70 protein, we have mapped the antigenic determinants of several HSP70-specific mAbs, and have found that the HSP70 antigenic determinants are all located in the carboxy terminus of the protein (unpublished observations).

One currently popular theory regarding the function of HSP70 is that the protein uses the energy of ATP hydrolysis to promote disaggregation or repair of cellular structures, such as nucleoli, damaged by various stress treatments. This stems from observations that ATP causes the release of HSP70, or other members of the HSP70 family, from various protein complexes *in vitro* (4, 5, 9, 17, 26, 37, 48); and that

in cells constitutively expressing high levels of HSP70, the heat-induced structural alterations visible in nucleoli are repaired much faster (31). Thus, we examined the recovery after heat shock of cells transiently transfected with either the WT protein or the various deletion constructs, particularly the mutant, which did not bind ATP (NF). As cells recover from heat shock the association of HSP70 with nucleoli diminishes and the protein accumulates in the cytoplasm (43). Using the intracellular distribution of HSP70 as a measure of cellular recovery, we found that cells transfected with the WT construct recovered much faster than the sur-

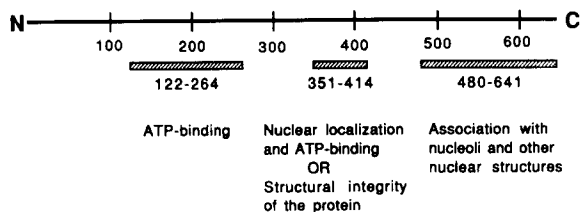


Figure 12. Domains of HSP70 identified by analysis of deletion mutants. The ATP-binding ability of the HSP70 protein was abolished by deletion of amino acids 122–264. Removal of amino acids 351–414 affected both the intracellular localization and ATP-binding capability of the HSP70 protein, probably through disruption of the tertiary structure. The association of HSP70 with nucleoli and other nuclear structures was dependent upon the presence of the carboxy-terminal region of the HSP70 protein.

rounding untransfected cells. However, cells transfected with the NF mutant, which cannot bind ATP, did not show accelerated recovery. In fact, some of the NF-transfected cells still displayed nucleolar localization of the NF protein when the surrounding untransfected cells had fully recovered. Apparently, the inability to bind ATP may have interfered with or slowed the release of the NF protein from the nucleolus. These results suggest that the inability to bind ATP affects the ability of HSP70 to dissociate from complexes *in vivo*. This is consistent with *in vitro* data indicating that ATP promotes dissociation of HSP70 from complexes (4, 5, 9, 17, 26, 37, 48).

Models have been proposed in which HSP70 binds ATP before its interaction with other components (17, 32). Our results indicate that this may not be absolutely necessary. Both the NF mutant and the carboxy-terminal fragment NSC displayed typical association with nucleoli and other nuclear structures, yet neither of these mutants was able to bind ATP. Hence, the inability to bind ATP did not appear to influence the ability of HSP70 to associate with nucleoli.

The recovery experiments have allowed us to draw some conclusions regarding the necessity of ATP-binding for the function of HSP70. However, they have also left us with many questions that illustrate the complexity of the recovery process. For instance, some of the HSP70 deletions had little or no effect on the ability of the mutant protein to accelerate the recovery of cells from heat shock, and in essence had no phenotype. Does this imply that these regions of the protein are unnecessary, or does our inability to detect a phenotype for these mutants stem from the limited functional assays and our lack of understanding of the function of HSP70? The SMA mutant, which, although present in the nucleus, did not associate with nucleoli after heat shock, was still capable of allowing transfected cells to recover faster than the surrounding untransfected cells. Besides the repair of nucleolar damage, what other processes in the nucleus might the SMA protein be facilitating to allow the accelerated recovery of transfected cells? The most perplexing result, however, is that the small carboxy-terminal fragment NSC also allowed the accelerated recovery of transfected cells. This mutant lacks the entire amino terminus and cannot bind ATP. Yet, unlike the NF protein, this small fragment somehow facilitated accelerated recovery and its ability to dissociate from nucleoli did not appear to be altered. Perhaps this peculiarity is due to the small size of the NSC fragment. We demon-

strated that the carboxy-terminal sequences present in NSC were responsible for directing HSP70 to the nucleolus, but we cannot rule out that another region of the protein not contained in the NSC fragment may also be involved in binding to nucleoli. This other binding site may in fact be what necessitates ATP-dependent release. How the NSC fragment can facilitate accelerated recovery is unclear. One possible model is that the HSP70 protein may be capable of both ATP-dependent and -independent interactions with substrates, and different regions of the protein may be used for these interactions. Both types of interactions may be necessary during the recovery of cells from stress treatments. By augmenting either one or both of these interactions by overexpressing wild-type HSP70 or the various deletion mutants, the recovery process may be accelerated.

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References

- Bardwell, J., and E. A. Craig. 1984. Major heat shock gene of *Drosophila* and the *Escherichia coli* heat-inducible dnaK gene are homologous. *Proc. Natl. Acad. Sci. USA.* 81:848–852.
- Burglin, T. R., and E. M. DeRobertis. 1987. The nuclear migration signal of *Xenopus laevis* nucleoplasm. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2617–2625.
- Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195–203.
- Chappell, T. G., B. B. Konforti, S. L. Schmid, and J. E. Rothman. 1987. The ATPase core of a clathrin uncoating protein. *J. Biol. Chem.* 262:746–751.
- Chappell, T. G., W. J. Welch, D. M. Schlossman, K. B. Palter, M. J. Schlesinger, and J. E. Rothman. 1986. Uncoating ATPase is a member of the 70kDa family of stress proteins. *Cell.* 45:3–13.
- Chen, C., and H. Okayama. 1987. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* 7:2745–2752.
- Chirico, W. J., M. G. Waters, and G. Blobel. 1988. 70K heat shock related proteins stimulate translocation into microsomes. *Nature (Lond.)* 332:805–810.
- Chu, G., and P. A. Sharp. 1981. SV40 DNA transfection of cells in suspension: analysis of the efficiency of transcription and translation of T-antigen. *Gene (Amst.)* 13:197–202.
- Clarke, C. F., K. Cheng, A. B. Frey, R. Stein, P. W. Hinds, and A. J. Levine. 1988. Purification of complexes of nuclear oncogene p53 with rat and *Escherichia coli* heat shock proteins: *in vitro* dissociation of hsc70 and dnaK from murine p53 by ATP. *Mol. Cell. Biol.* 8:1206–1215.
- Craig, E. A. 1985. The heat shock response. *CRC Crit. Rev. Biochem.* 18:239–280.
- Dang, C. V., and W. M. F. Lee. 1988. Identification of the human c-myc protein nuclear translocation signal. *Mol. Cell. Biol.* 8:4048–4054.
- Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman. 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature (Lond.)* 332:800–805.
- Hinds, P. W., C. A. Finlay, A. B. Frey, and A. J. Levine. 1987. Immunological evidence for the association of p53 with a heat shock protein, hsc70, in p53-plus-ras-transformed cell lines. *Mol. Cell. Biol.* 7:2863–2869.
- Hunt, C., and R. I. Morimoto. 1985. Conserved features of eukaryotic HSP70 genes revealed by comparison with the nucleotide sequence of human HSP70. *Proc. Natl. Acad. Sci. USA.* 82:6455–6459.
- Kalderon, D., W. D. Richardson, A. F. Markham, and A. E. Smith. 1984.

- Sequence requirements for nuclear location of simian virus 40 large-T antigen. *Nature (Lond.)*. 311:33-38.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
 17. Lewis, M. J., and H. R. B. Pelham. 1985. Involvement of ATP in the nuclear and nucleolar functions of the 70kD heat shock protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:3137-3143.
 18. Lindquist, S. 1986. The heat-shock response. *Annu. Rev. Biochem.* 55:1151-1191.
 19. Lindquist, S., and E. A. Craig. 1988. The heat-shock proteins. *Annu. Rev. Genet.* 22:631-677.
 20. Lyons, R. H., B. Q. Ferguson, and M. Rosenberg. 1987. Pentapeptide nuclear localization signal in adenovirus E1a. *Mol. Cell Biol.* 7:2451-2456.
 21. Messing, J. 1983. New M13 vectors for cloning. *Methods Enzymol.* 101C:20-78.
 22. Milarski, K. L., and R. I. Morimoto. 1986. Expression of human HSP70 during the synthetic phase of the cell cycle. *Proc. Natl. Acad. Sci. USA.* 83:9517-9521.
 23. Milarski, K. L., W. J. Welch, and R. I. Morimoto. 1989. Cell cycle-dependent association of HSP70 with specific cellular proteins. *J. Cell Biol.* 108:413-423.
 24. Millan, J. L., C. E. Driscoll, K. M. LeVan, and E. Goldberg. 1987. Epitopes of human testis-specific lactate dehydrogenase deduced from a cDNA sequence. *Proc. Natl. Acad. Sci. USA.* 84:5311-5315.
 25. Munro, S., and H. R. B. Pelham. 1984. Use of peptide tagging to detect proteins expressed from cloned genes: deletion mapping functional domains of *Drosophila* HSP70. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:3087-3093.
 26. Munro, S., and H. R. B. Pelham. 1986. An HSP70-like protein in the ER: identity with the 78kD glucose-regulated protein and the immunoglobulin heavy chain binding protein. *Cell.* 46:291-300.
 27. Munro, S., and H. R. B. Pelham. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell.* 48:899-907.
 28. Murakami, H., D. Pain, and G. Blobel. 1988. 70-kD heat shock-related protein is one of two distinct cytosolic factors stimulating protein import into mitochondria. *J. Cell Biol.* 107:2051-2057.
 29. Nakamaye, K. L., and F. Eckstein. 1986. Inhibition of restriction endonuclease NciI cleavage by phosphorothioate groups and its application to oligonucleotide-directed mutagenesis. *Nucleic Acids Res.* 14:9679-9698.
 30. O'Malley, K., A. Mauron, J. D. Barchas, and L. Kedes. 1985. Constitutively expressed rat mRNA encoding a 70-kilodalton heat shock-like protein. *Mol. Cell Biol.* 5:3476-3483.
 31. Pelham, H. R. B. 1984. HSP70 accelerates the recovery of nucleolar morphology after heat shock. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:3095-3100.
 32. Pelham, H. R. B. 1986. Speculations on the functions of the major heat shock and glucose-regulated proteins. *Cell.* 46:959-961.
 33. Pelham, H. R. B., and R. J. Jackson. 1976. Efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* 67:247-256.
 34. Pinhasi-Kimhi, O., D. Michalovitz, A. Ben-Zeev, and M. Oren. 1986. Specific interaction between p53 cellular tumor antigen and major heat shock proteins. *Nature (Lond.)*. 320:182-185.
 35. Richardson, W. D., B. L. Roberts, and A. E. Smith. 1986. Nuclear localization signals in polyomavirus large-T. *Cell.* 44:77-85.
 36. Siomi, H., H. Shida, S. H. Nam, T. Nosaka, M. Maki, and M. Hatanaka. 1988. Sequence requirements for nucleolar localization of human T cell leukemia virus type I pX protein, which regulates viral RNA processing. *Cell.* 55:197-209.
 37. Ungewickell, E. 1985. The 70-kD mammalian heat shock proteins are structurally and functionally related to the uncoating protein that releases clathrin triskelion from coated vesicles. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:3385-3391.
 38. Walter, G., A. Carbone, and W. J. Welch. 1987. Medium tumor antigen of polyomavirus transformation-defective mutant NG59 is associated with 73-kilodalton heat shock protein. *J. Virol.* 61:405-410.
 39. Watowich, S. S., and R. I. Morimoto. 1988. Complex regulation of heat shock- and glucose-responsive genes in human cells. *Mol. Cell Biol.* 8:393-405.
 40. Welch, W. J., and J. R. Feramisco. 1984. Nuclear and nucleolar localization of the 72,000-dalton heat shock protein in heat shocked mammalian cells. *J. Biol. Chem.* 259:4501-4513.
 41. Welch, W. J., and J. R. Feramisco. 1985. Rapid purification of mammalian 70,000-dalton stress proteins: affinity of the proteins for nucleotides. *Mol. Cell Biol.* 5:1229-1237.
 42. Welch, W. J., and L. A. Mizzen. 1988. Characterization of the thermotolerant cell. II. Effects on the intracellular distribution of heat shock protein 70, intermediate filaments and small nuclear ribonucleoprotein complexes. *J. Cell Biol.* 106:1117-1130.
 43. Welch, W. J., and J. P. Suhan. 1986. Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. *J. Cell Biol.* 103:2035-2052.
 44. White, E., D. Spector, and W. J. Welch. 1988. Differential distribution of the adenovirus E1A proteins and colocalization of E1A with the 70-kilodalton cellular heat shock protein in infected cells. *J. Virol.* 62:4153-4166.
 45. Wu, B. J., C. Hunt, and R. I. Morimoto. 1985. Structure and expression of the human gene encoding major heat shock protein HSP70. *Mol. Cell Biol.* 5:330-341.
 46. Wu, B. J., R. E. Kingston, and R. I. Morimoto. 1986. Human HSP70 promoter contains at least two distinct regulatory domains. *Proc. Natl. Acad. Sci. USA.* 83:629-633.
 47. Zimmerman, R., M. Sagstetter, M. J. Lewis, and H. R. B. Pelham. 1988. Seventy-kilodalton heat shock proteins and an additional component from reticulocyte lysate stimulate import of M13 procoat protein into microsomes. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2875-2880.
 48. Zyllicz, M., D. Ang, and C. Georgopoulos. 1987. The grpE protein of *Escherichia coli*. *J. Biol. Chem.* 262:17437-17442.