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Characterization of Nascent Polypeptides and Their Molecular Chaperones in Mammalian Cells

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CHARACTERIZATION OF NASCENT POLYPEPTIDES AND THEIR MOLECULAR CHAPERONES IN MAMMALIAN CELLS

Daryl K. Eggers
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Abstract. Folding of newly-synthesized proteins *in vivo* is believed to be facilitated by the cooperative interaction of a defined group of proteins known as molecular chaperones. The primary focus of the research presented in this thesis was to identify which chaperones form stable complexes with nascent polypeptides in the cytosol of mammalian cells. The antibiotic puromycin was used extensively in this work to generate a heterogenous population of tagged and truncated nascent polypeptides. By employing a combination of metabolic labeling and immunoprecipitation techniques, including the use of a polyclonal antibody to puromycin, hsp70 was found to be the predominant chaperone bound to the general population of nascent polypeptides. The interaction between hsp70 and nascent polypeptides was characterized as one of high affinity yet constant dynamics in the presence of millimolar concentrations of ATP or ADP. This dynamic interaction could be stabilized by one of three methods: (1) hydrolysis of endogenous nucleotides with apyrase during cell lysis; (2) lysing cells in the presence of 10 mM AMP; or (3) addition of a chemical crosslinker to living cells. The possibility that ATP hydrolysis is not required for the chaperone activity of hsp70 in mammalian cells is discussed. Nascent puromycyl-polypeptides exhibited a low solubility in the absence of detergent and a strong propensity for binding many proteins nonspecifically in cell lysates. It seems that the physical properties of a nascent polypeptide which confer a dependence on chaperones *in vivo* are the same properties which make it difficult to determine relevant protein interactions *in vitro*. Future work in this field must

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

2-D	two-dimensional (gel)
BSA	bovine serum albumin
CCT	cytosolic chaperonin containing TCP-1
CHX	cycloheximide
DMEM	Dulbecco's Modified Eagle's Medium
DOC	deoxycholic acid, sodium salt
DSP	dithio*bis*(succinimidyl-propionate)
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
HEPES	4(-2-hydroxyethyl)-1-piperazineethanesulfonic acid
hsp	heat shock protein
ID	inner diameter
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline (free of Ca^{2+}, Mg^{2+})
RAM	rabbit anti-mouse secondary antibody
RAR	rabbit anti-rat secondary antibody
RC-BSA	reduced and carboxymethylated bovine serum albumin
SDS	sodium dodecyl sulfate (lauryl sulfate)

1. BACKGROUND & INTRODUCTION

Protein folding is a fundamental and critical step in the maturation of all newly-synthesized proteins. Christian Anfinsen received the Nobel Prize in Chemistry in 1972 for demonstrating that the "information" for proper folding is encoded solely in the unique sequence of amino acid residues that define the primary structure of every protein (Anfinsen, 1973). Anfinsen showed that many model proteins may be unfolded by chemical denaturants and then refolded into functional proteins without the addition of any other factors. The initial folding of a newly-synthesized protein *in vivo*, however, may be a much more complicated process than the refolding of full-length model proteins in the test tube. In the cell, a nascent polypeptide just emerging from the ribosome has not acquired enough information (*i.e.* incorporated enough amino acids) to fold into a stable structure. In addition, a nascent polypeptide must contend with the crowded environment of the cell during its synthesis. Incidental binding between nascent polypeptides and their nearest neighbors could lead down a nonproductive folding pathway resulting in a nonfunctional protein. It often has been observed in heterologous expression systems (*eg.*, systems expressing human proteins in bacteria) that complete translation of a message corresponding to a known protein sequence does not ensure that the product will fold into a biologically active protein. Improper folding *in vivo* may result in protein aggregation (formation of inclusion bodies and plaques) or in high rates of degradation (protein deficiencies). Recognition of the fact that misfolding of specific proteins may lead to human disease states is an important motivating factor for understanding the folding process *in vivo* (Sifers, 1995; Thomas *et al.*, 1995).

A group of proteins, now referred to as molecular chaperones, is one of the factors believed to facilitate proper folding *in vivo* by shielding or stabilizing critical intermediates during protein synthesis. The two main classes of molecular chaperones implicated in the folding of nascent or newly-synthesized polypeptides are the hsp60 and hsp70 families. Members of both families are highly conserved, and the expression of some (but not all) members is increased upon different types of metabolic stress, including heat shock. Since metabolic stress oftentimes results in destabilization and aggregation of proteins, molecular chaperones are thought to play an important role in the renaturation and/or prevention of such aggregates in addition to their role in facilitating the folding of newly-synthesized proteins. The hsp60 class is referred to as the "chaperonins" and its members are characterized structurally as large complexes of two heptameric rings. The hsp70 chaperones, on the other hand, are believed to function as monomers. Members of both hsp60 and hsp70 families hydrolyze ATP in a reaction cycle which alters their conformation and, thereby, modulates their affinity for unfolded model substrates. The ATP reaction cycle for hsp70 (and its bacterial homologue, DnaK) may be mediated by other proteins *in vivo* including hsp40 (DnaJ in bacteria), p48 (also known as Hip), and GrpE (in bacteria and mitochondria only). The ATP reaction cycle for hsp60 (GroEL in bacteria) is mediated by the smaller ring complex known as hsp10 (GroES).

The following section critically reviews many of the published observations related to chaperone interactions with nascent or newly-synthesized proteins from *in vivo* sources (*i.e.* metabolically-labeled cells or *in vitro* translation experiments). The phrase "nascent polypeptide" is used to describe a protein which is still bound to the ribosome as peptidyl-tRNA or a polypeptide that was terminated prematurely and released from the ribosome. The phrase "newly-synthesized protein" is used to refer to a full-length translation product which

was recently released from the ribosome and which may or may not have acquired its final folded conformation. This mini-review excludes a discussion of *in vitro* refolding experiments and of proteins that facilitate secretion and/or targeting of nascent polypeptides to specific organelles (*e.g.* SRP, NAC, and SecB).

1.1. Review of Literature

1.1.1 Chaperone Interactions in Procaryotes

The list of suspected molecular chaperones in bacteria includes GroEL, GroES, DnaJ, DnaK, and trigger factor. GroEL/ES and DnaJ/K were all identified originally as gene products that are important for growth and assembly of bacteriophages. It was subsequently discovered that DnaJ and DnaK are necessary for DNA replication at elevated temperatures (see Georgopoulos and Welch, 1993, for an historical perspective). Trigger factor was identified originally as a translocation factor for proOmpA (Crooke and Wickner, 1987).

In contrast to the vast number of papers dealing with the refolding of proteins in the presence of GroEL, there are surprisingly few studies describing an interaction between GroEL and nascent or newly-synthesized proteins. It seems that the biological role of GroEL has been inferred largely from mitochondrial import studies involving the GroEL homologue, hsp60. In one of the earliest reports of a newly-synthesized protein bound to any molecular chaperone, it was observed that GroEL can be crosslinked to the N-termini of newly-synthesized (full length) pre-β-lactamase following *in vitro* translation in an *E. coli* extract (Bochkareva *et al.*, 1988). GroEL was crosslinked to a relatively small fraction of the total translated product, however, and radiolabeled pre-β-

lactamase was found in every fraction of a sucrose gradient following sedimentation. Although not discussed by the authors, this result suggested that the newly-synthesized protein was aggregating and/or binding other proteins in addition to GroEL, perhaps in a nonspecific manner. A similar aggregation problem was noted after sedimentation of newly-synthesized rhodanese which had been released from the ribosome with puromycin (Reid and Flynn, 1996). At least two studies have concluded that GroEL does not bind to nascent polypeptides cotranslationally since GroEL does not cosediment with ribosomes in sucrose gradients (Gaitanaris *et al.*, 1994; Reid and Flynn, 1996).

In perhaps the most relevant experiment performed thus far involving GroEL, Horwich and coworkers demonstrated that a subset of endogenous newly-synthesized (pulse-labeled) proteins becomes insoluble *in vivo* at 37 °C in the presence of a temperature-sensitive lethal mutation of GroEL (Horwich *et al.*, 1993). Presumably, the newly-synthesized (full length) proteins aggregated due to their own misfolding. However, it is also possible that the insoluble proteins were associated with other nascent polypeptides that had not completed synthesis. (Truncated nascent polypeptides will be shown to be highly susceptible to aggregation in the Results & Discussion.) Importantly, it was noted that preexisting (mature) proteins were unaffected by a shift to the nonpermissive temperature; only newly-synthesized proteins were found to aggregate in the presence of the defective chaperone. It would have been extremely satisfying if this study had gone further to show whether (or not) the same subset of insoluble proteins is normally bound to GroEL after synthesis in a wild-type strain (via immunoprecipitation of GroEL, for example). In fact, no one in the field has proven a direct interaction between GroEL and any newly-synthesized protein following a pulse of label *in vivo*. (The sedimentation

profiles reported in Gaitanaris *et al.*, 1994, were not convincing and were not confirmed by immunoprecipitation.)

Other studies in bacteria have shown that the levels of either DnaJ/K or GroEL/ES can influence a number of cellular events including the aggregation of mature proteins following heat shock at 42 °C (Gragerov *et al.*, 1992), the reactivation of heat-inactivated firefly luciferase (Schröder *et al.*, 1993), and the specific activity of the phage λ *c*1857 repressor (Gaitanaris *et al.*, 1994). The level of soluble protein recovered from a highly-expressed and aggregation-prone fusion protein was increased by overexpression of DnaJ/K but not by overexpression of GroEL/ES (Thomas and Baneyx, 1996). In addition, overexpression of GroEL/ES has been found to suppress defects in temperature-sensitive folding mutants of the phage P22 coat protein, but not folding mutants of the tailspike protein (Gordon *et al.*, 1994).

Hardesty and coworkers have reported that all of the bacterial chaperones may influence the release and activation of ribosome-bound rhodanese which accumulates as inactive full-length peptidyl-tRNA during cell-free translation (Kudlicki *et al.*, 1994a). It is not clear yet whether chaperones mediate this effect through interactions with nascent rhodanese or, alternatively, by facilitating the activity (or recycling) of translation termination factors (*i.e.*, RF-1, RF-2, and RF-3). Changes in conformation of rhodanese were probed by incorporation of a fluorescent coumarin derivative at the N-terminal methionines (Kudlicki *et al.*, 1994b). It was found that none of the chaperones (individually or in combination) altered the fluorescent properties of the tagged rhodanese in a manner comparable to that observed upon binding of an anti-coumarin antibody.

Unlike GroEL/ES, the chaperones DnaJ/K have been found to cofractionate with translating ribosomes following sucrose gradient centrifugation (Gaitanaris *et al.*, 1994; Vysokanov, 1995). Addition of purified

DnaJ/K had a marked effect on the recovery of active CAT trimers that were synthesized *in vitro* using an *E. coli* extract prepared from a strain bearing a deletion in DnaJ/K (Vysokanov, 1995). It was noted that DnaK alone provided the main contribution to the enhanced activity of CAT.

Trigger factor (TF) was implicated only recently as a possible generic chaperone in bacteria. By incorporating photo-reactive modified lysines into nascent polypeptides, TF was crosslinked to every ribosome-bound nascent polypeptide that was tested, independent of a secretory signal sequence (Valent *et al.*, 1995). Crosslinking of TF was greatly reduced or abolished by high salt and puromycin treatment. Thus, TF only interacted with nascent polypeptides in the context of the ribosome. A similar finding was reported upon crosslinking of nascent β-galactosidase (Hesterkamp *et al.*, 1996). The photocrosslinking approach also was employed to show that (exogenously added) DnaJ can bind cotranslationally to nascent polypeptides (Hendrick *et al.*, 1993). The crosslinking efficiency of DnaJ to nascent luciferase was very low, however, and other investigators have reported a failure to crosslink DnaJ to nascent polypeptides using wild-type lysate, TF-depleted lysate, lysate from a DnaJ overexpressing strain, or a lysate prepared from heat-shocked cells (Valent *et al.*, 1995).

1.1.2 Chaperone Interactions in Eucaryotes

The list of purported molecular chaperones in the eucaryotic cytosol includes hsp70, CCT (TRiC), hsp40, p48 (Hip), and hsp90. Cytosolic hsp70 was investigated as a potential chaperone because BiP, the hsp70 homolog in the ER, was reported to bind newly-translocated polypeptides in the lumen of the ER (reviewed in Welch *et al.*, 1997). The chaperonin CCT is a heteromeric complex containing TCP-1 and several other subunits (Kubota *et al.*, 1995). TCP-1 was

implicated originally as a member of the chaperonin family based on a sequence similarity search (Ellis, 1990). The chaperone activity of hsp40 has been inferred primarily from studies with the bacterial homolog, DnaJ. The 48-kDa protein, p48, was described originally as a transient component of steroid receptor complexes during assembly studies in a cell-free system (Prapapanich *et al.*, 1996). Hsp90 also participates in steroid receptor complexes, but the primary biological function of this highly expressed protein remains elusive. Although hsp90 has been shown to enhance the refolding of certain proteins *in vitro* (Jakob *et al.*, 1995; Freeman and Morimoto, 1996), there have been no reports of hsp90 in complexes with nascent or newly-synthesized proteins.

Most of the chaperone studies in eucaryotic systems have relied completely on immunoprecipitation techniques using antibodies to specific chaperones. By such an approach, it was found that both nascent and newly-synthesized proteins are coprecipitated with hsp70 in HeLa cell lysates (Beckmann *et al.*, 1990). The hsp70 interaction in this study was not observed unless ATP was depleted with the enzyme apyrase prior to immuno-precipitation. It was subsequently shown that hsp70 cosediments with polysomes from yeasts and that the association with ribosomes was greatly reduced upon release of the nascent polypeptides with puromycin (Nelson *et al.*, 1992). A similar study noted that the heat-inducible hsp72 may be associated with ribosomes at all times in mammalian cells, whereas the constitutive hsp73 may be associated with ribosomes through a direct interaction with nascent polypeptides (Beck and De Maio, 1994). Hsp70 also has been detected in large complexes with nascent polypeptides following cell-free translation of truncated CAT (Hansen *et al.*, 1994), truncated luciferase (Frydman *et al.*, 1994), and truncated actin (Frydman and Hartl, 1996), all in rabbit reticulocyte lysates.

CCT has been shown to cofractionate with newly-synthesized tubulin in reticulocyte lysate after sizing chromatography (Yaffe *et al.*, 1992). In addition, newly-synthesized actin, α-tubulin, and β-tubulin were coprecipitated from pulse-labeled CHO cells with the 23C antibody to the α-CCT subunit (Sternlicht *et al.*, 1993). It is a concern, however, that none of the nascent radiolabelled α-CCT subunit was observed in the immunoprecipitate with actin and tubulin. Also, this study used "no antibody" as a control immunoprecipitation which would not detect problems of nonspecific binding to the antibody itself. (Note: the CCT immunoprecipitations were performed in concentrated lysates, a condition which will be shown to promote nonspecific interactions in this thesis.) CCT has been found in complexes with nascent forms of firefly luciferase (Frydman *et al.*, 1994) and actin (Frydman and Hartl, 1996) and with assembly intermediates of the hepatitis B virus capsid in a cell-free system (Lingappa *et al.*, 1994).

Frydman and Hartl analyzed the binding of hsp40, hsp70, and CCT with nascent luciferase in three reticulocyte lysates, each one of which had been partially depleted of one of the chaperones (Frydman *et al.*, 1994). They interpreted the results of the depletion experiment as evidence of a "defined order of chaperone interactions" which essentially mimics the model put forth by the Hartl lab for describing the interactions of bacterial chaperones (Langer *et al.*, 1992; Schröder *et al.*, 1993; Szabo *et al.*, 1994). In their model, hsp40 (DnaJ, in bacteria) binds first to nascent polypeptides followed by hsp70 (DnaK) and finally CCT (GroEL). The results of the depletion studies in reticulocyte lysates are open to other interpretations, however, and the role of hsp40 is especially uncertain; so far, hsp40 has been found to bind only one nascent model protein, firefly luciferase.

Like hsp40, the data in support of p48 as a general molecular chaperone is greatly lacking. Antibodies to p48 were used to (weakly) coprecipitate a population of nascent luciferase polypeptides (Höhfeld *et al.*, 1995). This immunoprecipitation result is suspect, however, due to the fact that the lysate was not diluted before addition of the antibody and the stringency of the washing step was low (only the last of five washes contained detergent).

1.2. Scope of Thesis

Experimental approaches for studying protein folding have become more sophisticated in recent years in order to simulate the folding process *in vivo*. For example, refolding experiments have been performed in the presence of whole lysates (Schumacher *et al.*, 1994; Frydman and Hartl, 1996; Tian *et al.*, 1996; Melki *et al*, 1997) or purified molecular chaperones (Langer *et al.*, 1992; Schröder *et al.*, 1993; Szabo *et al.*, 1994; Jakob *et al.*, 1995; Freeman and Morimoto, 1996), and *in vitro* translation systems have been used to identify proteins which interact with ribosome-bound nascent polypeptides (reviewed in the preceding section). Both refolding experiments and *in vitro* translation experiments are dependent on the selection of a model protein, however, and the chaperone interactions which are detected in these systems are not necessarily general to the folding of all proteins.

For much of the work presented in this thesis, the drug puromycin was used to study the complex formed between nascent polypeptides and their molecular chaperones in the cytosol of mammalian cells. Puromycin is an inhibitor of protein synthesis which competes with aminoacyl-tRNAs for the A-site of ribosomes. Once bound to the ribosome, puromycin becomes covalently incorporated into the C-terminus of the polypeptide chain resulting in premature termination. This property of puromycin was exploited to "tag" nascent

polypeptides synthesized *in vivo* and to selectively isolate the polypeptides using a polyclonal antibody to puromycin. The feasibility of this approach was demonstrated in a previous communication (Hansen *et al.*, 1994). The truncated chains are referred to as "puromycyl-polypeptides", although "peptidyl-puromycins" may be (structurally) more correct. At low micromolar concentrations of puromycin, it is possible to accumulate a large population of nascent polypeptides derived from all the actively-translated mRNAs in the cell. This heterogenous population of puromycyl-polypeptides may yield information about molecular chaperone interactions which is more generally characteristic of the folding and maturation of all proteins. This approach differs significantly from previous studies in that the nascent polypeptide is the "hook" for capturing the complex and not the molecular chaperone; there is no bias as to the identity of any of the bound proteins prior to immunoprecipitation.

All of the experiments in this thesis started with metabolically-labeled HeLa cells. Protein synthesis in the presence of ^{35}S-methionine was carried out for short times to "pulse label" the nascent polypeptides, including puromycyl-polypeptides, or for longer times (typically overnight) to label all the mature proteins of the cell, including the chaperones. Physical properties of the complex between a nascent polypeptide and its bound chaperone(s) were examined by many methods including sizing chromatography, native gels, velocity sedimentation, and protease sensitivity. The puromycin antibody was used to identify those proteins which bind to puromycyl-polypeptides under immunoprecipitation conditions that maintain stable protein-protein interactions. In addition, a crosslinking reagent was employed for stabilizing the complex between nascent polypeptides and their chaperones *in vivo* before lysis of the cells. The crosslinking approach permitted analysis of complexes that were stabilized in the presence of physiological concentrations of nucleotides and total

protein, two parameters which may be critical for characterizing the roles of molecular chaperones *in vivo*.

A recurring theme will develop in the Results & Discussion section; nascent polypeptides have a strong propensity for aggregation and for binding other proteins in a nonspecific manner. Although this property of a nascent polypeptide seems logical and consistent with their dependence on molecular chaperones for proper folding in the cell, the consequences of nonspecific binding *in vitro* have been largely ignored or unappreciated by previous investigators. A key challenge in the present thesis work was to distinguish relevant chaperone interactions from the unavoidable nonspecific binding events that occur upon cell lysis.

2. MATERIALS & METHODS

2.1. Commercial Reagents

The EXPRE^{35}S^{35}S protein labeling mix was used for all labeling experiments (Dupont NEN #NEG-072, 1000 Ci/mmole). Puromycin, BSA, RC-BSA, α_s-casein, apyrase, and Sephacryl S-400 were all obtained from Sigma. Sepharose CL-4B, Protein A Sepharose CL-4B, and ampholines were purchased from Pharmacia. Electrophoresis grade Triton X-100 was a product of FisherBiotech. Many monoclonal antibodies, including N27, 1B5, 84a, 91a, 16F1, and 9D2, were obtained from Stressgen. Secondary antibodies, RAM and RAR, were purchased from Cappel and Nordic Immunology, respectively. The crosslinking reagent DSP was obtained from Pierce Chemicals. The proteasome inhibitor, MG132, and proteasome antibodies were obtained through A.L. Goldberg (Harvard). Antibodies to p48 were a gift from D.F. Smith (U. of Nebraska Medical Center, Omaha), and the P5 antibody to CCT was kindly donated by V. Bibor-Hardy (Institut du Cancer de Montréal, Montréal, Québec).

2.2. Metabolic Labeling, Puromycin Treatment, and Cell Lysis

HeLa cells were used for all experiments described in this work. The cells were grown in 10% calf serum/DMEM as monolayers on culture plates to 50-75% confluency. Typically, 250-500 µCi of ^{35}S-methionine in 5 ml of medium was used for metabolic labeling of a 10 cm plate of cells. "Pulse labeling" was performed in methionine-free DMEM for short times as indicated in the figure legends, and the cells were lysed immediately without a chase period. "Steady-

state labeling" was achieved by incubating the cells with label overnight (14-18 hours) in methionine-free DMEM supplemented with 5% complete DMEM and 2% calf serum (after sterilizing medium through an 0.2 micron syringe filter). Overnight labeling was followed by a minimum 4 hour chase period in complete medium containing 10% calf serum (but no label) to ensure that all labeled proteins were given time to mature before cell lysis. In those experiments utilizing puromycin, the drug was added to the medium for the indicated time (typically between 15-30 minutes) just prior to lysis of the cells. For pulse-labeling experiments, puromycin and ^{35}S-methionine often were added simultaneously. See figure legends for exact duration of pulse labeling and puromycin treatments. Unless noted otherwise, cells were washed with PBS and placed at 4 °C before being harvested by scraping into lysis buffer with the large end of a pipet tip. Typically, 1 ml of buffer A (20 mM HEPES, pH 7.4, 100 mM KCl, 2 mM $MgCl_2$, 1 mM DTT) containing 0.1% Triton X-100 was used per 10 cm plate for detergent lysis. For hypotonic lysis, the cells were incubated at 4 °C in 10 mM HEPES, 10 mM KCl, and 0.1 mM EDTA and allowed to swell at least 15 minutes while still on the plate. The cells then were scraped off the plate, and the lysate was adjusted with salts to achieve final buffer A concentrations. Lysates were extruded through a 26g needle, incubated for 10 minutes at 4 °C with 10 units/ml apyrase to deplete ATP, and clarified in a microcentrifuge for 10 minutes. In some experiments, the lysates were supplemented with MgATP in place of the apyrase treatment (see figure legends). Some preparations of commercial apyrase were found to be contaminated with a protease activity which could be reduced by preincubation with soybean trypsin inhibitor at a concentration of 200 ng/unit apyrase.

2.3. Immunoprecipitation Techniques

For immunoprecipitation under "denaturing conditions", cell lysates were adjusted with Laemmli sample buffer (final 1% SDS) and heated at 95 °C for 5-10 minutes. Following clarification in a microcentrifuge, the lysate was diluted in RIPA buffer (1% Triton X-100, 1% deoxycholate in PBS) such that the final concentration of SDS was always less than 0.05%. All samples were pre-adsorbed with Sepharose CL-4B for 30 minutes before addition of antibody. Incubations with the primary antibody were carried out for a minimum of 2 hours at 4 °C followed by a 1-hour incubation with rabbit anti-mouse (RAM) and rabbit anti-rat (RAR) secondary antibodies, when appropriate. The resulting complexes were captured with Protein A Sepharose CL-4B and washed four times with RIPA buffer supplemented with 0.1% SDS.

For examining protein-protein interactions, immunoprecipitation under "native" or "nondenaturing conditions" was performed. In this case, the cells were both lysed and diluted in buffer A + 0.1% Triton at 4 °C (no SDS, no heating step). Antibody incubations and immunoprecipitations were the same as described above, except that the final precipitates were washed four times with buffer A supplemented with 1% Triton X-100 and 1% sodium deoxycholate.

2.4. Antibodies

The polyclonal antibody to puromycin was developed by conjugating the molecule to a mixture of keyhole limpet hemocyanin and hen egg white lysozyme and combining with complete Freund's adjuvant prior to injection of rabbits (completed by W.J. Hansen). The resulting immune serum was used for

all precipitations of puromycyl-polypeptides without further purification of the antibody. A mixture of antibodies was used for precipitation of hsp72/hsp73 which included four mouse monoclonal antibodies (N6, N15, N21, N33) (Milarski *et al.*, 1989), a rabbit peptide antibody to hsp73 (Brown *et al.*, 1993), and a rat monoclonal antibody 1B5. The mouse monoclonal antibody N27 was added to the hsp70 antibody mixture for analysis of denatured samples. A mixture of mouse antibodies was used to capture p48 (2G6, 1A6, 10D1) (Prapapanich *et al.*, 1996). The P5 antibody which recognizes the γ-subunit of CCT was used for nondenatured samples only (Joly *et al.*, 1994). For immunoprecipitation of CCT from denatured samples, a mixture of a rabbit polyclonal antibody made against a synthetic peptide of mouse TCP-1 (Lingappa *et al.*, 1994) and the rat monoclonal antibodies 84a & 91a (Willison *et al.*, 1989) was used. A rabbit polyclonal antibody to hsp40 was prepared in the Welch laboratory using recombinant human hsp40 as immunogen. A rabbit polyclonal antibody to hsp90 was used for immunoprecipitation of nondenatured samples (W.J.W., unpublished) and was supplemented with the rat monoclonal antibodies 16F1 and 9D2 (Lai *et al.*, 1984) for the analysis of denatured samples.

2.5. Electrophoresis & Western Blotting

Protein samples were separated by SDS-PAGE and fluorographed with diphenyloxazole (15% PPO in DMSO) in gels containing a constant product of acrylamide and bis-acrylamide (%A x %B = 1.3) (Blattler *et al.*, 1972). The isoelectric gradient for all two-dimensional gels was developed using ampholines of 70% pH 5-7 and 30% pH 3-10. A 12.5% acrylamide gel was used for the second dimension.

For western blotting, proteins were transferred from gels to nitrocellulose followed by staining of the molecular weight standards with Ponceau S or amido black solution. The stain was removed after trimming the nitrocellulose and cutting individual strips as desired. The nitrocellulose was blocked with 3% BSA in PBS for at least 2 hours (and sometimes overnight) to minimize nonspecific binding of antibodies. Primary antibodies were diluted in 1% BSA/PBS and layered on top of the nitrocellulose for 30-60 minutes. Alkaline phosphatase-conjugated goat anti-antibodies were employed for colorimetric detection of the primary antigen. Both primary and secondary antibody incubations were washed extensively with 0.05% Tween in PBS.

2.6. Blue Native Gels

Blue native gels were made following the buffer and gel recipes of Schägger and von Jagow (Schägger and von Jagow, 1991; Schägger *et al.*, 1994). Briefly, a 5-10% acrylamide gradient was poured on top of a (pre-polymerized) bottom layer of 15% acrylamide. The bottom layer represented about 20% of the total migration path thru the gel. The uppermost stack region of the gel was made up of 5% acrylamide and was added before the gradient polymerized. All of the gel solutions were made in 150 mM Bistris buffer, pH 7.0. Typically, proteins were electrophoresed in these gels overnight at 300-500 volts (in the cold room). The cathode (top) buffer contained 50 mM Tricine, 15 mM Bistris, and 0.002% Coomassie blue G-250, pH 7.0. The anode buffer contained 50 mM Bistris, also at pH 7.0. In one experiment, the cathode buffer was replaced after 3 hours with the same buffer containing no Coomassie such that most of the dye had exited the gel prior to transfer of the protein to nitrocellulose.

2.7. Sizing Chromatography

Sizing chromatography was performed in a 1.0 cm ID x 20 cm column packed with Sephacryl S-400 which was equilibrated in buffer A at 5 °C (set up in a cold room). Samples to be analyzed were added directly to the top of the column bed (without glycerol) and allowed to enter the resin before topping the bed with buffer A and connecting the buffer reservoir. The speed of elution was controlled by the elevation of the running buffer. In general, the best protein separations were obtained when the flow rate was maintained near a drop per second which resulted in chromatography runs lasting 3-6 hours. The excluded volume of the column was measured by the elution of phage lambda DNA followed by ethidium bromide staining. The included volume of each run was checked for reproducibility by adding a trace amount of the blue dye xylene cyanole FF to each protein sample.

2.8. Velocity Sedimentation

Sedimentation profiles were achieved by layering lysate samples on top of a 9 ml linear gradient of 10-40% sucrose in buffer A with a 1 ml bottom cushion of 60% sucrose in the same buffer (14 x 89 mm ultra-clear centrifuge tubes, Beckman). The gradient was prepared using a two-chamber gradient maker (Hoefer Scientific). Centrifugation was carried out typically in a SW-41 rotor (Beckman) for 24 hours at 39,000 rpm and 4 °C. Fractions were collected from the bottom of each tube using a 200 µl glass pipet connected to 1/16 inch ID tubing. Fractions containing the 20 S proteasome were determined by immunoprecipitation using a polyclonal antibody against one of the proteasome subunits. Other

sedimentation markers were visualized by Coomassie stain following SDS-PAGE as shown in **Fig. 1**.

For isolation of polysomes, cells were treated with 0.1 mg/ml of cycloheximide prior to hypotonic lysis. Nuclei and intact organelles were removed by centrifugation at 14,000 rpm for 15 minutes in a JA-20 rotor (Beckman). The supernatant was layered on top of 5 ml of 20% sucrose in buffer A + cycloheximide with a 2 ml cushion of 70% sucrose in the same buffer. Polysomes were found near the interface of the two sucrose layers following a 2 hour centrifugation at 39,000 rpm. A typical pool of polysomes obtained in this manner is shown in **Fig. 2**, lanes 3, -/+ ATP. There was a clear reduction in the amount of actin and myosin which co-fractionated with the polysomes in the presence of ATP, as well as a reduction in some of the other less prominent proteins.

2.9. Crosslinking Experiments

Following labeling, HeLa cells were incubated with 0.1 mg/ml of cycloheximide for 2 minutes at 37 °C to stabilize polysomes. The cells were rinsed with PBS containing 0.1 mg/ml cycloheximide and incubated for 10 minutes at ambient temperature (20-22 °C) in the same solution supplemented with 2 mM DSP, a cell permeable crosslinker (Safiejko-Mroczka and Bell, 1996). After removal of the DSP solution, the reaction was quenched by incubating the cells in 50 mM glycine and 50 mM Tris, pH 7, for 10 minutes. The cells then were lysed in Laemmli sample buffer without any reducing agent and heated for 10 minutes at 75 °C. Immunoprecipitation under denaturing conditions was performed using antibodies against the different molecular chaperones as indicated. The final immunoprecipitates were boiled in Laemmli sample buffer containing extra

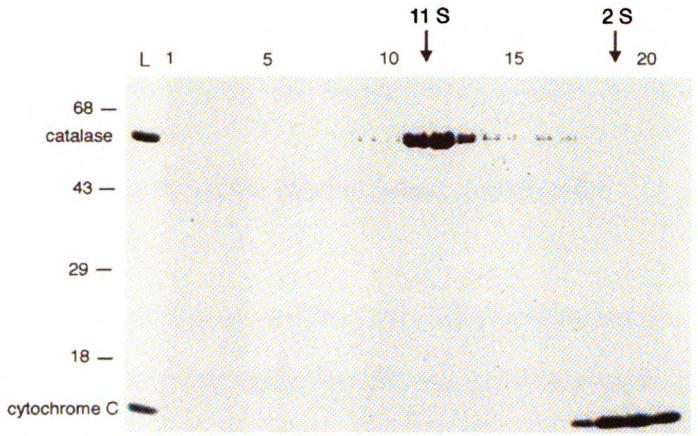

Figure 1 Typical Sedimentation Profile of Native Size Markers

Cytochrome C (0.2 mg) and catalase (0.4 mg) were mixed in a total sample volume of 0.4 ml and layered on top of a typical 10-40% sucrose gradient. After centrifugation for 20 hours at 39,000 rpm, an aliquot of each gradient fraction was analyzed by SDS-PAGE on a 12.5% acrylamide gel. Shown is the Coomassie-stained gel after drying in cellophane. Lane designations at top of figure correspond to gradient fractions from the bottom (1) to the top (20). L = original sample applied to top of gradient.

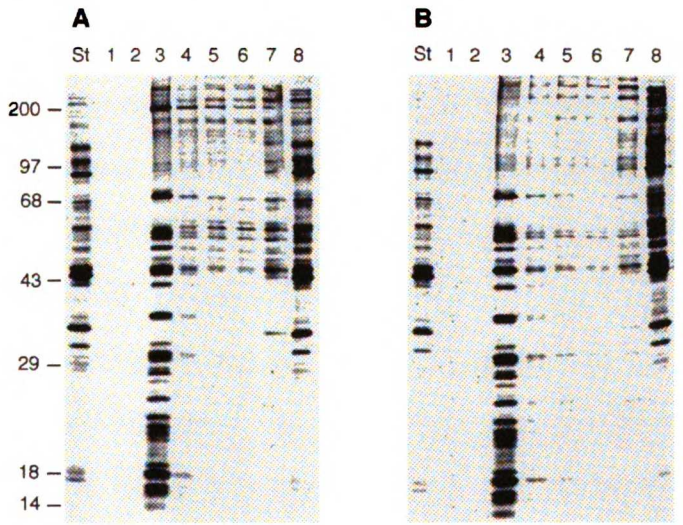

Figure 2 Isolation of Polysomes (-/+ ATP)

Steady-state labeled cells were lysed hypotonically, adjusted to buffer A salt concentrations, and split into two samples. One sample was depleted of ATP with apyrase, and the other was supplemented with 10 mM MgATP. Both lysates were sedimented for 2 hours at 39,000 rpm over a 5 ml layer of 20% sucrose followed by a step change to 3 ml of 70% sucrose (bottom layer). (**A**) Sucrose fractions from the -ATP lysate (lane 1 corresponds to bottom fraction). (**B**) Sucrose fractions from the +ATP lysate. St = starting lysate before sedimentation. Only 10% of the sample volume was loaded in lane 8 relative to the other lanes of each gel.

reducing agent (0.25 M DTT and 0.25 M β-mercaptoethanol) to reverse the crosslinks.

3. RESULTS & DISCUSSION

3.1. Characterization of Anti-hsp70 Monoclonal Antibodies

A number of different monoclonal antibodies were compared on the basis of their ability to precipitate the cytosolic hsp70s under three different lysis conditions (**Fig. 3**). Note that the highly stress-inducible hsp72 (also referred to as hsp70) and the constitutive hsp73 (also known as hsc70) are both present in unstressed HeLa cells. The lysates were either (A) supplemented with ATP, (B) treated with apyrase (-ATP), or (C) denatured by heating in the presence of SDS. The mouse monoclonal antibodies N6, N15, N21 and N33 appeared to precipitate hsp72 specifically, and worked well under all three lysis conditions. The rat monoclonal 1B5 appeared to be specific for hsp73 and worked best under native conditions following apyrase treatment (lane 10). The antibody mixture designated "B3" contained a pool of different antibodies which were able to precipitate both hsp72 and hsp73 under all conditions. A similar "cocktail" of antibodies was used in all subsequent experiments reported in this work where immunoprecipitation of hsp70 was desired (see Section 2.4). The monoclonal antibodies C96, N4, and N27 were not effective for precipitating hsp70 under any of the conditions shown in Fig. 3. However, it is possible that the particular aliquots of these antibodies that were used had been degraded or mishandled in some way. N27 is known to be an excellent reagent for immunoblot analysis of both hsp72 and hsp73, and, therefore, one would expect N27 to precipitate its antigen(s) under denaturing conditions.

One should note the small molecular weight protein of about 26 kD size which coprecipitates with the hsp70s under native conditions following apyrase

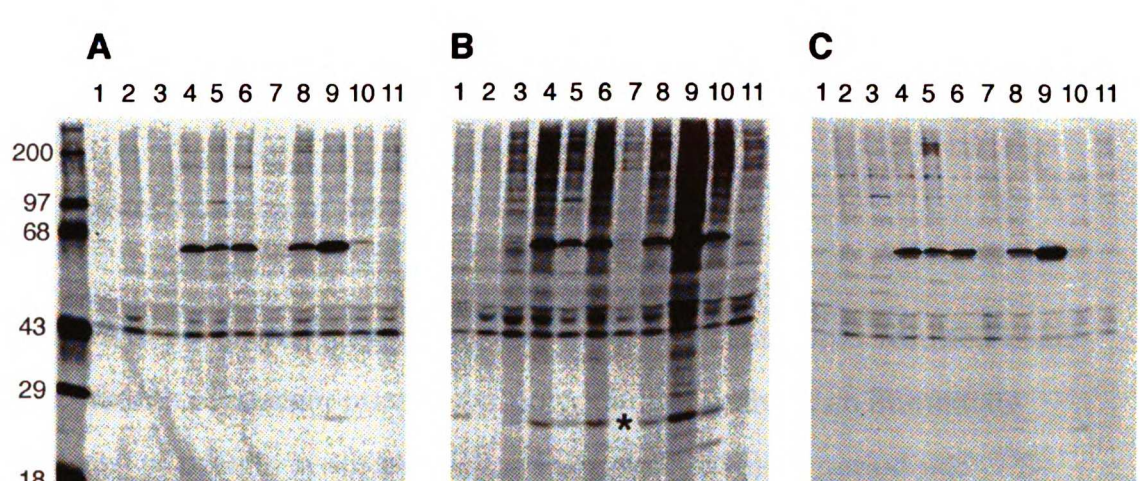

Figure 3 Characterization of Monoclonal Antibodies to Hsp72/Hsp73

Three plates of HeLa cells were labeled with ^{35}S-methionine for 30 minutes and lysed in disruption buffer containing (**A**) 5 mM ATP; (**B**) apyrase; or (**C**) Laemmli sample buffer. The lysate from plate C was boiled immediately. All three lysates were diluted 1:20 in RIPA buffer (-SDS), and 1-ml aliquots were used for immunoprecipitation with each of the following antibodies: (1) preimmune serum; (2) C96; (3) N4; (4) N6; (5) N15; (6) N21; (7) N27; (8) N33; (9) mixture "B3"; (10) 1B5; (11) no primary antibody. Primary incubations were for 90 minutes at 1:500 dilution. RAM secondary antibody was added to samples 2-9 & 11, and RAR was added to sample 10, all at 1:333 dilution for 45 minutes. Starting lysates looked identical for all three plates by autoradiography. Molecular weight standards are indicated at left of figure in kDaltons. (*) Indicates the position of a possible cofactor for hsp72 and hsp73.

treatment (see * in Fig. 3B). This unidentified protein may represent an important cofactor of the hsp70 family. Antibodies to hsp70 also are able to coprecipitate protein constituents of the ribosome which account for much of the "background" precipitation below the 29 kD size marker in Fig. 3B. The ability to coprecipitate ribosomes will be shown more clearly in a Section 3.12.

3.2. *Characterization of Anti-puromycin Antibody*

Addition of puromycin to growing cells at low concentrations resulted in an accumulation of nascent puromycyl-polypeptides without an immediate shutdown of protein synthesis. Following a short labeling period with ^{35}S-methionine in the presence of puromycin, the puromycyl-polypeptides could be examined by immunoprecipitation with the rabbit polyclonal antibody to puromycin. When analyzed by SDS-PAGE, the puromycin-released polypeptides appeared as a "smear" of radiolabeled proteins migrating throughout the gel (**Fig. 4A**). This heterogeneous appearance is what should be expected for a population of polypeptides which were derived from all of the active mRNAs in the cell by puromycin incorporation at random points in translation. Regardless of the brevity of the labeling period, a number of discrete bands always was observed against this background "smear" of puromycyl-polypeptides in the total cell lysates. These discrete bands represent full-length translation products that were terminated normally in the cells without incorporation of the antibiotic (lanes 1-2). Consistent with this interpretation is the fact that few, if any, full-length radiolabeled proteins were immunoprecipitated using the anti-puromycin antibody (lanes 3-9). Production of puromycyl-polypeptides showed little dependence on incubation times beyond 15 minutes when using a concentration of 2 μM puromycin (lanes 4-5), but a strong dependence on puromycin

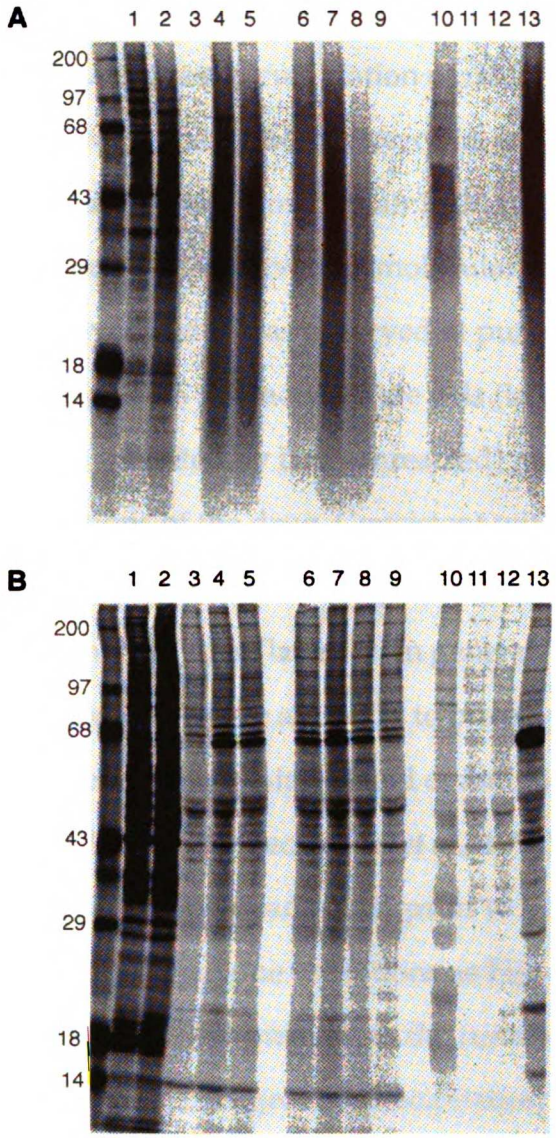

Figure 4 Characterization of the Polyclonal Antibody to Puromycin

HeLa cells were **(A)** pulse-labeled for 20 minutes or **(B)** steady-state labeled and incubated with puromycin as described below prior to cell lysis in the presence of apyrase (-ATP). Lanes 1-2 are starting lysates before immunoprecipitation; lanes 3-9,11 & 12 are immunoprecipitations with the puromycin antibody under nondenaturing conditions. (1) total lysate, no puromycin; (2) total lysate, 2 μM puromycin for 30 min; (3) no puromycin treatment; (4) 2 μM puromycin for 15 min; (5) 2 μM puromycin for 30 min; (6) 0.4 μM puromycin for 30 min; (7) 2 μM puromycin for 30 min; (8) 10 μM puromycin for 30 min; (9) 50 μM puromycin for 30 min; (10) immunoprecipitation under denaturing conditions, 2 μM puromycin for 30 min; (11) blocked antibody control, 100 μM puromycin added to diluted lysate prior to antibody; (12) control, preimmune serum; (13) coprecipitation with antibodies to hsp72/hsp73 under nondenaturing conditions using 5-fold more volume of the same lysate used in lane 7.

concentration for a given incubation time (lanes 6-9). In general, 2 μM puromycin for 15-30 minutes yielded the highest accumulation of released nascent polypeptides which exhibited a broad distribution of molecular sizes (lane 7). Concentrations of puromycin higher or lower than 2 μM resulted in nascent polypeptides of either smaller or larger average molecular mass, respectively. No accumulation of nascent polypeptides was observed at puromycin concentrations above 50 μM, as analyzed by 12.5% polyacrylamide gels (lane 9). It is possible that immunoprecipitation is inhibited by free (unreacted) puromycin in lysates treated with high concentrations of the drug. Consistent with this interpretation, prior incubation of the immune serum with free puromycin effectively blocked the capture of puromycyl-polypeptides (lane 11). In general, puromycin-released nascent chains that were captured by the antibody to puromycin were indistinguishable from those captured using a pool of antibodies to the cytosolic chaperones hsp72 and hsp73 (lane 13) (Beckmann *et al.*, 1990).

When the cells were labeled to steady-state prior to puromycin treatment and immunoprecipitation under the same conditions as Fig. 4A, a number of discrete bands was observed to coprecipitate with the (unlabeled) puromycyl-polypeptides (**Fig. 4B**). Relative to background precipitation from the lysate that was never treated with puromycin, the major coprecipitating proteins seem to be near the 68 kD size marker (compare lanes 3 and 4 of Fig. 4B). These proteins were not coprecipitated under denaturing conditions (lane 10) and were specifically bound to puromycyl-polypeptides since they were not precipitated by the antibody when it was blocked with free puromycin (lane 11). The identity of these proteins will be determined by 2-D gels in Section 3.8.

3.3. Analysis of Nascent Chain Complexes by Gel Filtration

The antibody to puromycin was used to characterize some of the physical properties of nascent polypeptides and their complexes with chaperones. It was first thought that the native size of the complex might be dominated by a defined, stoichiometric group of molecular chaperones, independent of the length of the nascent chain. With this in mind, a pulse-labeled puromycin-treated lysate was fractionated by sizing chromatography to isolate the putative "folding machine" (**Fig. 5A**). When the individual fractions were analyzed for the presence of puromycyl-polypeptides by immunoprecipitation, a wide size distribution of nascent polypeptides was observed (**Fig. 5B**). In general, the native size of a polypeptide by sizing chromatography was much larger than its molecular mass by SDS-PAGE. This indicated that many of the puromycyl-polypeptides were bound by other proteins, some of which may include molecular chaperones. Notice in Fig. 5A that some radiolabeled protein was eluted immediately following the void volume of the column. These proteins probably represent nascent polypeptides still bound to the ribosome (*i.e.* not released by puromycin). Consistent with this hypothesis, no radiolabeled protein was observed in these fractions after immunoprecipitation with the anti-puromycin antibody (Fig. 5B).

By fractionating a steady-state labeled lysate on the same column, it was possible to detect the mature proteins which were bound to the puromycyl-polypeptides after sizing chromatography (**Fig. 6**). The lysate fractions (Fig. 6A) were analyzed by immunoprecipitation with the pool of antibodies against hsp70 (Fig. 6B) and with the polyclonal antibody to puromycin (Fig. 6C). The resulting immunoprecipitation profiles were similar, but not identical, suggesting that hsp70 is one of the major proteins bound to puromycyl-polypeptides.

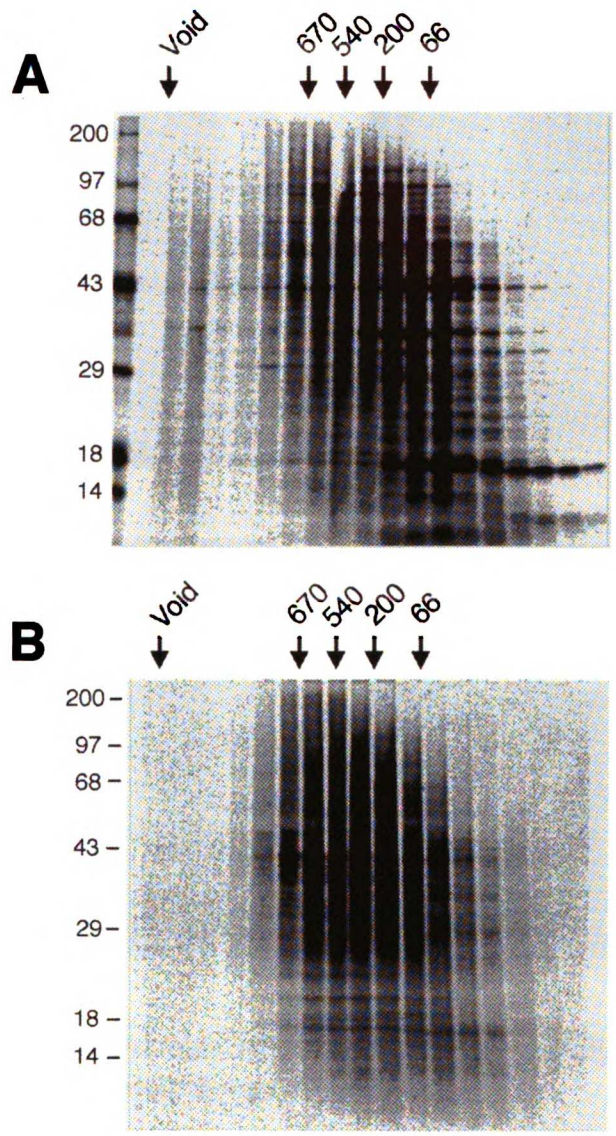

Figure 5 Distribution of Puromycyl-polypeptides following Sizing Chromatography

(**A**) Chromatography profile of an ATP-depleted lysate which had been pulse-labeled in the simultaneous presence of 2 μM puromycin for 20 minutes. Elution off the S-400 column is from left to right. Native size markers are thyroglobulin, 670 kD; urease, 540 kD; β-amylase, 200 kD; and hemoglobin, 66 kD. (**B**) Immunoprecipitation analysis of the fractions in (A) using the puromycin antibody under denaturing conditions.

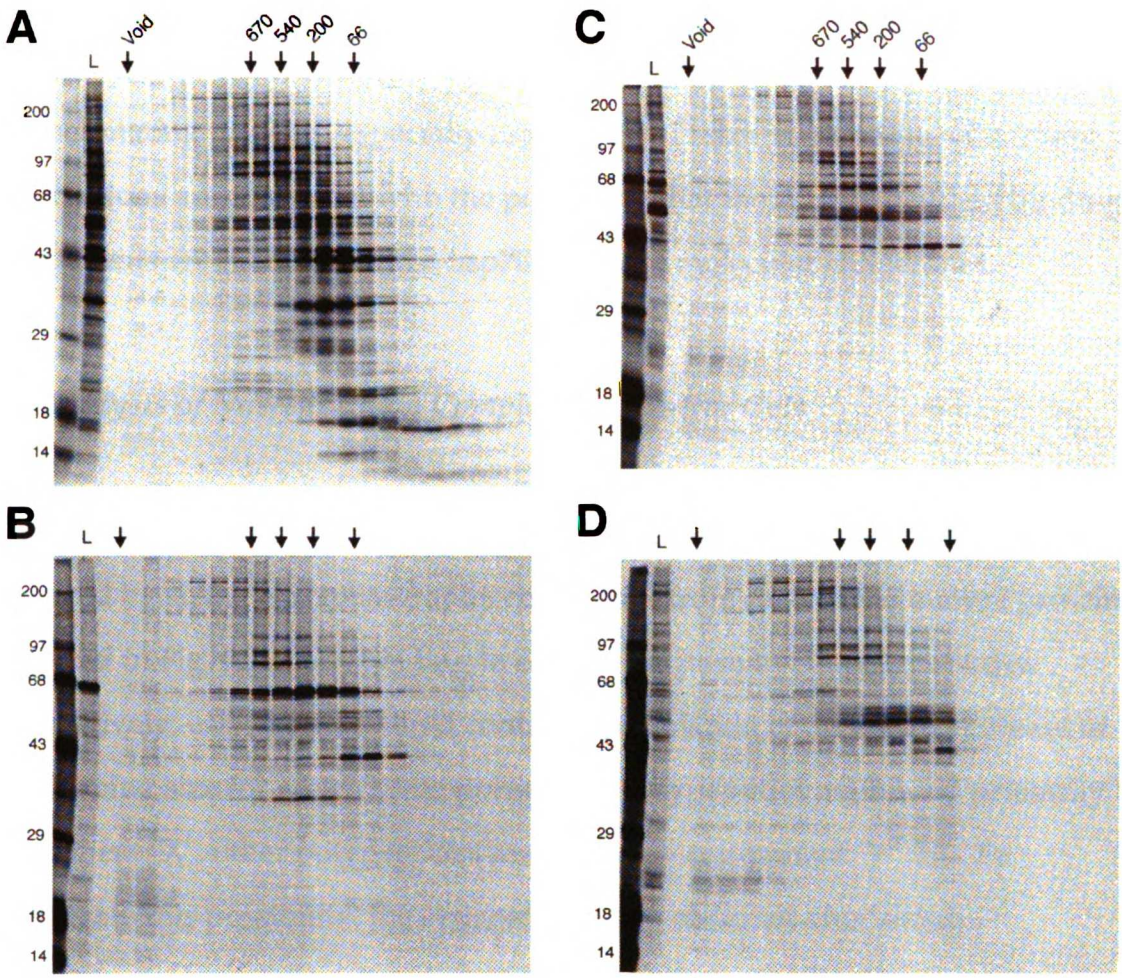

Figure 6 Detection Of Proteins Bound To Puromycyl-Polypeptides After Sizing Chromatography (-/+ ATP)

Steady-state labeled puromycin-treated lysates were eluted from an S-400 column under the same conditions used in Fig.5. (**A**) Fractions following chromatography of an ATP-depleted lysate. (**B**) Immunoprecipitation of the corresponding fractions in (A) with antibodies to hsp72/hsp73 under nondenaturing conditions. (**C**) Immunoprecipitation of the corresponding fractions in (A) with the antibody to puromycin under nondenaturing conditions. (**D**) Immunoprecipitation with the antibody to puromycin using fractions obtained from a different lysate which was prepared in 5 mM MgATP and eluted in the presence of 10 mM MgATP. L = lysate loaded to column (or corresponding immunoprecipitation).

When cells were steady-state labeled and lysed in the presence of ATP (instead of apyrase) before fractionation by sizing chromatography, immunoprecipitation of the puromycyl-polypeptides resulted in fewer coprecipitating proteins, especially near the 68 kD size marker (Fig. 6D). These observations are consistent with the possibility that the ATP-dependent binding components are members of the hsp70 family of molecular chaperones.

3.4. Analysis of Nascent Chain Complexes by Native Gels

The relatively large native mass of the puromycyl-polypeptide complexes observed by sizing chromatography could arise from each of the mature proteins (detected in Fig. 6C & 6D) binding to each of the puromycyl-polypeptides. Alternatively, each fraction could contain a population of defined complexes of overlapping size for which some puromycyl-polypeptides are bound primarily to component "X", other polypeptides are bound by component "Y", etc. To investigate this possibility, the original fractions obtained after sizing chromatography were analyzed using blue native electrophoresis instead of (denaturing) SDS-PAGE (**Fig. 7**). Blue native gels employ Coomassie dyes to introduce a charge shift on proteins prior to electrophoresis at neutral pH (Schägger and von Jagow, 1991; Schägger *et al.*, 1994).

As shown in Fig. 7A, only a few discrete bands were observed when the fractions from a steady-state labeled lysate were analyzed by blue native electrophoresis. A large portion of the labeled protein remained near the top of the gel. Nevertheless, at least three different complexes could be discerned. The largest (and most prominent) complex migrated near the 670 kD native gel marker. Thus, the size of the complex by native electrophoresis corresponded nicely with the size determined by chromatography (indicated at the top of the

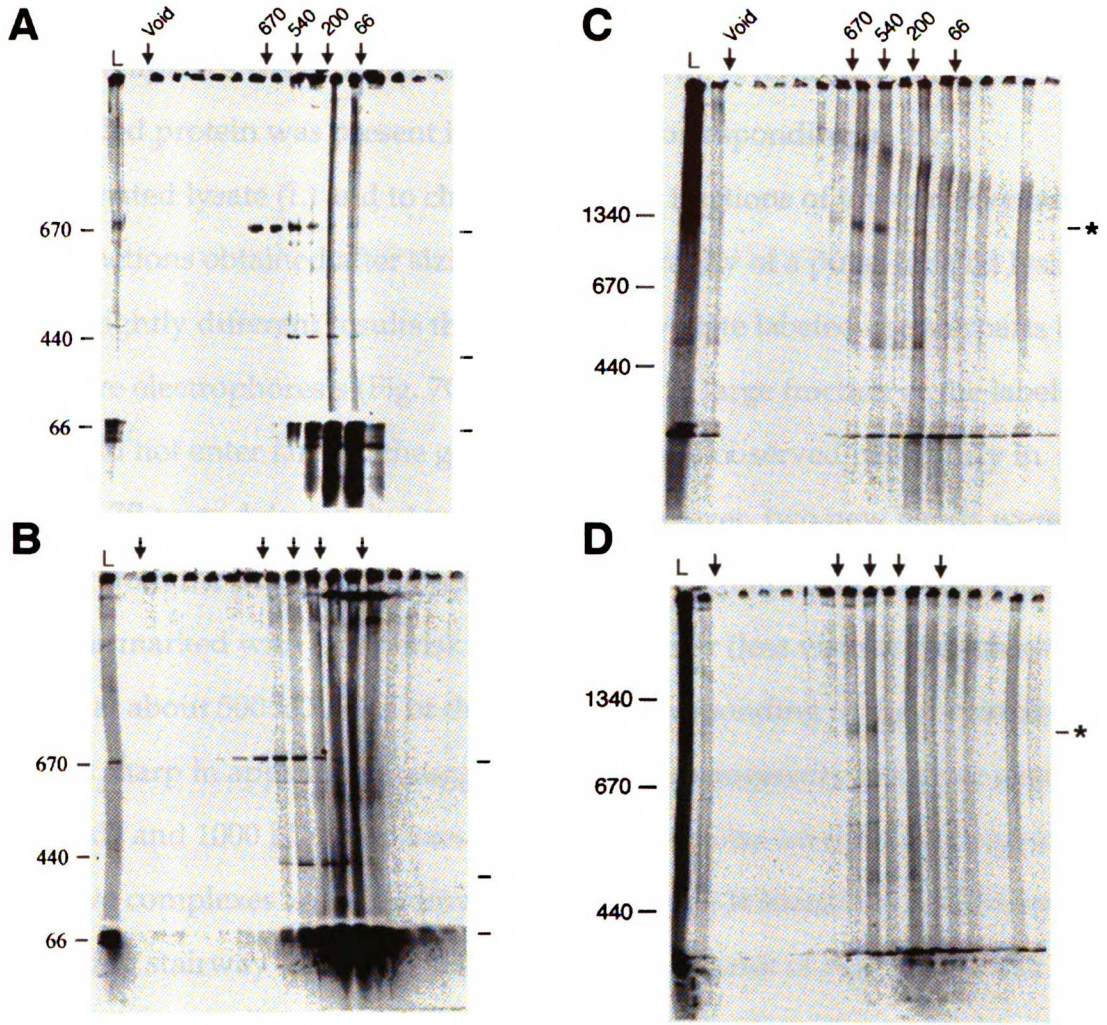

Figure 7 Native Gel Electrophoresis of Fractions from Sizing Chromatography (-/+ ATP)

Four different lysates were fractionated by sizing chromatography and a portion of each fraction was analyzed on a blue native gel. (**A**) Fractions from a steady-state labeled lysate, -ATP (same fractions as shown in Fig. 6A). (**B**) Steady-state labeled lysate, +ATP. (**C**) Pulse-labeled lysate, -ATP. (**D**) Pulse-labeled lysate, +ATP. Native gel markers on the y-axis are BSA (66 kD), ferritin (440 kD), thyroglobulin (670 kD), and a thyroglobulin dimer complex (1340 kD). The band denoted by an asterisk (*) in (C) and (D) does not correspond in size with any of the bands from the steady-state labeled fractions in (A) and (B).

gel). Complexes of approximately 500 kD and 600 kD also were detected. None of these complexes were disrupted by ATP (Fig. 7B). A noticeable smear of radiolabeled protein was present in those lanes corresponding to the unfractionated lysate (L) and to chromatography fractions of lower native mass.

Fractions obtained after sizing chromatography of a pulse-labeled lysate yielded slightly different results than their steady-state labeled counterparts by blue native electrophoresis (Fig. 7C & 7D). Again, a large fraction of the labeled protein did not enter far into the gel. The complexes observed previously in Fig. 7A & 7B were detected, but only weakly. However, two new bands were apparent from the pulse-labeled fractions. One new complex of approximately 1000 kD is marked with an asterisk in Fig. 7. Another (less visible) complex was detected at about 500 kD. Both of the "bands" corresponding to these complexes were less sharp in appearance, suggesting some heterogeneity in size or charge. The 500 kD and 1000 kD complexes observed after pulse-labeling may represent true native complexes with puromycyl-polypeptides. It should be noted that the "descending stairway" of counts visible in the top quarter of Fig. 7C was not reproducible and is likely an artifact due to a crease in the native gel during the drying process.

In order to try and identify some of the protein components of these native complexes, whole lysate samples were subjected to blue native electrophoresis followed by western blot analysis. This procedure was complicated by the need to remove the Coomassie dye from the gel before transferring the proteins to nitrocellulose (see Section 2.6). As shown in **Fig. 8**, the end result was not very informative. Still, there was an indication that the 1000 kD complex detected in Fig. 7 might contain a subunit of the cytosolic chaperonin, CCT (see * in Fig. 8). Overall, native gel analysis was found to be unsuitable for characterizing the heterogenous population of nascent polypeptides from HeLa cells.

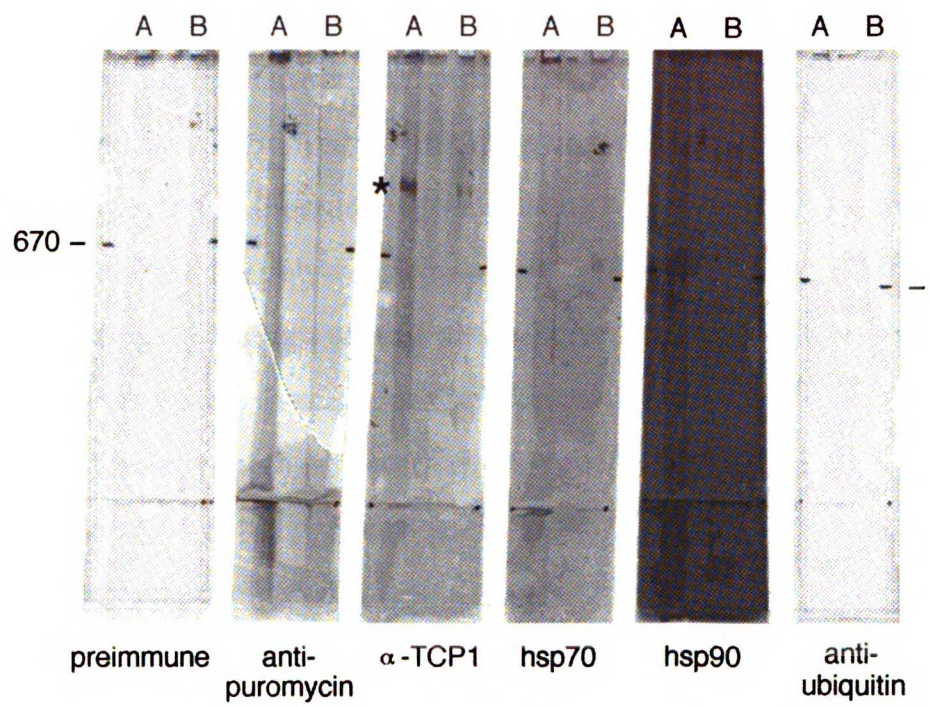

Figure 8 Western Blot Analysis of Lysates following Native Gel Separation

Two differently labeled lysates (A & B) were loaded in 6 pairs of lanes prior to electrophoresis on a blue native gel. The protein complexes were transferred to nitrocellulose and immunoblotted with antibodies which recognize the proteins indicated under each blot in the figure. The one (possible) positive result is denoted by an asterisk (*) in the strip blotted for TCP-1.

3.5. Analysis of Nascent Chain Complexes by Velocity Sedimentation

As yet another approach for analyzing some of the physical properties of nascent polypeptides and puromycin-released polypeptides, radiolabeled HeLa lysates were fractionated by velocity sedimentation using sucrose gradients. In the first experiment employing sedimentation, the starting lysate was not treated with puromycin, but rather the polysome complexes were stabilized by addition of cycloheximide (**Fig. 9**). Using a steady-state labeled lysate, protein constituents of the ribosome were visible throughout the lower two-thirds of the sucrose gradient after a (relatively short) centrifugation of 90 minutes at 39,000 rpm. Although the location of the polysomes is not discernible in the film exposure of Fig. 9A, ribosomal proteins can be seen in the monosome peak (lanes 13-14, Fig. 9A). When a pulse-labeled lysate was analyzed in the same manner, the polysome-containing fractions were revealed (lanes 1-8, Fig. 9B). Notice how the larger polysomes (bottom fractions) are synthesizing the larger nascent polypeptides (by SDS-PAGE). This is consistent with the idea that the number of ribosomes bound to a mRNA is limited by the length of the coding region for that message.

A much longer sedimentation time was required to develop a reasonable profile of puromycin-released polypeptides. As shown in **Fig. 10A**, most of the nascent or newly-synthesized proteins from a pulse-labeled puromycin-treated lysate remained in the top half of a sucrose gradient after 25 hours at 39,000 rpm. When these same fractions were immunoprecipitated with the antibody to puromycin, the majority of the puromycyl-polypeptides were found between the 2 S and 11 S sedimentation markers (**Fig. 10B**). A trend for larger polypeptides (by SDS-PAGE) to sediment at larger S-values was evident.

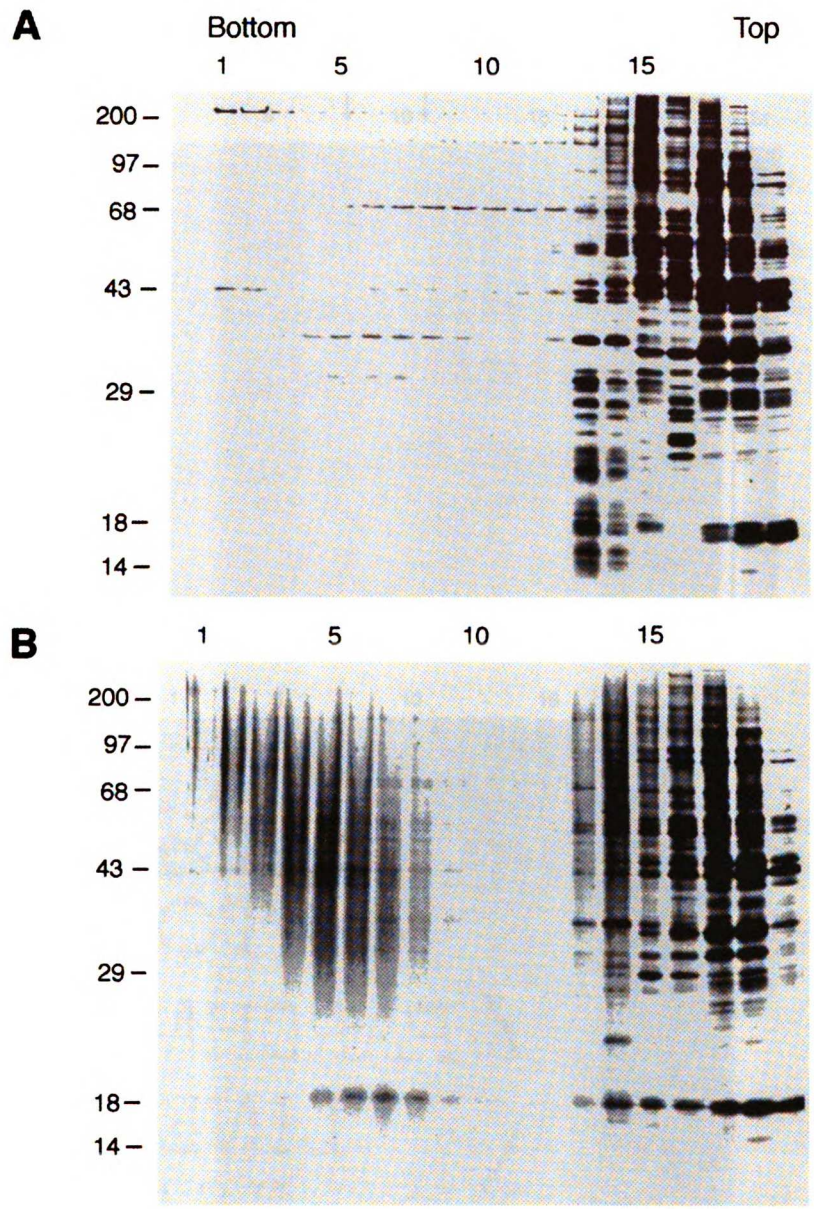

Figure 9 Profiles of Polysomes and Their Nascent Polypeptides following Sedimentation in a Sucrose Gradient

Two differently labeled lysates were fractionated by velocity sedimentation at 39,000 rpm for 90 minutes on a 10-40% sucrose gradient. Following centrifugation, the samples were removed from the bottom of each tube in approximately 0.5 ml fractions. A portion of each fraction was analyzed by SDS-PAGE on a 12.5% acrylamide gel as shown. (**A**) Steady-state labeled lysate. (**B**) Pulse-labeled lysate.

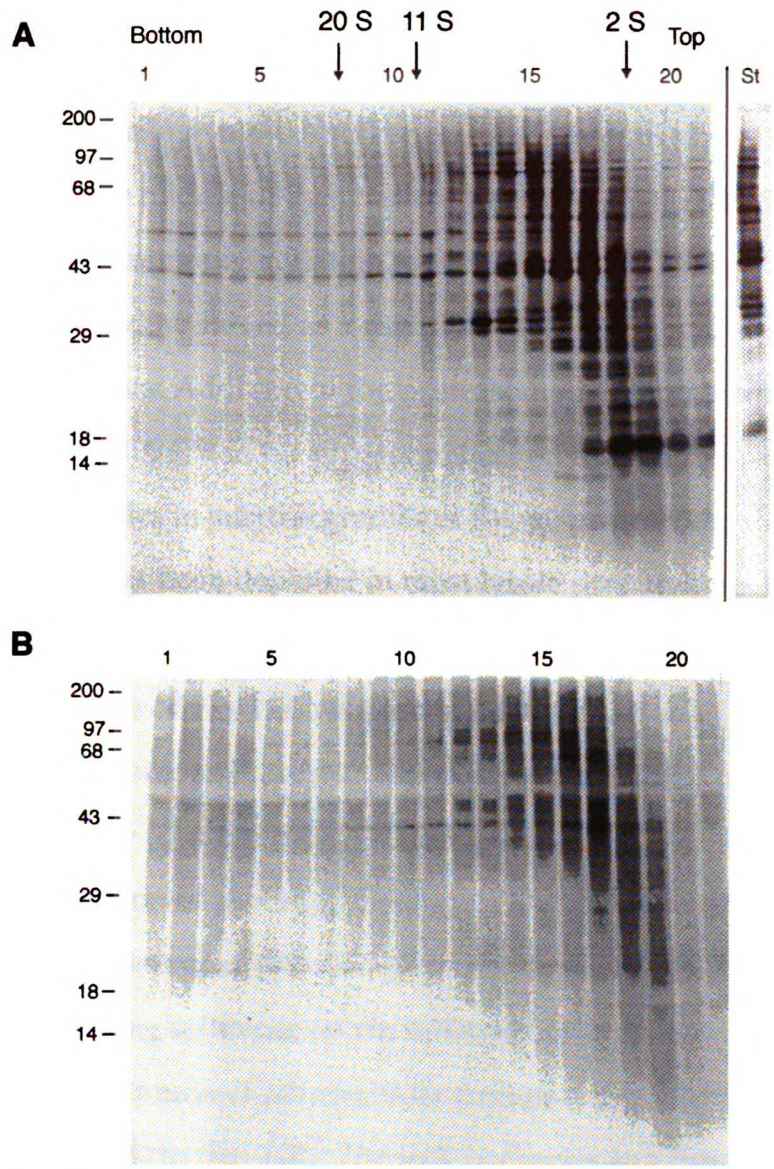

Figure 10 Sedimentation Profile of Nascent Polypeptides after Release from the Ribosome with Puromycin

(**A**) A pulse-labeled puromycin-treated lysate was sedimented through a 10-40% sucrose gradient for 25 hours at 39,000 rpm. The fraction numbers and sedimentation markers are shown at the top of the figure. St = starting lysate. (**B**) Immunoprecipitation of each of the corresponding fractions in (A) with the antibody to puromycin under denaturing conditions. An equal volume of each fraction was heated to 95°C in the presence of 0.5% SDS prior to dilution and immunoprecipitation.

A steady-state labeled puromycin-treated lysate was analyzed under similar sedimentation conditions as its pulse-labeled counterpart (**Fig. 11**). In this case, the ribosomal proteins were not seen because they had formed a pellet in the bottom of the centrifuge tube. Another curious subset of proteins, however, was observed near the middle of the gradient (lanes 8-9, Fig. 11A). These proteins were identified by immunoprecipitation as members of the 20 S proteasome. The location of the proteasome served as a useful sedimentation marker in subsequent experiments. Addition of the proteasome inhibitor MG132 (similar to MG115 in Rock *et al.*, 1994) had no apparent effect on the sedimentation of puromycyl-polypeptides in sucrose gradients. (Note: proteasome degradation requires ATP which has been depleted in most lysate preparations.) Immunoprecipitation of the (unlabeled) puromycyl-polypeptides in the sedimentation fractions of Fig. 11A resulted in coprecipitation of many proteins of the same molecular size as those proteins observed to coprecipitate following sizing chromatography (compare Fig. 11B to Fig. 6C).

The sedimentation of puromycyl-polypeptides also was examined in the presence of nonionic detergent and ATP (**Fig. 12**). Neither 0.1% Triton X-100 nor 10 mM ATP had a strong influence on the sedimentation profile of newly-synthesized and/or puromycyl-polypeptides (compare Fig. 12A & 12B to Fig. 10A, Fig. 12C & 12D to Fig. 10B). The lack in change as a function of ATP indicates that the sedimentation of puromycyl-polypeptides is not influenced by the binding of (ATP-dependent) molecular chaperones (Fig. 12D).

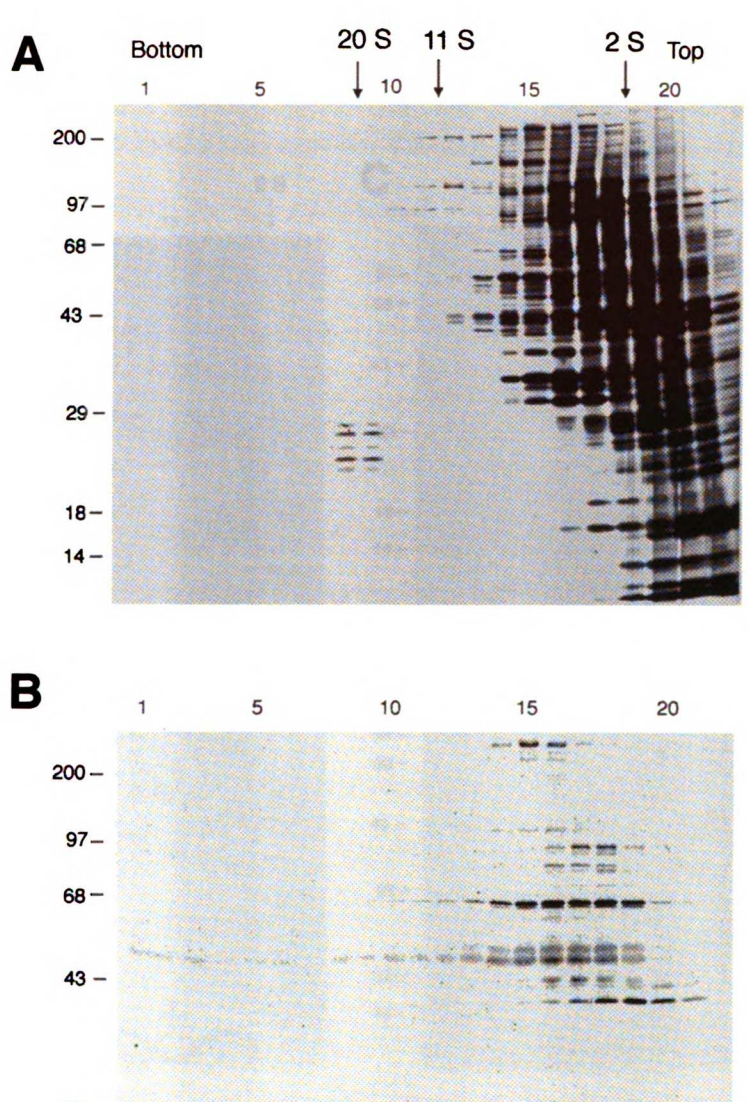

Figure 11 Detection of Proteins Bound to Puromycyl-polypeptides after Velocity Sedimentation

A sedimentation analysis similar to Fig.10, except that the starting lysate was obtained from cells which were steady-state labeled prior to puromycin treatment. (**A**) Fractionation of lysate after 20 hours at 39,000 rpm through a 10-40% sucrose gradient. (**B**) Immunoprecipitation with the puromycin antibody under nondenaturing conditions for each of the corresponding fractions in (A).

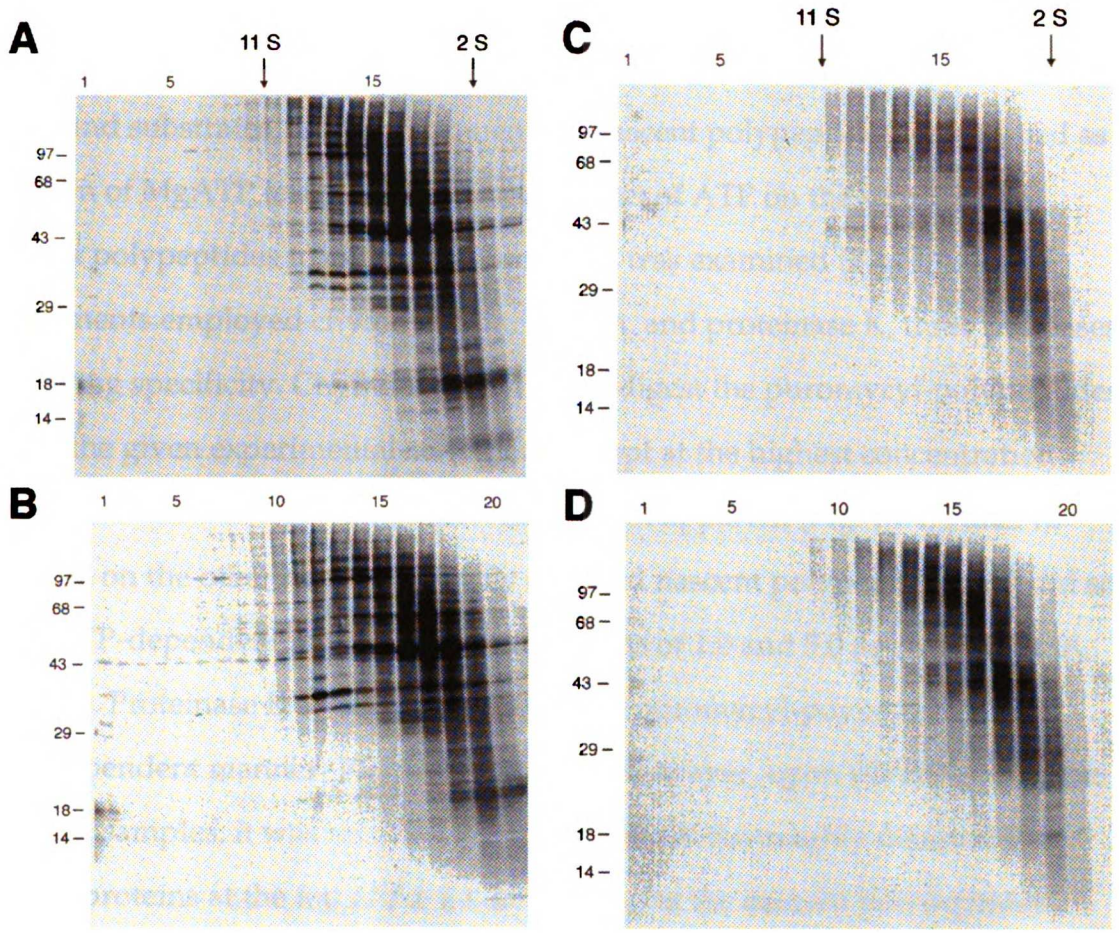

Figure 12 Effects of Triton and ATP on the Distribution of Puromycyl-polypeptides in Sedimentation Gradients

Two different pulse-labeled puromycin-treated lysates were fractionated in a 10-40% sucrose gradient by sedimentation for 24 hours at 39,000 rpm. Unlike all previous sedimentation experiments, the gradient contained 0.1% Triton X-100 in both cases. (**A**) Fractionation of lysate depleted of ATP. (**B**) Fractionation of a lysate supplemented with 10 mM MgATP in both the lysis buffer and gradient. (**C**) Immunoprecipitation with the puromycin antibody under denaturing conditions for each of the corresponding fractions in (A), -ATP. (**D**) Immunoprecipitation with the puromycin antibody under denaturing conditions for each of the corresponding fractions in (B), +ATP.

3.6. Sensitivity of Nascent Polypeptides to Proteases in the Presence of MgATP

Since members of the hsp60 and hsp70 chaperone families are known to have a weak ATPase activity which modulates their conformation and affinity for bound substrates, the "environment" of nascent polypeptides was probed as a function of MgATP levels. To do this, the effect of ATP on the sensitivity of nascent polypeptides to exogenous proteases was examined (**Fig. 13**). Initial experiments employed chymotrypsin, trypsin, and proteinase K, three proteases of varying specificity. Chymotrypsin did not digest the puromycyl-polypeptides under the given experimental conditions except at the highest concentration employed where a dependence on ATP was not apparent (Fig. 13A, Panel I). Trypsin, on the other hand, effectively digested nascent polypeptides and did so in an ATP-dependent manner at concentrations of 1.0 and 5.0 μg/ml (Fig. 13A, Panel II). Proteinase K appeared also to digest puromycyl-poypeptides in an ATP-dependent manner (Fig. 13A, Panel III). However, upon silver-staining the digested samples, it was revealed that proteinase K thoroughly digested the mature proteins at the top of the gel in addition to the nascent polypeptides (Fig. 13B, Panel III). Proteolysis experiments are not diagnostic for chaperone-mediated protection of nascent polypeptides if the chaperones themselves also are degraded by the protease.

The trypsin experiment was repeated many times with reproducible results. The observed ATP-dependent digestion was not due to an unusual susceptibility by puromycyl-polypeptides since nascent polypeptides prepared from cells which were treated with cycloheximide instead of puromycin showed a similar pattern of digestion (**Fig. 14A**). Notice how the discrete bands representing full-length radiolabeled proteins were not degraded at any of the three trypsin concentrations employed, independent of ATP manipulation.

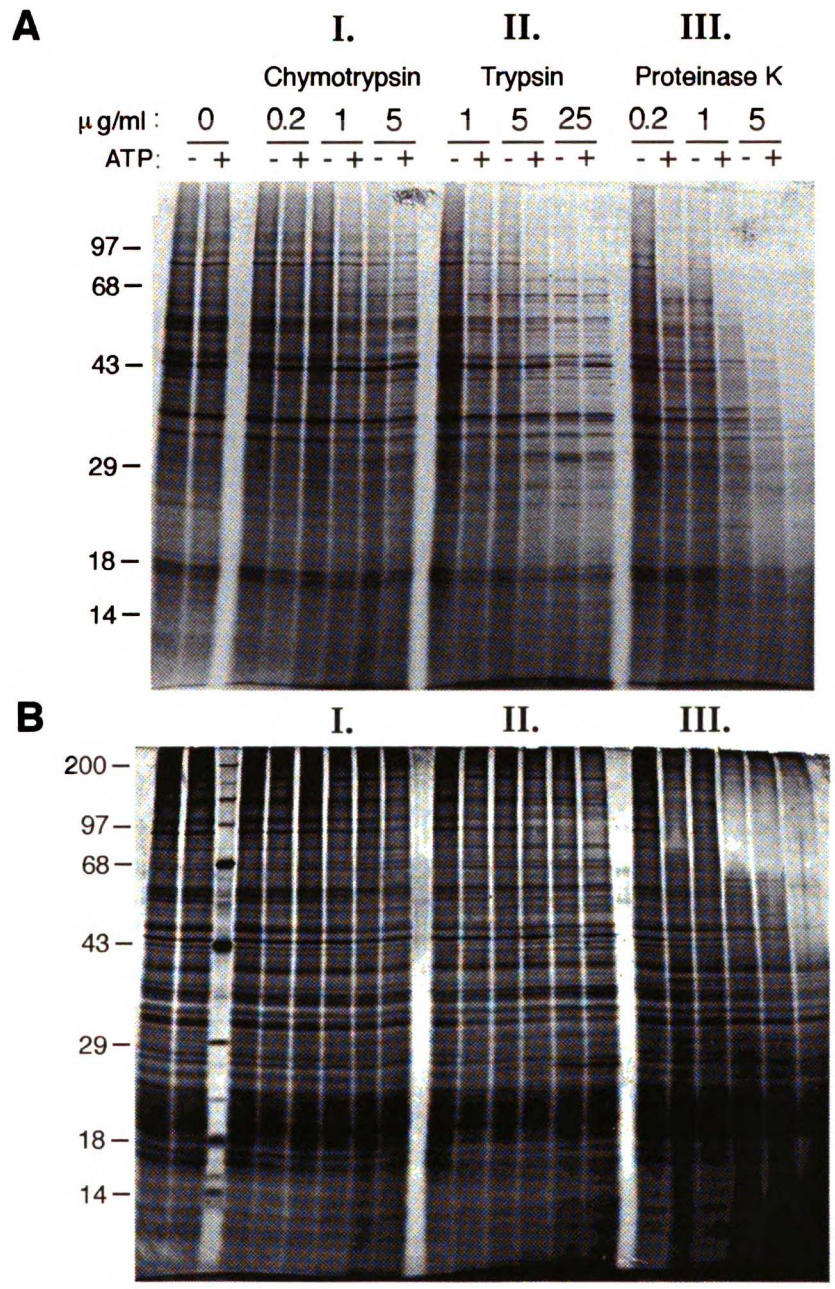

Figure 13 Digestion of Puromycyl-polypeptides in Whole Lysates by Three Different Proteases as a Function of ATP Levels

Two plates of HeLa cells were labeled with ^{35}S-methionine for the last 10 minutes of a 20 minute incubation with 2 µM puromycin. One plate was lysed in the presence of apyrase (-ATP), and the other plate was lysed with 5 mM MgATP in the buffer (+ATP). A stock solution of (**I.**) chymotrypsin, (**II.**) trypsin, or (**III.**) proteinase K was added at the indicated concentrations to 20 µl aliquots of lysate and incubated for 30 minutes at 22°C. Proteolysis was terminated by boiling the samples in 3 x Laemmli sample buffer and analyzed in duplicate on 12.5% acrylamide gels. (**A**) Autoradiograph of nascent and newly-synthesized proteins. (**B**) Silver-stained gel revealing all proteins.

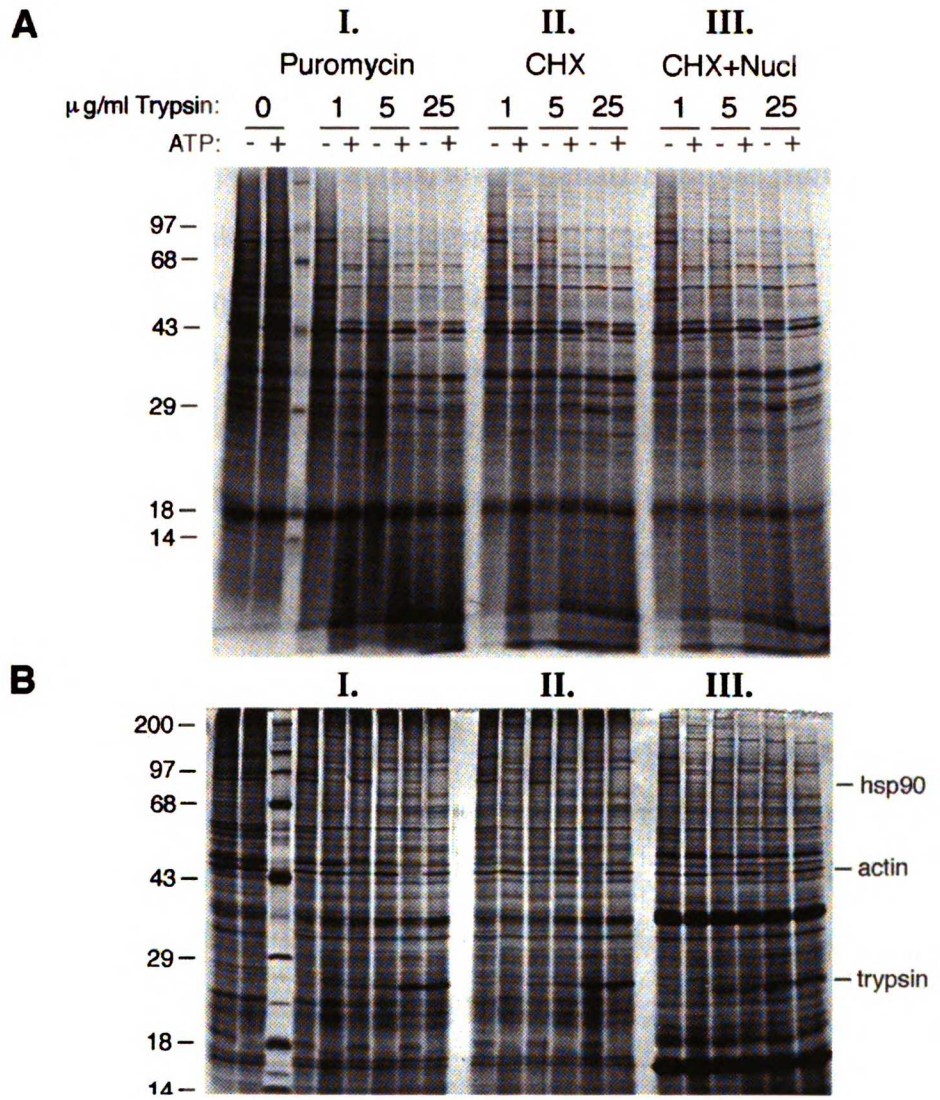

Figure 14 Trypsin Digestion of Nascent Polypeptides before and after Release from the Ribosome

Four plates of cells were pulse-labeled for 10 minutes. For Panel **I.**, two plates were treated with puromycin as in Fig. 13 and lysed in the presence of apyrase (-) or 5 mM MgATP (+). For Panels **II.** and **III**, the two remaining plates were incubated with 0.5 mM cycloheximide (CHX) during the last 5 minutes of the 10-minute labeling period and lysed in buffer containing CHX and apyrase (-) or ATP (+). All four lysates were divided into equal aliquots and incubated with trypsin at the indicated concentrations (30 minutes at 22°C) followed by inactivation of the protease by boiling as in Fig. 13. For panel **III**, the denatured trypsin digests were subsequently incubated with a mixture of nucleases (DNase & RNase) for 30 minutes at 22°C before analysis by SDS-PAGE. (**A**) Autoradiograph of nascent and newly-synthesized proteins. (**B**) Silver-stained gel revealing all proteins.

Instead, the primary target of the added protease was the "smear" of radioactive material which represents the nascent polypeptides. In the case of cycloheximide treatment, proteolysis was harder to distinguish by SDS-PAGE since this inhibitor of translation elongation does not allow the accumulation of truncated polypeptides as seen with puromycin (*i.e.*, lower signal). Samples from the cycloheximide-treated lysate also were digested with nucleases (after inactivation of trypsin) to see whether removal of the tRNA from peptidyl-tRNAs affected the digestion pattern by SDS-PAGE, but no change was observed (Fig. 14A, Panel III). Global degradation of mature proteins was not apparent in any of the trypsin lysates, although hsp90 appeared to be more susceptible to trypsin in the presence of ATP, and actin was more susceptible to trypsin in ATP-depleted lysates (**Fig. 14B**). ATP-sensitive proteolysis also has been reported for a single nascent polypeptide species that was translated from a truncated message *in vitro* (Hansen *et al.*, 1994). These results suggest that nascent chains may exist in a protective environment governed by the binding of one or more ATP-dependent chaperones. However, one can not rule out that part of the observed protease protection is due to nonspecific interactions with other proteins (*i.e.*, actin and tubulin) which also are characterized by nucleotide-dependent changes in conformation.

3.7. Pulse-chase Studies on the Fate of Puromycyl-polypeptides in Vivo

In the early stages of this work, there was concern that the use of puromycin would result in the production of nascent polypeptides which would become immediate targets of intracellular proteases. This might be expected since the majority of puromycyl-polypeptides are unlikely to fold into native structures. The half-life of puromycyl-polypeptides was examined by pulse-chase analysis

(**Fig. 15**). In these experiments, the cells were not lysed immediately after puromycin treatment, but rather, the puromycin and radiolabel were removed, and the cells were chased in normal growth medium. At designated time points, a plate of cells was harvested, and the amount of puromycyl-polypeptides in the lysate was determined by immunoprecipitation. As shown by SDS-PAGE in Fig. 15A, puromycyl-polypeptides persisted in cells on a time scale of hours to days. The decay was quantified by liquid scintillation of the immunoprecipitates (Fig. 15B and other data not shown). The half-life of puromycyl-polypeptides was estimated to be between 2-3 hours although some fraction of the puromycyl-polypeptides appeared to decay much slower and could still be detected after 48 hours of chase (data not shown).

Although ubiquitination of puromycyl-polypeptides was never demonstrated, the effect of chasing the cells in the presence of an inhibitor of the proteasome (MG132) was investigated. As shown in Fig. 15B, the proteasome inhibitor caused a substantial delay in the onset of proteolysis of puromycyl-polypeptides, although the subsequent decay rate was very similar to cells chased in the absence of inhibitor. When this experiment was repeated but the cells lysed in a different manner, it became evident that the decrease in decay (+MG132) corresponded with an increase in detergent-insoluble puromycyl-polypeptides (Fig. 15C). Thus, inhibition of the proteasome may lead to aggregation of those proteins that are normally turned over in the cell by proteolysis.

It is important to note that protein constituents of the proteasome were never observed to coprecipitate with puromycyl-polypeptides. For example, when a chase experiment was performed with steady-state labeled cells instead of pulse-labeled cells, the pattern of coprecipitating bands (mature proteins) was unchanged regardless of the chase time (data not shown). Perhaps, once a

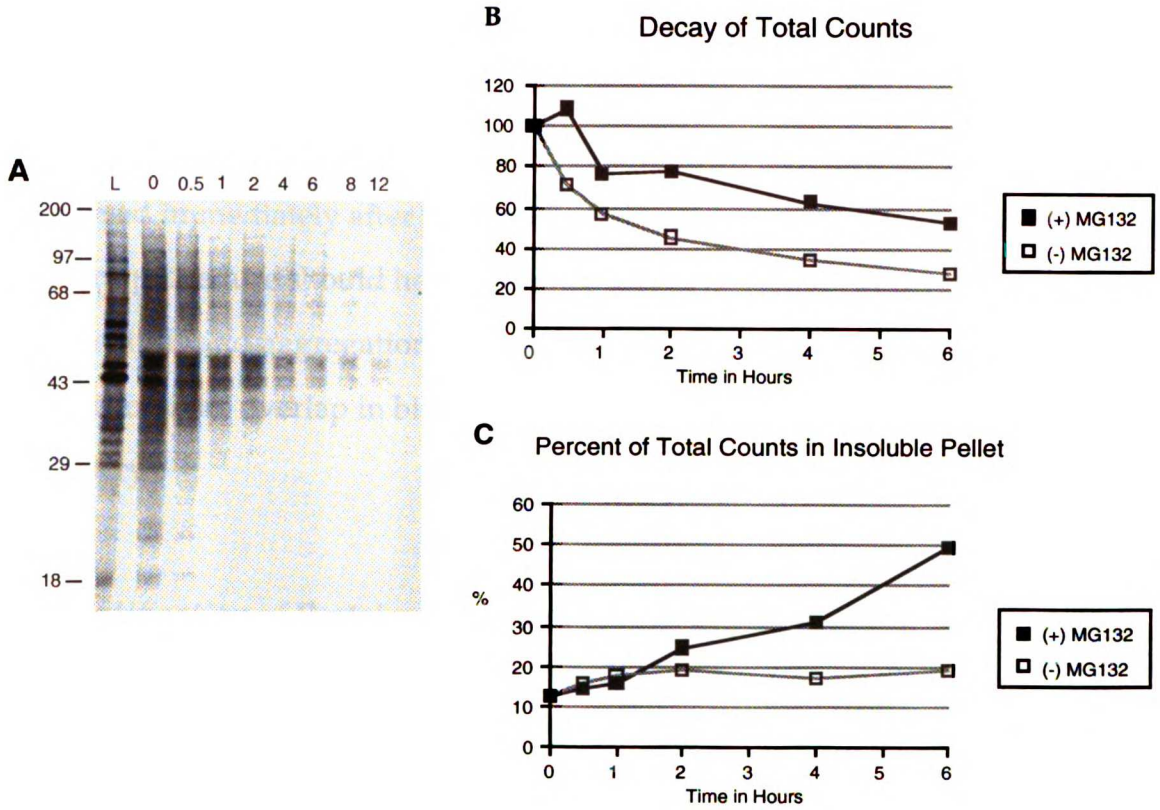

Figure 15 Degradation of Puromycyl-polypeptides *in Vivo* during Pulse-Chase Experiments (-/+ Proteasome Inhibitor)

(**A**) Eight plates of HeLa cells were metabolically labeled for the last 15 minutes of a 25-minute incubation with 2 µM puromycin (pulse). Next the medium was replaced with 10% calf serum in DMEM and chased in the absence of label or puromycin. At each of the times indicated at the top of the figure (in hours), one plate was lysed in Laemmli sample buffer. The resulting lysates were immunoprecipitated under denaturing conditions with the antibody to puromycin. Shown is the SDS-PAGE of the immunoprecipitations and a typical whole lysate (L). For the graphs in (**B**) and (**C**), eleven plates of cells were labeled for 20 minutes in the simultaneous presence of 2 µM puromycin. One plate was harvested immediately (T=0 hours). Five plates were chased in 10% calf serum/DMEM, and the remaining five plates were chased in the same medium supplemented with 50 µM MG132 to inhibit proteasome activity. All plates were lysed in the presence of 0.1% Triton X-100 and clarified in a microcentrifuge for 10 minutes. Both the supernatant and pellet fractions of each lysate were immuno-precipitated with the puromycin antibody under denaturing conditions and quantified by liquid scintillation. In graph (B), the total (immunoprecipitated) counts obtained from addition of the supernatant and pellet fractions are shown as a function of MG132 addition. The counts (y-axis) were standardized relative to the cells that received no chase period (T=0). In graph (C), the amount of counts in the Triton-insoluble pellet of each lysate is given as a percent of the total counts that were immunoprecipitated for that plate.

puromycyl-polypeptide becomes engaged with the proteolytic machinery, the C-terminal puromycin "tag" is efficiently removed, and, thus, the clipped polypeptide can not be precipitated with the puromycin antibody. All of the results reported in this work (outside of Fig. 15) were obtained from cells that were lysed immediately after puromycin treatment (*i.e.*, no chase period). This standard procedure should help minimize loss of puromycyl-polypeptides due to proteolysis and aggregation, as well as minimize confusion in interpretation due to (possible) overlap in binding of "folding machinery" and "proteolysis machinery".

3.8. Identification of Proteins Bound to Puromycyl-polypeptides by Two-dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was used to identify the proteins that coprecipitated with puromycyl-polypeptides (**Fig. 16**). A typical 2-D profile of an immunoprecipitate from a steady-state labeled lysate is shown in Fig. 16A along with a one-dimensional SDS-PAGE profile of the same sample. The individual proteins have been numbered for reference (#1-#11). The identity of most of the proteins could be verified by immunoprecipitation using an antibody specific to the suspected protein and comparing the position of the antigen on another 2-D gel (**Fig. 17**, for examples). The major chaperones that were identified as coprecipitating with puromycyl-polypeptides were members of the hsp70 family, especially the cytosolic members, hsp73 (#3) and hsp72 (#4). One hsp70 member known to reside within the endoplasmic reticulum, BiP (#1), and two residents of mitochondria, grp75 and hsp60 (#2 and #6), also were detected from cells lysed in the presence of detergent. The three compartmentalized chaperones were not coprecipitated when the cells were lysed hypotonically followed by removal of

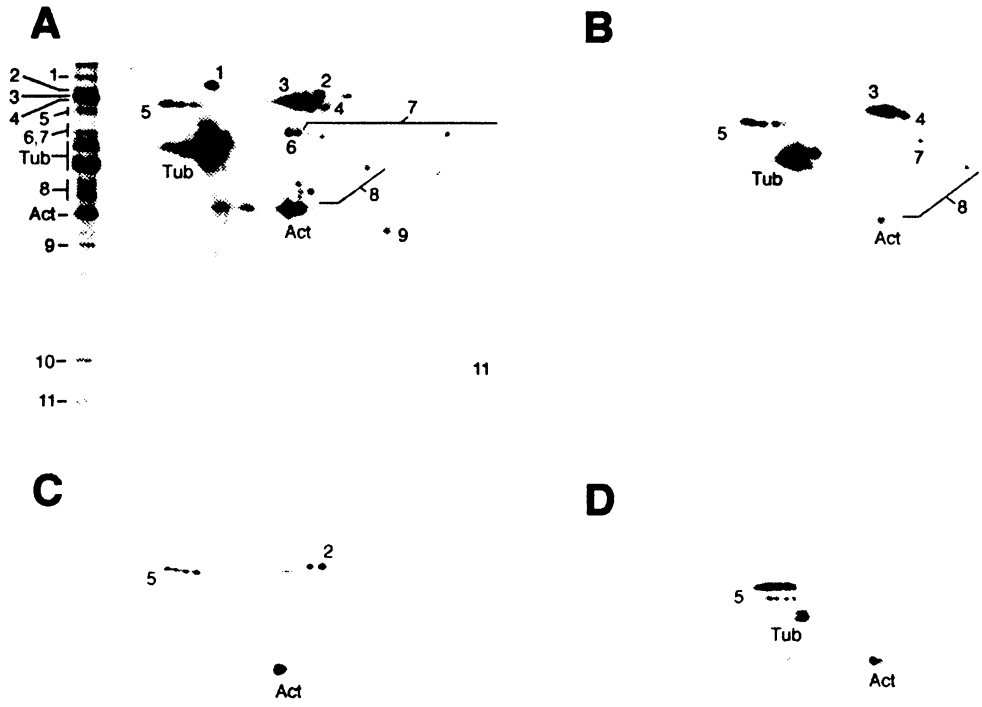

Figure 16 Two-dimensional Gel Electrophoresis of Proteins which Coprecipitate with Puromycyl-polypeptides

(**A**) HeLa cells were labeled with ^{35}S-methionine overnight, chased for 4 hours, and treated for 30 minutes with 2 μM puromycin prior to detergent lysis and immunoprecipitation with the anti-puromycin antibody under nondenaturing conditions. A one-dimensional profile of the same sample from another gel is shown at left. (**B**) Mature proteins were labeled and treated with puromycin as in (A), but the cells were lysed hypotonically to remove the organelles before immunoprecipitation with the anti-puromycin antibody. (**C**) Nonspecific precipitation from detergent-lysed cells using a preimmune serum. (**D**) Nonspecific precipitation from hypotonically-lysed cells which were not treated with puromycin following incubation with the puromycin immune serum. Proteins of more basic isoelectric points are seen on the right side of each 2-D gel. The identified proteins are as follows: (1) BiP; (2) grp75; (3) hsp73; (4) hsp72; (5) unknown; (6) hsp60; (7) CCT; (8) cytokeratins; (9-11) unknown; (Tub) α- and β-tubulin; (Act) actin.

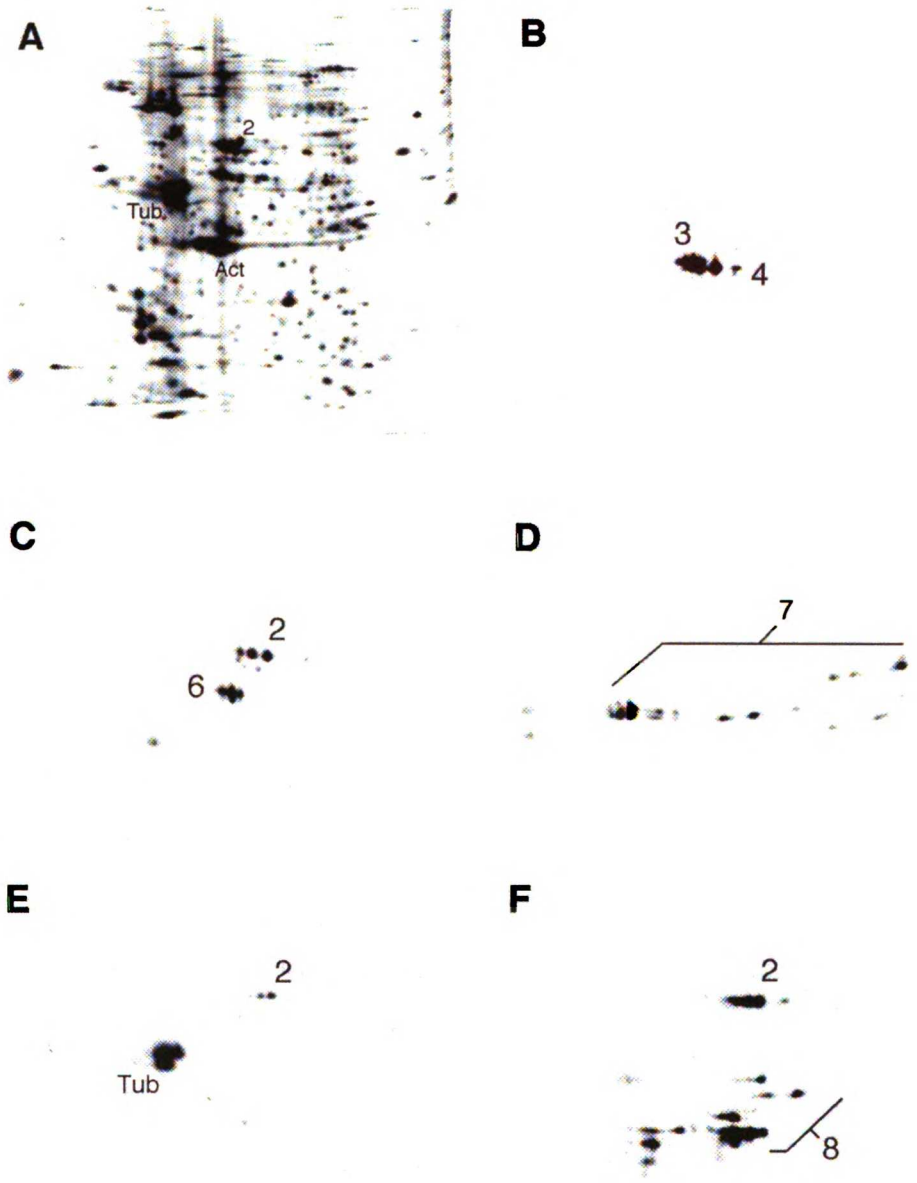

Figure 17 Identification of the Proteins Which Bind to Puromycyl-polypeptides by 2-D Gel Electrophoresis

(**A**) Typical whole lysate after detergent lysis. Specific proteins were immunoprecipitated from steady-state labeled lysates similar to that shown in (A) under nondenaturing conditions with antibodies that recognize the following antigens: (**B**) hsp72 and hsp73; (**C**) mitochondrial hsp60; (**D**) CCT; (**E**) α-tubulin; (**F**) cytokeratins. Individual proteins are numbered as in Fig. 16.

the intact organelles by centrifugation prior to immunoprecipitation (Fig. 16B). A low but detectable amount of the complex containing TCP-1, CCT (#7), was observed but required long exposure times to verify the presence of all its subunits relative to a CCT particle precipitated with an antibody to the γ-subunit (Fig. 17D). The small unknown protein of about 26 kD (#11) is likely the same protein found to coprecipitate with hsp70 under nondenaturing conditions (see Fig. 3). The isoelectric point for hsp40 is too basic to have been resolved on this gel (pI=8.9), but its absence was confirmed by non-equilibrium 2-D gels (H. Nagata, in preparation). The unidentified species marked (#5), (#9), and (#10) were binding nonspecifically since they also were precipitated using preimmune serum (Fig. 16C) and/or precipitated with the puromycin antibody from lysates that had never been treated with puromycin (Fig. 16D, for example). In detergent-treated lysates, nonspecific binding of grp75 to antibodies was observed frequently (see Fig. 17C, 17E & 17F, for examples). Of the other proteins that coprecipitated with puromycyl-polypeptides, many were identified as structural subunits of the cytoskeleton, including actin, tubulin, and some intermediate filament proteins (#8). Conditions for reducing the binding of these structural proteins and for differentiating between a specifically-bound and a nonspecifically-bound protein are discussed in later sections.

3.9. Side-by-side Comparison of Chaperone Interactions with Nascent Polypeptides using Antibodies to the Individual Chaperones

As a complementary approach to identify molecular chaperones which interact with nascent polypeptide chains, immunoprecipitation studies were carried out using antibodies specific for a selected set of molecular chaperones. The specificity of the various antibodies was confirmed by immunoprecipitation of

steady-state labeled lysates under nondenaturing conditions (**Fig. 18A**). A cocktail of antibodies against cytosolic hsp70 precipitated both the constitutive and inducible forms (lane 1). An antibody to the γ–subunit of CCT resulted in the capture of a number of proteins of approximately 55-65 kDa size by SDS-PAGE, consistent with the heteromeric nature of the cytosolic chaperonin particle (lane 2, and seen by 2-D gel analysis in Fig. 17D). A recently prepared antibody to hsp40 resulted in its isolation along with another prominent protein of approximately 65 kDa, the latter which may represent another DnaJ homologue present in animal cells (H. Nagata, in preparation). Antibodies to p48/Hip brought down an approximately 48 kDa antigen, while the polyclonal hsp90 antibody precipitated hsp90 along with minor amounts of two other proteins. When the relative interaction of these different molecular chaperones was examined with pulse-labeled nascent polypeptides released *in vivo* by puromycin treatment, a significant amount of the puromycyl-polypeptides was observed to coprecipitate with cytosolic hsp70 (**Fig. 18B**, lane 1). In contrast, antibodies to CCT, hsp40, p48, and hsp90, while capturing their respective full-length newly-synthesized antigens, failed to coprecipitate the "smear" of puromycin-released nascent chains (Fig. 18B). Typically, coprecipitation of puromycyl-polypeptides with the pool of anti-hsp70 antibodies yielded 15-20% of the total polypeptides captured with the polyclonal antibody to puromycin (as quantified by liquid scintillation).

3.10. Propensity of Puromycyl-polypeptides for Binding Nonspecifically to Many Proteins upon Cell Lysis

All of the results presented so far indicate that members of the hsp70 family represent the most prominent molecular chaperones which interact with nascent

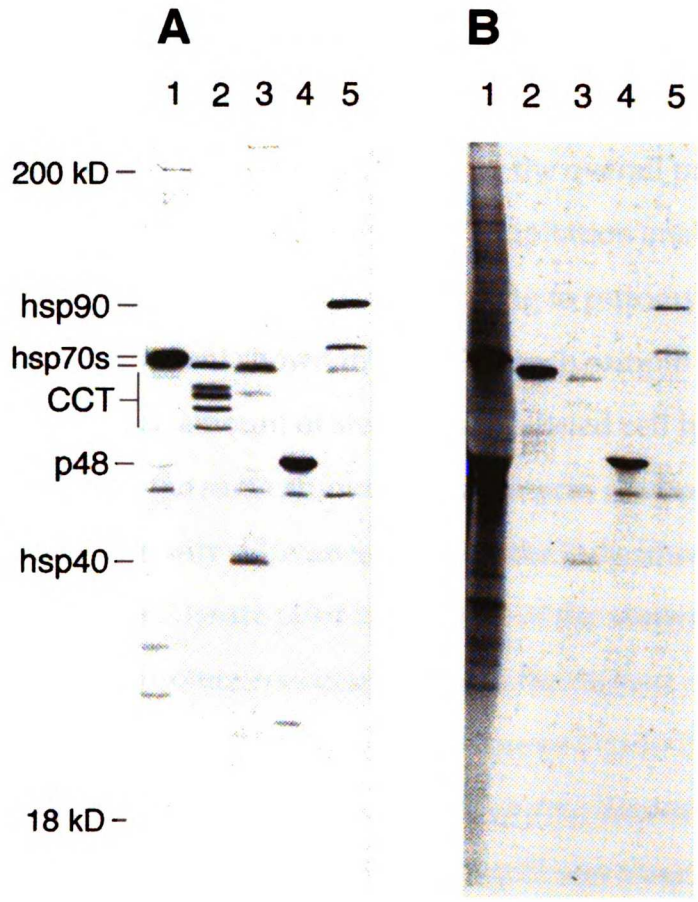

Figure 18 Ability of Anti-chaperone Antibodies to Coprecipitate Puromycyl-polypeptides

Immunoprecipitations were performed with antibodies which recognize the following antigens: (1) hsp72/hsp73; (2) CCT; (3) hsp40; (4) p48; (5) hsp90. See Materials and Methods for description of antibodies employed. (**A**) HeLa cells were labeled overnight with ^{35}S-methionine, chased 4 hours, treated with 2 µM puromycin for 20 minutes, and immunoprecipitated under nondenaturing conditions. (**B**) HeLa cells were simultaneously pulse-labeled and treated with 2 µM puromycin for 20 minutes followed by immunoprecipitation under nondenaturing conditions.

polypeptides. However, significant amounts of both actin and tubulin consistently were observed to coprecipitate with the puromycin-released nascent chains. The coprecipitation of these two abundant proteins may originate from nonspecific hydrophobic interactions with the nascent polypeptides since many of the truncated polypeptides are unable to fold into stable structures. To further investigate this phenomenon, the effect of reducing the overall protein concentration of the cell lysate prior to immunoprecipitation analysis was examined for its consequences on nonspecific binding to puromycyl-polypeptides. In the experiment shown in **Fig. 19A**, each sample was programmed with the same amount of steady-state labeled cell lysate, immunoprecipitated with the same amount of puromycin antibody, and washed using the same buffer. The only difference between the individual reactions was the initial dilution of the cell lysate prior to addition of the antibody. In the sample where the overall protein concentration was the highest (1:2 dilution), the greatest amount of coprecipitating proteins was observed (lane 2). Further lysate dilutions of 1:6, 1:20, and 1:60 resulted in a corresponding decrease in all coprecipitating proteins. At the highest dilution, hsp70 was clearly the major protein bound to puromycyl-polypeptides (lane 5). The other coprecipitating proteins were associated directly with the nascent polypeptides, in fact, and not binding nonspecifically to the antibody itself since they were not precipitated when the antibody was blocked with free puromycin (compare lanes 6 and 7). The increased detection of these proteins in more-concentrated samples may be attributed to both an increase in nonspecific binding per nascent chain and an increase in the number of nascent chains captured under conditions of higher antibody/antigen concentrations (data not shown).

The stringency of the dilution buffer and washing buffer following immunoprecipitation with Protein A Sepharose also was examined for effects on

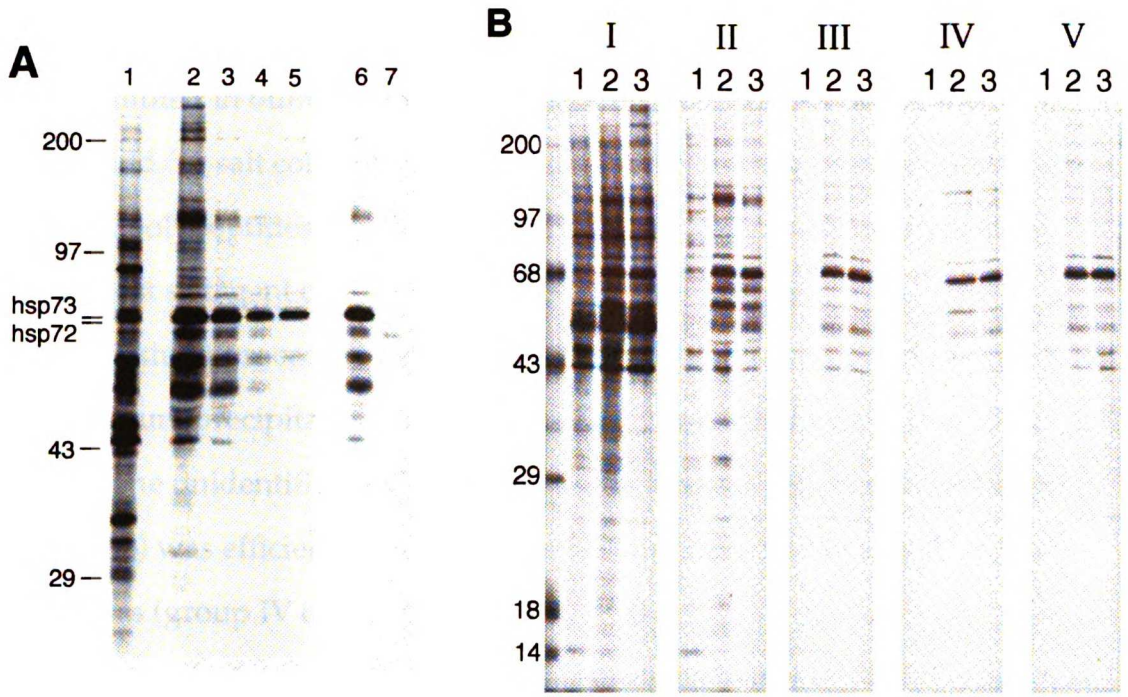

Figure 19 Effects of Lysate Dilution and Wash Stringency on Coprecipitation of Proteins with Puromycyl-polypeptides

(**A**) HeLa cells were steady-state labeled and treated with 2 µM puromycin for 15 minutes. For each immunoprecipitation (lanes 2-7), 50 µl of the same lysate was diluted in buffer A + 0.1% Triton before addition of the anti-puromycin antibody. All precipitates were washed four times with 1% Triton and 1% DOC in buffer A. (1) starting lysate; (2) immunoprecipitation after 1:2 dilution; (3) 1:6 dilution; (4) 1:20 dilution; (5) 1:60 dilution; (6) identical sample as lane 3, 1:6 dilution; (7) 1:6 dilution with addition of 100 µM puromycin to block antibody.

(**B**) A lysate prepared identically to that in (A) was diluted 1:24 into five buffers of differing stringency: (**I**) buffer A alone; (**II**) buffer A + 0.1% Triton; (**III**) buffer A + 1% Triton + 1% DOC; (**IV**) buffer A + 0.1% Triton + 1.0 M NaCl; (**V**) buffer A + 1% Triton + 1% DOC + 0.1% SDS. The diluted lysates were divided into three equal volumes followed by (lane 1) addition of preimmune serum, or (lanes 2 & 3) addition of puromycin antibody. In lanes 1 & 2 of each group, the resulting immunoprecipitates were washed with the same buffer as that used for the initial dilution. A different buffer was used for the wash step in **lane 3** of each group: (I, II & IV) buffer A + 1% Triton + 1% DOC; (III) same + 0.1% SDS; (V) PBS.

nonspecific binding of proteins to puromycyl-polypeptides (**Fig. 19B**). The highest occurrence of nonspecific binding was observed in the lysate samples that were diluted in buffer A with no detergent (group I). Increasing the detergent and/or salt concentration resulted in less binding of many proteins to puromycyl-polypeptides. The binding of hsp70, however, was unaffected by even the most stringent conditions. Lane 3 of group II corresponds to the standard washing procedure used in all other experiments reported in this work (*i.e.*, for immunoprecipitation under nondenaturing conditions). It should be noted that one unidentified ~65 kD species which was often present in 2-D gels (#5 of Fig. 16) was efficiently removed from immunoprecipitates by 0.5-1.0 M NaCl washes (group IV of Fig. 19B).

Nonspecific binding of many cellular proteins to nascent polypeptides in concentrated lysates has important implications on research in the field of molecular chaperones. Other investigators have classified certain proteins as "chaperones" based on their ability to coprecipitate nascent polypeptides that were made by *in vitro* translation (Frydman *et al.*, 1994; Höhfeld *et al.*, 1995). This type of analysis could lead to erroneous conclusions if the immunoprecipitations were done under conditions that allow nonspecific binding. For example, antibodies to α-tubulin can coprecipitate just as many puromycyl-polypeptides as the antibody cocktail to hsp70 when starting from a concentrated HeLa lysate (data not shown). One might argue from this observation that tubulin is a chaperone (if one didn't already have an understanding of its biological role and structure). Nonspecific binding at high protein concentrations probably has gone unnoticed in studies where the results were obtained by immunoblot analysis or by employing *in vitro* translation since these methods generally are used for the detection of a single protein. It should be noted that the proteins identified in the 2-D gel of Fig. 16A were coprecipitated from a lysate dilution of only 1:5, which

explains the large amount of tubulin present in that particular immuno-precipitate.

3.11. Insolubility of Nascent Polypeptides following Release from the Ribosome

Early attempts to release nascent polypeptides from isolated polysomes *in vitro* with puromycin were nonproductive due to low recovery of the nascent chains. When polysomes were treated with puromycin in "high salt" conditions (optimized by Blobel and Sabatini, 1971), less than 10% of the nascent polypeptides remained soluble following a high-speed spin to remove the ribosomal subunits (**Fig. 20A**). It was subsequently discovered that recovery of puromycyl-polypeptides could be greatly enhanced by addition of detergents (Triton and DOC). Recovery of soluble protein was not greatly affected by addition of ATP or addition of a HeLa lysate (Fig. 20A). In control samples where polypeptides were not released with puromycin, a low but significant level of radiolabeled protein remained in the 50K rpm supernatant under all conditions. This could indicate a problem with spontaneous release of peptidyl-tRNA from the ribosome. The levels of soluble nascent polypeptides in Fig. 20A were directly proportional to the levels of radiolabeled protein that were coprecipitated from supernatants with antibodies to hsp70 (data not shown). The solubility results were reproducible over several experiments using different preparations of polysomes. The highest recovery of puromycin-released polypeptides in the presence of detergent varied between 40-60%. A similar detergent-enhanced solubility was observed when immunoprecipitated puromycyl-polypeptides were released from their Protein A-bound antibodies with free puromycin (data not shown).

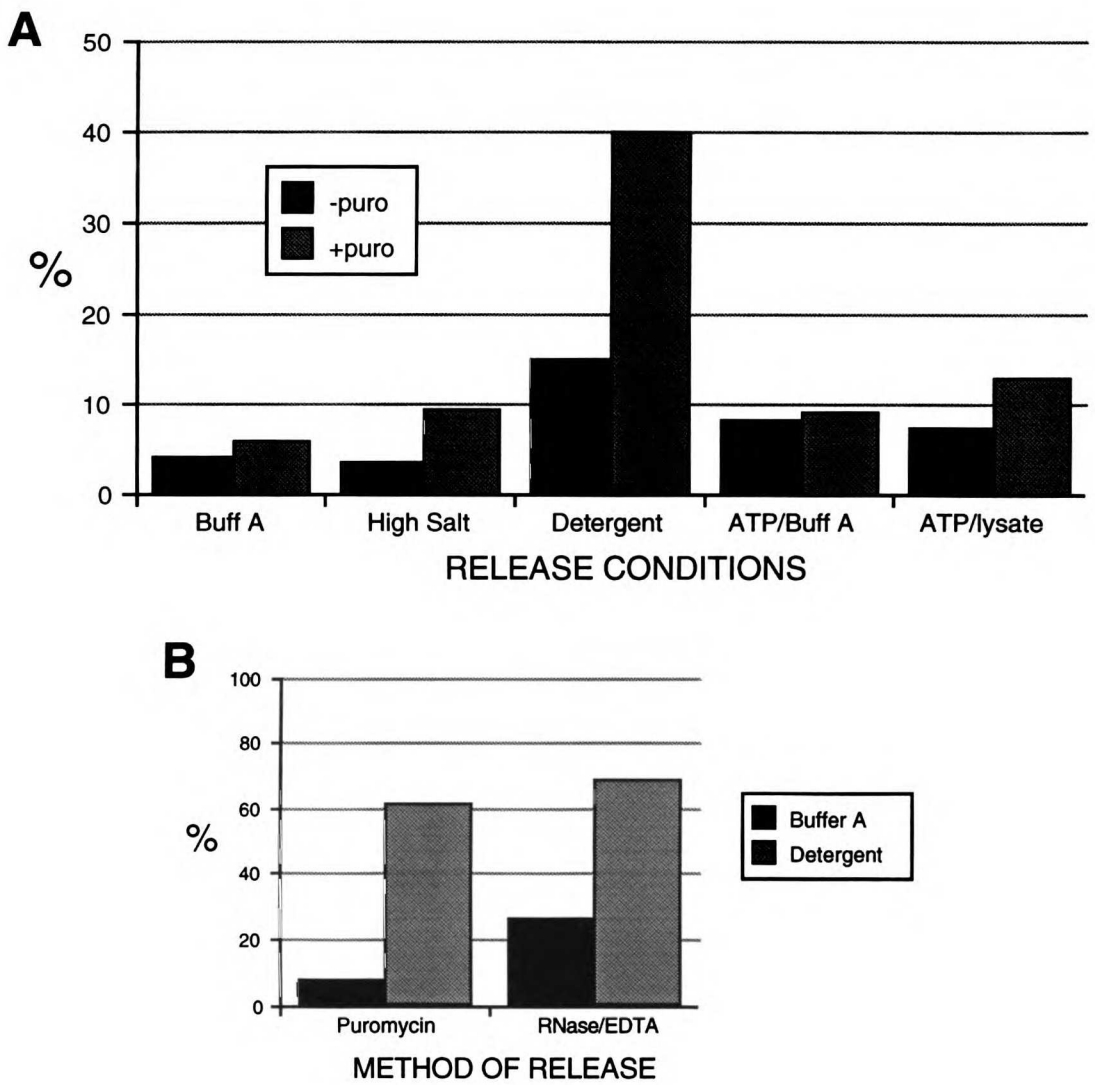

Figure 20 Solubility of Nascent Polypeptides after Release from Isolated Polysomes

HeLa cells were pulse-labeled, and the polysomes were isolated by sedimentation through 20% sucrose (see Materials & Methods). (**A**) The enriched polysomes were diluted 1:14 into five different solutions, all containing buffer A and 35 mM CHX with the following additions: "High Salt"= 500 mM KCl and 10 mM $MgCl_2$; "Detergent"= 1% Triton + 1% DOC; "ATP"= 5 mM MgATP; and "ATP/lysate"= 5 mM MgATP + concentrated HeLa lysate (S-100), prepared by WJW. Puromycin (0.5 mM) was added to half of each polysome sample to release nascent polypeptides (in vitro). Ribosome subunits and insoluble protein were pelleted at 50,000 rpm for 1.5 hours, 4°C (Beckman Ti-70 rotor). Solubility is given as a percent of total counts remaining in supernatant following centrifugation. (**B**) A different preparation of polysomes was treated similarly, except that some of the samples were treated with RNase for 30 minutes at 22°C and cooled to 4°C before addition of 10 mM EDTA. In this experiment, sedimentation was for 2 hours at 50,000 rpm.

To examine whether the puromycin moiety itself was responsible for the apparent aggregation phenomenon, puromycin treatment was compared to polypeptide release via disruption of the ribosomes by RNase/EDTA treatment (**Fig. 20B**). Nascent polypeptides released with RNase/EDTA treatment also exhibited a propensity to aggregate in the absence of detergent. Thus, the solubility of puromycyl-polypeptides may be an intrinsic property of their sequence and structure and not an artifact of the C-terminal puromycin "tail".

The solubility issue also was investigated in whole lysates following puromycin release *in vivo* (**Fig. 21**). Lysates were prepared in buffers containing ATP, ADP, AMP, or apyrase since the nucleotide-bound state of hsp70 and hsp60 governs the binding kinetics of the chaperones and (presumably) their ability to prevent aggregation of nascent polypeptides. With this in mind, different ratios of ATP:ADP were examined, expecting to see some correlation with solubility. As somewhat of a surprise, the solubility of puromycyl-polypeptides showed little dependence on ATP versus ADP. Even more surprising, the lowest solubility was observed in lysates treated with apyrase or 10 mM AMP, two conditions which promote stable binding of hsp70 to polypeptides (discussed in section 3.14). The differences in solubility as a function of nucleotide content were magnified by incubating the lysates at 45 °C (Fig. 21B) instead of 37 °C (Fig. 21A).

3.12. Attempts to Study Nascent Polypeptides from Isolated Polysomes

One caveat of using puromycin to study nascent polypeptides released from the ribosome *in vivo* is that one can not determine whether the observed protein interactions (*i.e.*, binding of chaperones) were initiated before or after release (cotranslational *versus* posttranslational). In order to address the issue of

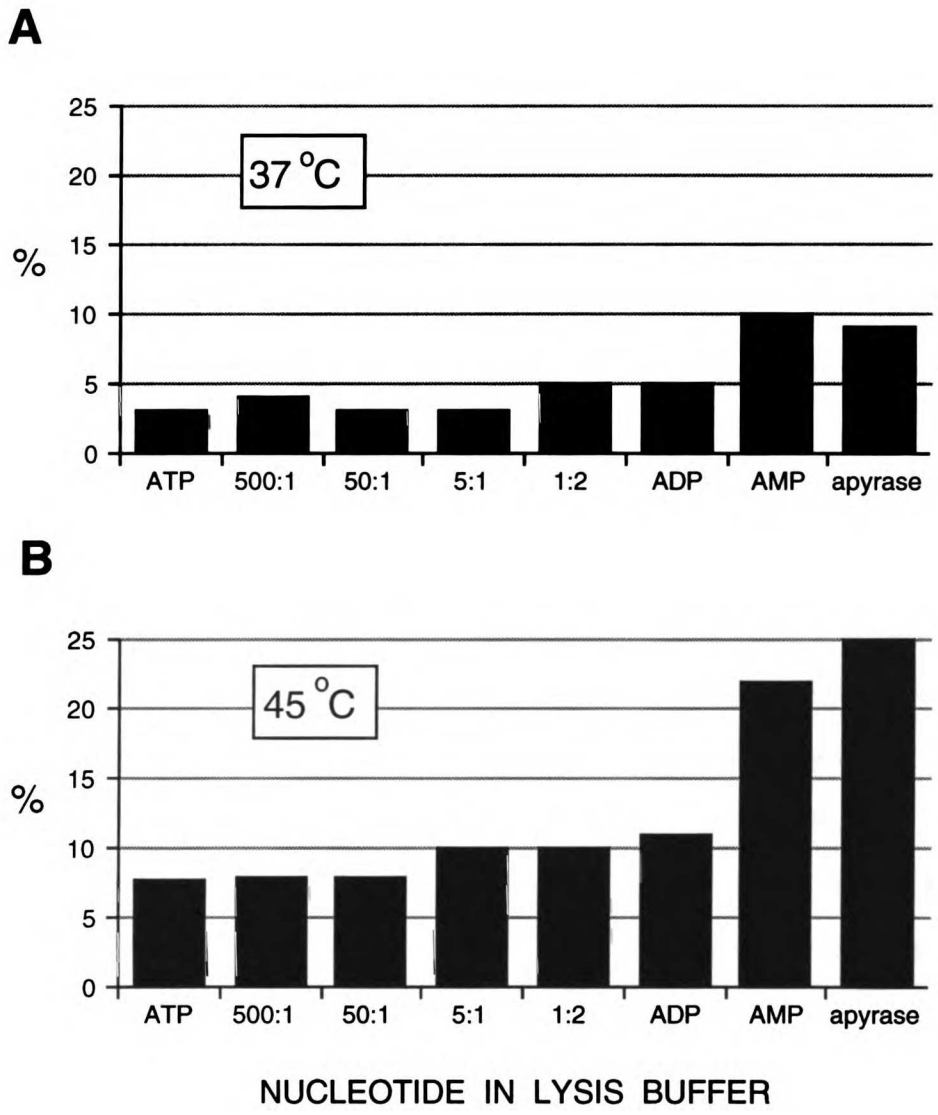

Figure 21 Insolubility of Puromycyl-polypeptides in Lysates as a Function of Temperature and Nucleotide Content

(**A**) Eight different plates of cells were pulse-labeled and treated with puromycin in vivo. The cells were lysed at 22°C in buffer A + 0.1% Triton and 10 mM of each of the nucleotides indicated in the figure. In some lysates, a ratio of ATP:ADP was employed (10 mM total nucleotide). The "apyrase" sample was supplemented with the enzyme only. Lysates were placed at 37°C for 10 minutes and then moved to ice before clarification in a microcentrifuge at maximum speed. The pellet and supernatant fractions were immunoprecipitated with the puromycin antibody under denaturing conditions and quantified by liquid scintillation. The results are given as percent of the total immunoprecipitated counts in the pellet fraction (insoluble puromycyl-polypeptides). (**B**) Identical procedure as in (A), except lysates were placed at 45°C for 20 minutes before moving to ice and clarifying in the microcentrifuge.

cotranslational interactions, cycloheximide-stabilized polysomes were isolated by velocity sedimentation and treated with puromycin *in vitro*. Unfortunately, most attempts to immunoprecipitate the released polypeptides were unsuccessful. These experiments were complicated by the need to remove "free" unreacted puromycin prior to immunoprecipitation. It was shown in Fig. 4 that free puromycin can effectively block the antibody to puromycin from binding puromycyl-polypeptides. Many research hours were devoted to finding a way of removing the inhibitory puromycin. Gel filtration, dialysis, and organic extraction with ethyl acetate and/or xylene were all found to be ineffective. It may be that the (somewhat hydrophobic) puromycin molecule has a strong affinity for exposed hydrophobic regions of the (unstably-folded) puromycyl-polypeptides. As described in the previous section, puromycyl-polypeptides have a propensity to aggregate and require the presence of detergent for maximal solubility. Contributing further complexity to the problem, cycloheximide, which is required to stabilize polysomes during isolation, has been reported to inhibit the puromycin reaction at low puromycin concentrations (Baliga *et al.*, 1970). Nevertheless, some interesting observations were made following immunoprecipitation analyses of isolated polysomes, as summarized in the next three figures.

For the experiments shown in **Fig. 22**, nascent polypeptides were released from isolated polysomes with 1 mM puromycin followed by extensive dialysis to reduce the concentration of unreacted puromycin. When the ribosomal subunits were subsequently removed by velocity sedimentation, several proteins remained in the soluble supernatant (lane "S" of Fig. 22A). The upper band of the doublet found near the 68 kD size marker of lane S probably corresponds to the poly-A binding protein, a species slightly larger than hsp70 which is abundant in isolated polysomes. The lower band of the doublet is hsp70, as revealed by 2-D

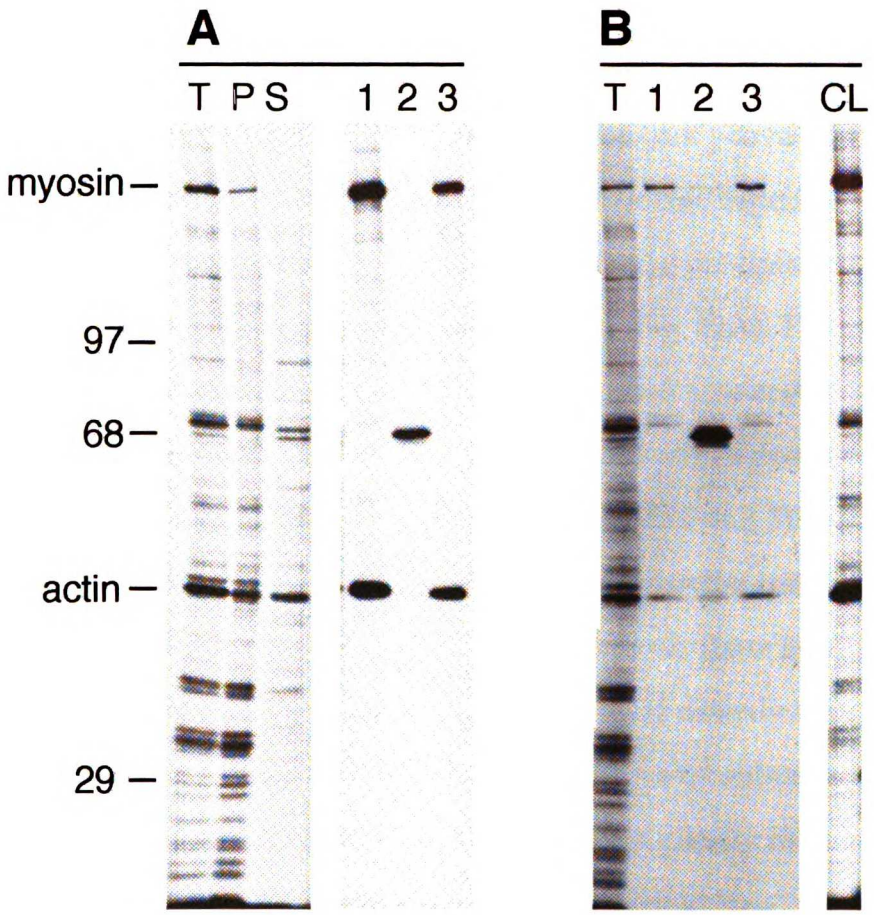

Figure 22 Attempts to Coprecipitate Chaperones with Puromycyl-polypeptides following Release from Isolated Polysomes

Polysomes were isolated from steady-state labeled cells as descibed in Materials & Methods. (**A**) Nascent polypeptides were released in vitro with 1 mM puromycin after adjusting the buffer to 500 mM KCL, 10 mM $MgCl_2$, and 0.1% Triton. The sample was dialyzed against three changes of buffer A + 0.2% Triton (1 ml sample to 200 ml dialysis buffer for several hours each change). The ribosomes were pelleted for 2 hours at 50,000 rpm, and the resulting supernatant was used for immunoprecipitation analyses. Gel lanes: T= total polysome sample; P= pellet of 50K rpm spin; S= supernatant after 50K rpm spin; (1) precipitation with puromycin antibody; (2) precipitation with hsp 70 monoclonal antibodies using the supernatant from (1) after removal of Protein A-Sepharose; (3) precipitation with rabbit preimmune serum. (**B**) A different polysome preparation was incubated with puromycin and dialyzed as in (A). Instead of a high-speed spin to pellet ribosomal subunits, the sample was pre-adsorbed with Sepharose CL-4B and used directly for immunoprecipitation. Lanes are labeled as in (A), CL= proteins that were adsorbed during incubation with CL-4B.

gel analysis (data not shown). It is important to note that hsp70 was found in the 50K rpm supernatant *regardless* of whether or not the polysomes were incubated with puromycin (data not shown). Thus, this result does not prove that hsp70 was bound to nascent polypeptides. When the ribosome-free supernatant was analyzed by immunoprecipitation, only two proteins were precipitated with the antibody to puromycin, actin and myosin heavy chain (Fig. 22A). The identification of these two proteins was inferred from their size and association. The precipitation of actin and myosin also was observed with a preimmune serum, indicating a nonspecific interaction (compare lanes 1 and 3). Hsp70 could be immunoprecipitated with hsp70-specific antibodies from the residual proteins in the supernatant after removal of the puromycin antibody (lane 2). A similar result was obtained when the polysome fraction was used directly for immunoprecipitation without prior removal of the ribosomal subunits (Fig. 22B). In this experiment, the binding of actin and myosin were greatly reduced by preadsorption onto Sepharose CL-4B (see Sepharose pellet in lane "CL").

In a slightly different approach, nascent polypeptides were released from isolated polysomes with low (micromolar) concentrations of puromycin, diluted in buffer, and immunoprecipitated with the puromycin antibody without attempting to remove the free puromycin. In this case, several proteins in addition to actin and myosin were precipitated (**Fig. 23A**). These proteins appeared to be specifically bound to puromycyl-polypeptides since no protein was precipitated in the absence of the puromycin antibody or at the highest puromycin concentration which may have blocked all the antibody (lanes 4 & 5 of Fig. 23A). The species marked "x" and "y" likely correspond to α- and β-tubulin, respectively. The band marked "z" is unknown but could represent a keratin or other intermediate filament protein. All three proteins (x, y & z) were clearly evident in the starting polysome fraction (lane T), but were not always so

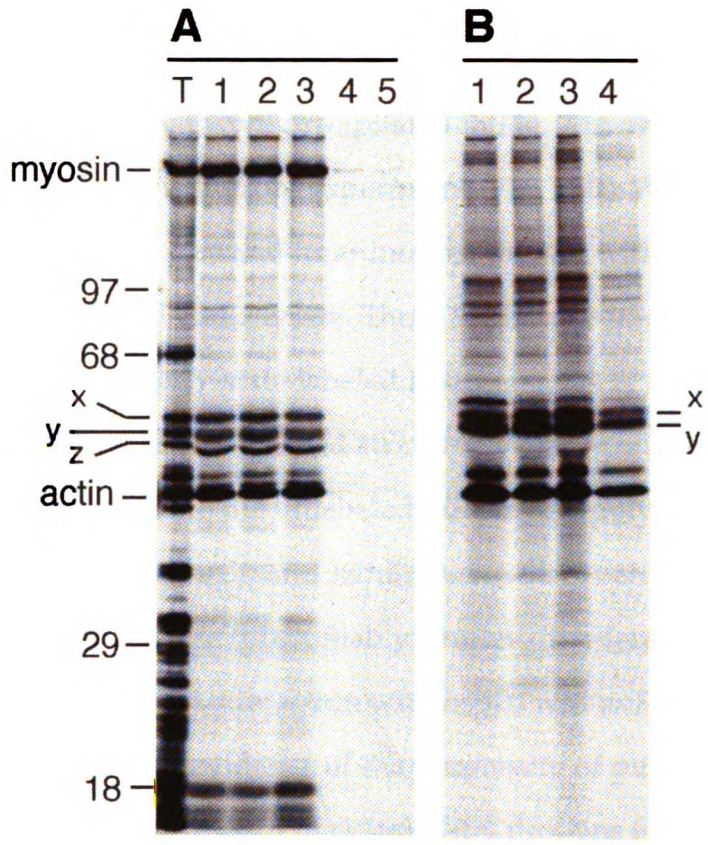

Figure 23 Further Attempts to Coprecipitate Chaperones with Puromycyl-polypeptides after Release *in Vitro*

(**A**) Aliquots of an enriched polysome fraction were incubated with low micromolar concentrations of puromycin, diluted 1:20 in buffer A + 0.1% Triton, and immunoprecipitated with the puromycin antibody. T= total polysome fraction. Lanes 1-5 are immunoprecipitates from samples treated with the following concentrations of puromycin (before 1:20 dilution). (1) 2 µM; (2) 0.2 µM; (3) 0.02 µM; (4) none; (5) 20 µM.

(**B**) Puromycyl-polypeptides were immunoprecipitated from an unlabeled HeLa lysate (released in vivo). The Sepharose-bound protein was washed normally, diluted into buffer A + 0.1% Triton, and split into two tubes. Both Sepharose pellets were washed an additional three times with dilution buffer, but the buffer for one tube contained 10 mM MgATP to strip ATP-dependent chaperones. The samples were split again and incubated for 20 minutes with steady-state labeled hypotonic lysates made in the presence of apyrase or ATP (-/+ ATP). Apyrase was then added to all samples, and the Sepharose pellets were washed four times in buffer A + 1% Triton + 1% DOC. Shown are the resulting precipitates from the following combinations of nucleotide-treated lysates (unlabeled prep/ labeled prep): (1) -ATP/-ATP; (2) -ATP/+ATP; (3) +ATP/-ATP; (4) +ATP/+ATP.

evident in other polysome preparations. Precipitation of x, y & z was not reproducible with the puromycin antibody.

The set of proteins that were precipitated in Fig. 23A were similar to that observed following a "reconstitution" experiment (**Fig. 23B**). Puromycyl-polypeptides were made *in vivo* and immunoprecipitated with the puromycin antibody from an unlabeled HeLa lysate. These Sepharose-bound polypeptide chains were mixed with steady-state labeled lysates (-/+ ATP). The lysates were removed and the Sepharose was washed stringently before analyzing which labeled proteins were bound to the unlabeled puromycyl-polypeptides. Once again, significant amounts of actin and tubulin were discovered to bind puromycyl-polypeptides. These (presumably) irrelevant interactions were not affected by ATP levels, and an interaction with hsp70 was not detected under any condition. This is further evidence of the propensity of puromycyl-polypeptides to bind nonspecifically to cytoskeletal proteins following cell lysis (first discussed in section 3.10).

Still other experiments using isolated polysomes were performed to confirm and expand on the original reports of Beckmann *et al.*, 1990. Using antibodies to hsp70, it was possible to immunoprecipitate nascent polypeptides from a pulse-labeled polysome fraction before and after release with puromycin (**Fig. 24A**). Interestingly, coprecipitation of nascent polypeptides was reduced significantly when the ribosomes were disrupted by RNase/EDTA treatment (group III). Perhaps EDTA chelated all the magnesium which consequently affected the structure and binding of hsp70.

Isolated polysomes from steady-state labeled lysates also were examined by immunoprecipitation with antibodies to specific proteins. In addition to hsp70, antibodies to α-tubulin and cytokeratin were analyzed (**Fig. 24B**). Ribosomes were coprecipitated equally well with hsp70 (lanes 2) and tubulin

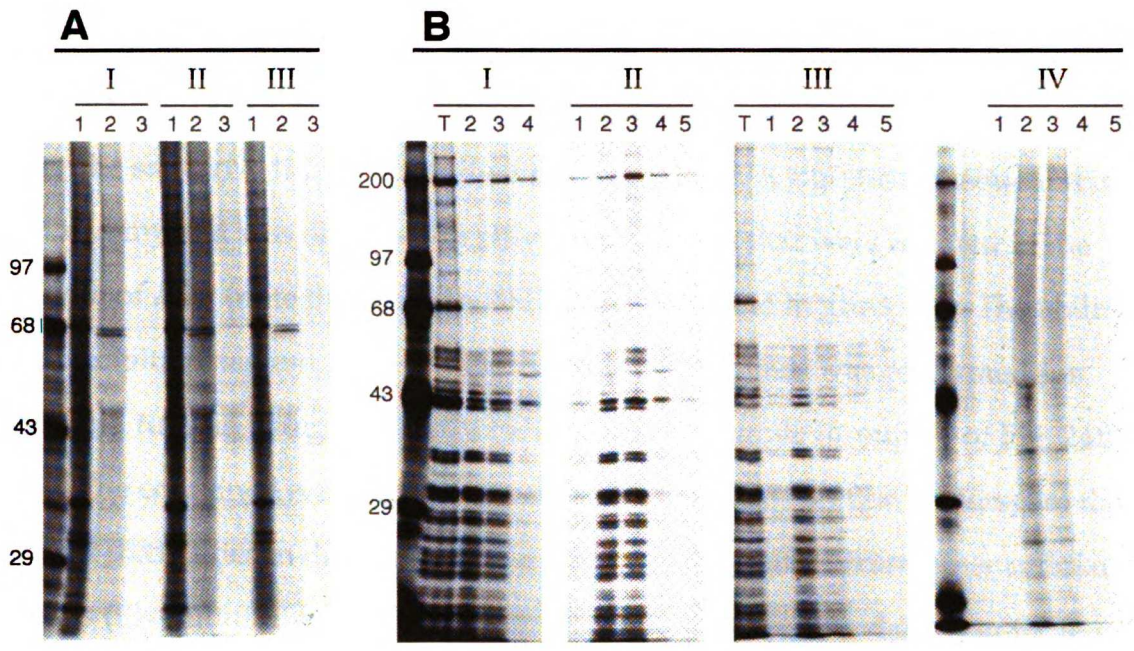

Figure 24 Ability to Coprecipitate Nascent Polypeptides and Ribosomes with Antibodies to Hsp70 or Tubulin

(**A**) Isolated polysomes from a pulse-labeled lysate were dialyzed for 2 hours to reduce the sucrose concentration. The sample was subdivided into three tubes and treated as follows: (I) no further treatment; (II) 1 mM puromycin; (III) RNase for 30 minutes at 22°C, then 25 mM EDTA at 4°C. Lane: (1) starting sample; (2) precipitation with antibodies to hsp70; (3) control precipitation with secondary antibody only (RAM).

(**B**) Polysomes were isolated and treated as follows:

(I) steady-state labeling, -ATP, no puromycin;

(II) steady-state labeling, -ATP, 0.2 µM puromycin (released in vitro);

(III) steady-state labeling, +ATP, 0.2 µM puromycin;

(IV) pulse labeling, -ATP, 0.2 µM puromycin.

The polysomes were diluted into buffer A + 0.1% Triton before immuno-precipitation with antibodies which recognize the following antigens: (1) puromycin; (2) hsp70; (3) α-tubulin; (4) pan-cytokeratins; (5) secondary antibody only (RAM). Lane designations correspond with antibodies. T= total polysome fraction used for immunoprecipitation.

(lanes 3). This coprecipitation was independent of puromycin treatment (compare groups I and II) and independent of ATP levels (group III). The ability to coprecipitate ribosomes with the hsp70 antibody in the presence of ATP was not anticipated since puromycyl-polypeptides are not coprecipitated with hsp70 under the same conditions (examined in section 3.14). A significant reduction in actin and myosin was observed in polysome samples that were isolated in the presence of ATP (note the lack of a 200 kD myosin band in group III). The ability to coprecipitate nascent polypeptides bound to polysomes was confirmed for hsp70 and tubulin using a pulse-labeled polysome sample (group IV of Fig. 24B).

The combined results of all the experiments in this section underscore the inherent difficulties in distinguishing a relevant "chaperone" from other cellular proteins (like tubulin) which bind to nascent polypeptides in whole lysates. It seems likely that those physical properties of a nascent polypeptide which confer a dependence on molecular chaperones *in vivo* are the same properties that are responsible for the low solubility and nonspecific binding interactions observed for puromycyl-polypeptides *in vitro*.

3.13. Affinity of Hsp70 for Puromycyl-polypeptides in the Presence of Exogenous (Non-native) Proteins

To further examine the affinity of hsp 70 for nascent polypeptides as a function of ATP levels, cells were disrupted in the presence of exogenous proteins, both native and abnormally-folded (**Fig. 25A**). It was rationalized that high concentrations of a non-native protein, such as reduced and carboxymethylated BSA (RC-BSA) or α_S-casein, should compete with puromycyl-polypeptides for binding of hsp70 under conditions that facilitate dynamic interactions. In those samples where ATP levels were depleted immediately with apyrase during cell

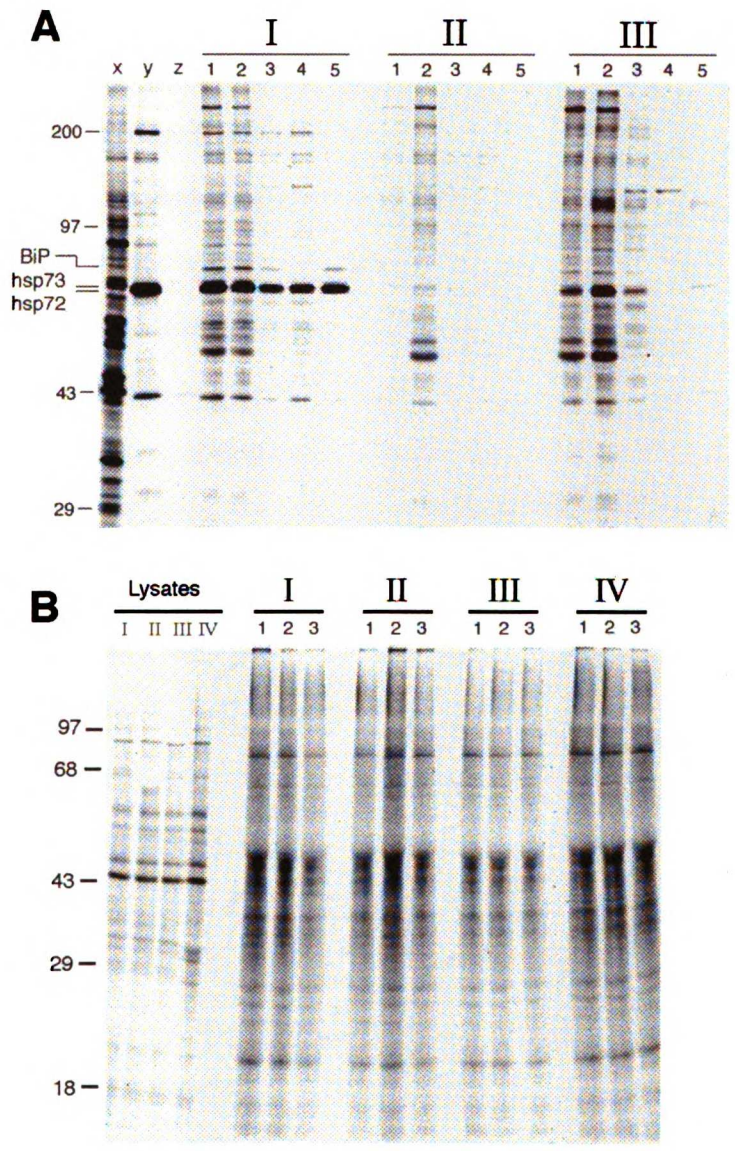

Figure 25 Affinity of Hsp70 for Puromycyl-polypeptides in the Presence of Exogenous Proteins

(**A**) Cells were steady-state labeled and treated with 2 μM puromycin for 30 minutes before lysis in buffer A + 0.1% Triton containing exogenous proteins (numbered 1-5) and incubated with either (I) apyrase, (II) 10 mM MgATP, or (III) 10 mM MgATP for 10 minutes followed by apyrase treatment. Lanes: (x) typical starting lysate; (y) immunoprecipitation with antibodies to hsp70; (z) puromycin antibody blocked with free puromycin; (1-5) anti-puromycin immunoprecipitations performed after 1:40 dilution using lysates which contain the following: (1) no exogenous protein; (2) 10 mg/ml BSA; (3) 10 mg/ml RC-BSA; (4) 10 mg/ml α_S-casein; (5) 6-fold excess of unlabeled HeLa protein from cells untreated with puromycin. (**B**) Cells were pulse labeled for 20 minutes in the presence of 2 μM puromycin and lysed in the same buffers as used in (A). (I) no exogenous protein; (II) BSA; (III) RC-BSA; and (IV) casein. Lysates are shown in the left panel and the corresponding immunoprecipitations with the puromycin antibody are numbered as follows: (1) -ATP; (2) +ATP; (3) +ATP, then apyrase.

lysis, significant amounts of hsp70 were observed to coprecipitate with the puromycin-released nascent chains, independent of the presence of the different protein supplements (group I of Fig. 25 A). A significant reduction in the "background" of other proteins which coprecipitated with the released nascent chains was observed, however, from the lysates containing RC-BSA, casein, or a 6-fold excess of unlabeled HeLa cell lysate (lanes 3-5). The reduction in background coprecipitation was not observed from a sample where RC-BSA had been added subsequent to cell lysis and ATP depletion (data not shown). When present during cell disruption (*i.e.*, in the lysis buffer), RC-BSA and casein appear to intercept many of the proteins which coprecipitated with nascent polypeptides in a concentration-dependent, nonspecific manner (see section 3.10). These observations support the idea that hsp70 is the major relevant protein interacting with puromycyl-polypeptides in the cytosol (prior to lysing the cells).

When ATP was present during cell lysis, labeled hsp70 no longer was observed to coprecipitate with the puromycin-released chains (group II of Fig. 25A). Instead, only those proteins shown to bind nonspecifically were coprecipitated. The amount of puromycin-released chains that was captured with the puromycin antibody was unaffected by ATP depletion or by the presence of any of the exogenous proteins, as quantified in a parallel experiment using pulse-labeled lysates (**Fig. 25B**). In those lysates where ATP was initially present and then depleted after a 10-minute incubation, rebinding of the radiolabeled hsp70 chaperone to the puromycin-released nascent chains was observed. The extent of rebinding, however, varied as a function of the particular protein supplement (group III of Fig. 25A). For example, in those lysates supplemented with either nothing or native BSA, hsp70 was observed to coprecipitate again with the puromycin-released chains (group III, lanes 1 & 2). In contrast, much less radiolabeled hsp70 was observed to coprecipitate with the puromycin-released

chains in those lysates supplemented with 10 mg/ml of either RC-BSA or α_S-casein (group III, lanes 3 & 4). This is consistent with the idea that these non-native protein supplements provide a vast excess of substrate for the hsp70 chaperone. In the case where unlabeled HeLa cell lysate was present (lane 5), a significant reduction of the radiolabeled hsp70 chaperone was expected since the 6-fold excess of unlabeled hsp70 could compete for rebinding to the puromycin-released nascent chains. Overall, the results of Fig. 25 are consistent with the hypothesis that the interaction between members of the hsp70 family and nascent polypeptides is one of high affinity yet constant dynamics in the presence of physiological concentrations of ATP.

Since RC-BSA was observed to reduce nonspecific binding of many proteins to puromycyl-polypeptides, the native size of complexes containing puromycyl-polypeptides was re-examined using lysates made in the presence of RC-BSA. Nonspecific interactions with proteins like tubulin and actin might contribute significantly to the native size of puromycyl-polypeptides when analyzed by chromatography (as in Section 3.3) or by sedimentation (as in Section 3.5). Surprisingly, the addition of RC-BSA caused puromycyl-polypeptides to elute sooner from an S-400 sizing column, indicating larger native complexes (**Fig. 26**). Likewise, puromycyl-polypeptides in RC-BSA lysates were found to sediment further into sucrose gradients than lysates made without RC-BSA (data not shown).

When fractions from the sizing chromatography experiment of Fig. 26 were separated by SDS-PAGE and stained with Coomassie, RC-BSA could be seen in every fraction of the elution profile including those fractions which immediately followed the void volume of the column. Thus, it seems likely that puromycyl-polypeptides eluted as larger native complexes due to direct interactions with RC-BSA. This interaction was investigated using an antibody to

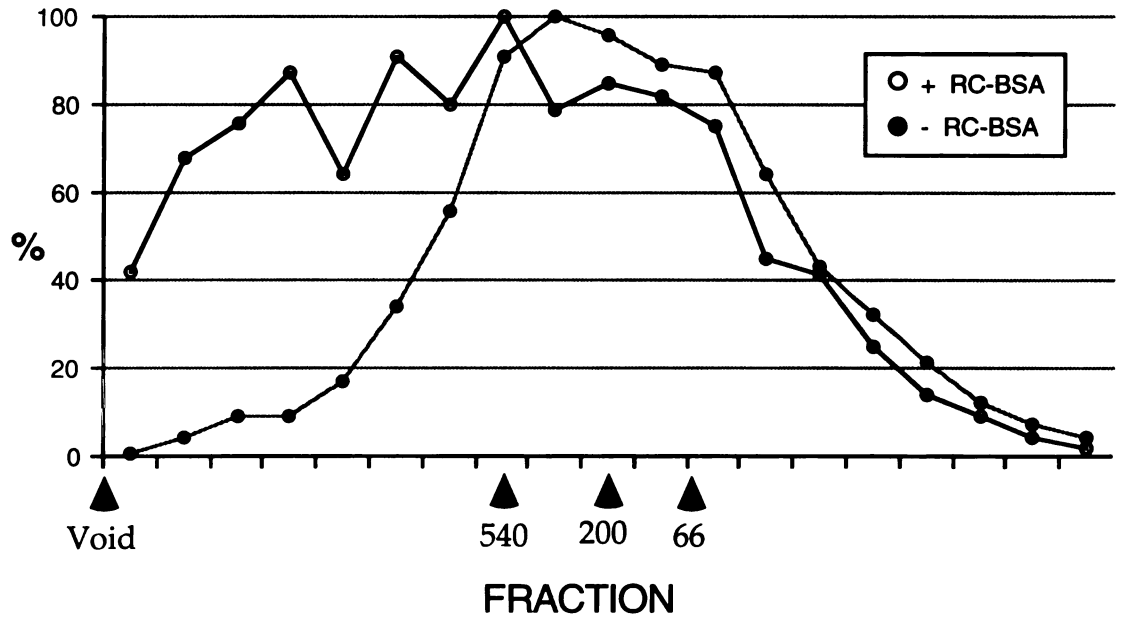

Figure 26 Effect of RC-BSA on Elution of Puromycyl-polypeptides by Sizing Chromatography

Two HeLa lysates were fractionated on the same S-400 column. Both plates of cells were pulse labeled and treated with puromycin before lysis in buffer A + 0.1% Triton and apyrase. One lysis buffer also contained 10 mg/ml RC-BSA (added before scraping cells off plate). Following elution from the column, each fraction was analyzed by immunoprecipitation with the puromycin antibody under denaturing conditions. The amount of puromycyl-polypeptides in each fraction was quantified by liquid scintillation of the immunoprecipitates. The results are plotted as a percentage of the counts detected in the peak fraction for each chromatography run (+/- RC-BSA). Elution peaks of native size markers are indicated at the bottom of the figure (in kDaltons).

(native) BSA to coprecipitate puromycyl-polypeptides. Unfortunately, this approach was unsuccessful since the BSA antibody precipitated more pulse-labeled puromycyl-polypeptides in the absence of RC-BSA than in the presence of RC-BSA (or BSA), presumably due to more nonspecific binding to the antibody in the absence of an exogenous protein.

In Fig. 25A, it was shown that hsp70 can be displaced from nascent polypeptides in the presence of ATP and that subsequent rebinding of hsp70 is greatly reduced in the presence of RC-BSA or casein (group III of Fig. 25A). This is consistent with the idea that RC-BSA and casein are competitive ligands for the binding of chaperones when ATP is present to promote a dynamic cycle of binding and release. However, the results discussed above make it clear that a three-way interaction may exist between the nascent polypeptide, the chaperone(s), and the non-native "chaperone competitor". Although a nonspecific binding interaction between two non-native proteins like RC-BSA and (truncated) puromycyl-polypeptides is easy to imagine, such an interaction may not have been considered in other investigations where non-native proteins were employed (Langer *et al.*, 1992; Schumacher *et al.*, 1994; Szabo *et al.*, 1994; Yonehara *et al.*, 1996). In the case of refolding experiments performed in the presence of proteins like casein and RC-BSA, a direct interaction between the protein being refolded and these permanently unfolded proteins also seems likely, which leads to a much different interpretation of the results.

3.14. Nucleotide Effects on the Ability to Capture Hsp70 in Stable Complexes with Puromycyl-polypeptides

Hsp70 coprecipitates with puromycyl-polypeptides after depletion of ATP by apyrase treatment, as clearly depicted in Fig. 25A. In the presence of ATP, hsp70

may still interact with puromycyl-polypeptides (and nascent polypeptides, in general), but this interaction is apparently too dynamic to capture by immunoprecipitation. It has been generally assumed that apyrase treatment stabilizes the interaction between hsp70 and its ligands by "locking" the chaperone into its ADP-bound conformation. If this hypothesis were correct, then lysing cells in a vast excess of ADP (relative to ATP) should have the same effect as apyrase treatment. As shown in **Fig. 27**, this is not the case. In the presence of 10 mM ADP, it was not possible to coprecipitate hsp70 with puromycyl-polypeptides (compare lanes 2). Coprecipitation of hsp70 was not greatly enhanced when the ADP-containing buffer was supplemented with glycerol kinase and hexokinase to consume endogenous levels of ATP, although a trace amount of hsp70 was now visible (see ADP+, lane 2). In contrast, addition of 10 mM AMP to the lysis buffer resulted in significant coprecipitation of hsp70 with puromycyl-polypeptides, equal in intensity to that observed in the apyrase-treated lysate. It is known that apyrase can hyrolyze ADP to AMP as well as ATP to ADP. These results are consistent with the observation that hsp70 interactions are dynamic in the presence of both ATP and ADP, although the kinetics of binding (and affinities for a given substrate as measured at equilibrium) may differ substantially for the two nucleotide-bound states of the chaperone (Schmid *et al.*, 1994; McCarty *et al.*, 1995; Theyssen *et al.*, 1996).

The ability of hsp70 to bind AMP and to bind other protein ligands in the presence of AMP has not been well characterized. It is tempting to speculate that AMP may promote polymerization of hsp70 and that this oligomer of hsp70 is the form which binds to puromycyl-polypeptides following apyrase treatment in whole lysates. This may explain why ATP-depletion *in vivo* (using drugs which uncouple mitochondrial synthesis of ATP, like CCCP) results in less coprecipitation of hsp70 with puromycyl-polypeptides relative to apyrase

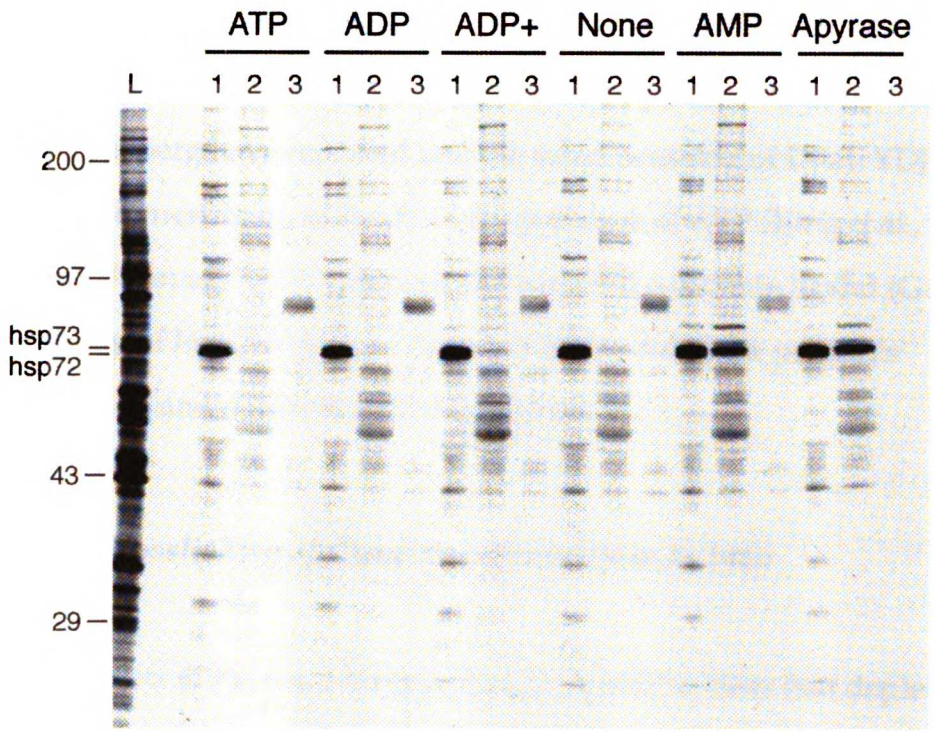

Figure 27 Coprecipitation of Hsp70 with Puromycyl-polypeptides as a Function of Nucleotide Content

Six plates of cells were labeled overnight, chased 4 hours without label, and treated with 2 μM puromycin for 20 minutes. Each plate was lysed in buffer A + 0.1% Triton with one of the following additions (indicated at the top of the figure): **ATP** = 10 mM MgATP; **ADP** = 10 mM MgADP; **ADP+** = 10 mM MgADP + 325 mM glycerol + 70 mM glucose + mixture of glycerol kinase/hexokinase (25:1); **None** = no addition; **AMP** = 10 mM MgAMP; **Apyrase** = 10 units/ml. An equal volume of each lysate was diluted 1:40 in buffer A + 0.1% Triton before immunoprecipitation under nondenaturing conditions with the following antibodies: (1) hsp70 cocktail; (2) puromycin antibody; (3) control using puromycin antibody blocked with free puromycin. The protein corresponding to the broad band in each of the blocked control lanes (3) is unknown and probably an artifact due to use of an old serum aliquot in those immunoprecipitations. L= typical whole lysate.

treatment (data not shown). Chemical manipulation of ATP levels in the cell would not necessarily result in complete hydrolysis of ADP to AMP. It should be noted that oligomers of hsp70 have been detected by sizing chromatography in the absence of nucleotide (Palleros *et al.*, 1991). Oligomers of hsp70 also have been observed by native gels and sedimentation analysis (Benaroudj *et al.*, 1995). Greene and Eisenberg have reported that the yeast homolog of DnaJ, YDJ1, catalyzes the polymerization of hsp70 in the presence of ATP (King *et al.*, 1995). This polymeric form of hsp70, however, did not bind a peptide ligand (Gao *et al.*, 1996). The ability of hsp70 to polymerize may be crucial to the protein's biological function and deserves further attention.

3.15. Chemical Crosslinking of Chaperone Interactions in Vivo

From the discussion in the preceding section, it should be clear that depletion of ATP by apyrase treatment likely results in non-physiological concentrations of nucleotides, *i.e.* ATP may need to be hydrolyzed completely to AMP in order to stabilize the (presumably) dynamic interaction between hsp70 and nascent polypeptides. To investigate the interaction between chaperones and nascent polypeptides in the presence of more (physiologically) relevant concentrations of nucleotides, chaperones, and total macromolecules, a membrane-permeable crosslinker was added directly to living cells. Following incubation with the crosslinker, the cells were lysed under denaturing conditions which maintain the integrity of the crosslinks (heated in the presence of SDS) and analyzed by immunoprecipitation. The crosslinks in the immunoprecipitates were cleaved by heating in the presence of reducing agents prior to SDS-PAGE (see Section 2.9 for details). The chemical crosslinking approach has several potential advantages: (1) crosslinking should trap dynamic interactions with certain chaperones which

may be hard to detect in the absence of crosslinking; (2) crosslinking should stabilize detergent-sensitive interactions which may have been lost in the presence of detergents during lysis and immunoprecipitation in previous experiments; and (3) crosslinking should provide a means for studying cotranslational interactions with nascent polypeptides still bound to the ribosome (*i.e.*, in the absence of puromycin treatment). Some possible limitations of this approach include nonspecific crosslinking of chaperones to all cellular proteins (high background) and the complication of using puromycin in conjunction with crosslinking since the unreacted puromycin molecules contain a primary amino group which could be nonspecifically linked to any protein.

DSP was chosen as the membrane-permeable and thiol-cleavable reagent for crosslinking experiments. DSP has been reported to be a favored reagent for preserving soluble and filamentous proteins in the cell prior to detergent extraction and analysis by fluorescence microscopy (Safiejko-Mroczka and Bell, 1996). The first experiments with DSP were done with steady-state labeled cells to confirm that antibodies to the selected chaperones were still capable of precipitating their respective antigens after the crosslinking treatment. (DSP reacts with the ε-amino groups of lysines leaving the residues covalently modified after cleavage of the crosslink.) As shown in **Fig. 28**, the crosslinking treatment did not reduce the immunoprecipitation of a selected set of molecular chaperones (the same set of chaperones investigated in Fig. 18A under nondenaturing conditions in the absence of crosslinking). Modification of lysines on the chaperones was evident following SDS-PAGE as a shift in mobility to slightly larger molecular masses. Excessive crosslinking of cellular proteins to any one chaperone was not apparent (low background). In the case of CCT precipitation, antibodies to the α-subunit (TCP1) appeared to coprecipitate the other subunits of the heterogenous particle, although the α-subunit was

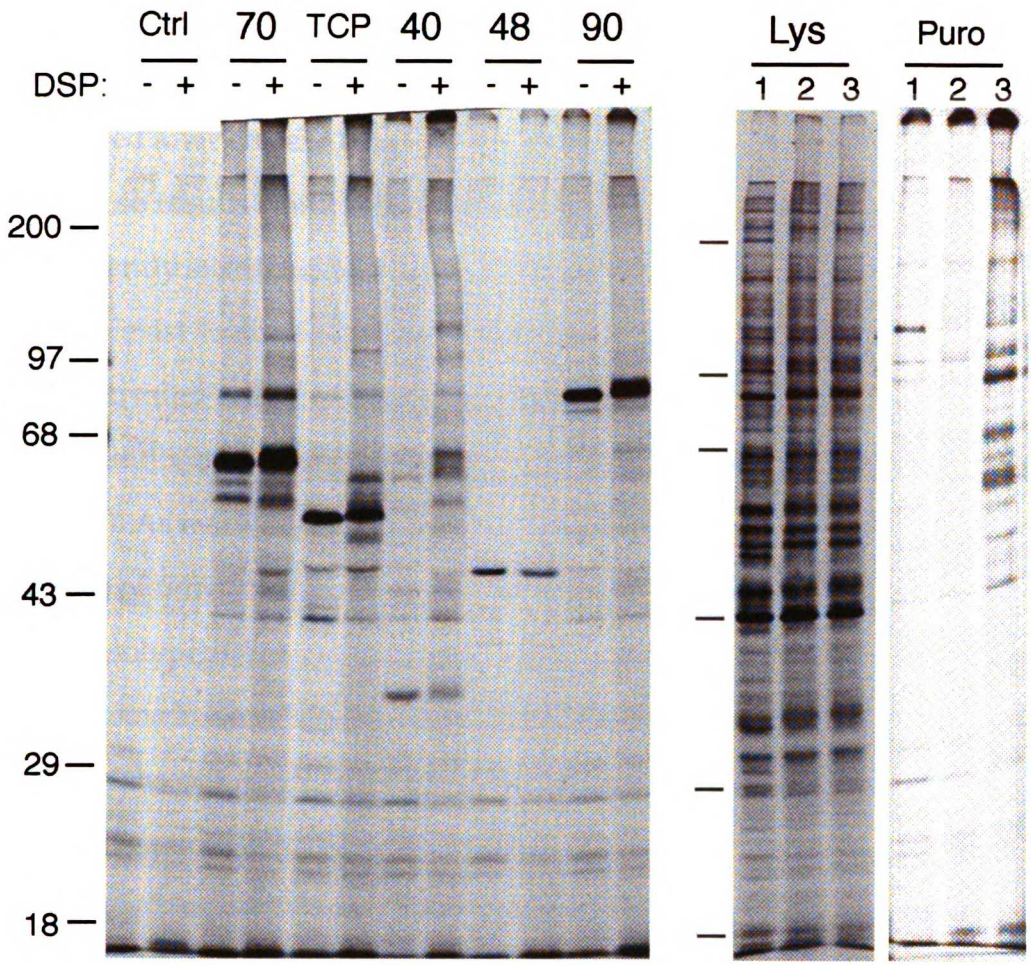

Figure 28 Ability to Precipitate Selected Chaperones after Crosslinking in Vivo

Three plates of HeLa cells were labeled overnight, chased without label for 5 hours, and treated as follows: (1) uncrosslinked lysate, no DSP; (2) crosslinked with 2 mM DSP; (3) 10 minutes with puromycin before crosslinking, no cycloheximide until DSP incubation. See Materials & Methods for important details of crosslinking experiments. Lysates (1) and (2) were used for immunoprecipitation of chaperones under denaturing conditions in the absence (-) or presence (+) of DSP, respectively. Antibodies employed: **Ctrl** = control precipitation with secondary antibodies only; **70** = hsp70 cocktail plus N27; **TCP** = mixture to α-subunit of CCT; **40** = rabbit polyclonal to hsp40; **48** = mixture to p48/Hip; **90** = hsp90 mixture including 16F1, 9D2, and rabbit polyclonal. **Puro** = immunoprecipitations with the puromycin antibody using lysates (**Lys**) 1-3, respectively. Note that the stacking portion of the gel was left intact to monitor the amount of protein that was unable to enter the gel following reversal of the crosslinks.

predominant (compare TCP lanes, -/+ DSP). When an antibody for the γ-subunit of CCT was employed (P5 antibody), the other subunits again were coprecipitated after crosslinking but the γ-subunit was predominant (data not shown). These results could mean that not all the heteromeric CCT molecules were sufficiently stabilized by crosslinking, or that homomeric particles of one subunit also exist *in vivo*. When the crosslinking approach was applied to puromycin-treated cells and analyzed by immunoprecipitation with the puromycin antibody, the precipitate looked much like the total lysate (Fig. 28, Puro, lane 3). As mentioned previously, such a result could be due to nonspecific crosslinking of unreacted puromycin. On the other hand, it is possible that puromycyl-polypeptides are never completely "shielded" from contacting other cellular proteins because chaperone interactions are transient and dynamic.

When the same crosslinking analysis was performed on pulse-labeled cells, antibodies to hsp70 were found to be the most efficient in coprecipitating the radiolabeled nascent chains (two experiments shown in **Fig. 29**). The crosslinking of pulse-labeled nascent polypeptides to each chaperone was quantified by liquid scintillation under conditions of excess antibody for five independent experiments. The relative counts obtained with each chaperone-specific antibody was found to fluctuate, but the general order of chaperones ranked in terms of highest to lowest coprecipitation of nascent polypeptides was the same:

hsp70 > hsp90, CCT > hsp40 >> p48.

The crosslinking results indicate that hsp70 and hsp90 may bind to the general population of nascent polypeptides in a cotranslational manner. Note in Fig. 29B, however, that coprecipitation of nascent polypeptides with hsp90 was not remarkable when compared to the control sample in every experiment.

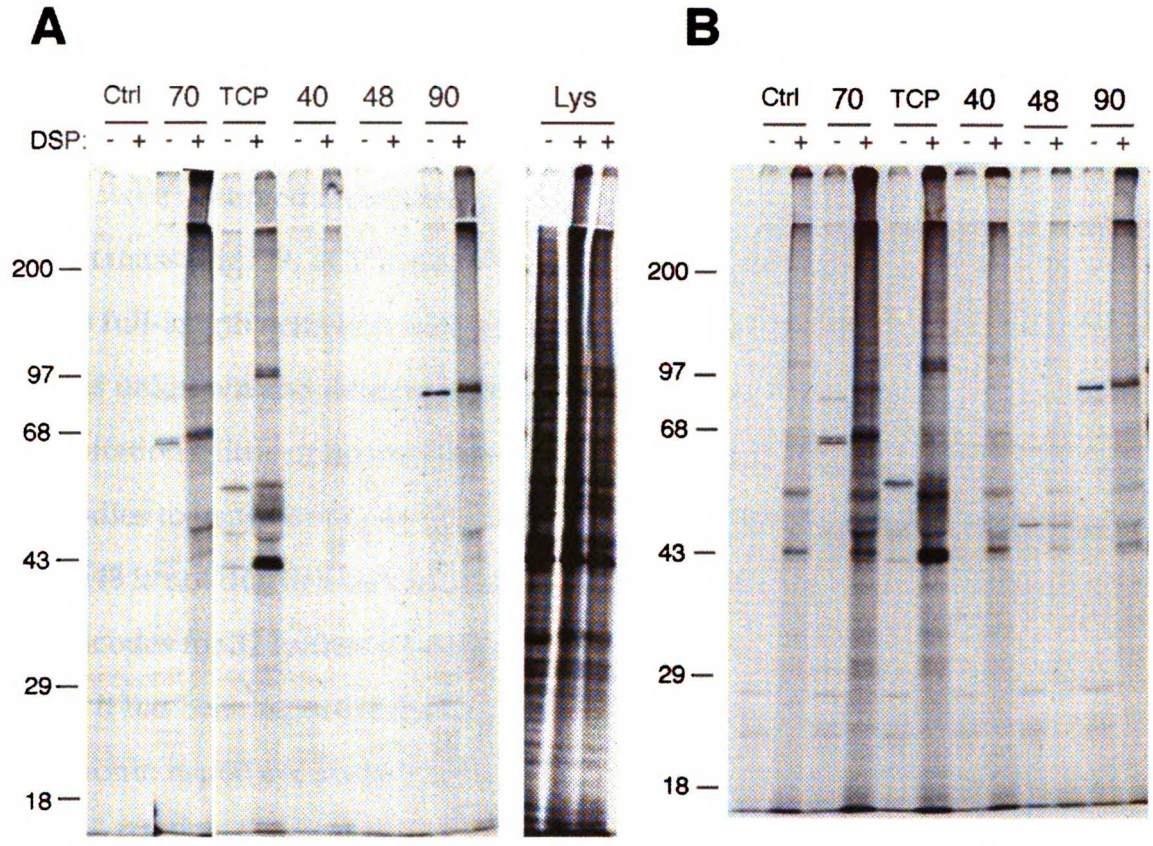

Figure 29 Ability to Coprecipitate Nascent Polypeptides with Selected Chaperones after Crosslinking in Vivo

Two plates of cells were pulse labeled for just 5 minutes before addition of cycloheximide. One plate was used as the uncrosslinked lysate (-DSP), and the other was crosslinked as described in Materials & Methods (+DSP).
(**A**) Immunoprecipitations using the same antibodies to the same chaperones indicated in Fig. 28. The sample in the lane corresponding to the first crosslinked lysate (Lys +) was not reduced before loading onto the gel; the second (+) lysate was reduced under the same conditions used for the immunoprecipitates.
(**B**) An independent experiment using two new plates of cells and following the exact same protocol as in (A). Note that the background precipitation (control, +DSP) was higher in experiment (B) than in (A).

Coprecipitation with hsp90 was not affected by treating the cells with 2 μg/ml of herbimycin A for 60 minutes before crosslinking (data not shown). In the case of CCT, the majority of the coprecipitating polypeptides did not appear as a smear of random truncated polypeptides by SDS-PAGE. On the contrary, discrete bands were observed representing proteins of approximately 45, 50-55, and 100 kDa in mass (Fig. 29, TCP lanes, +DSP). The two smaller proteins correspond in size to full-length actin and tubulin (see section 3.17). The nature of the 100 kD band is unknown and deserves further investigation. When compared to control precipitations, little or no radioactive material was observed to coprecipitate with antibodies to hsp40 or to p48 (Fig. 29). The absence of any proteins in complex with p48 is not due to a lack of DSP-reactive residues since the DNA sequence of p48 encodes for 32 lysines (Prapapanich *et al.*, 1996).

It has been reported that some complexes with the mitochondrial chaperonin hsp60 are dissociated in the presence of Triton X-100 (Rospert and Hallberg, 1995). A similar sensitivity to detergent may explain why complexes between hsp90 and nascent polypeptides were detected by immunoprecipitation after crosslinking but never in the absence of crosslinking.

3.16. Hsp70 Depletion Experiments

Since hsp70 was found to be the predominant chaperone bound to puromycyl-polypeptides, the functional significance of this interaction was investigated by depletion of hsp70 in whole lysates. Presumably the binding of hsp70 helps to maintain the solubility of unstably-folded nascent polypeptides by preventing "off-pathway" folding events, including irreversible aggregation with other macromolecules. Experimentally, off-pathway folding events might be realized

as reduced activity, reduced solubility, or increased binding of other (nonspecific) proteins to the nascent polypeptides.

In the case of puromycyl-polypeptides synthesized *in vivo*, it should be possible to detect changes in solubility or nonspecific binding following depletion of hsp70 from the cell lysate. However, the solubility of puromycyl-polypeptides may be difficult to interpret after immunodepletion of hsp70 because those polypeptides of marginal solubility will likely be removed with the Sepharose-bound antibodies during the low-speed centrifugation step. In one experiment with pulse-labeled puromycyl-polypeptides, the Sepharose pellets were removed in the microcentrifuge at a low speed, and the resulting supernatant was given a second spin at the maximum speed. No pellet was visible in the hsp70-depleted sample, and no difference was detected (relative to undepleted samples) in the amount of puromycyl-polypeptides that remained in the supernatant as quantified by immunoprecipitation with the puromycin antibody (data not shown). One possible flaw with this experiment is that chaperone interactions may not be critical for the solubility of nascent polypeptides in the presence of 0.1% Triton. As discussed in section 3.11, detergent is a necessary reagent for studying puromycyl-polypeptides *in vitro*.

The effect of hsp70 depletion on the extent of nonspecific binding to puromycyl-polypeptides was examined using steady-state labeled lysates (**Fig. 30**). Puromycyl-polypeptides were immunoprecipitated from three different lysates: (1) a lysate depleted of hsp70 in the presence of ATP; (2) an undepleted control lysate made in the presence of ATP; and (3) an undepleted lysate made in the presence of apyrase to stabilize hsp70 interactions. Since nonspecific binding has been shown already to be a concentration-dependent phenomenon (Fig. 19A), nonspecific binding was compared in the hsp70-depleted and undepleted lysates at two different dilutions. It should be noted that the capture

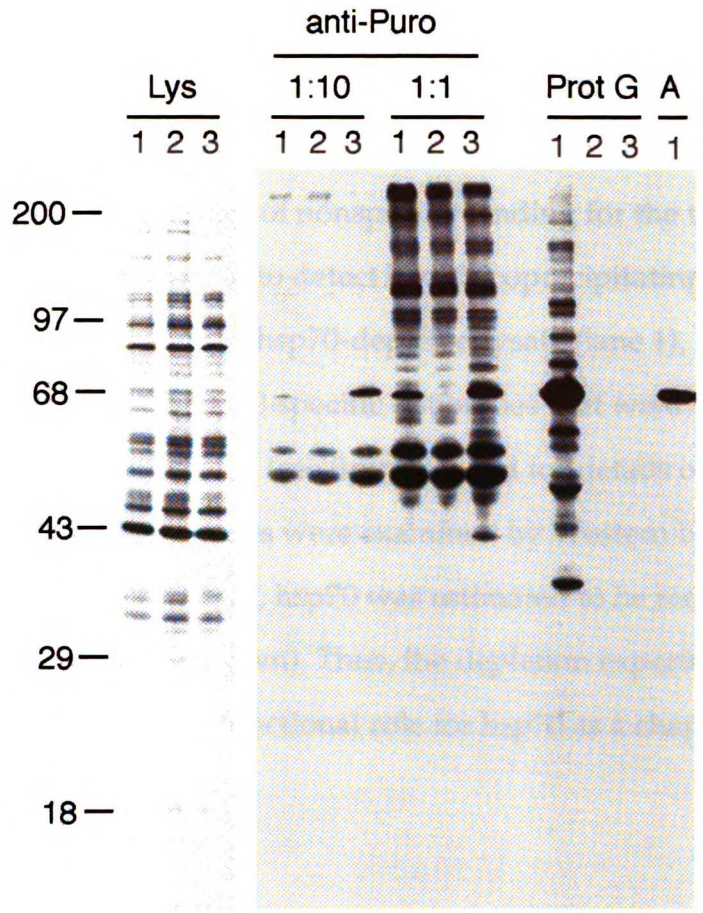

Figure 30 No Change Detected in the Extent of Nonspecific Protein Binding to Puromycyl-polypeptides upon Depletion of Hsp70

Three plates of cells were labeled overnight, chased for 4 hours in the absence of label, and treated with 2 μM puromycin for 15 minutes. The cells in plates (1) & (2) were lysed in the presence of 10 mM MgATP, whereas plate (3) was lysed with apyrase (-ATP). The usual cocktail of antibodies to hsp70 was incubated overnight with Protein G Sepharose which had been preadsorbed with 1% BSA in buffer A + 0.1% Triton to reduce nonspecific binding. The Sepharose-bound antibody was washed with buffer A + 0.1% Triton before addition to lysate (1). The other two lysates were incubated with Protein G Sepharose having no bound antibody, and all three samples were rotated for 30 minutes at 4°C. Protein G was removed by low-speed centrifugation, and the supernatants were incubated another 30 minutes with Protein A Sepharose to help remove residual hsp70 antibodies. The final supernatants were analyzed by immunoprecipitation under nondenaturing conditions with the antibody to puromycin. A volume of 100 μl of each lysate was diluted (1:10) in buffer A + 0.1% Triton, or used directly for analysis without dilution (1:1). Shown are the starting lysates (**Lys**), the corresponding immunoprecipitations (**anti-Puro**), and the corresponding Sepharose pellets prior to immunoprecipitation of the puromycyl-polypeptides (**Prot G** or **A**).

of puromycyl-polypeptides was two times greater at the 1:1 dilution compared to the 1:10 dilution, as determined in a parallel experiment with pulse-labeled lysates treated in an identical manner. For a given dilution, there was no observed difference in the amount of nonspecific binding for the three lysates (Fig. 30). Although it was possible to detect hsp70 "coprecipitating" with the puromycyl-polypeptides from the hsp70-depleted lysate (lane 1), this hsp70 was likely captured with residual hsp70-specific antibodies that were inadequately removed during the depletion step (see figure legend for details of depletion experiment). When the three lysates were examined by Western blot analysis with the monoclonal antibody N27, hsp70 was estimated to be reduced 90% in the depleted sample (data not shown). Thus, the depletion experiments were unsuccessful in elucidating any functional role for hsp70 as a chaperone for puromycyl-polypeptides.

3.17. Identification of Newly-synthesized Proteins with CCT

In earlier experiments, antibodies to the cytosolic chaperonin CCT were found to coprecipitate a number of newly-synthesized polypeptides following crosslinking (Fig. 29, TCP lanes). To identify these newly-synthesized proteins, similar immunoprecipitates were analyzed by 2-D gel electrophoresis (**Fig. 31**). For these experiments, two plates of HeLa cells were labeled overnight and chased for 4.5 hours. One plate was harvested immediately. Culture medium containing fresh ^{35}S-methionine was added to the other plate, and newly-synthesized proteins were labeled for 15 minutes before lysing the cells. Immunoprecipitation reactions were performed under nondenaturing conditions using the antibody to the γ-subunit of CCT. From the steady-state labeled cells, only the subunits comprising the cytosolic chaperonin were precipitated

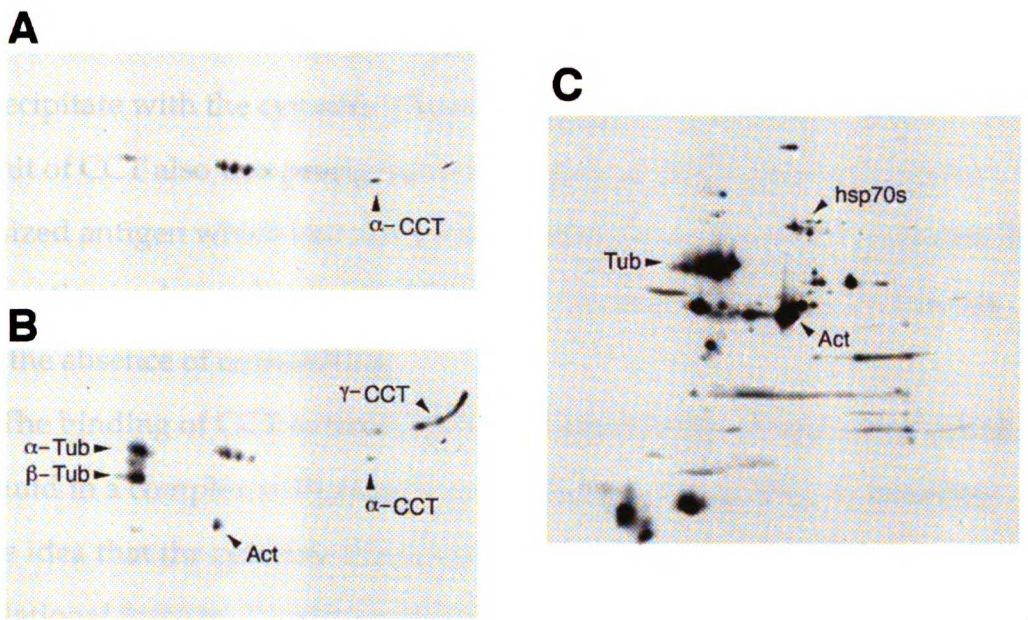

Figure 31 Coprecipitation of Newly-synthesized (Full-length) Actin and Tubulin with CCT

For A and B, two plates of cells were labeled overnight and chased for 4.5 hours in the absence of label. One plate was lysed immediately in the usual buffer (0.1% Triton and apyrase). The other plate was pulse labeled for 15 minutes just prior to lysis. Both lysates were diluted 1:20 and analyzed by immunoprecipitation under nondenaturing conditions with the P5 antibody to the γ-subunit of CCT. Shown are the resulting immunoprecipitates after 2-D gel electrophoresis. (**A**) Immunoprecipitation of CCT from the steady-state labeled lysate, no pulse. (**B**) Immunoprecipitation of CCT from the lysate that received a subsequent 15-minute pulse of label. (**C**) Two-dimensional profile of isolated polysomes from HeLa cells which were steady-state labeled before hypotonic lysis (see Materials and Methods for details). Note that most of the basic ribosomal proteins do not focus in the pH range used for the first dimension of the 2-D gel.

(Fig. 31A). Those subunits of CCT having more basic isoelectric points did not focus well in the pH range used for the first dimension. Only a trace amount of mature tubulin was observed to coprecipitate with CCT under these conditions. From those cells provided the second 15-minute labeling period, however, discernible amounts of newly synthesized actin and (α,β)–tubulin were observed to coprecipitate with the cytosolic chaperonin (Fig. 31B). As expected, more of the γ-subunit of CCT also was precipitated, consistent with the capture of newly-synthesized antigen which had not yet assembled into mature CCT particles. The newly-synthesized protein of ~100 kD which was observed in Fig. 29 was not seen in the absence of crosslinking.

The binding of CCT to full-length actin and tubulin, along with the lack of CCT found in a complex with puromycin-released polypeptides, is consistent with the idea that the cytosolic chaperonin does not bind to its substrates in a cotranslational fashion. To address this issue directly, polysomes were isolated by velocity sedimentation and examined for the presence of co-sedimenting chaperones. As expected, most of the basic ribosomal proteins were not resolved when analyzed by an equilibrium 2-D gel using ampholines in a slightly acidic pH range (Fig. 31C). (A typical one-dimensional SDS-PAGE profile of isolated polysomes was shown in Fig. 2.) Proteins which fractionated with the polysomes included the cytosolic forms of hsp70 and a number of cytoskeletal proteins. The different protein constituents of CCT were not apparent within the isolated polysomes, suggesting that the cytosolic chaperonin does not interact with nascent polypeptides during their synthesis. This is contrary to results obtained with reticulocyte lysate (Frydman *et al.*, 1994).

3.18. Aggregation of Puromycyl-polypeptides in Vivo upon Heat Shock

Since molecular chaperones are believed to play an important role in cell survival during periods of physical or metabolic stress, HeLa cells were subjected to heat shock to examine the effects of thermal stress on the interaction between hsp70 and puromycyl-polypeptides. Pulse-labeled plates of cells were harvested after different times of incubation at 45 °C. The soluble and insoluble proteins from each lysate were analyzed by immunoprecipitation to determine the distribution of puromycyl-polypeptides (**Fig. 32**). The fraction of puromycyl-polypeptides in the insoluble pellet increased upon heat shock treatment in a time-dependent manner such that nearly 50% of the puromycyl-polypeptides had precipitated after just 30 minutes. This is noteworthy since other investigators have stated that the precipitation of mature proteins after stress (including chaperones) is unlikely to result from interactions with nascent polypeptides (Kabakov and Gabai, 1994). Cycloheximide was routinely added to cells during heat shock experiments to avoid further synthesis of puromycyl-polypeptides during the chase at 45 °C. The use of cycloheximide did not appear to affect the final results as demonstrated in Fig. 32B (compare 30 minute time points for shaded bars and hatched bars).

In one variation of the heat shock experiment, the cells were lysed in the presence of low detergent (0.1% Triton), and the resulting pellet was extracted in high detergent (1% Triton + 1% DOC). In this case, the distribution of puromycyl-polypeptides was quantified for all three "fractions": the supernatant, the detergent-solubilized pellet, and the insoluble pellet (**Fig. 33A**). The sum of the two pellet fractions increased with time of heat shock, but the main contribution to the increase was observed to originate from the insoluble pellet. An identical experiment was performed with steady-state labeled cells and

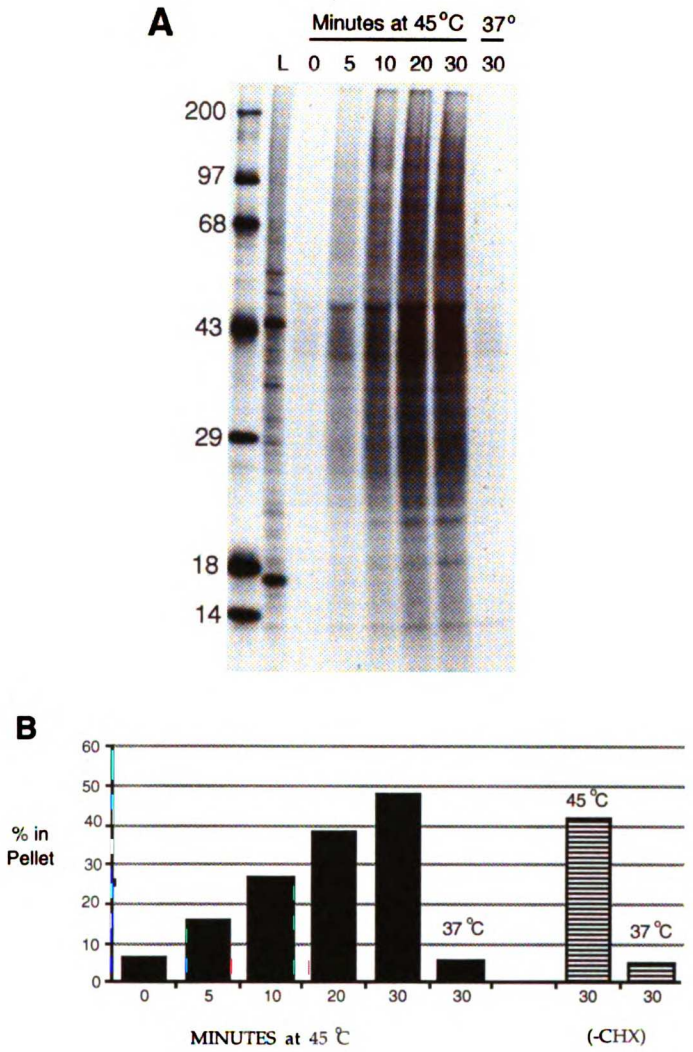

Figure 32 Rapid Aggregation of Puromycyl-polypeptides upon Heat Shock at 45 °C

Six plates of HeLa cells were labeled for 15 minutes in the simultaneous presence of 2 μM puromycin. The medium was supplemented with 0.5 mM CHX for 2 min before replacing the medium with fresh DMEM + 0.1 mM CHX. One plate was harvested immediately (t=0). Four plates were placed in contact with a 45 °C water bath for the indicated times. The last plate was left at 37 °C for 30 min. Plates were not washed with PBS prior to harvest to avoid loss of detached cells. All cells were lysed in buffer A + 1% Triton + 1% DOC (no apyrase). Insoluble proteins were separated in a microcentrifuge at maximum speed for 20 min. The pellet was boiled in 2x Laemmli sample buffer. The puromycyl-polypeptides in the supernatant and pellet were immunoprecipitated with the antibody to puromycin under denaturing conditions. (**A**) Autoradiograph of the insoluble puromycyl-polypeptides which were immunoprecipitated from the pellets. L= total pellet from lysate at t=0. (**B**) Aggregation of puromycyl-polypeptides was quantified by liquid scintillation of the immunoprecipitates. Shown is the amount of insoluble polypeptides as a percent of the total from each plate (supernatant + pellet). The two data points at the far right were obtained under identical conditions as in (A), except that cycloheximide treatment was omitted (-CHX).

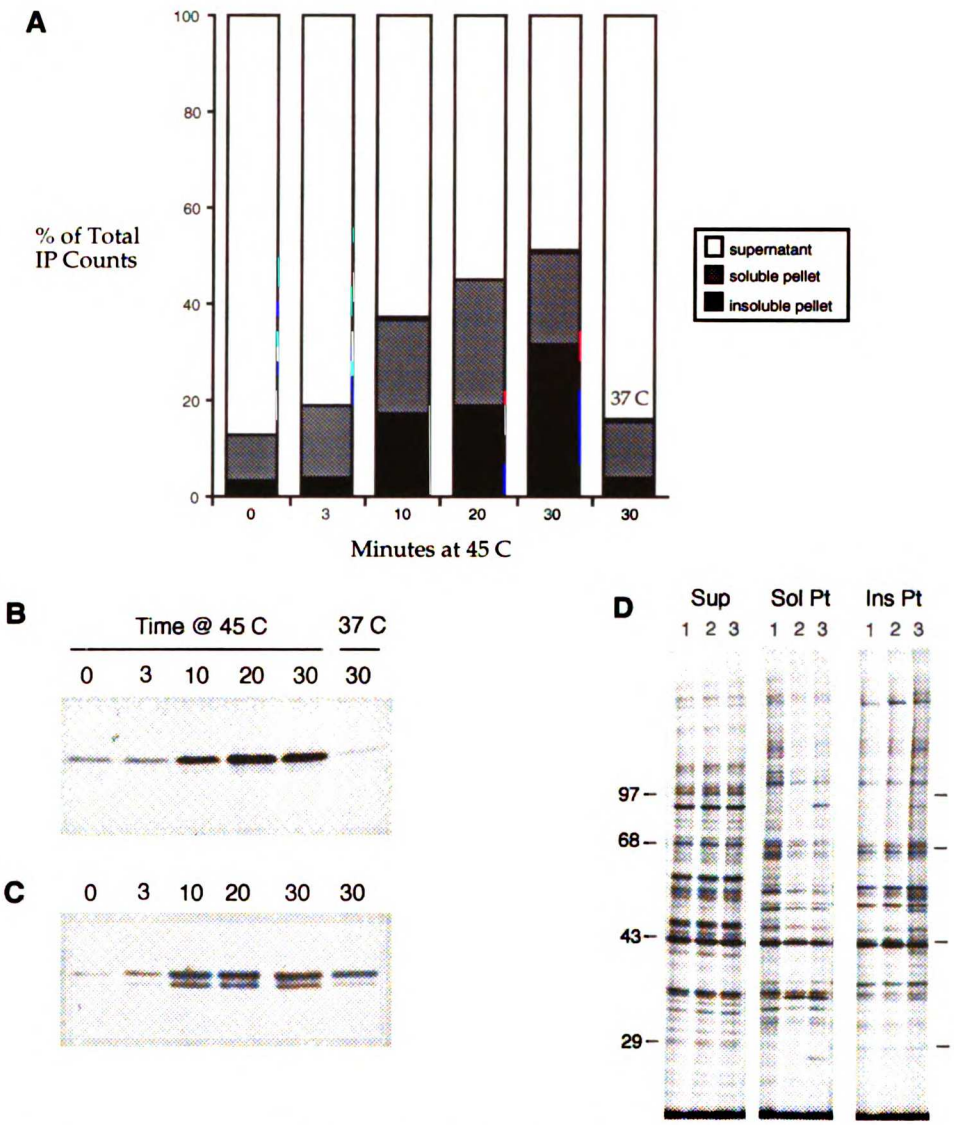

Figure 33 Precipitation of Hsp70 and Puromycyl-polypeptides upon Heat Shock at 45 °C

Six plates of cells were pulse-labeled and treated with puromycin before heat shock treatment as in Fig. 32. In this experiment, however, the cells were lysed in buffer A + 0.1% Triton with apyrase. After centrifugation, the initial pellet was resuspended in buffer A + 1% Triton + 1% DOC followed by a second spin in the microcentrifuge. The detergent-extracted proteins were designated as the "soluble pellet" and the remaining protein as the "insoluble pellet". The amount of puromycyl-polypeptides in each fraction of each plate was quantified by immunoprecipitation and liquid scintillation as described in Fig. 32. (**A**) Bar chart showing distribution of puromycyl-polypeptides relative to the total counts obtained for each plate. (**B**) Immunoprecipitation of hsp70 from the insoluble pellets of a parallel experiment in which the cells were labeled to steady-state before puromycin treatment and heat shock as in (A). (**C**) Western blot analysis of hsp70 in the insoluble pellets of (A). N27 was used as the primary antibody (1:1000). (**D**) Autoradiograph of the total proteins in each fraction after heat shock at 45 °C. The cells in this experiment were steady-state labeled as in (B). **Sup** = initial supernatant; **Sol Pt** = detergent-soluble pellet; **Ins Pt** = insoluble pellet. Time points: (1) t = 0; (2) t = 10 min; (3) t = 60 min.

analyzed by immunoprecipitation of hsp70 under denaturing conditions. As seen with puromycyl-polypeptides, a time-dependent increase in hsp70 was observed in the insoluble pellets (**Fig. 33B**). This result was confirmed by western blot analysis of hsp70 in the insoluble pellets (**Fig. 33C**). It was not possible to determine whether hsp70 was precipitating due to its interaction with puromycyl-polypeptides because protein-protein interactions were disrupted during solubilization of the pellet (heating in SDS). All of the proteins in the three fractions were examined by SDS-PAGE using steady-state labeled cells (**Fig. 33D**). No significant change in the identity of insoluble proteins was observed between 0, 10, and 60 minutes of heat shock (*i.e.*, no difference in the pattern of protein bands in the insoluble pellet). The use of the puromycin antibody in conjunction with pulse-labeled cells may be a superior method for quantifying the effect of cellular stress on protein solubility (as opposed to measuring increases in total protein aggregation or aggregation of one specific protein).

One of the presumed functions of molecular chaperones is to maintain the solubility of marginally-stable and/or unfolded proteins. If so, overexpression of molecular chaperones should mitigate the observed aggregation of puromycyl-polypeptides upon heat shock. With this in mind, HeLa cells were "pre-stressed" for 90 minutes at 43 °C to induce higher expression of hsp72 and other heat shock proteins (Beckmann *et al.*, 1992). After recovery under normal growth conditions, these "thermotolerant" cells were treated with puromycin and subjected to a more severe heat shock at 45 °C, as done in the previous heat shock experiments. Surprisingly, the time course of aggregation of puromycyl-polypeptides was no different for thermotolerant cells than for control cells (**Fig. 34**). In the same study, some plates of cells were depleted of ATP *in vivo* by addition of the mitochondrial uncoupler CCCP and nonmetabolizable 2DG, 2-

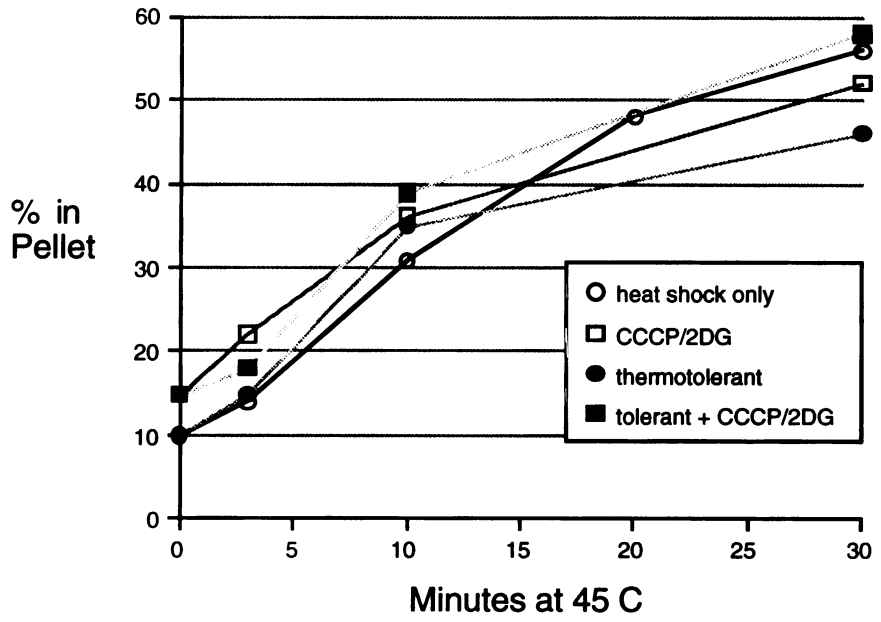

Figure 34 Aggregation of Puromycyl-polypeptides in Thermotolerant Cells and in Cells Depleted of ATP prior to Heat Shock

Half of the culture plates were made thermotolerant by incubating the cells at 43 °C for 90 minutes followed by a recovery period overnight at 37 °C. All plates were then pulse-labeled and treated with puromycin as described in Fig. 32. In addition, some plates were depleted of ATP after puromycin treatment by addition of 20 µM CCCP and 12.5 mM 2DG for 5 min in glucose-free DMEM (see Beckmann et al., JCB, 1992). Next the cells were heat shocked at 45 °C for the indicated times. Lysates were made in buffer A + 0.1% Triton. The "pellet" in this experiment corresponds to the sum of the "soluble pellet" + "insoluble pellet" of Fig. 33 (no second extraction in high detergent). The insoluble puromycyl-polypeptides were quantified by immunoprecipitation and liquid scintillation as described previously.

deoxyglucose (Gronostajski *et al.*, 1985). The purpose of ATP depletion was to try and stabilize the interaction (slow down the dynamics) between hsp70 and puromycyl-polypeptides which might influence the extent of aggregation. Once again, however, addition of CCCP/2DG had no significant effect on the aggregation of puromycyl-polypeptides at 45 °C (Fig. 34).

None of the heat shock results support the hypothesized role of molecular chaperones in reducing aggregation of nascent or mature proteins. Clearly, further studies are required to discern the functional significance of protein interactions with molecular chaperones. Perhaps puromycyl-polypeptides are not a good model for stress experiments because these truncated polypeptides may aggregate in a nonreversible manner, whereas mature full-length proteins may be "resurrected" following aggregation. Still, it is confusing that there is an increase in insoluble hsp70 during heat shock and that no enhanced interaction is observed between hsp70 and soluble proteins after heat shock (this work and Beckmann *et al.*, 1992).

4. SUMMARY OF RESULTS & CONCLUDING REMARKS

Previous work in the field of molecular chaperones has led to the hypothesis that folding and maturation of newly-synthesized proteins is facilitated by the cooperative interaction of multiple chaperone classes including the hsp40, hsp70, and hsp60 families. In this thesis, complexes between nascent polypeptides and chaperones were studied in a mammalian cell line using a combination of metabolic labeling and immunoprecipitation techniques. A polyclonal antibody to puromycin was found to be an exceptional tool for analyzing protein interactions with nascent polypeptides. The use of puromycin differs from other approaches used to study the binding of proteins to nascent polypeptides in two significant ways: (1) nascent puromycyl-polypeptides are a population of randomly truncated polypeptides obtained from all the proteins being synthesized in the cell, and, therefore, the resulting complexes with puromycyl-polypeptides should represent an average of all the chaperone interactions that occur *in vivo*; (2) the puromycin antibody makes it possible to specifically isolate nascent polypeptides without any experimental bias as to the identity of the bound chaperones.

It was anticipated that immunoprecipitation with the puromycin antibody might lead to the discovery of new chaperones that had not been implicated previously in the process of protein folding and maturation. Instead, the well-known members of the hsp70 family were found to be the predominant species that coprecipitated with puromycyl-polypeptides (Fig. 16). The higher degree of hsp70 binding to puromycyl-polypeptides relative to other purported chaperones was corroborated by the more traditional approach of using chaperone-specific antibodies to coprecipitate the nascent polypeptides (Fig. 18).

Antibodies to hsp70 also coprecipitated the most nascent polypeptides following crosslinking *in vivo* without the use of puromycin (Fig. 29). In addition, hsp90 was found to interact with nascent polypeptides following crosslinking. It seems probable that hsp90 interactions are disrupted by detergent (in the absence of crosslinking) which would explain why hsp90 has not been reported previously in such a complex by immunoprecipitation analysis.

Another key finding of this work was the fact that nascent polypeptides have a strong tendency for aggregation and for nonspecific binding to proteins in whole cell lysates. The dilution experiment of Fig. 19A highlights one advantage of using cell culture to metabolically label all the mature proteins prior to cell lysis and immunoprecipitation; nonspecific binding at high protein concentrations probably has gone unnoticed in studies where the results were obtained by western blot analysis or by employing *in vitro* translation since these methods generally are used for the detection of a single protein (or products of a single mRNA). Not surprisingly, protein constituents of the cytoskeleton were among the most prominent proteins found to bind puromycyl-polypeptides in a nonspecific manner (esp. actin and tubulin). It is easy to imagine an unstably-folded nascent polypeptide becoming physically adsorbed onto or entangled with fragments of the cytoskeleton upon cell lysis. The observation that high concentrations of RC-BSA, casein, or unlabeled HeLa proteins in the lysis buffer could intercept the binding of these structural proteins (without affecting the binding of hsp70) strongly supports the conclusion that nascent polypeptides bind to cytoskeletal proteins as an artifact of cell disruption (Fig. 25A). It also was shown that nascent polypeptides themselves may associate with "chaperone traps" like RC-BSA (Fig. 26). An interaction between denatured full-length model proteins and permanently-unfolded chaperone traps (RC-BSA, RCMLA, etc.) may not have been considered in previously reported refolding experiments

which included molecular chaperones (Langer *et al.*, 1992; Szabo *et al.*, 1994; Yonehara *et al.*, 1996). Refolding experiments performed in the presence of whole lysates also may be subject to some nonspecific protein interactions which might be difficult to distinguish from the relevant interactions that occur *in vivo* (Schumacher *et al.*, 1994; Frydman and Hartl, 1996; Tian *et al.*, 1996; Melki *et al*, 1997).

Upon first examination, the coprecipitation of chaperones from the ER (BiP) and mitochondria (grp75 and hsp60) with puromycyl-polypeptides might be used as evidence of cotranslational translocation into these organelles (Fig. 16A). It seems likely, however, that these three proteins are binding to cytosolic nascent chains only upon their liberation from the organelles during detergent lysis. As observed with subunits of the cytoskeleton, coprecipitation of BiP and grp75 with puromycyl-polypeptides is reduced in the presence of other unfolded protein traps. Casein was especially effective in reducing the capture of BiP (Fig. 25A, lane 4), and both casein and RC-BSA abolished the capture of grp75 (not easily discerned by SDS-PAGE).

The puromycin antibody was used to characterize some of the physical properties of nascent polypeptides in addition to identifying molecular chaperones. The native size distribution of puromycyl-polypeptides was found to be large and heterogenous by both sizing chromatography (Fig. 5) and velocity sedimentation (Fig. 10). Although previous investigators have pointed to the large native size of nascent polypeptides as evidence of complexes with molecular chaperones (Frydman *et al.*, 1994; Hansen *et al.*, 1994), this large native size may be totally dominated by nonspecific interactions and aggregation problems. In support of this hypothesis, the sedimentation profile of puromycyl-polypeptides was unaffected by the addition of ATP, a condition which should promote release of the major chaperones, including hsp70 (Fig. 12). Regardless of

the contribution of nonspecific binding, nearly all the native complexes formed with puromycyl-polypeptides are smaller than the reported native size of CCT, both by sizing chromatography (900 kD) and sedimentation (20 S) (Lewis *et al.*, 1992; Yaffe *et al.*, 1992). This is consistent with the lack of CCT observed in complexes with puromycyl-polypeptides. Since the chromatography and sedimentation fractions were analyzed for pulse-labeled puromycyl-polypeptides under denaturing conditions, one can not argue that CCT-bound complexes were missed due to sequestration of the antigen inside the central cavity of CCT.

Although puromycyl-polypeptides exhibited an ATP-dependent sensitivity to exogenous proteases (Fig. 13 & 14), this result also could be due to nonspecific interactions with nucleotide-binding cytoskeletal proteins. The enzyme apyrase hydolyzes all the nucleotides in the lysate (not just ATP) which likely affects the properties of tubulin (a GTPase) as well as actin (an ATPase). In regard to this point, myosin heavy chain was often found to coprecipitate nonspecifically with puromycyl-polypeptides from ATP-depleted lysates (200 kD band), but was undetectable in immunoprecipitates from ATP-supplemented lysates. ATP also affected the cosedimentation of actin and myosin with isolated polysomes (Fig. 2 & 24B, Panel III).

A major criticism of using puromycyl-polypeptides as a model of nascent polypeptides is that one can not determine whether the observed protein interactions (*i.e.*, binding of chaperones) were initiated before or after release from the ribosome. In order to focus on this issue, cycloheximide-stabilized polysomes were isolated by sedimentation, the nascent polypeptides were released with puromycin *in vitro*, and the antibody to puromycin was added to try and capture the resulting complexes. Unfortunately, this approach was hindered by a number of complications: (1) drugs like cycloheximide, which

stabilize polysomes during isolation by inhibiting the translocation step of translation elongation, may also inhibit the puromycin reaction at low (μM) puromycin concentrations (Baliga *et al.*, 1970); (2) at higher (mM) concentrations of puromycin, it was difficult to remove the unreacted puromycin by any method prior to immunoprecipitation, possibly due to hydrophobic interactions between puromycin and the unstably-folded nascent polypeptides; (3) nascent polypeptides were highly aggregation-prone upon release from isolated polysomes (Fig. 20), and addition of detergents to enhance the solubility of released polypeptides also resulted in stripping some of the hsp70 from the ribosome, independent of puromycin addition. Furthermore, it was found that antibodies to α-tubulin were just as efficient as antibodies to hsp70 for coprecipitating ribosomes and their associated nascent polypeptides (Fig. 24). The strongest evidence of cotranslational binding of chaperones was obtained by the crosslinking approach in the absence of puromycin treatment (Fig. 29).

The inability to coprecipitate hsp70 with puromycyl-polypeptides in the presence of ATP (Fig. 25 & 27) or in the presence of ADP (Fig. 27) suggest that the interaction of hsp70 with nascent polypeptides is highly dynamic under physiological conditions. Studies with bacterial DnaK indicate that the kinetic rate constants for both binding and release of model peptide ligands are higher when this hsp70 homolog is in the ATP-bound conformation (Schmid *et al.*, 1994; McCarty *et al.*, 1995; Theyssen *et al.*, 1996). Thus, it is possible that hsp70 functions as a chaperone *in vivo* through multiple rounds of binding and release with nascent polypeptides while still bound to the same molecule of ATP. Multiple binding events have been observed for the bacterial chaperonin, GroEL, with some of its model substrates (Todd *et al.*, 1994; Weissman *et al.*, 1994). Importantly, hsp70 could be crosslinked to nascent polypeptides *in vivo* without manipulating the endogenous levels of nucleotides (Fig. 29). At this time, it is not

clear whether ATP hydrolysis is a prerequisite for the chaperone function of hsp70 *in vivo*. It has been demonstrated that the binding and release of DnaK is too fast to be coupled stoichiometrically to its measured ATPase activity (Schmid *et al.*, 1994). Furthermore, it has been reported that hsp70 can protect cells from thermal stress after deletion of its ATP-binding domain (Li *et al.*, 1992). Perhaps stable binding to the hsp70 family as a result of ATP hydrolysis is reserved for specific ligands (*e.g.* translocating polypeptides) or special circumstances (*e.g.* cellular stress). For studying ATP-dependent chaperone interactions with nascent polypeptides, the use of puromycyl-polypeptides generated *in vivo* may be more appropriate than the use of actin and luciferase, two proteins which themselves bind ATP. As shown in Fig. 14B, mature actin becomes degraded by trypsin following apyrase treatment, indicating that the conformational stability of actin has a strong nucleotide dependence. Using native gel analysis, nucleotide-dependent conformations also have been observed for firefly luciferase (W.J. Hansen, unpublished results).

Lysing cells in the presence of AMP or apyrase (but not ADP) resulted in similar coprecipitations of hsp70 with puromycyl-polypeptides (Fig. 27). Little has been reported about the conformation of hsp70 when bound to AMP or no nucleotide at all, but one might speculate that AMP induces oligomerization of hsp70. This would be consistent with the observation that apyrase treatment or AMP reduced the solubility of puromycyl-polypeptides in whole lysates relative to ATP or ADP (Fig. 21). It has been reported that AMP (or no nucleotide) addition does not allow refolding of β-galactosidase in the presence of hsp70 and hdj1 (Freeman and Morimoto, 1996). Addition of ADP, on the other hand, was about 67% as efficient as ATP in the β-gal refolding assay.

The purported chaperones hsp40 and p48 (Hip) were not observed to bind to nascent polypeptides by any approach in this thesis. Although these two

proteins have been shown to affect the ATP hydrolysis cycle of hsp73 (Höhfeld *et al.*, 1995; Minami *et al.*, 1996), their ability to bind to nascent truncated polypeptides has been demonstrated following *in vitro* translation of only one protein, firefly luciferase (Frydman *et al.*, 1994; Höhfeld *et al.*, 1995). Further studies are needed to determine the cellular context in which hsp40 and p48 interact with members of the hsp70 family. If it were possible for the cytosolic forms of hsp70 to function as chaperones without hydrolyzing ATP, then cofactors which affect the ATP hydrolysis cycle of hsp70 would not be obligated to participate in complexes with nascent polypeptides. It has been established that hsp70 and p48 are bound simultaneously to certain unactivated forms of steroid hormone receptors in reticulocyte lysates (Prapapanich *et al.*, 1996). A direct interaction between these two proteins using antibodies against p48 to coprecipitate hsp70 was not confirmed in HeLa cell lysates, with or without crosslinking (Fig. 18A & 28). It should be noted that the level of p48 in rabbit reticulocytes is at least 20-fold higher than that in HeLa cells as determined by western blot analysis (W.J. Hansen, unpublished observation).

The prevailing model of protein folding *in vivo* enlists the sequential and cooperative binding of members from three different chaperone families: hsp40 (DnaJ), hsp70 (DnaK), and CCT (hsp60, GroEL). The results presented in this thesis indicate that members of the hsp70 family of molecular chaperones are the major proteins bound to nascent polypeptides in the cytosol of a mammalian cell line. The interaction of hsp70 with nascent polypeptides is apparently dynamic and may be initiated during translation. For the first time, results were obtained *in vivo* which also suggest a chaperone function for hsp90 with nascent polypeptides. Immunoprecipitation studies supported the viewpoint that CCT is a chaperone specific for newly-synthesized actin and tubulin (Fig. 31). The roles

of hsp40 and p48, as related to the maturation of nascent polypeptides, remain unclear.

Although the binding of hsp70 to puromycyl-polypeptides has been well characterized by the experiments in this thesis, the functional relevance of this interaction is still obscure. Depletion of hsp70 from cell lysates had no effect on nonspecific binding to puromycyl-polypeptides (Fig. 30). Also, increasing the levels of chaperones by mild heat shock had no effect on the aggregation of puromycyl-polypeptides *in vivo* upon a subsequent heat shock at 45 °C (Fig. 34). The upregulation of heat shock proteins often has been cited as indirect evidence for the role of hsps as molecular chaperones. Inconsistent with this theory, it has been demonstrated that no (soluble) mature proteins coprecipitate with hsp70 following heat shock (Beckmann *et al.*, 1992). On the contrary, a significant amount of hsp70 partitions to the insoluble fraction following heat shock and remains insoluble for at least 6 hours (Beckmann *et al.*, 1992). In the present work, the time-dependent aggregation of hsp70 was shown to correlate with the aggregation of puromycyl-polypeptides upon heat shock (Fig. 33).

In spite of the lack of data relating a functional significance to hsp70 binding, one may speculate that, through constant dynamic interactions, hsp70 provides a protective environment around the nascent polypeptide without impeding the folding pathway of the polypeptide and without denying access to the polypeptide by other factors (including proteins which may target the nascent polypeptide to a specific organelle). In theory, dynamic binding interactions would allow protein folding to be initiated cotranslationally, depending on the relative kinetics of protein folding, chaperone binding, and translation elongation. **Figure 35** illustrates a possible sequence of events during the folding of a hypothetical protein. As translation elongation proceeds from the N-terminus to the C-terminus, the nascent polypeptide may exist in one of many

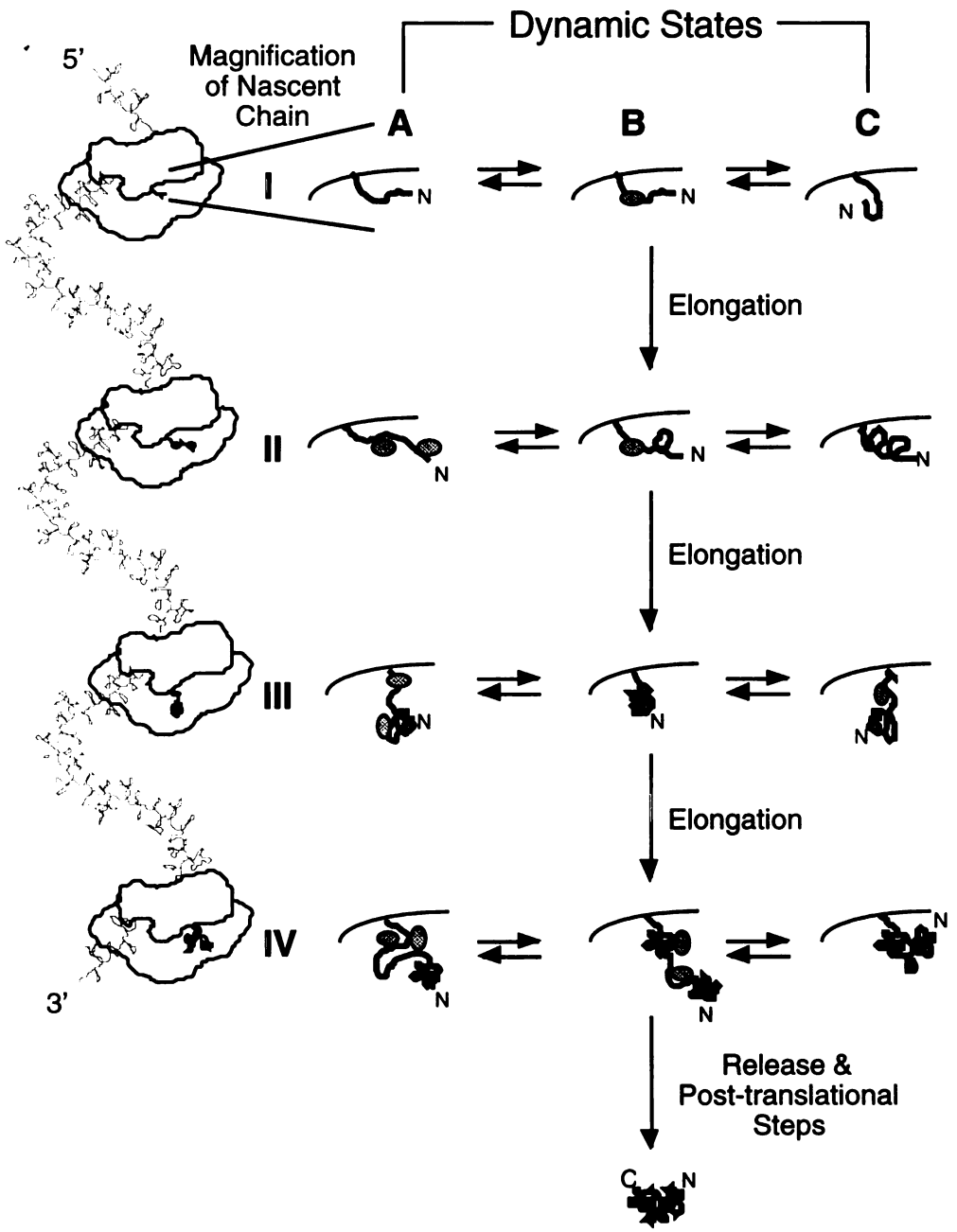

Figure 35 Cotranslational Folding of a Hypothetical Nascent Polypeptide

different "dynamic states" for each point in elongation. Three of these dynamic states (A, B, C) are shown at four stages in elongation (I-IV). Early in translation, the nascent chain may not expose enough primary sequence information to form any stable secondary structures upon leaving the ribosome (Stage I). However, chaperones may be able to recognize and to bind this unstructured sequence (Stage I, B). During protein synthesis, the nascent chain may be released and rebound by chaperones many times allowing the nascent chain to "search" for its most stable conformation. As elongation proceeds and more folding information exits the ribosome, the nascent chain may start to collapse into stable intermediates where secondary structure (Stage II) and tertiary structure (Stage III) become apparent. Late in translation, stably-folded domains may be present although chaperones are still needed to protect the hydrophobic surfaces of the domains from aggregating with its cytosolic neighbors (Stage IV).

Interestingly, it has been shown that engineered constructions of rhodanese and firefly luciferase can fold into enzymatically-active proteins while tethered from the ribosome by C-terminal extensions (Kudlicki *et al.*, 1995; Makeyev *et al.*, 1996). A possible corollary of this result is that individual domains of a multi-domain protein also fold cotranslationally (as depicted in Fig. 35) and that such vectorial folding is important for acquisition of the final structure of many proteins.

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