

A Eukaryotic Cytosolic Chaperonin Is Associated with a High Molecular Weight Intermediate in the Assembly of Hepatitis B Virus Capsid, a Multimeric Particle

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Abstract. We have established a system for assembly of hepatitis B virus capsid, a homomultimer of the viral core polypeptide, using cell-free transcription-linked translation. The mature particles that are produced are indistinguishable from authentic viral capsids by four criteria: velocity sedimentation, buoyant density, protease resistance, and electron microscopic appearance. Production of unassembled core polypeptides can be uncoupled from production of capsid particles by decreasing core mRNA concentration. Addition of excess unlabeled core polypeptides allows the chase of the unassembled polypeptides into mature capsids. Using this cell-free system, we demonstrate that assembly of capsids proceeds by way of a novel

high molecular weight intermediate. Upon isolation, the high molecular weight intermediate is productive of mature capsids when energy substrates are manipulated. A 60-kD protein related to the chaperonin t-complex polypeptide 1 (TCP-1) is found in association with core polypeptides in two different assembly intermediates, but is not associated with either the initial unassembled polypeptides or with the final mature capsid product. These findings implicate TCP-1 or a related chaperonin in viral assembly and raise the possibility that eukaryotic cytosolic chaperonins may play a distinctive role in multimer assembly apart from their involvement in assisting monomer folding.

MOLECULAR chaperones are a class of proteins that mediate the correct folding and assembly of other proteins, but do not become part of the final product (Rothman, 1989; Ellis and van der Vies, 1991; Hartl et al., 1992). To date, two major groups of molecular chaperones, represented by members of the heat shock protein (hsp)¹ 70 and hsp 60 families, have been implicated in polypeptide folding and assembly. In eukaryotes, members of the hsp 70 family bind to nascent polypeptide chains either during their synthesis or during their translocation into organelles (Nelson et al., 1982; Haas and Wabl, 1983; Bole et al., 1986; Copeland et al., 1986; Kozutsumi et al., 1988; Beckman et al., 1990; Kang et al., 1990; Mizzen et al., 1991). On the basis of such studies, it has been proposed that binding of hsp 70 retards protein folding and plays a role in stabilizing polypeptide chains (Nelson et al., 1982; Roth-

man, 1989; Beckman et al., 1990; Ellis and van der Vies, 1991). The 60-kD molecular chaperones, referred to as chaperonins, also bind to unfolded polypeptides. Members of this family are thought to facilitate the proper folding of these maturing polypeptides (Bochkareva et al., 1988; Hemmingsen et al., 1988; Goloubinoff et al., 1989; Ostermann et al., 1989; Langer et al., 1992; reviewed in Hendrick and Hartl, 1993). In fact, it has been proposed that these two families of molecular chaperones can function in tandem to facilitate protein maturation. For example, studies have shown that nascent polypeptides bound to a member of the hsp 70 family are handed over to a member of the chaperonin family at which time folding commences (Kang et al., 1990; Manning-Krieg et al., 1991; Mizzen et al., 1991; Langer et al., 1992).

Some of the structural and functional details of how chaperonins mediate monomeric folding have been characterized in vitro (Martin et al., 1993a and b; for review see Ellis and van der Vies, 1991; Hendrick and Hartl, 1993). Evidence has also accumulated in favor of a second role for the chaperonins of eukaryotic organelles and prokaryotes, namely assembly of monomers into multimeric complexes. For example, mutations in genes encoding different chaperonins result in a failure of proper oligomer assembly (Georgopoulos et

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1. *Abbreviations used in this paper:* CC 60, a TCP-1-related cytosolic chaperonin; GRP 94, glucose-regulated protein of 94 kD; HBV, hepatitis; TCP-1 B virus; hsp, heat shock protein; TCP-1, t-complex polypeptide 1.

al., 1973; Cheng et al., 1989, 1990; Horwich et al., 1993; also see reviews by Georgopoulos et al., 1990; Hartl et al., 1992). It has been difficult, however, to demonstrate that there exists a distinct role for chaperonins in assembly of multimeric proteins, separate from their role in monomer folding. Any apparent role of chaperonins in assembly might simply be a consequence of their requirement for facilitating correct monomer folding, which is a prerequisite for multimer assembly. Alternatively, chaperonins may have an additional role in directly orchestrating the oligomerization process.

While the chaperonins of eukaryotic organelles and prokaryotes have been studied for a number of years, the eukaryotic cytosolic homologues of the 60-kD molecular chaperone family have only recently been identified (Ellis, 1990; Trent et al., 1991; Frydman et al., 1992). One of these homologues, t-complex polypeptide 1 (TCP-1), exhibits only weak sequence similarity to the chaperonins of eukaryotic organelles and prokaryotes. However, its double-stacked ring appearance by negative staining electron microscopy strongly resembles that of the other 60-kD chaperonins (Frydman et al., 1992; Lewis et al., 1992). Like its prokaryotic and organellar homologues, TCP-1 binds to denatured proteins *in vitro* and prevents their aggregation (Frydman et al., 1992). In addition TCP-1 and TCP-like proteins appear to mediate the proper folding of a number of cytoplasmic polypeptides, including actin, tubulin, and related proteins (Ursic and Culbertson, 1991; Frydman et al., 1992; Gao et al., 1992; Yaffe et al., 1992; Melki et al., 1993; Sternlicht et al., 1993), as well as firefly luciferase (Frydman et al., 1992) and plant phytochrome (Mummert et al., 1993).

We have established a system to address questions concerning the role of eukaryotic cytosolic chaperonins in the assembly of multimeric proteins. The multimer we have chosen to study is the capsid (or core particle) of hepatitis B virus (HBV). These capsids are composed of 180 monomers of the 21-kD core polypeptide (Onodera et al., 1982) and are assembled within the cytoplasm of virally infected hepatocytes. During viral morphogenesis, core polypeptides first assemble into capsids in the cytoplasm, incorporating within them the viral polymerase and genomic RNA. These nucleocapsids then acquire a lipoprotein envelope bearing the viral surface glycoproteins by budding into the endoplasmic reticulum. They are exported out of the cell by the constitutive secretory pathway (Ganem, 1991). Core protein is the only viral protein required for capsid assembly. Recombinant expression of the core gene alone in *Escherichia coli*, yeast, *Xenopus* oocytes, or cultured cell lines results in the production of morphologically correct capsids that lack polymerase and genomic RNA (Burrell et al., 1979; Edman et al., 1981; Cohen and Richmond, 1982; Knisern et al., 1986; Roosinck et al., 1986; Nassal, 1988; Zhou et al., 1992). To date neither HBV capsids nor infectious HBV viral particles have been produced in cell-free systems using cellular extracts. A recent report describing the synthesis of infectious polio virus (Molla et al., 1991) in a cell-free system suggests, however, that this should be possible.

In this paper, we demonstrate assembly of HBV capsids in a cell-free system. We find that newly synthesized core polypeptides chase from an unassembled form into completed capsids by way of a novel high molecular weight com-

plex. Evidence is presented that a TCP-1-related 60-kD cytosolic chaperonin (CC 60) is associated with core polypeptides in this high molecular weight intermediate. In contrast, neither the initial unassembled core polypeptides nor the final mature capsids are found to be associated with CC 60.

Materials and Methods

Plasmids

Plasmids were derived from SP64 (Promega Corp., Madison, WI), into which the 58-bp 5' untranslated region of *Xenopus* globin (Krieg and Melton, 1984; Melton et al., 1984) was inserted in the HindIII site. HBV core cDNA was engineered downstream from the SP6 promoter and the globin untranslated region. Concentration of cDNA was adjusted to 2.3 mg/ml by measuring absorbency at 260 nm. Prolactin and GRP 94 (glucose regulated protein of 94 kD) were similarly engineered downstream from the SP6 promoter and the untranslated region.

Cell-free Transcription and Translation

In vitro transcription using SP6 polymerase, and translation of the transcription products in wheat germ extract (Erickson and Blobel, 1983) containing [³⁵S]methionine translabel (ICN Biomedicals, Costa Mesa, CA) were performed essentially as described previously (Yost et al., 1983). The transcription reaction was incubated for 60 min at 40°C and translation for 120 min at 24°C. Instead of using the standard 2.0 mM MgAc concentration for translation, we used 4.0 mM MgAc, since this was found to be optimal for HBV core translation and assembly (J. R. Lingappa, unpublished observations).

In pulse-chase experiments, [³⁵S]cysteine (Amersham Corp., Arlington Heights, IL) was used for translation instead of radiolabeled methionine. 10 min into translation, 3 mM unlabeled cysteine was added to the translation and the incubation was continued at 24°C for varying periods of time. The total amount of labeled core polypeptide produced after a 10-, 35-, 50-, and 170-min chase period was the same, as judged by densitometry of total product bands seen on SDS-PAGE (data not shown).

In transcript dilution experiments, HBV core transcript was produced by the usual method and then diluted to 50% or less as specified by adding mock transcript (a transcription reaction containing 20 mM Tris buffer, pH 7.4, in the place of DNA). This diluted transcript was then used for translation.

To chase unassembled core polypeptides into capsids, translation of 50% core transcript was performed for the standard 120 min. A five- to ninefold excess of an unlabeled translation mix was added to the first mix and incubation was continued for either 45 or 120 min. The unlabeled translation was made by performing a translation using 100% core transcript with unlabeled methionine instead of [³⁵S]methionine. This mixture was preincubated for 45 min. In controls, other unlabeled translations, made using a similar procedure, were added. These included either a translation of prolactin transcript, a translation of 50% core transcript, or a translation of mock transcript (see above).

Sucrose, CsCl, and Glycerol Gradient Analysis

Except where noted, all gradients described below were centrifuged in a TL-100 Beckman centrifuge (Beckman Instrs., Inc., Carlsbad, CA) using a TLS55 swinging bucket rotor. For sucrose gradients, 10–100 μ l of translation products (or protein standards) were diluted to 200 μ l in a sample buffer containing 1% NP-40 buffer (10 mM TrisAc, pH 7.4, 100 mM NaCl, 4 mM MgAc, 50 mM KAc, and 1% NP-40) as well as 1 mM DTT and 1 mM PMSF. Samples were layered onto 2.0-ml 10–50% sucrose gradients, containing 1% NP-40 buffer. Gradients were centrifuged at 55,000 rpm (\sim 200,000 g) for 1 h at 4°C. 200- μ l fractions were removed from top to bottom of the gradients by hand. The pellet was resuspended in 200 μ l of 1% NP-40 buffer. 100- μ l aliquots were precipitated in an equal volume of 50% TCA, and washed with 1:1 ethanol-ether. Pellets were solubilized in SDS sample buffer (100 mM Tris, pH 8.9, 15.0% glycerol, 4.0% SDS, 2.0 mM EDTA, 0.01% bromophenol blue). Fractions were analyzed by SDS-PAGE on 12–17% gradient gels and visualized by Coomassie staining (to see unlabeled protein standards and recombinant capsids) as well as autoradiography. Lanes 1–11 on gels correspond to gradient fractions from top to

bottom, and lane 12 corresponds to the pellet. Standards of less than 12 S (5–15 μg of bovine serum albumin, cytochrome C, and catalase) were analyzed under these gradient conditions (in parallel gradients as well as the same gradients used for translation products) and were found to migrate in the first three fractions. To look at migration of authentic core particles, we used recombinant core particles produced by transfection of *E. coli* and isolation on CsCl gradients (provided by P. Valenzuela, Chiron Corporation, Emeryville, CA). Protein standards and authentic core were visualized by Coomassie staining of gels.

Similar results were obtained using a variety of other detergent types and concentrations in the sample dilution buffer described above, including 0.1% NP-40, 0.1% Nikkol, 0.01% Nikkol, and buffer containing no detergent (data not shown).

In some controls, 10 mM EDTA was added at the completion of translation. In those experiments, 5 mM EDTA was also added to the sample buffer and the sucrose gradient buffer.

Fractions from sucrose gradients, diluted in 1% NP-40 buffer, were further analyzed on CsCl gradients under equilibrium sedimentation conditions. Gradients were made using a CsCl solution of 472.4 mg/ml and were centrifuged at 50,000 rpm for 26 h at 25°C. Fractionation and analysis of samples was as described above. For the purpose of quantitating the percentage of total translation product that comigrated with recombinant capsids, fractions from the middle of the sucrose gradients were centrifuged along with recombinant capsids on 14-ml CsCl gradients using an SW40 Ti rotor in a Beckman L8 70 ultracentrifuge for 36 h. 44 300- μl samples were removed from each gradient and analyzed as described above. Quantitation of band densities is described below.

Translation products from translations using 50% of the standard transcript concentration were also analyzed on 5–25% glycerol gradients containing 1% NP-40 buffer. Gradients were centrifuged at 4°C for 14 h at 55,000 rpm and fractions were analyzed as above.

Proteinase K Digestion

In vitro translation was performed for 75 min, and 120 μl of translation products were diluted in 1% NP-40 buffer and centrifuged for 1 h on a 10–50% sucrose gradient as described above. 20- μl aliquots of fractions 1 and 2 (top) or 6 and 7 (middle) were incubated at 22°C for 1 h (according to the method of Zhou et al., 1992) either with tris buffer or with proteinase K (at a final concentration of 10, 100, and 250 $\mu\text{g}/\text{ml}$). (Proteinase K stock [5 mg/ml in 10 mM Tris, pH 8] was preincubated for 10 min at 37°C and stored at –80°C until use.) Proteinase K was inactivated by addition of PMSF to 5 mM, and samples were TCA precipitated and processed as described above. Samples were solubilized in SDS sample buffer containing 5 mM PMSF and analyzed by SDS-PAGE and autoradiography.

Electron Microscopy

100% core transcript and transcript of GRP 94 truncated with the restriction enzyme NcoI were translated for 150 min and then treated with 10 mM EDTA to disassemble ribosomes. 45 μl of each translation as well as 3.0 μg of authentic capsids were each diluted to 200 μl using 1% NP-40 buffer, and centrifuged separately on CsCl gradients as described above. Fraction 6 of each gradient was collected and half (100 μl) was diluted to 200 μl with water and sedimented in an Airfuge (Beckman Instrs.) at 28 psi for 40 min to pellet capsids. The supernatants were removed and the pellets were resuspended in 20 μl of buffer containing 2 mM Hepes, 4 mM MgAc₂, and 50 mM KAc, pH 7.4. In a single blind study, the resuspended pellets were prepared for EM by negative staining with uranyl acetate (Dubochet et al., 1971). Samples were correctly identified by the microscopist as either containing capsids or being blank in three separate trials.

Isolation and Chase of Pellet

To chase the isolated pellet, translation of 100% core transcript was performed for 30 min and products were diluted to 200 μl in 0.01% Nikkol buffer containing the same salt concentrations as described for the NP-40 buffer above. Samples were centrifuged on 10–50% sucrose gradients, containing 0.01% Nikkol, as described above. The pellet of a 10- μl translation was resuspended in pellet buffer (containing 1.25 μl of standard wheat germ translation compensating buffer (20 mM Hepes–KOH, pH 7.6, 140 mM KAc, 2 mM MgAc₂, 0.4 mM spermidine) that had been diluted to 4.65 μl in 12.5 mM TrisAc, pH 7.4, and 4.3 mM MgAc). The following components were added to the resuspended pellet: creatine kinase (0.125 μl of a 4 mg/ml stock in 50% glycerol and 10 mM TrisAc, pH 7.5), bovine liver tRNA (1.25

μg), and RNase inhibitor (2.5 U). In addition, selected additional ingredients were added to certain samples in various combinations as specified in the text. These included: 5.0 μl of standard unlabeled translation energy mix (5 mM ATP, 5 mM GTP, 60 mM CP, 0.2 mM of each amino acid, pH 7.4, with Tris Base), 5.0 μl of wheat germ extract (in 40 mM Hepes, 100 mM Kac, 5 mM MgAc, and 4 mM DTT), 0.1 mM Emetine (Sigma Immunochemicals, St. Louis, MO), and 10 U of Apyrase (grade VIII; Sigma Immunochemicals). 10 μg of BSA, as a source of nonspecific protein, was added to all samples that did not contain wheat germ extract. In all samples the volume of incubation was adjusted to 20 μl with 20 mM TrisAc, pH 7.4. Incubations were performed at 25°C for time periods of 90–180 min. The reactions were then prepared as described above for centrifugation on 10–50% sucrose gradients in 1% NP-40 buffer.

Generation of Polyclonal Antisera

A conserved peptide region between mouse TCP-1, yeast TCP-1, and *Sulfolobus shibatae* TF55 (Trent et al., 1991) was synthesized and cross-linked to keyhole limpet hemocyanin or to chicken ovalbumin with m-maleimido-benzoyl-N-hydroxysuccinimide ester (Pierce Chem. Co., Rockford, IL). The proteins cross-linked to the peptide were used to generate a polyclonal serum in New Zealand White rabbits. A second version of the antiserum was made by cross-linking the conserved peptide to itself to form a structure that was branched eight times creating a multiple antigen presenter (Tam, 1988). Both antibodies were used in immunoprecipitations as well as immunoblotting and gave similar results.

Immunoprecipitation Using Steady State-labeled HeLa Cell Extracts

HeLa cells growing in a 10-cm culture dish were labeled overnight at 37°C with 250 μCi ³⁵S-translabel (Amersham Corp.) in DME containing 2% calf serum. After labeling, the cells were washed with PBS, solubilized in Laemmli sample buffer, and heated at 100°C for 5 min. The lysate was then clarified to remove insoluble material and the supernatant was adjusted to 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS in PBS for immunoprecipitation. Immunoprecipitations were performed as described below.

Immunoblotting

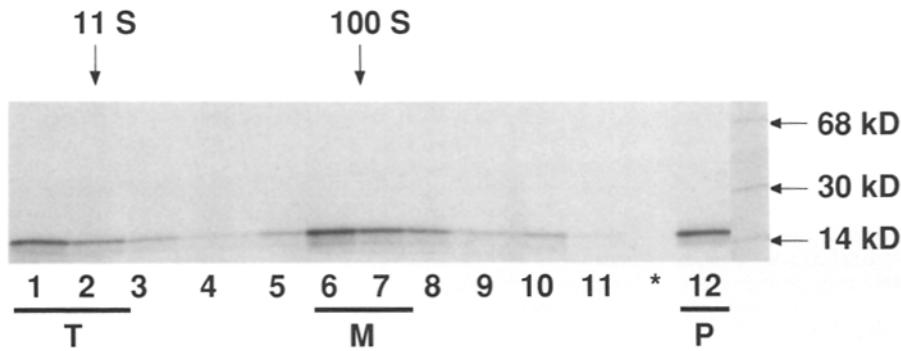
2 μl of wheat germ extract and 2 μl of rabbit reticulocyte extract were diluted to 200 μl in 1% NP-40 buffer (described above) and applied to separate 10–50% sucrose gradients containing 1% NP-40 buffer and centrifuged as described above. The gradients were fractionated from the top and analyzed by Western blotting using the ECL system (Amersham Corp.) (Fig. 6, C). The primary antiserum (anti 60) was used at a dilution of 1:250 and the secondary antibody was used at a dilution of 1:1,000–1:2,000.

Immunoprecipitations of Cell-free Translation Products

Sucrose gradient fractions were divided into three 25- μl aliquots and were diluted to 250 μl in 1% NP-40 buffer (the same buffer as described above except it contained more Tris [0.1 M, pH 8.0] and 1 mM PMSF). 3 μl of antiserum was added to each tube and incubated for 12–48 h at 4°C then 20 μl Protein A Affigel was added and incubated with constant mixing for 3 h at 4°C. The beads were washed three times with 1% NP-40 buffer containing 0.1 M Tris, pH 8.0, and 1 mM PMSF, and twice in wash buffer (0.1 M NaCl, 0.1 M Tris, pH 8.0, 4 mM MgAc) containing 1 mM PMSF. Proteins were eluted from the beads by boiling in 10 μl SDS sample buffer, and were visualized by SDS-PAGE and autoradiography. The antisera used were: polyclonal rabbit antiserum against human HBV core (Dako Corp., Carpinteria, CA), anti 60 (preparation described above), and nonimmune serum.

Image Analysis and Quantitation

Autoradiographs were quantitated following digitization using an AGFA Focus Color Plus scanner and Adobe Photoshop software (Adobe Systems Incorporated, Mountain View, CA). Mean band densities were determined, adjusted for band size, and background subtracted to obtain quantitative values for comparison with other regions of autoradiographs.



autoradiography to detect the radiolabeled 21-kD core polypeptide band. Two minor HBV core bands of lower molecular weight are seen (in both in vitro translations as well as in core protein produced by transfecting *E. coli*). These are thought to be either degradative products or the result of initiation of translation at internal methionines. Positions of molecular weight standards are shown. The position of catalase, an 11-S standard, in this type of gradient (as determined by Coomassie staining) is shown with an arrow. Likewise the migration of recombinant core particles, known to have a sedimentation coefficient of ~ 100 S, is shown with an arrow. Radiolabeled HBV core polypeptides migrate in three regions of this gradient: top (*T*) corresponding to fractions 1 and 2; middle (*M*) corresponding to fractions 6 and 7; and pellet (*P*) corresponding to fraction 12, as shown with dark bars.

Results

HBV Core Cell-free Translation Products Migrate in Three Positions upon Velocity Sedimentation

To synthesize radiolabeled HBV core polypeptides, HBV core DNA was transcribed in vitro and translated for 120 min in a heterologous cell-free system containing wheat germ extract (see Materials and Methods). The radiolabeled translation products were analyzed for formation of HBV core multimers by rate sedimentation on 10–50% sucrose gradients at 200,000 *g* for 1 h. Following fractionation of the gradients, the migration of radiolabeled core proteins was determined using SDS-PAGE, Coomassie staining, and autoradiography. Under these conditions, unlabeled protein standards of less than 12 S, such as catalase, migrated in the first three fractions. Mature core particles produced in recombinant *E. coli* (referred to as authentic capsids) were found predominantly in fractions 5–7 (~ 100 S). Radiolabeled cell-free translation products were found to migrate in three distinct positions using these gradient conditions, as shown in Fig. 1. The first region, at the top of the gradient (*T*) corresponds to the position of monomeric and small oligomeric core polypeptides, while the second region, in the middle of the gradient (*M*), corresponds to the position of authentic capsids. The third region, in the pellet (*P*), represents very high molecular weight structures. The possibility that either the pellet or the middle fraction consists of completed chains not yet released from ribosomes was ruled out by treatment of the translation products after completion of synthesis with EDTA, which is known to disassemble ribosomes (Sabatini et al., 1966). Both pellet and middle fractions were largely unaffected by EDTA treatment (data not shown). Taken together, these results raised the possibility that capsid-like particles were being assembled from newly synthesized core polypeptides in this cell-free system.

Cell-free Translation Products in the Middle of the Gradient Are Resistant to Protease Digestion

It has been shown previously that HBV capsids assembled in *Xenopus* oocytes are relatively resistant to digestion by

proteinase K, whereas unassembled core polypeptides are digested (Zhou et al., 1992). For this reason, we compared the protease resistance of material from the top (*T*) and middle (*M*) of the sucrose gradients described above. Radiolabeled chains from the top (representing unassembled core polypeptides) were digested at all concentrations of proteinase K that were tested (Fig. 2 lanes 1–4). In contrast, core polypeptides from the middle of the gradient were relatively resistant to proteinase K (Fig. 2, lanes 5–8). Thus, the radiolabeled chains that migrate with authentic capsids in the middle of the sucrose gradient are resistant to protease digestion as has been shown for authentic capsids produced in cellular systems. Core polypeptides in the pellet were also resistant to proteinase K at all of the above concentrations (J. R. Lingappa, unpublished observations).

A Portion of the Cell-free Translation Products Has the Same Buoyant Density and Electron Microscopic Appearance as Authentic Capsids

To determine whether some of the translation products that migrated in the middle of the sucrose gradients resembled

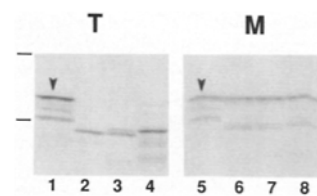


Figure 2. Core polypeptides in the middle fraction are resistant to digestion by protease. HBV core was translated for 75 min, centrifuged on a sucrose gradient and fractionated as described in Fig. 1. Fractions 1 and 2 were pooled (*T*), as were fractions 6 and 7 (*M*). Equal aliquots of *T* and *M* were incubated for 60 min at 22°C either in the absence of protease (lanes 1 and 5) or in the presence of Proteinase K at the following concentrations: lanes 2 and 6, 250 $\mu\text{g/ml}$; lanes 3 and 7, 100 $\mu\text{g/ml}$; lanes 4 and 8, 10 $\mu\text{g/ml}$. Samples were processed as described in Materials and Methods. Arrowheads point to the full-length radiolabeled core polypeptide present in the absence of protease. Horizontal bars indicate positions of 30- and 14-kD MW markers. Protease treatment results in digestion of the radiolabeled core chains present in the top of the gradient, but not those present in the middle of the gradient.

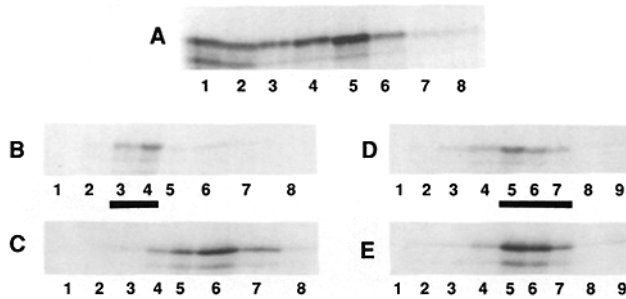


Figure 3. Fidelity of core particles assembled in a cell-free system as determined by sequential equilibrium centrifugation. In vitro translation was performed for 150 min followed by sucrose gradient fractionation of translation products as described in Fig. 1. Material from fractions 6 and 7 of the sucrose gradient (labeled as *M* in Fig. 1) was pooled and layered onto a 2.0 ml 1.35 g/cm³ isopycnic CsCl gradient along with 12.5 μg of authentic core particles, and centrifuged for 26 h. 12 fractions were removed from top to bottom of the gradient and analyzed by SDS-PAGE followed by Coomassie staining and autoradiography. This isopycnic equilibrium centrifugation resulted in banding of authentic core particles in a narrow region of the gradient corresponding to fractions 5–7 as seen by Coomassie staining (data not shown). Radiolabeled core polypeptides from the middle of the sucrose gradient migrated in a broad band spanning fractions 1–6 (*A*). Thus, a portion of the HBV core translation products appeared to comigrate with authentic core particles. To test whether this subfraction of HBV core translation runs true under equilibrium centrifugation conditions, fractions 6 and 7 of the CsCl gradient were pooled and centrifuged on a second CsCl gradient, along with 12.5 μg of authentic core particles. The gradient was fractionated and analyzed by SDS-PAGE. The Coomassie stain and autoradiograph of this gel are shown in *D* and *E*, respectively. The radiolabeled core polypeptides migrated in a narrow band encompassing lanes 5–7 (*D*, *dark bar*) that corresponded exactly to the position of authentic core particles (*E*). In contrast, when fractions 3 and 4 from the first CsCl gradient were centrifuged along with authentic core particles on a second CsCl gradient, the radiolabeled core polypeptides migrated in lanes 3 and 4 (*B*, *dark bar*), and did not correspond to the position of authentic core particles as seen by Coomassie staining of the same gel (*C*).

authentic core particles, we examined material present in fractions 6 and 7 of the sucrose gradients described above by equilibrium centrifugation in CsCl. Under equilibrium conditions, recombinant core particles band with a buoyant density of 1.34–1.36 g/cm³ in CsCl (Cohen and Richmond, 1982). After centrifugation for 26 h in an isopycnic CsCl gradient, fractions were analyzed using SDS-PAGE, Coomassie staining, and autoradiography. Radiolabeled core polypeptides were present in a broad distribution in fractions 1 through 6 (Fig. 3 *A*), while unlabeled recombinant core particles in the same gradient migrated in a narrow band corresponding to fractions 5–7 (data not shown). Thus, the products of cell-free translation appear to be heterogeneous in composition, with some material comigrating with authentic capsids and some material of lower density. Quantitation of band density revealed that ~20% of the total core polypeptide synthesized at 120 min in the cell-free system had assembled into particles that comigrated with authentic HBV capsids under these equilibrium gradient conditions (data not shown). To determine if the portion of the cell-free translation products that comigrates with authentic core runs true upon repeated equilibrium sedimentation, this material

(fractions 6 and 7 from the CsCl gradient) was run on a second identical CsCl gradient and was found to band exclusively in fractions 5–7 (Fig. 3 *D*), comigrating with authentic capsid (Fig. 3 *E*). In contrast, when fractions 3 and 4 of the first CsCl gradient were centrifuged on a second CsCl gradient, the radiolabeled material again appeared in fractions 3 and 4 (Fig. 3 *B*), while the authentic capsid standard migrated in fractions 5–7 (Fig. 3 *C*).

As further confirmation of the authenticity of the capsids produced in the cell-free system, relevant fractions were examined by EM. The products of cell-free translation of HBV core (Fig. 4, *CELL-FREE*) and of cell-free translation of an unrelated protein (GRP 94) (Fig. 4, *CONTROL*) as well as recombinant HBV capsids (Fig. 4, *AUTHENTIC*) were treated with EDTA to disassemble ribosomes and then centrifuged to equilibrium on CsCl gradients. Fractions 6 and 7 of each of these gradients were collected and concentrated in an Airfuge. Electron micrographs of the resuspended pellets examined by a microscopist in single blinded fashion revealed particles indistinguishable from authentic capsids in the products of HBV core cell-free translation. In contrast, no particles resembling capsids were seen in the equivalent fractions of the cell-free translation of an unrelated protein. Thus by four criteria—velocity sedimentation, buoyant density, protease resistance, and electron microscopy—a portion of the HBV core translation products assembles into bona fide HBV capsids.

Progression of Newly Synthesized Core Polypeptides into Multimeric Particles

To determine the order of appearance of labeled core polypeptides in top, middle, and pellet fractions of the sucrose gradient described in Fig. 1, we performed cell-free translations using a 10-min pulse of [³⁵S]cysteine, followed by a chase for varying lengths of time in the presence of excess unlabeled cysteine. Translation products were sedimented through sucrose gradients and analyzed by SDS-PAGE and autoradiography. After a 10-min chase period, a time at which essentially all of the labeled chains have completed translation, the cohort of chains synthesized in the presence of labeled cysteine was found predominantly in the top of the gradient (Fig. 5 *A*). (At even shorter chase times, all of the completed chains were located in the top fraction, while incomplete chains sedimented in polysomes in other gradient fractions [data not shown].) Upon extending the chase period to 35 min, a significant amount of material was found in both the pellet and the middle of the gradient (Fig. 5 *B*). Following a chase period of 50 min, there were very few labeled chains present at the top of the gradient. Rather, increasing amounts of label had accumulated in the pellet and middle fractions (Fig. 5 *C*). After a 170-min chase period, the amount of radiolabeled material in the middle underwent a further increase with a decrease in labeled material in both the pellet and top fractions (Fig. 5 *D*). Quantitation of autoradiographs, shown next to the corresponding gels, confirmed that the labeled material at the top of the gradient diminished dramatically over time. The material in the pellet initially increased and then decreased, while the material in the middle accumulated progressively over the course of the chase period. Thus, the data indicate that newly synthesized core polypeptides chase over time into HBV capsids, and it

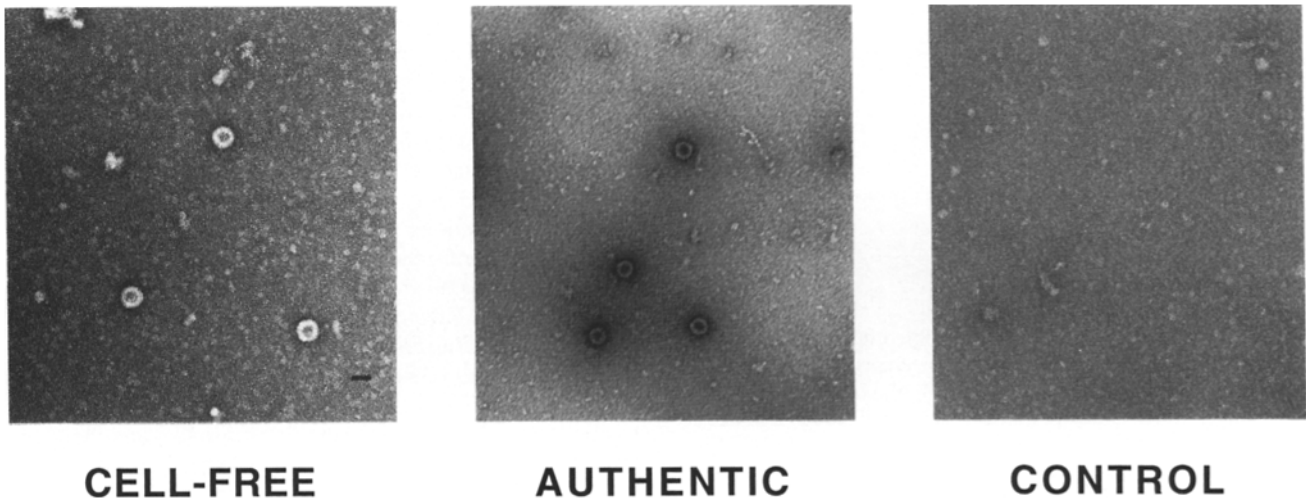


Figure 4. Electron micrographs of capsids produced in a cell-free system. Translation of HBV core transcript (*CELL-FREE*) as well as translation of an unrelated protein (GRP-94 truncated at NcoI, referred to here as *CONTROL*) were performed for 150 min and these products as well as recombinant capsids (*AUTHENTIC*) were centrifuged to equilibrium on separate CsCl gradients. Fraction 6 from each gradient was collected and further sedimented in an Airfuge. In single blinded fashion the pellet of each was collected, resuspended, and prepared for EM by negative staining. Identity of samples was correctly determined by the microscopist. No particles resembling capsids were seen in the control samples. Bar, 34 nm.

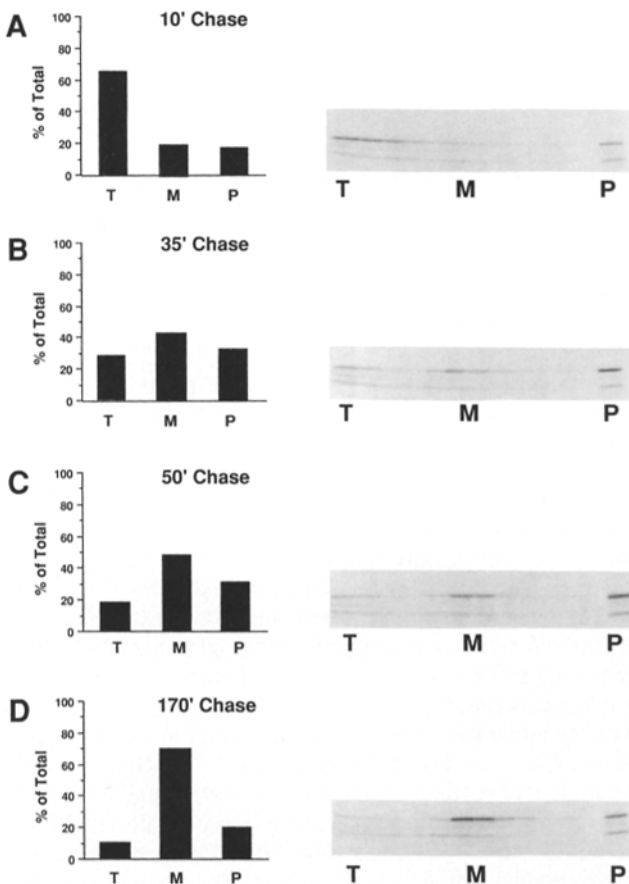


Figure 5. Pulse chase analysis of assembly of HBV core particles. In vitro transcription and translation were performed as described in Materials and Methods with an initial 10-min pulse of [³⁵S]cysteine followed by a chase with unlabeled cysteine for either 10 (A), 35 (B), 50 (C), or 170 min (D). Translation products were layered on sucrose gradients, centrifuged, fractionated, and analyzed by

is likely that they do so, at least in part, by way of a high molecular weight complex contained within the pellet. Definitive confirmation that the pellet contains an intermediate in the formation of completed capsids is presented below (see Fig. 10).

CC 60 Is Associated with Intermediates in the Assembly of HBV Capsids

A polyclonal rabbit antiserum (anti 60) was raised against a peptide sequence of TCP-1 (Fig. 6 A). Studies by others have shown that TCP-1 is a protein of ~60 kD that migrates as a 20-S particle (Gao et al., 1992; Yaffe et al., 1992). From total extracts of steady state-labeled HeLa cells, our anti 60 antiserum immunoprecipitated a single 60-kD protein under denaturing conditions (Fig. 6 B, lane 1). The same 60-kD protein was immunoprecipitated by anti 60 under native conditions (Martin, R., and W. J. Welch, manuscript in preparation). When either rabbit reticulocyte lysate or wheat germ extract was fractionated on a 10–50% sucrose gradient, the anti 60-reactive material migrated as a 20-S particle as revealed by immunoblotting of gradient fractions (Fig. 6 C, top and bottom, respectively). Furthermore, a 60-kD polypeptide component of a 20-S particle (purified from reticulocyte lysate) that is known to be recognized by a previously described antibody to TCP-1 (Willison et al., 1989)

SDS-PAGE and autoradiographed as previously described. Autoradiographs are shown to the right of the respective bar graphs that quantitate density of bands present in the top (T), middle (M), and pellet (P) of the respective autoradiographs. The total amount of radiolabeled full-length core polypeptide present at each time point is the same, as determined by quantitation of band densities of 1- μ l aliquots of total translation (data not shown). Labeled core polypeptides chase from the top to the pellet and finally to the middle of the gradient over time.

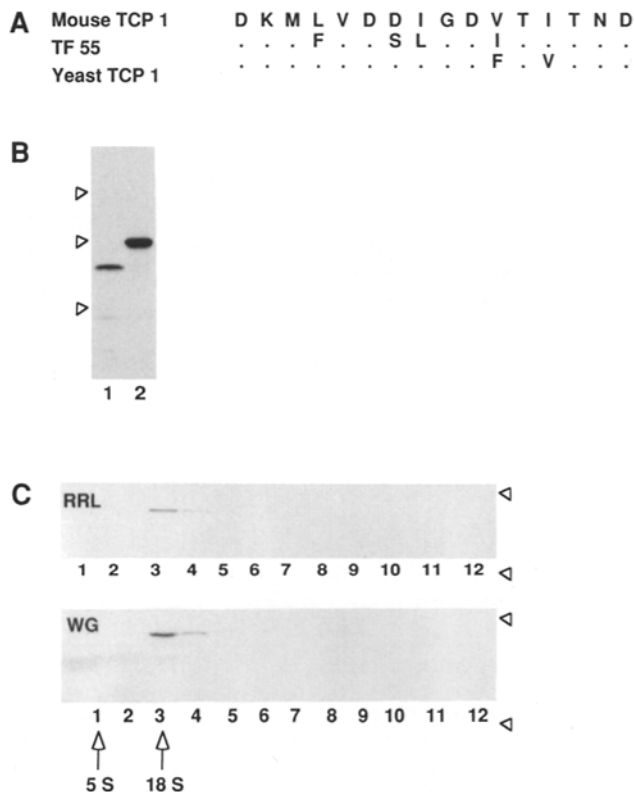


Figure 6. Preparation and characterization of a polyclonal antiserum against a cytosolic chaperonin. *A* shows alignment of an amino acid sequence present within mouse TCP-1 (positions 42–57) (Lewis et al., 1992), *S. shibatae* TF55 (a heat shock protein of a thermophilic archaeobacterium) (positions 55–70) (Trent et al., 1991), and yeast TCP-1 (positions 50–65) (Ursic and Culbertson, 1991). Amino acids identical to those in the mouse sequence are designated by (:). A synthetic peptide was synthesized corresponding to amino acids 42–57 from mouse TCP-1 because of the high degree of homology in this region. This peptide was conjugated to carrier protein or cross-linked to itself and used to generate rabbit polyclonal antisera (anti 60). Immunoprecipitations were performed with this antiserum under denaturing conditions on whole cell extracts of steady state, [³⁵S]methionine-labeled HeLa cells. A protein of ~60 kD was precipitated by anti 60, shown in *B*, lane 1. As a control, *B*, lane 2 shows an immunoprecipitation under denaturing conditions done with antiserum to hsp 70 in the same experiment. Molecular weight markers (92, 68, and 45 kD) are indicated to the left with open arrowheads. Under native conditions, anti 60 also immunoprecipitates a 60-kD protein in solubilized HeLa cells (Martin, R., and W. J. Welch, manuscript in preparation). To further characterize the antigen recognized by this antiserum, rabbit reticulocyte extract and wheat germ extract were layered onto 10–50% sucrose gradients, centrifuged at 55,000 rpm for 60 min in a TL-100 Beckman ultracentrifuge, fractionated, and analyzed by SDS-PAGE. The proteins were transferred to nitrocellulose and were immunoblotted with anti 60 as shown in *C*. To determine S values, protein standards were centrifuged in a separate gradient tube at the same time and fractions were visualized by Coomassie staining of SDS-PAGE gels. The positions of these markers (BSA and α -macroglobulin) are indicated with arrows. Molecular weight markers (68 and 45 kD) are indicated to the right with open arrowheads. In both immunoblots, only a single band was recognized, representing a 60-kD protein, migrating in the 20-S position. Thus, anti 60 appears to recognize a 60-kD protein (CC 60) that migrates in the 20-S region and is likely to be either TCP-1 or homolog.

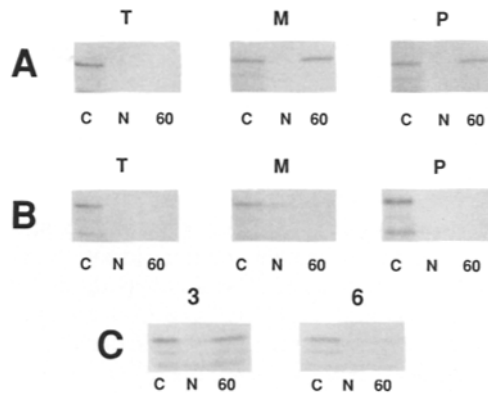


Figure 7. Immunoprecipitations of HBV core translation products. HBV core was translated *in vitro* for 60 min. Translation products were centrifuged on sucrose gradients and fractionated as described in Fig. 1. Fractions from the top (*T*), middle (*M*), and pellet (*P*) regions were divided into equal aliquots and immunoprecipitations were performed as described in Materials and Methods under either native (*A*) or denaturing (*B*) conditions using either anti-core antiserum (*C*), nonimmune serum (*N*), or anti 60 (*60*). Immunoprecipitated labeled core protein was visualized by SDS-PAGE and autoradiography. *C* shows a separate experiment in which native immunoprecipitations were performed on HBV core translation products following equilibrium density centrifugation. In this experiment, HBV core was translated for 150 min and centrifuged on sucrose gradients as described in Fig. 1. Material from the middle (lanes 6 and 7) of sucrose gradients was pooled and centrifuged on CsCl equilibrium gradients. Fractions 3 and 6 were collected, divided into equal aliquots, and immunoprecipitated under native conditions using either anti-core antiserum (*C*), nonimmune serum (*N*) or anti 60 (*60*). Exposure times for autoradiographs were identical for each of the three lanes (*C*, *N*, and *60*) within a set, but vary between sets.

also reacted to the anti 60 antisera described here (H. Sternlicht, personal communication). Mitochondrial hsp 60, in contrast, failed to be recognized by anti 60 (data not shown). The 20-S particle recognized by anti 60 also was recognized by an antibody (provided by J. Trent, Argonne National Laboratory, Argonne, IL) (see Trent et al., 1991) against TF 55, the hsp 60 homolog found in the thermophilic archaeobacterium *Sulfolobus shibatae* (data not shown). Thus, anti 60 appears to be recognizing either TCP-1 or a closely related eukaryotic cytosolic protein, which we refer to as CC 60.

To determine whether CC 60 is associated with HBV core in our cell-free assembly system, we examined whether anti 60 (Fig. 7, *60*) was able to coprecipitate newly synthesized HBV core polypeptides from various fractions of the sucrose gradients. Control immunoprecipitations were performed using nonimmune serum (Fig. 7, *N*) as well as polyclonal rabbit antiserum to HBV core polypeptide (Fig. 7, *C*). Fig. 7 *A* shows that under native conditions anti 60 coprecipitated radiolabeled core polypeptides present within the middle (*M*) and the pellet (*P*) of the sucrose gradients, but did not coprecipitate core polypeptides from the top (*T*). Similarly, antibody to TF 55 (see above) coprecipitated core polypeptides in the pellet and the middle of the gradients (data not shown). As expected, when immunoprecipitations were performed after denaturation of samples by boiling in SDS, anti 60 no longer coprecipitated core polypeptides from any of these gradient fractions (Fig. 7 *B*). In contrast, antiserum to

core polypeptide recognized labeled core protein in all three of these fractions under both native and denaturing conditions (Fig. 7, *A* and *B*). Based on these observations, it appears that CC 60 is not associated with unassembled forms of HBV core protein, but is associated with multimeric forms of the protein. These results raised the possibility that CC 60 plays a role in the assembly of HBV core particles.

If CC 60 were to play a role in assembly, one might expect this chaperonin to dissociate from the multimeric core particle once assembly is complete. To test this hypothesis we performed immunoprecipitations on material from the middle of sucrose gradients that had been further fractionated on a CsCl gradient. As described above (see Fig. 3), using such an equilibrium centrifugation method we can separate mature capsids (found in fractions 5–7 under these conditions) from core multimers that have a density less than that of true capsids (found in fractions 1–4 of the CsCl gradients) and are possibly incomplete assembly intermediates. Fig. 7 *C* shows that under native conditions, anti 60 precipitates HBV core polypeptides present in fraction 3 from CsCl gradients (corresponding to incomplete capsids) but fails to precipitate core polypeptides present in fraction 6 from the same gradients (corresponding to completed capsids). Antiserum to core polypeptide recognizes core protein in both fractions. Thus it appears that CC 60 is associated with partially assembled capsids, but is not associated with mature capsids.

As further confirmation that CC 60 is only transiently associated with core polypeptides in the process of assembly, immunoblots of gradient fractions were performed with antiserum to CC 60 at different times during translation (Lingappa, J. R., W. J. Welch, and V. R. Lingappa, manuscript in preparation). These immunoblots revealed the presence of a large amount of CC 60 in the pellet at early time points during translation of HBV core transcript but not during translation of mock transcript. In contrast, at later times during the core translation and assembly reaction, all of the CC 60 was located in the 20-S position with none remaining in the pellet. In these experiments the total amount of CC 60 was essentially unchanged over the course of translation.

Core Polypeptide Production Can Be Uncoupled from Core Particle Assembly

To distinguish between a role for CC 60 in folding of core monomers versus a role in assembly of multimers, we attempted to uncouple production of core polypeptides from core particle assembly. In *Xenopus* oocytes, assembly of core particles is known to be exquisitely dependent on the concentration of core polypeptide chains (Seifer et al., 1993). We observed an equally striking concentration dependence in our system. When we decreased the concentration of HBV core transcript to 50% or less of the standard concentration used in our cell-free system, HBV capsid assembly was virtually abolished (Fig. 8 *A*), while total core polypeptide synthesis was diminished in a roughly linear fashion (data not shown). These conditions resulted in the accumulation of a population of unassembled, full-length core polypeptides that migrated at the top of the previously described sucrose gradients (Fig. 8 *A*). Even when incubated for a long time (6 h), these unassembled chains remained at the top of the gradient indicating that assembly does not occur even at a slow rate under these conditions (data not shown). When centrifuged on a 5–25% glycerol gradient for 14 h, the unas-

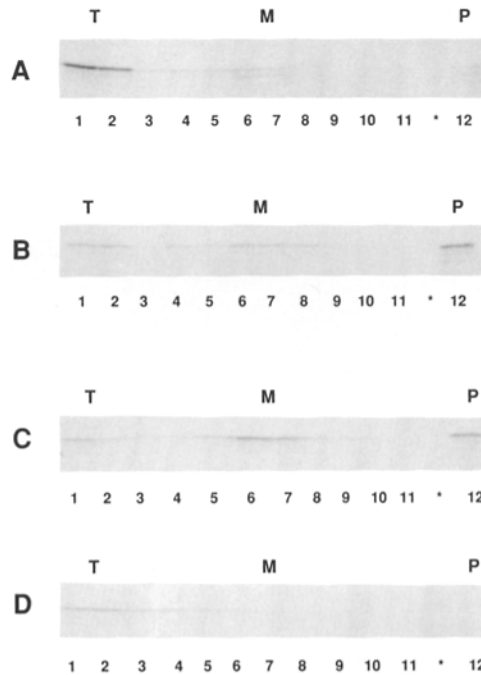


Figure 8. Unassembled core polypeptides can be chased into multimeric particles. HBV core transcript was diluted by 50% with mock transcript, and then translated for 120 min as described in Materials and Methods. Translation products were divided into three aliquots. One aliquot was put on ice (*A*). To a second aliquot was added a translation of HBV core polypeptides that was made using 100% transcript and only unlabeled amino acids that had been incubated for 45 min. This mixture was then further incubated for either 45 (*B*) or 120 min (*C*). To a third aliquot was added a translation of mock transcript that had been incubated for 45 min, and this mixture was further incubated for 120 min (*D*). All four samples were then centrifuged on sucrose gradients and fractions were removed and analyzed by SDS-PAGE and autoradiography as previously described. Unassembled core polypeptides shown in *A* are found to move first into the pellet and then into the middle over time (*B* and *C*, respectively) with the addition of a high concentration of (unlabeled) HBV core polypeptide chains. In contrast, with addition of mock translation (*D*), core polypeptides remain at the top of the gradient.

sembled core polypeptides migrated in the approximate region expected for folded globular dimers of core, based on the position of protein standards (data not shown). Thus, the data indicate that the unassembled material at the top of the gradient does not consist of unfolded polypeptides. Rather, this material likely represents core polypeptide dimers, or a mixture of monomers and dimers. Dimers are known to be capsid assembly precursors in vivo (Zhou and Standing, 1992).

To determine whether the unassembled core polypeptides present at the top of the gradient are in fact competent for assembly into capsids, we asked if they could be chased into capsids in the presence of excess unlabeled core chains. To do this we added to these unassembled radiolabeled chains an excess of an unlabeled translation mix that had been programmed with 100% core transcript for 45 min. The 45-min time point was chosen because it represents a point at which the newly synthesized core chains are present in roughly equal proportions in the top, middle, and pellet regions of

our standard sucrose gradients (data not shown). After mixing the labeled, unassembled chains with the unlabeled translation, incubation was continued at 24°C for either 45 or 120 min and the mixture was then layered onto sucrose gradients, centrifuged, fractionated, and analyzed by SDS-PAGE and autoradiography as previously described. After a 45-min incubation, the labeled polypeptides were found primarily in the pellet (*P*) with a small amount in the middle of the gradient (*M*) (Fig. 8 *B*), while after 120 min a significant quantity of labeled chains was present in the middle of the gradient (Fig. 8 *C*). When material from the middle of that sucrose gradient (Fig. 8 *C*) was subsequently centrifuged on CsCl, the radiolabeled chains were found to comigrate with authentic core particles in amounts comparable to that shown in Fig. 2, confirming that completed capsids were produced during the chase (data not shown).

When an unlabeled mock translation was preincubated for 45 min and added to the unassembled core polypeptides, the radiolabeled core polypeptides at the top of the gradient failed to chase into either the pellet or the middle (Fig. 8 *D*). A similar result was obtained when a translation programmed with bovine prolactin, an unrelated protein, was added to the unassembled core polypeptides. Likewise, when an unlabeled translation of 50% of the standard core transcript was added to the unassembled radiolabeled core polypeptides, the radiolabeled chains remained at the top of the gradient (data not shown). In the latter experiment, the concentration of HBV core chains was maintained at 50% of the standard concentration, and thus failed to rise to the necessary threshold for assembly. Thus, under the appropriate conditions, unassembled chains appear to be competent to form mature capsids.

Unassembled Core Polypeptides Are Not Associated with CC 60

Having established conditions that allow us to arrest newly synthesized core polypeptides in an unassembled form, we then proceeded to ask whether these unassembled core polypeptides are associated with CC 60. Under native conditions anti 60 failed to coprecipitate those core polypeptides present at the top of the gradient (Fig. 9, *A* and *B*). In the same experiment, native immunoprecipitations were performed after the core polypeptides at the top of the gradient had been chased into both the high molecular weight (*P*) and capsid (*M*) forms by the addition of a high concentration of newly synthesized unlabeled chains (Fig. 9, *C* and *D*). Only the core polypeptides that had been chased into the pellet and the middle fractions of the gradient were observed to coprecipitate with anti 60. Again, this antiserum failed to coprecipitate the small amount of unassembled material that remained at the top of the gradient (*T*). Thus, CC 60 does not associate, under any of our conditions, with unassembled core polypeptides.

Completed Capsids Can Be Released by Manipulation of the Isolated Pellet

Having found an association of CC 60 with multimeric complexes, we wished to determine whether any of these complexes constitute intermediates in the assembly of the final capsid product and whether energy substrates play a role in the progression of such intermediates. Molecular chaper-

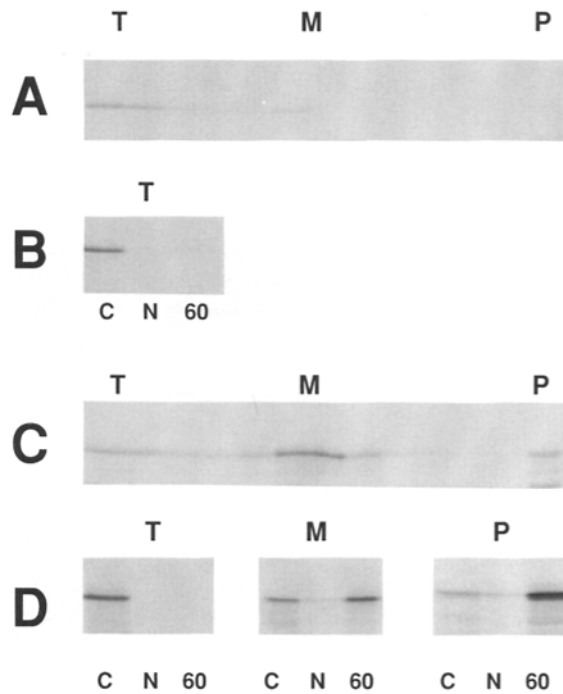


Figure 9. Native immunoprecipitations before and after chase of unassembled core polypeptides into mature capsids. HBV core transcript was diluted by 50% with mock transcript, and then translated for 120 min as described in Materials and Methods. Translation products were divided into two aliquots. One aliquot (unassembled material) was put on ice and the second aliquot (chased material) was incubated for 120 min with an excess of preincubated core translation made with unlabeled amino acids and 100% core transcript (similar to the chase described in Fig. 8). Both aliquots were subsequently centrifuged on a sucrose gradient, fractionated, and analyzed by SDS-PAGE and autoradiography as previously described (*A* and *C*, respectively). Immunoprecipitations under native conditions were performed on selected fractions (top [*T*], middle [*M*], or pellet [*P*]) of the sucrose gradients of the unassembled polypeptides (*B*) and the chased material (*D*) using anti-core antiserum (*C*), nonimmune serum (*N*), or anti 60 (*60*). Exposure times for the autoradiographs are: (*A*) 5 h; (*B*) 2.5 d; (*C*) 11 h; (*D*, *T*, and *M*) 2.5 d; (*P*) 20 d.

ones are known to be involved in solubilizing aggregates of misfolded protein as well as in facilitating correct folding and assembly of polypeptides as discussed above. Thus, CC 60 could be associated with multimeric complexes in the pellet and middle fractions either because these complexes represent “dead end pathways” consisting of aggregates of misfolded or misassembled protein, or because these complexes represent productive intermediates along the pathway towards assembly of completed capsids. To address this, pellet material was isolated by fractionating the products of a 30-min translation of HBV core on a sucrose gradient and resuspending the pellet in buffer. The resuspended pellet was divided into equal aliquots and treated either with apyrase or with buffer for 90 min at 24°C. Radiolabeled material from the pellet chased to the middle with apyrase treatment (Fig. 10 *A*, top), but not with incubation in buffer (Fig. 10 *A*, bottom). When fractions 6 and 7 were collected after apyrase treatment and centrifuged to equilibrium on a CsCl gradient, most of the radiolabeled material was found to

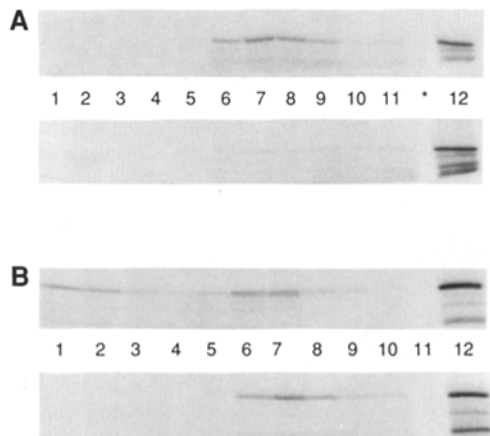


Figure 10. Completed capsids are released from the isolated pellet. Following translation of HBV core transcript for 30 min, the translation product was diluted in 0.01% Nikkol buffer and centrifuged on a 10–50% sucrose gradient as described in Materials and Methods. The supernatant was removed and the pellet was resuspended in buffer and divided into equal aliquots. To one aliquot was added apyrase (*A, top*) while the control was incubated in buffer alone (*A, bottom*). Incubations were done at 25°C for 90 min. Reaction mixtures were then centrifuged on standard 10–50% sucrose gradients. Fractions were analyzed by SDS-PAGE and autoradiography. In a separate experiment (*B*) the pellet was isolated and resuspended in identical fashion. To one aliquot was added wheat germ extract as well as unlabeled energy mix (*B, top*); to the second aliquot was added wheat germ extract and apyrase (*B, bottom*). See Materials and Methods for details of reaction conditions. The reactions were incubated at 25°C for 180 min and centrifuged as described for *A*. Treatment with apyrase (with or without wheat germ extract) resulted in release of radiolabeled material that migrated in the middle of the gradient. That this material represents complete capsids along with authentic capsid as a control as described in Fig. 3 (data not shown). In contrast, treatment with wheat germ extract and energy mix resulted in generation of radiolabeled material that migrated in the top as well as the middle of the gradient. The material in the middle of these gradients was also shown to include completed capsids by centrifugation on CsCl along with authentic capsid as a marker.

comigrate with authentic core particles (data not shown). Thus, apyrase treatment of isolated pellet material results in release of completed capsids from the pellet.

When the isolated pellet was treated with the energy mix used in cell-free translations (containing ATP, GTP, and creatine phosphate) along with wheat germ extract, radiolabeled core polypeptides in the pellet were found to chase into both middle and top fractions (Fig. 10 *B, top*). Once again, when the radiolabeled material in the middle was examined by equilibrium sedimentation, a small portion had a buoyant density identical to that of authentic capsids (data not shown). Treatment of the isolated pellet with either wheat germ extract or energy mix alone resulted in chase of a much smaller amount of radiolabeled material to the middle of the gradient (data not shown). Treatment of the isolated pellet with apyrase and wheat germ extract (Fig. 10 *B, bottom*) produced the same result as treatment with apyrase alone (Fig. 10 *A, top*). Thus, the addition of energy substrates results in release of both unassembled core polypeptides as well as assembled capsids from the pellet. Additional data demon-

strated that polysomes do not play a role in the pellet: (*a*) the protein synthesis inhibitor emetine did not affect the results of treatment of the isolated pellet with energy substrates or apyrase; and (*b*) as previously mentioned, treatment of translation products with 10 mM EDTA had no effect on relative distribution of labeled core polypeptides in the top, middle, and pellet regions of the gradients (data not shown). The ability of the pellet to chase into completed capsids with various manipulations of energy substrates indicates that some of the material in the pellet constitutes an intermediate in the pathway to completed capsids.

Discussion

We present evidence that in a cell-free system HBV core polypeptides assemble into multimeric particles that are indistinguishable from authentic capsids produced in cellular systems, as determined by the following techniques: equilibrium and velocity sedimentation, protease resistance, and EM morphology. As judged by buoyant density criteria, 20% of the core translation product appears to be fully assembled. This number is not surprising, since cell-free systems, which have a limited supply of factors, would not be expected to result in highly efficient production of a complex end product.

In fact, this relative inefficiency may provide distinct experimental advantages, since intermediates that exist only transiently *in vivo* may accumulate *in vitro*, where they can be trapped, isolated, characterized, and manipulated. To date, *in vivo* studies of HBV core assembly have failed to identify assembly intermediates beyond core dimer formation, suggesting that within cells such assembly reactions are indeed extremely rapid (Zhou and Standring, 1992; Zhou et al., 1992). To look for intermediates in capsid assembly, we performed pulse chase experiments that revealed that newly synthesized chains can be chased into both a high molecular weight complex as well as into mature capsids. The results of these experiments are consistent with the idea that unassembled core polypeptides in the top fractions of our gradients chase first into high molecular weight structures in the pellet, and then into a variety of multimeric structures, including mature capsids, that are located in the middle of the gradient. We cannot exclude the possibility that some core polypeptides chase directly from the top of the gradient into the middle fractions (Fig. 10). However, experiments done with the isolated pellet, discussed in greater detail below, confirm that the pellet contains an intermediate from which completed capsids can be generated.

Native immunoprecipitations reveal that the pellet as well as a subfraction of the material in the middle of our sucrose gradients contain CC 60 in association with newly synthesized core polypeptides. The association of core polypeptides with CC 60 raises questions concerning the role of cytosolic chaperones in this system. One possibility is that the chaperones are only present to mediate correct folding of unassembled polypeptide chains and play no distinct role in assembly. Alternatively, these chaperones may play a role in assembly distinct from any role they may play in monomer folding. To address this question we made use of the concentration dependence of core particle assembly in our system to uncouple synthesis of completed core polypeptides from their subsequent assembly. By reducing the concentration of

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polypeptide chains synthesized, we can prevent assembly of core particles. Under these conditions, completed core polypeptides are arrested in an unassembled state but are competent to form capsids when provided with a high concentration of newly synthesized core polypeptides.

When the core polypeptides arrested at the top of the gradient were sedimented on glycerol gradients, they migrated in the position of an ~50-kD complex. Based on these results, we suggest that the unassembled polypeptides consist either of folded dimers, or a mixture of folded monomers and dimers. Studies by others have shown that the major assembly unit for HBV capsid production in *Xenopus* oocytes is likely to consist of core dimers (Zhou and Standring, 1992). Hence, the basic unit of HBV capsid assembly in our cell-free system may be similar to that found in vivo. By native immunoprecipitation, these newly synthesized core polypeptide monomers or dimers are not associated with CC 60. Thus, at least in the case of HBV capsid assembly in our cell-free system, there appears to be a role for this cytosolic chaperonin in multimer assembly, likely distinct from any role it may have in monomer folding. Whether or not CC 60 also participates in the earlier events of core monomer folding or dimer production remains to be determined.

The association of CC 60 with multimeric complexes could be for the purpose of assisting in oligomerization of competent assembly units in the pathway to formation of completed capsids, or for arresting the progression of incompetent dead end by-products, resulting from misfolding or aberrant assembly. The chase of radiolabeled core polypeptides from the isolated pellet into completed capsids by manipulation of energy conditions demonstrates that at least some of the material in the pellet represents a true intermediate in the assembly pathway. Thus, it is quite possible that the chaperonin present in the pellet is playing a role in the assembly process. Definitive evidence, however, that CC 60 or any chaperonin is essential for capsid assembly will require a more detailed biochemical characterization of the species present in the pellet, as well as an assembly assay in which CC 60 is inactivated or depleted.

The mechanism by which completed capsids are generated from this high molecular weight intermediate is not yet clear. The experiments presented here indicate that the mechanism is complex. Treatment with nucleotide triphosphate-containing energy mix together with cytosolic extract appears to release core polypeptides as a spectrum of both unassembled and assembled forms. Unexpectedly, depletion of energy substrates by treatment with apyrase drives conversion of core polypeptides from the pellet into completed capsids in the middle of the gradient. This unusual finding argues against the idea that the pellet is simply an aggregate that requires a chaperone in order to be solubilized. Previous studies using a different protein showed that resolubilization of aggregates by hsp 70 requires ATP hydrolysis (Skowrya et al., 1990). Further investigation will be required to produce a model for the actions of energy substrates in our system. Nevertheless, the results are consistent with the known abilities of energy charge to affect the activity of molecular chaperones (Ellis and van der Vies, 1991). The seemingly paradoxical results of manipulations of energy charge in this system may ultimately be explained by the coexistence of a number of different energy-requiring interactions in the function of molecular chaperone complexes. It is known, for

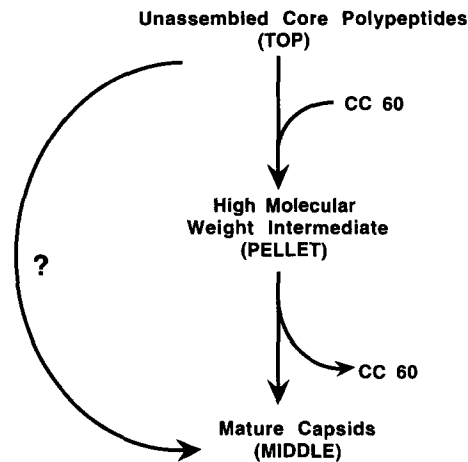


Figure 11. CC 60 plays a role in the assembly of HBV capsid, a homomultimeric particle. Unassembled core polypeptides (present at the top of the gradient) chase into a novel high molecular weight intermediate (located in the pellet of the gradient). This intermediate appears to be associated with CC 60, a cytosolic chaperonin of eukaryotic cells. From this intermediate mature capsids are generated. Neither the unassembled core polypeptides (starting material) nor the mature capsids (final product) are associated with CC 60. See text for details.

example, that energy-dependent interactions take place between molecular chaperones and their substrates, while at the same time there exist energy-dependent associations between individual subunits of the chaperonin complexes. Thus, release of substrates from either hsp 70 or GroEL in vitro is facilitated by addition of ATP (Ellis and van der Vies, 1991; Hartl et al., 1992). In the case of the prokaryotic chaperonin, the purified GroES and GroEL subunits interact with one another only when ATP is present (Chandrasekhar et al., 1986). These examples illustrate that the role of energy as it relates to molecular chaperones is complex. The effects of energy and its depletion on CC 60-associated steps in capsid biogenesis may be viewed in this light and clearly warrant further study.

Unlike the assembly intermediates, the final mature capsid was not associated with CC 60 in our system. This is consistent with the idea that once assembly of the capsid is complete, the chaperonin dissociates from the final product. It has been suggested that 60-kD chaperonins play an important role in assembly of viral particles based on early studies showing that bacteriophages fail to assemble properly in *E. coli* strains carrying mutations in GroEL, the prokaryotic chaperonin (Coppo et al., 1973; Georgopoulos et al., 1973; Sternberg, 1973; Georgopoulos and Horn, 1978; Pato et al., 1987). We believe that the system described here will allow for a detailed examination of the mechanism by which such chaperonins are involved in viral particle biogenesis.

In conclusion, the data presented here indicate that a cytosolic chaperonin resembling TCP-1 is associated with an intermediate in the assembly of a multimeric viral particle in

a cell-free system (summarized in Fig. 11). Confirmation that this is a general property of eukaryotic cytosolic chaperones will require that similar results be obtained in cellular systems. The system presented here, however, should be useful for studying the actions of cytosolic chaperonins in detail. Indeed, further characterization of the high molecular weight chaperonin-associated intermediate described here may reveal new forms of cellular machinery that are involved in protein assembly. Likewise, by examining whether the capsids produced in this system are competent to package and be enveloped, we may be able to ask whether molecular chaperones mediate still higher order assembly events involving association of protein with nucleic acid or lipid.

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References

- Beckman, R. B., L. A. Mizzen, and W. J. Welch. 1990. Interactions of HSP 70 with newly synthesized proteins: implications for protein folding and assembly. *Science (Wash. DC)*. 248:850-854.
- Bochkareva, E. S., N. M. Lissin, and A. S. Girshovich. 1988. Transient association of newly synthesized unfolded proteins with the heat-shock GroEL protein. *Nature (Lond.)*. 336:254-257.
- Bole, D. G., L. M. Hendershot, and J. F. Kearney. 1986. Posttranslational associations of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol.* 102:1558-1566.
- Burrell, C. J., P. Mackay, P. J. Greenaway, P. H. Hofschneider, and K. Murray. 1979. Expression in *Escherichia coli* of hepatitis B virus DNA sequences cloned in plasmid pBR322. *Nature (Lond.)*. 279:43-47.
- Chandrasekhar, G. N., K. Tilly, C. Woolford, R. Hendris, and C. Georgopoulos. 1986. Purification and properties of the GroES morphogenetic protein of *E. coli*. *J. Biol. Chem.* 261:12414-12419.
- Cheng, M. Y., F.-U. Hartl, J. Martin, R. A. Pollock, F. Kalousek, W. Neupert, E. M. Hallberg, R. L. Hallberg, and A. L. Horwich. 1989. Mitochondrial heat-shock protein hsp 60 is essential for assembly of proteins imported into yeast mitochondria. *Nature (Lond.)*. 337:620-625.
- Cheng, M. Y., F.-U. Hartl, and A. L. Horwich. 1990. The mitochondrial chaperonin hsp 60 is required for its own assembly. *Nature (Lond.)*. 346:455-458.
- Cohen, B. J., and J. E. Richmond. 1982. Electron microscopy of hepatitis B core antigen synthesized in *E. coli*. *Nature (Lond.)*. 296:677-678.
- Copeland, C. S., R. W. Doms, E. M. Bolzau, R. G. Webster, and A. Helenius. 1986. Assembly of hemagglutinin trimers and its role in intracellular transport. *J. Cell Biol.* 103:1179-1191.
- Coppo, A., A. Manzi, J. F. Pulitzer, and H. Takashi. 1973. Abortive bacteriophage T4 head assembly in mutants of *Escherichia coli*. *J. Mol. Biol.* 76:61-87.
- Dubochet, J., M. Ducommun, M. Zollinger, and E. Kellenberger. 1971. A new preparation method for dark-field electron microscopy of biomacromolecules. *J. Ultrastruct. Res.* 35:147-167.
- Edman, J. C., R. A. Hallewell, P. Valenzuela, H. M. Goodman, and W. J. Rutter. 1981. Synthesis of hepatitis B surface and core antigens in *E. coli*. *Nature (Lond.)*. 291:503-506.
- Ellis, R. J. 1990. Molecular chaperones: the plant connection. *Science (Wash. DC)*. 250:954-959.
- Ellis, R. J., and S. M. van der Vies. 1991. Molecular chaperones. *Annu. Rev. Biochem.* 60:321-347.
- Erickson, A. H., and G. Blobel. 1983. Cell-free translation of messenger RNA in a wheat germ system. *Methods Enzymol.* 96:38-50.
- Frydman, J., E. Nimmesgern, H. Erdjument-Bromage, J. S. Wall, P. Tempst, and F.-U. Hartl. 1992. Function in protein folding of TRiC, a cytosolic ring complex containing TCP-1 and structurally related subunits. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:4767-4778.
- Ganem, D. 1991. Assembly of hepadnaviral virions and subviral particles. *Curr. Top. Microbiol. Immunol.* 168:62-83.
- Gao, Y., J. O. Thomas, R. L. G.-H. L. Chow, and N. J. Cowan. 1992. A cytoplasmic chaperonin that catalyzes β -actin folding. *Cell*. 69:1043-1050.
- Georgopoulos, C. P., and B. Horn. 1978. Identification of a host protein necessary for bacteriophage morphogenesis (the groE gene product). *Proc. Natl. Acad. Sci. USA*. 75:131-135.
- Georgopoulos, C., R. W. Hendrix, S. R. Casjens, and A. D. Kaiser. 1973. Host participation in bacteriophage lambda head assembly. *J. Mol. Biol.* 76:45-60.
- Georgopoulos, C., D. Ang, K. Liberck, and M. Zylicz. 1990. Properties of the *Escherichia coli* heat shock proteins and their role in bacteriophage lambda growth. In *Stress Proteins in Biology and Medicine*. E. A. Morimoto, editor. Cold Spring Harbor Press, Cold Spring Harbor, NY 191-222.
- Goloubinoff, P., J. J. Christella, A. A. Gatenby, and G. H. Lorimer. 1989. Reconstitution of active dimeric ribulose biphosphate carboxylase from an unfolded state depends on two chaperonin proteins and Mg-ATP. *Nature (Lond.)*. 342:884-890.
- Haas, I. G., and M. Wabl. 1983. Immunoglobulin heavy chain binding protein. *Nature (Lond.)*. 306:387-389.
- Hartl, F.-U., J. Martin, and W. Neupert. 1992. Protein folding in the cell: the role of molecular chaperones Hsp 70 and Hsp 60. *Annu. Rev. Biophys. Biomol. Struct.* 21:293-322.
- Hemmingsen, S. M., C. Woolford, S. van der Vies, K. Tilly, D. T. Dennis, C. P. Georgopoulos, R. W. Hendrix, and R. J. Ellis. 1988. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature (Lond.)*. 333:330-334.
- Hendrick, J. P. and F.-U. Hartl. Molecular chaperone functions of heat-shock proteins. 1993. *Annu. Rev. Biochem.* 62:349-384.
- Horwich, A. L., K. B. Low, W. A. Fenton, I. N. Hirshfield, and K. Furtak. 1993. Folding in vivo of bacterial cytoplasmic proteins: role of GroEL. *Cell*. 74:909-917.
- Kang, P., J. Osterman, J. Shilling, W. Neupert, E. Craig, and N. Pfanner. 1990. Requirement for hsp 70 in the mitochondrial matrix for translocation and folding of precursor proteins. *Nature (Lond.)*. 348:137-143.
- Knisern, P. J., A. Hagopian, D. L. Montgomery, P. Burke, N. R. Dunn, K. J. Hofmann, W. J. Miller, and R. W. Ellis. 1986. Unusually high-level expression of a foreign gene (hepatitis B virus core antigen) in *Saccharomyces cerevisiae*. *Genet. (Amst.)*. 46:135-141.
- Kozutsumi, Y., M. Segal, K. Normington, M. J. Gething, and J. Sambrook. 1988. The presence of misfolded proteins in the endoplasmic reticulum signals the induction of glucose regulated proteins. *Nature (Lond.)*. 332:462-464.
- Kreig, P. A., and D. A. Melton. 1984. Functional messenger RNAs are produced by SP6 in vitro transcription of cDNAs. *Nucleic Acids. Res.* 12:7057-7070.
- Langer, T., C. Lu, H. Echols, J. Flanagan, M. Hayer, and F.-U. Hartl. 1992. Successive action of Dna K, Dna J, and GroEL along the pathway of chaperone-mediated protein folding. *Nature (Lond.)*. 356:683-689.
- Lewis, V. A., G. M. Hynes, D. Zheng, H. Saibil, and K. Willison. 1992. T-complex polypeptide-1 is a subunit of a heteromeric particle in the eukaryotic cytosol. *Nature (Lond.)*. 358:249-252.
- Manning-Krieg, U. C., P. E. Scherer, and G. Schatz. 1991. Sequential action of mitochondrial chaperones in protein import into the matrix. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:3273-3280.
- Martin, J., M. Mayhew, T. Langer, and F.-U. Hartl. 1993a. The reaction cycle of GroEL and GroES in chaperonin-assisted protein folding. *Nature (Lond.)*. 366:228-233.
- Martin, J., S. Germanos, P. Tempst, and F.-U. Hartl. 1993b. Identification of nucleotide-binding regions in the chaperonin proteins GroEL and GroES. *Nature (Lond.)*. 366:279-282.
- Melki, R., I. E. Vainberg, R. L. Chow, and N. J. Cowan. 1993. Chaperonin-mediated folding of vertebrate actin-related protein and γ -tubulin. *J. Cell Biol.* 122:1301-1310.
- Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* 12:7035-7056.
- Mizzen, L. A., A. Kabling, and W. J. Welch. 1991. The two mitochondrial stress proteins, GRP 75 and hsp 58, transiently interact with newly synthesized proteins. *Cell Regul.* 2:165-179.
- Molla, A., A. V. Paul, and E. Wimmer. 1991. Cell-free, de novo synthesis of poliovirus. *Science (Wash. DC)*. 254:1647-1651.
- Mummert, E., R. Grimm, V. Speth, C. Eckerskorn, E. Schiltz, A. A. Gatenby, and E. Schafer. 1993. A TCP 1-related molecular chaperone from plants refolds phytochrome to its photoreversible form. *Nature (Lond.)*. 363:644-648.
- Nassal, M. 1988. Total chemical synthesis of a gene for hepatitis B virus core protein and its functional characterization. *Gene (Amst.)*. 66:279-294.
- Nelson, R. J., T. Ziegelhoffer, C. Nicolet, M. Werner-Washburne, and E. A. Craig. 1982. The translation machinery and 70 kD heat shock protein cooperate in protein synthesis. *Cell*. 71:97-105.
- Onodera, S., O. Hitoshi, M. Yamaki, and N. Ishida. 1982. Electron microscopy of human hepatitis B virus cores by negative staining-carbon film technique. *J. Med. Virol.* 10:147-155.
- Ostermann, J., A. L. Horwich, W. Neupert, and F.-U. Hartl. 1989. Protein folding in mitochondria requires complex formation with hsp 60 and ATP hydrolysis. *Nature (Lond.)*. 341:125-130.

- Pato, M., M. Banerjee, L. Desmet, and A. Toussaint. 1987. Involvement of heat shock proteins in bacteriophage Mu development. *J. Bacteriol.* 169: 5504-5509.
- Roossinck, M. J., S. Jameel, S. H. Loukin, and A. Siddiqui. 1986. Expression of hepatitis B viral core region in mammalian cells. *Mol. Cell Biol.* 6: 1393-1400.
- Rothman, J. E. 1989. Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. *Cell.* 59:591-601.
- Sabatini, D. D., Y. Tashiro, and G. E. Palade. 1966. On the attachment of ribosomes to microsomal membranes. *J. Mol. Biol.* 19:503-524.
- Seifer, M., S. Zhou, and D. N. Standring. 1993. A micromolar pool of antigenically distinct precursors is required to initiate cooperative assembly of hepatitis B virus capsids in *Xenopus* oocytes. *J. Virol.* 67:249-257.
- Skowrya, D., C. Georgopoulos, and M. Zylicz. 1990. The *E. coli dnaK* gene product, the hsp 70 homolog, can reactivate heat-inactivated RNA polymerase in an ATP hydrolysis-dependent manner. *Cell.* 62:939-944.
- Sternberg, N. 1973. Properties of a mutant of *Escherichia coli* defective in bacteriophage lambda head formation (groE). *J. Mol. Biol.* 76:25-44.
- Sternlicht, H., G. W. Farr, M. L. Sternlicht, J. K. Driscoll, K. Willison, and M. B. Yaffe. 1993. The t-complex polypeptide 1 complex is a chaperonin for tubulin and actin in vivo. *Proc. Natl. Acad. Sci. USA.* 90:9422-9426.
- Tam, J. P. 1988. Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. *Proc. Natl. Acad. Sci. USA.* 85:5409-5413.
- Trent, J. D., E. Nimmegern, J. S. Wall, F.-U. Hartl, and A. L. Horwich. 1991. A molecular chaperone from a thermophilic archaebacterium is related to the eukaryotic protein t-complex polypeptide-1. *Nature (Lond.).* 354: 490-493.
- Ursic, D., and M. R. Culbertson. 1991. The yeast homolog to mouse TCP-1 affects microtubule-mediated processes. *Mol. Cell Biol.* 11:2629-2640.
- Willison, K., V. Lewis, K. S. Zuckerman, J. Cordell, C. Dean, K. Miller, M. F. Lyon, and M. Marsh. 1989. The t complex polypeptide 1 (TCP-1) is associated with the cytoplasmic aspect of golgi membranes. *Cell.* 57: 621-632.
- Yaffe, M. B., G. W. Farr, D. Miklos, A. L. Horwich, M. L. Sternlicht, and H. Sternlicht. 1992. TCP-1 complex is a molecular chaperone in tubulin biogenesis. *Nature (Lond.).* 358:245-248.
- Yost, S. C., J. Hegpath, and V. R. Lingappa. 1983. A stop transfer sequence confers predictable transmembrane orientation to a previously secreted protein in cell-free systems. *Cell.* 34:759-766.
- Zhou, S., and D. N. Standring. 1992. Hepatitis B virus capsid particles are assembled from core-protein dimer precursors. *Proc. Natl. Acad. Sci. USA.* 89:10046-10050.
- Zhou, S., S. Q. Yang, and D. N. Standring. 1992. Characterization of hepatitis B virus capsid particle assembly in *Xenopus oocytes*. *J. Virol.* 66: 3086-3092.