JOURNAL OF VIROLOGY, Mar. 1992, p. 1520–1527
0022-538X/92/031520-08$02.00/0

Vol. 66, No. 3

Association of Heat Shock Protein 70 with Enterovirus Capsid Precursor P1 in Infected Human Cells

DENNIS G. MACEJAK AND PETER SARNOW*

Molecular Biology Program, Department of Biochemistry, Biophysics and Genetics, and Department of Microbiology and Immunology, University of Colorado Health Sciences Center, Denver, Colorado 80262

Received 12 August 1991/Accepted 20 November 1991

Members of the human heat shock (HSP) family of related proteins are involved in the intracellular folding, transport, and assembly of proteins and protein complexes. We have observed that human heat shock protein 70 (HSP70) is associated with the capsid precursor P1 of poliovirus and coxsackievirus B1 in infected HeLa cells. Antiserum generated against HSP70 coimmunoprecipitated the poliovirus protein P1, an intermediate in capsid assembly. Similarly, α-virion serum coimmunoprecipitated HSP70 from virus-infected cell extracts, but not from mock-infected cell extracts. The HSP70-P1 complex was stable in high-salt medium but was sensitive to incubation with 2 mM ATP, which is a characteristic of other known functional complexes between HSP70 and cellular proteins. The P1 in the complex was predominantly newly synthesized, and the half-life of complexed P1 was nearly twice as long as that of total P1. The HSP70-P1 complex was found to sediment at 3S to 6S, suggesting that it may be part of, or a precursor to, the "5S promoter particles" thought to be an assembly intermediate of picornaviruses. The finding that HSP70 was associated with the capsid precursors of at least two enteroviruses may suggest a functional role of these complexes in the viral life cycles.

Poliovirus, a member of the picornavirus family, has a 7,500-nucleotide positive-stranded RNA genome encapsidated in an icosahedral protein shell. The genomic RNA contains a single long open reading frame which encodes a 247-kDa polyprotein (29). This polyprotein is cotranslationally cleaved first by the virus-encoded protease 2A, releasing the capsid precursor P1 (34). The capsid precursor P1 is subsequently cleaved by the virus-encoded protease 3CD (36), to yield a 5S promoter particle containing one copy each of VP1, VP3, and VP0. Five of these promoter particles are then thought to assemble in the cytoplasm of the infected cells into a 14S pentamer (27, 29), which can then assemble into 75S empty capsids (27, 29). Subsequently, the viral RNA may then be encapsidated by either the 75S empty capsids or the 14S pentamers, resulting in the formation of a 125S to 150S provirion containing 60 copies each of VP0, VP1, and VP3. The final processing of the provirion into the mature virion requires cleavage of VP0 into VP2 and VP4, presumably by a virion-catalyzed mechanism (2).

It is striking that many processes in the viral life cycle, such as RNA replication (3, 4), translation (8, 28), and capsid assembly (7, 26, 35), appear to be localized on membranes derived from the endoplasmic reticulum (ER). For example, the RNA replication complexes are thought to be anchored on the membrane surface by the hydrophobicity of 2C, 2BC, and 3AB viral proteins (3, 4, 33) required for RNA synthesis. However, the mechanism for targeting poliovirus mRNA translation to ER membranes is not clear, because the poliovirus polyprotein has no known targeting signal sequence. Similarly, it is unknown why viral assembly takes place on membranes, because poliovirions do not contain glycoproteins.

There is evidence that proteins can be targeted to membrane-bound organelles derived from the ER by an association with members of the 70-kDa heat shock protein (HSP70) family, which presumably maintain the associated proteins in an unfolded, translocationally competent conformation. For example, both the bacterial plasma membrane M13 coat protein precursor (37) and the yeast preproalpha factor (9, 12) can be targeted to membranes by association with HSP70. Similarly, heat shock proteins have been reported to associate with steroid hormone receptor and may target the activated receptor to the nucleus (15). Besides playing a role in intracellular targeting, HSP70 is involved in the folding and translocation of proteins into the mitochondrial matrix (19). Another HSP70-like molecule, BiP, is localized in the lumen of the ER and is known to bind to newly synthesized immunoglobulin heavy chains during the course of their folding and assembly with immunoglobulin light chains (5, 16).

Evidence that HSP70 may be involved in the assembly of virions has been provided by Macejak and Luftig (22), who reported that HSP70 is associated with the fiber protein of adenovirus. Because members of the HSP70 family facilitate both the proper folding and targeting of proteins within the cell, we examined whether HSP70 was specifically associated with poliovirus proteins in infected cells. We report here the association of HSP70 with the capsid precursor P1 of both poliovirus type 1 and coxsackievirus B1 and suggest a possible role for this interaction in viral assembly.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% bovine serum (Irvine Scientific). Wild-type poliovirus type 1 was isolated from a single plaque derived from HeLa cells that had been transfected with RNA molecules made by in vitro transcription of a full-length poliovirus cDNA-containing plasmid as previously described (30). Coxsackievirus B1 was obtained from Harley Rotbart, University of Colorado Health Sciences Center, Denver.

Preparation of cell extracts and analysis of proteins by

* Corresponding author.

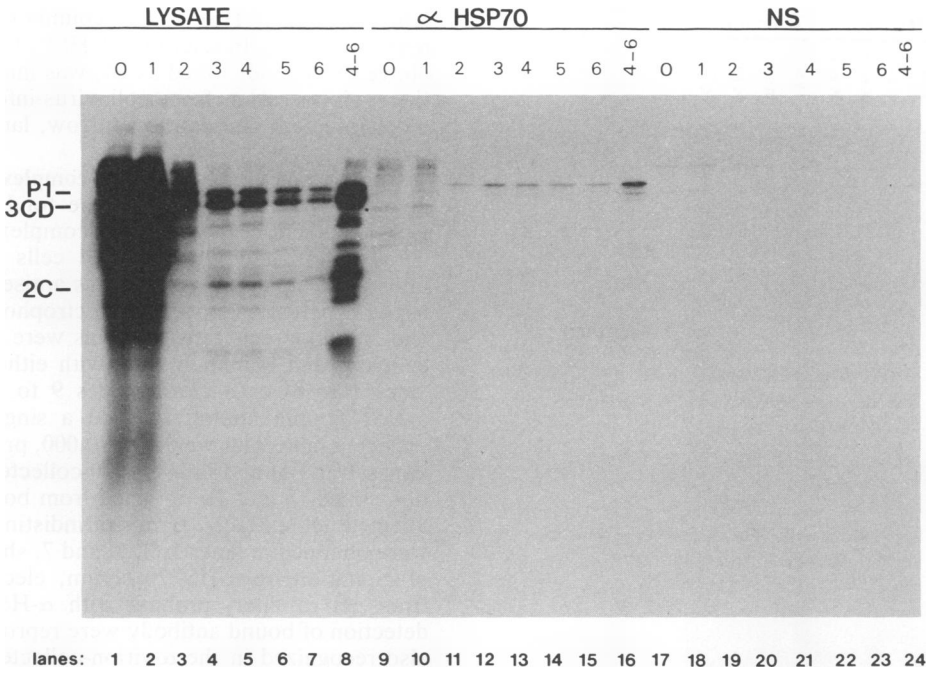

FIG. 1. Immunoprecipitation of P1 by α-HSP70 serum in poliovirus-infected HeLa cells. Infected cells were pulse-labeled with [^{35}S]methionine-[^{35}S]cysteine for 20 min at the times postinfection as indicated in hours above the autoradiograph. Cytoplasmic extracts were prepared and analyzed by SDS-PAGE. An autoradiograph is shown displaying labeled proteins obtained from the total lysate (LYSATE; lanes 1 to 8), after immunoprecipitation with α-HSP70 serum (HSP70; lanes 9 to 16), or after immunoprecipitation with normal rabbit serum (NS; lanes 17 to 24). The positions of poliovirus proteins P1, 3CD, and 2C are indicated. The asterisk (adjacent to lane 9) denotes the position of HSP70 in lanes 9 and 10.

immunoprecipitation and immunoblot techniques. HeLa cell monolayers were infected with wild-type poliovirus as previously described (32), except that a multiplicity of infection of 50 was used. At different times after infection, cells were incubated in methionine-free, cysteine-free medium and subsequently labeled with 100 μCi of [^{35}S]methionine and [^{35}S]cysteine (Trans-label; New England Nuclear) for 10 min (unless otherwise indicated) as described previously (18). In prelabeling experiments, cells were incubated with 100 μCi of Trans-label for 3 h prior to virus infection. For pulse-chase experiments, cells were labeled for 10 min as described above, subsequently washed three times in Dulbecco's modified Eagle's medium supplemented with 0.15 mg of methionine per ml and 0.04 mg of cysteine per ml (10-fold greater than the normal concentrations in Dulbecco's modified Eagle's medium), and incubated for various times at 37°C in Dulbecco's modified Eagle's medium supplemented with the 10-fold concentrations of methionine and cysteine.

Cytoplasmic cell extracts were prepared and immunoprecipitation analysis was performed as described previously (31). Proteins were analyzed on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels. The gels were treated with 1 M salicylate and dried, and autoradiographs were prepared at −70°C.

Immunoblot analysis with α-HSP70 antisera was performed as described previously (22). The α-HSP70 serum immunoprecipitated quantitatively HSP70 from cell extracts. This was determined by immunoprecipitation of various amounts of HSP70 purified either from HeLa cell extracts or from *Escherichia coli* strains which overexpressed HSP70 (21).

Analysis of viral and subviral particles. Preparation of cell extracts and separation of 75S and 150S particles by sedimentation through 15 to 30% sucrose gradients made in RSB (10 mM Tris [pH 7.4], 10 mM NaCl, 1.5 mM $MgCl_2$) were performed as described previously (11), except that cells were labeled with [^{35}S]methionine and [^{35}S]cysteine for 30 min at 3 h after infection. The 5S and 14S particles were separated by sedimentation through 5 to 20% sucrose gradients as described previously (23), except that the gradients were made in RSB.

RESULTS

Coimmunoprecipitation of poliovirus capsid precursor P1 with human HSP70 in infected HeLa cells. To determine whether HSP70 interacts with any poliovirus proteins in infected cells, HeLa cells were infected with wild-type poliovirus and pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine hourly from 0 to 6 h postinfection. Cytoplasmic extracts were prepared and immunoprecipitated, with either antiserum raised against HSP70 (α-HSP70) or normal rabbit serum. Figure 1 shows that poliovirus infection resulted in the expected inhibition of host cell translation (29) and the production of virus-specific proteins by 2 h postinfection (lane 3). Concurrently with the production of poliovirus proteins, α-HSP70 serum immunoprecipitated a protein from infected cell extracts which comigrated with capsid precursor P1 (lanes 11 to 15). In extracts prepared from cells that were continuously labeled from 4 to 6 h after infection, an additional protein comigrating with 3CD was also immunoprecipitated by the α-HSP70 serum (lane 16); however, this protein was also immunoprecipitated by normal serum (lane 24) and thus was not specifically immunoprecipitated by the HSP70 antiserum. Because of the virus-induced inhibition of cellular translation, cellular HSP70 could be labeled only at

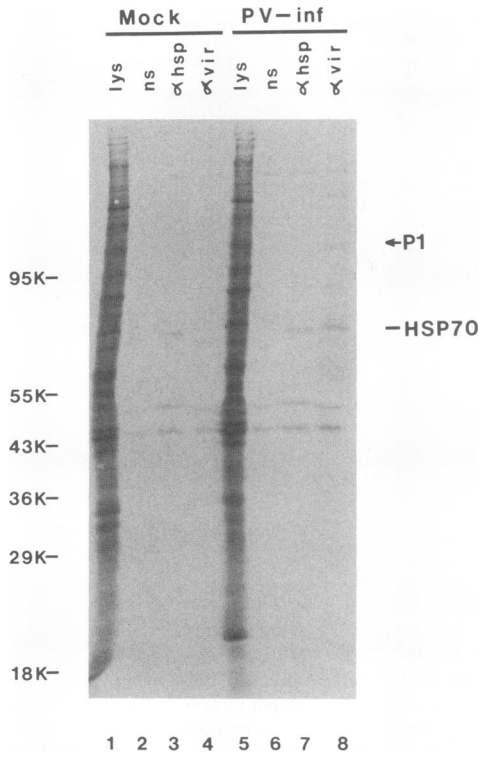

FIG. 2. Immunoprecipitation of HSP70 by α-virion serum in poliovirus-infected (PV-inf) HeLa cells. Cells were labeled with [^{35}S]methionine-[^{35}S]cysteine for 3 h prior to infection. Extracts were prepared 3 h after infection and analyzed by SDS-PAGE. An autoradiograph is shown displaying labeled proteins obtained from the extracts (lys) and after immunoprecipitation with normal rabbit serum (ns), α-HSP70 serum (αhsp), or α-virion serum (αvir). The migration of prestained marker proteins (Diversified Biotech) is indicated (in thousands) on the left. Note that the migration of the 70-kDa HSP70 protein was slightly retarded in lane 8 owing to excess immunoglobulin heavy chain migrating slightly below the 70-kDa protein. The position of HSP70 is indicated at right. The arrow denotes P1 immunoprecipitated specifically by the α-virion serum in infected cell extracts.

1 and 2 h after infection (lanes 9 and 10). The additional labeled proteins immunoprecipitated at that time most probably represent cellular proteins that are normally associated with HSP70 (25).

To test whether antiserum directed against poliovirions coimmunoprecipitated HSP70, we labeled HeLa cells for 3 h prior to infection with poliovirus. Cell extracts were then prepared at 3 h after infection and immunoprecipitated with antiserum directed against either HSP70 or virions. Figure 2 shows that the patterns of labeled proteins from mock- and virus-infected cell extracts were indistinguishable (lanes 1 and 5); no poliovirus-specific proteins could be seen under these labeling conditions. The α-HSP70 serum specifically immunoprecipitated the expected 70-kDa protein from both uninfected (lane 3) and infected (lane 7) extracts, as well as a few other proteins which may be present in complex with HSP70 in the cell extract. In addition, several other proteins were nonspecifically immunoprecipitated by both the α-HSP70 and the normal rabbit serum (lane 2). The α-virion serum immunoprecipitated a 70-kDa protein from infected cell extracts but not from mock-infected cell extracts (lanes 8 and 4, respectively). This indicated that the 70-kDa protein, presumably HSP70, was coimmunoprecipitated by the α-virion serum. In addition to HSP70, a protein migrating above 95 kDa, identified as P1, was immunoprecipitated by the α-virion serum from poliovirus-infected, but not from mock-infected, cell extracts (arrow, lanes 8 and 4, respectively).

Quantitation of the HSP70-P1 complex in infected cells. To determine the percentage of the total amount of P1 and HSP70 present in the observed complex, lysates from unlabeled mock- and virus-infected cells were prepared and immunoprecipitated with various antisera. After separation by SDS-polyacrylamide gel electrophoresis (PAGE), total and immunoprecipitated proteins were transferred to nitrocellulose and immunoblotted with either α-HSP70 (Fig. 3, lanes 1 to 8) or α-virion (lanes 9 to 16) antibodies. The α-HSP70 immunoblot revealed a single protein with the expected molecular weight of 70,000, present in total lysates (lanes 1 and 5) and in α-HSP70-collected immunoprecipitations (lanes 3 and 7), obtained from both mock- and virus-infected cell extracts. Bands of indistinguishable intensities were obtained in lanes 1, 3, 5, and 7, showing that immunoprecipitation by α-HSP70 serum, electrophoretic transfer from gel to filter, probing with α-HSP70 antibody, and detection of bound antibody were reproducible. HSP70 was also recognized in the α-virion-collected immunoprecipitations obtained from virus-infected extracts (lane 8) but not from mock-infected extracts (lanes 4). Quantitation by densitometry (32) showed that as much as 50% of total cellular HSP70 was immunoprecipitated by the α-virion serum.

The blot on the right in Fig. 3 shows that α-virion serum detected viral capsid precursors P1 and VP0 and capsid proteins VP1 and VP3 in lysates and in α-virion-collected immunoprecipitations from virus-infected (lanes 13 and 16) but not mock-infected (lanes 9 and 12) cells. Furthermore, the precursor protein P1 was detected in the α-HSP70-collected immunoprecipitation from virus-infected (lane 15) but not mock-infected (lane 11) cell extracts. Quantitation by densitometry revealed that approximately 1% of the total P1 was immunoprecipitated by the α-HSP70 serum.

Again, this experiment substantiated that HSP70 and P1 were not individually recognized by both antisera. Rather, HSP70 and P1 could be coimmunoprecipitated in infected cell extracts and thus appeared to be in a physical complex with each other. This complex was found to remain stable after incubation in 500 mM NaCl–1% Nonidet P-40–0.2% SDS (30 min at 0°C), but was sensitive to freezing at −20°C (21). Furthermore, the association of HSP70 appeared to be specific for P1 and not for the P1 cleavage products VP0, VP1, or VP3 or any other poliovirus proteins.

To gain further evidence for a physical association of HSP70 with P1, we cross-linked extracts from poliovirus-infected cells with both *N*-succinimidyl-6-(4′-azido-2′-nitrophenylamino)hexonate (SANPAH; Pierce) and bis(sulfosuccinimidyl)suberate (BS_3; Pierce). Both HSP70 and P1 appeared to redistribute into heterogeneous high-molecular-weight complexes in SDS-polyacrylamide gels after cross-linking (21). However, it was not conclusive whether these high-molecular-weight complexes contained HSP70-P1 complexes. If only 1% of total P1 was associated with HSP70 (Fig. 3), and if the efficiency of cross-linking was 50% or less, a HSP70-P1 complex may not be displayed by this approach.

HSP70-associated P1 has a longer half-life than total P1 does. To examine potential functional roles of the HSP70-P1 complex in infected cells, we first compared the stability of total P1 and HSP70-complexed P1. Poliovirus-infected cells were pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine 3

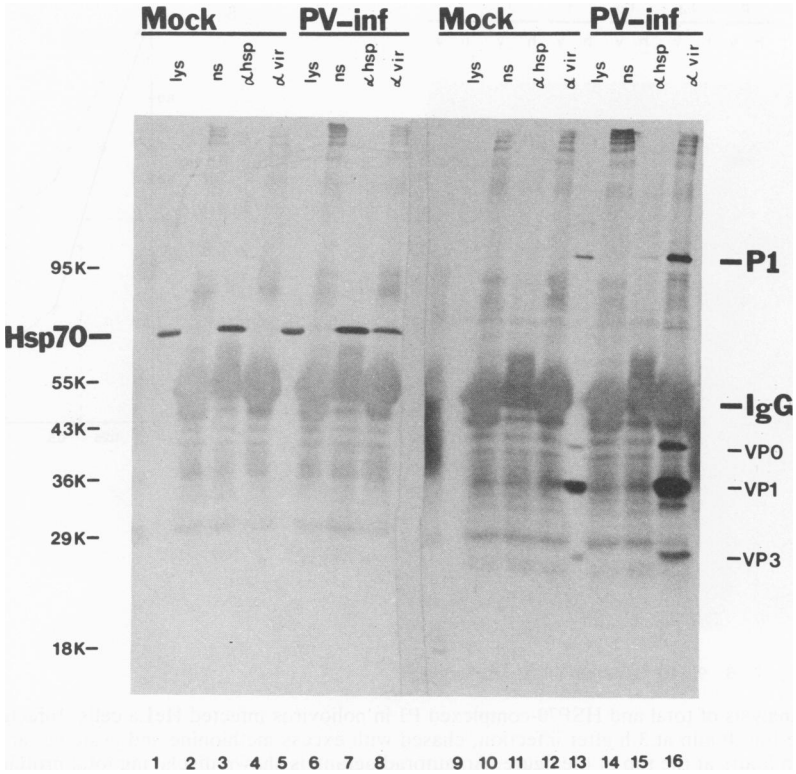

FIG. 3. Immunoblot analysis of capsid proteins and HSP70 in mock- and poliovirus-infected (PV-inf) cell extracts. Proteins from extracts (lys) and after immunoprecipitation with normal serum (ns), α-HSP70 serum (αhsp), or α-virion serum (αvir) sera were separated by SDS-PAGE and subsequently transferred to nitrocellulose filters. The filter represented on the left was incubated with α-HSP70 serum (lanes 1 to 8). The filter represented on the right was incubated with α-virion serum (lanes 9 to 16). The positions of HSP70 and marker proteins are indicated (in thousands) on the left. The positions of P1, cleavage products VP0, VP1, and VP3, and immunoglobulin heavy chain (IgG) are indicated on the right.

h after infection, excess unlabeled methionine-cysteine was added, and the processing and/or degradation of total and HSP70-complexed P1 was monitored by immunoprecipitation analysis. Figure 4A shows that pulse-labeled P1 was chased into P1 cleavage products VP0, VP1, and VP3 (lanes 1 to 6). P1 and its cleavage products could be immunoprecipitated with the α-virion antibody (lanes 8, 10, 12, 14, 16, and 18). The P1 precursor, but not the P1 cleavage products, could be immunoprecipitated by α-HSP70 serum and, thus, represented the HSP70-associated form of P1 (lanes 7, 9, 11, 13, 15, and 17).

Quantitation of pulse-labeled P1 (lanes 7 and 8) revealed that 15% of newly synthesized P1 was immunoprecipitated by the α-HSP70 serum. In contrast, only 1% of the total amount of P1 was complexed to HSP70 (Fig. 3). Thus, it appeared that HSP70 preferentially associated with newly synthesized P1 and that the half-life of P1 associated with HSP70 was longer (43 min) than that of total P1 (23 min) (Fig. 4B). The increased half-life of HSP70-associated P1 could be due to either slower processing of P1 by 3CD or slower overall decay of P1, or both. The individual contributions of processing and of overall decay on the stability of P1 could be studied by using extracts prepared from 3C mutant poliovirus-infected cells.

Sensitivity of the HSP70-P1 complex to incubation with ATP. Many known HSP70-protein complexes, such as HSP70-p53 and BiP-immunoglobulin heavy chain (reviewed in reference 25), are disrupted by incubation in buffers containing ATP (25). The precise role of the ATP is not clear. However, members of the HSP70 family are known to bind ATP with high affinity (25). Pelham has therefore suggested that ATP hydrolysis may provide the energy to change the conformation of the heat shock protein-protein complex, thereby inducing the disruption of the complex (25). As shown in Table 1, 84% of newly synthesized P1 that is associated with HSP70 (15% of newly synthesized P1 is associated with HSP70 in the cell extract) could be released from HSP70-P1 complexes after incubation of the infected cell extract with 2 mM ATP. Thus, the HSP70-P1 complex displayed the ATP sensitivity characteristic of many HSP70-protein complexes.

The HSP70-P1 complex sediments at 3S to 6S. The assembly intermediates of poliovirus have defined sedimentation values (27). To determine whether the HSP70-P1 complexes were associated with any of these particles, we separated infected cell extracts by sucrose density sedimentation. The separation of 75S and 150S particles in 15 to 30% sucrose gradients is shown in Fig. 5A and the proteins present in the individual fractions of the gradient are shown in Fig. 5B. Most of the radiolabeled capsid proteins were at the top of the gradient in fractions 1, 2, and 3, containing 31, 20, and 9% of labeled P1, respectively. The 150S peak (fractions 13 and 14) contained processed capsid proteins VP0, VP1, VP2, and VP3. Immunoprecipitation of alternate fractions with α-HSP70 serum showed that HSP70-associated P1 was found primarily in fraction 3 (44% of total radiolabeled P1) (Fig. 5C).

To resolve the HSP70-P1 complex further, we analyzed

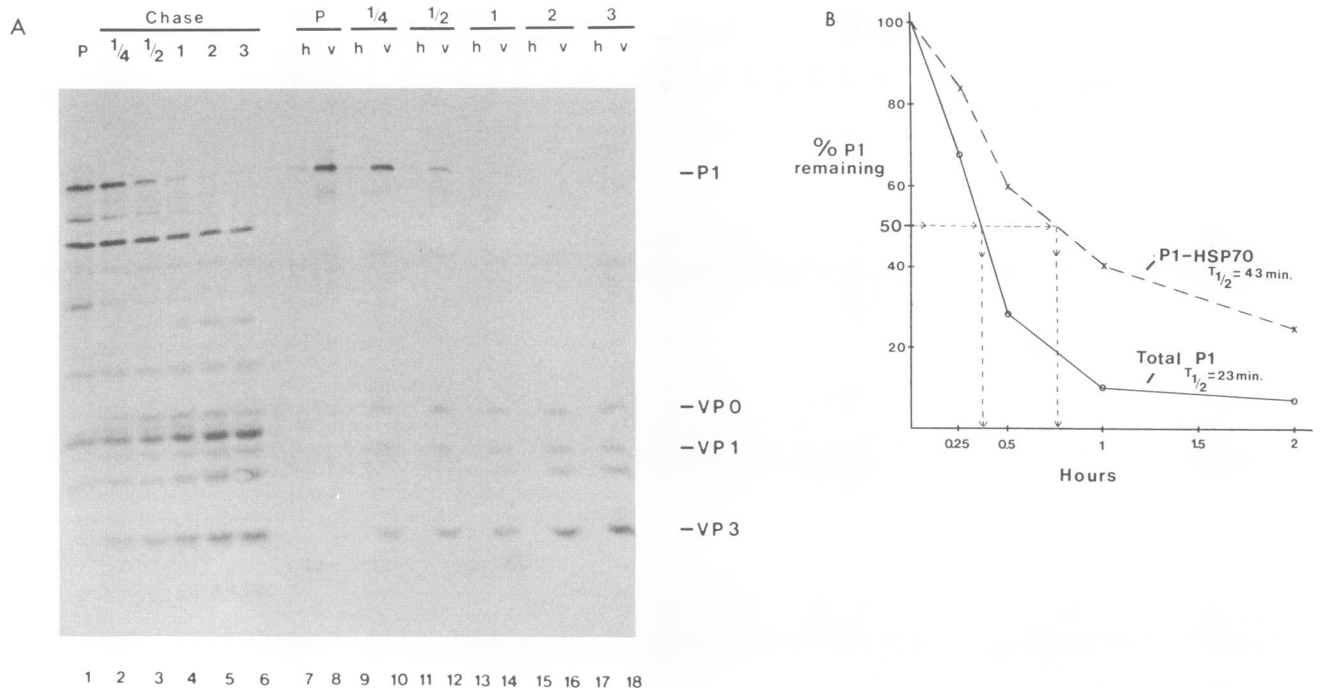

FIG. 4. (A) Pulse-chase analysis of total and HSP70-complexed P1 in poliovirus-infected HeLa cells. Infected cells were pulsed (P) with [^{35}S]methionine-[^{35}S]cysteine for 10 min at 3 h after infection, chased with excess methionine and cysteine, and harvested at various times after the chase as indicated in hours at the top of the figure. An autoradiograph is shown displaying total proteins (lanes 1 to 6) and proteins immunoprecipitated with α-HSP70 (h) or α-virion (v) serum. The positions of P1 and P1 cleavage products (VP0, VP1, and VP3) are indicated. (B) Quantitation of total and HSP70-complexed P1. P1 in panel A was quantitated by densitometric analysis. The amount of radioactivity in the immunoprecipitated P1 bands after the pulse-label was set to 100%. The percentages of P1 that could be immunoprecipitated at various times after the chase are shown.

infected cell extracts by sedimentation through 5 to 20% sucrose gradients (23). Fractions were immunoprecipitated with α-HSP70 serum and separated by SDS-PAGE. The gel was subsequently immunoblotted with α-virion serum. The P1 immunoprecipitated by α-HSP70 serum was found to be at the top of the gradient, particularly in fractions 3 and 4 (Fig. 5D). In a parallel gradient, the sedimentation of hemoglobin (9S) was monitored as a marker. The approximate S value of the HSP70-P1 complex was determined in this way to be 3S to 6S. Immunoprecipitations of the same fractions followed by immunoblotting with α-HSP70 serum indicated that HSP70 was present in fractions 3 and 4 as well (21). Thus, it appeared that the HSP70-P1 complex was not found primarily in large aggregates. Rather, this complex, sedimenting at 3S to 6S, may be part of a precursor, or the entire precursor, to the 5S particle, which is thought to be an intermediate in viral assembly.

TABLE 1. ATP sensitivity of the HSP70-P1 complex

Reaction condition[a]	Amt of P1 associated with HSP70[b]	
	OD_{655}	Relative amt
No addition	542	1.0
10 mM $MgCl_2$ added	546	1.0
10 mM $MgCl_2$ plus 2 mM ATP added	86	0.16

[a] Extracts were prepared in TENN buffer (50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40), and different samples from the same extract were incubated for 10 min at 25°C with the additions as indicated.

[b] Determined by densitometric analysis of the autoradiograph. The amount of P1 under reaction conditions with no addition, after collection by immunoprecipitation with α-HSP70 serum, was set at 1.0. OD_{655}, optical density at 655.

Coimmunoprecipitation of coxsackievirus B1 capsid precursor P1 with HSP70 in infected HeLa cells. To determine whether the HSP70-P1 interaction was seen in other enterovirus-infected cells, extracts were prepared from HeLa cells infected with coxsackievirus B1 and were subjected to immunoprecipitation analysis. As shown in Fig. 6A, α-HSP70 serum immunoprecipitated a 100-kDa protein from coxsackievirus B1-infected cell extracts (lane 4), as well as the 70-kDa HSP70 from mock-infected extracts (lane 3). The 100-kDa protein comigrated with coxsackievirus P1 recognized by α-virion serum (lane 6). As shown in Fig. 6B, the P1 protein of coxsackievirus B1 was observed to migrate more slowly than the analogous P1 protein of poliovirus type 1. The data in Fig. 6A and B suggest that the association of HSP70 with P1 is conserved among at least two enteroviruses; these capsid precursor proteins share about 60% homolgy (17).

DISCUSSION

We have examined the association of HSP70 with poliovirus structural proteins in infected HeLa cell extracts by using immunoprecipitation and immunoblot analysis with sera generated against either human HSP70 or poliovirus virions. The α-HSP70 serum coimmunoprecipitated the capsid precursor P1 in cytoplasmic extracts from infected cells. Conversely, the α-virion serum coimmunoprecipitated HSP70 from virus-infected but not mock-infected cell ex-

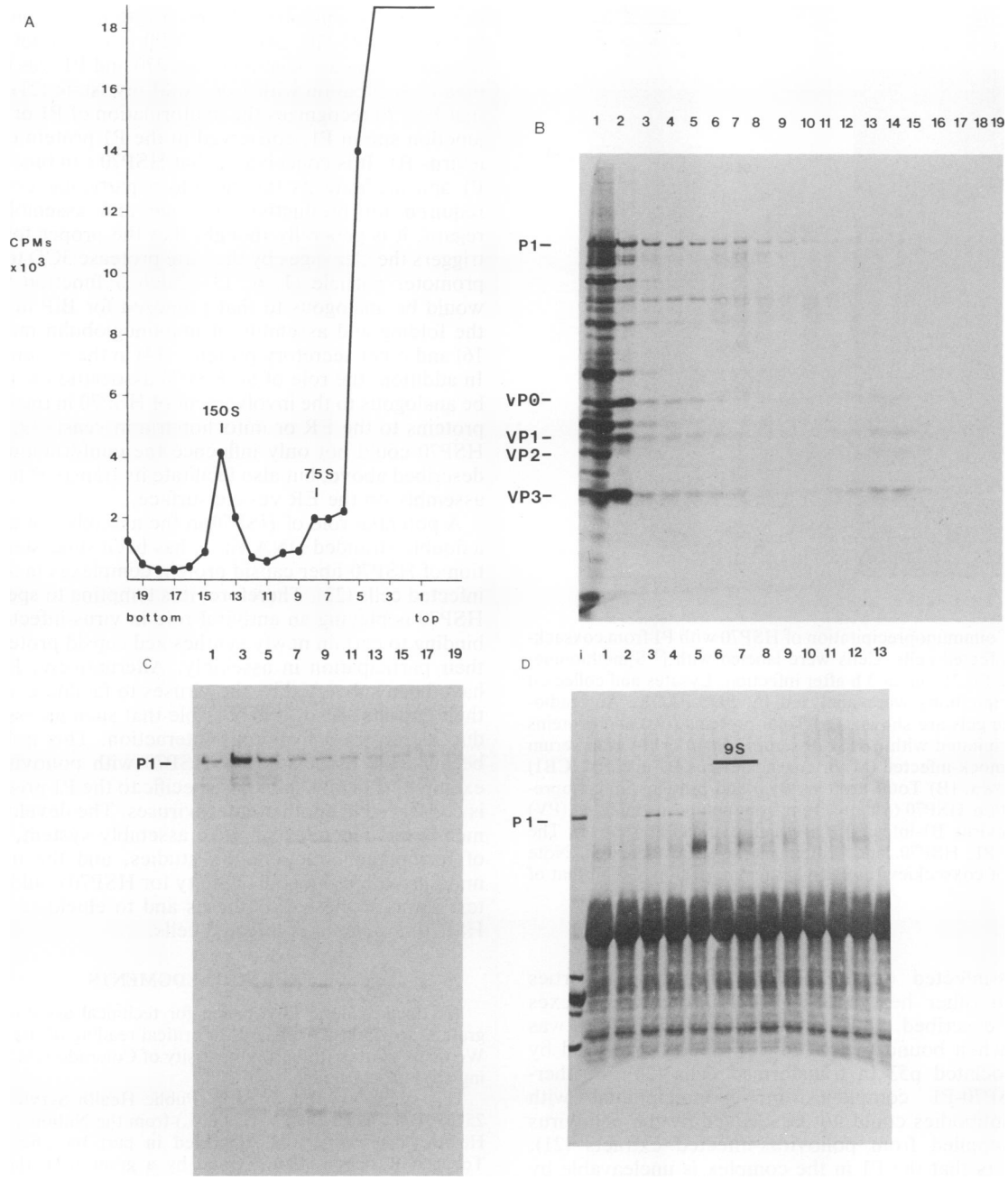

FIG. 5. Analysis by sucrose gradient sedimentation of HSP70-associated viral and subviral particles. (A) Profile of [^{35}S]methionine-[^{35}S]cysteine-labeled cell extracts from poliovirus-infected cells fractionated in a 15 to 30% sucrose gradient. Fractions are numbered from top to bottom. Counts per minutes (CPM) are indicated. (B) SDS-PAGE analysis of radiolabeled proteins in individual fractions of the sucrose gradient shown in panel A. An autoradiograph is shown. (C) Alternate fractions of the sucrose gradient shown in panel A were immunoprecipitated with α-HSP70 serum and analyzed by SDS-PAGE. An autoradiograph is shown. The coimmunoprecipitation of the low-molecular-weight proteins was also seen in samples collected after immunoprecipitation with normal serum, and thus is nonspecific. (D) Immunoblot analysis of proteins from poliovirus-infected cell extracts. Extracts were separated by sedimentation in 5 to 20% sucrose gradients, and individual fractions were immunoprecipitated with α-HSP70 serum. After SDS-PAGE, proteins were transferred to nitrocellulose and the blot was incubated with α-virion serum. The position of hemoglobin (9S), separated from rabbit reticulocyte lysate in a parallel gradient, is indicated. P1 in the unfractionated infected extract is also shown (lane i).

tracts. We have shown that the antibodies did not cross-react, and thus it appears that HSP70 and P1 are physically associated in infected cells.

The HSP70-P1 complex was stable in 500 mM NaCl and 0.2% SDS, but was sensitive to incubation with 2 mM ATP or to freezing at −20°C. As reviewed by Pelham (25), complexes between HSP70 and p53 or between BiP and immunoglobulin heavy chain are all resistant to high salt concentrations, but are released in an ATP-dependent manner. Thus, the HSP70-P1 complex that we have observed in

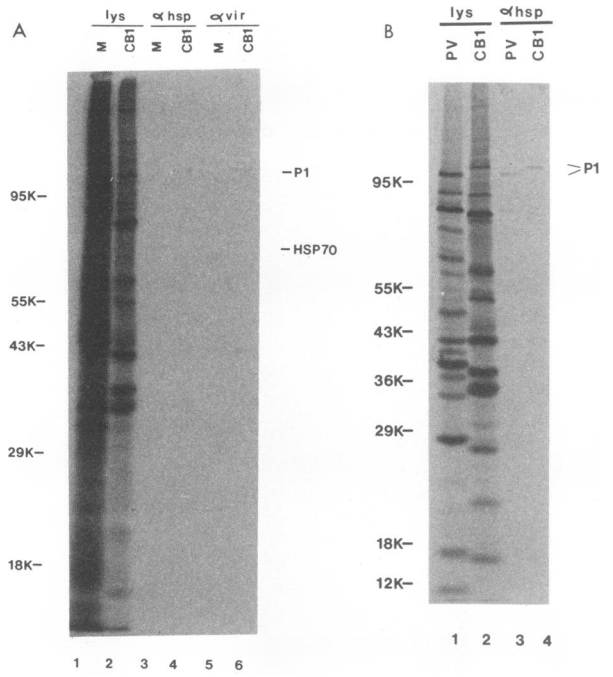

FIG. 6. Coimmunoprecipitation of HSP70 with P1 from coxsackievirus B1-infected cells. Cells were labeled with [^{35}S]methionine-[^{35}S]cysteine for 20 min at 3 h after infection. Lysates and collected immunoprecipitations were analyzed by SDS-PAGE. Autoradiographs of the gels are shown. (A) Total proteins (lys) and proteins immunoprecipitated with α-HSP70 serum (αhsp) or α-virion serum (αvir) from mock-infected (M) or coxsackievirus B1-infected (CB1) cells are shown. (B) Total proteins (lys) and proteins immunoprecipitated with α-HSP70 (αhsp) serum from poliovirus-infected (PV) or coxsackievirus B1-infected (CB1) cell extracts are shown. The positions of P1, HSP70, and protein markers are indicated. Note that the P1 of coxsackievirus B1 migrates more slowly than that of poliovirus.

enterovirus-infected cells shares biochemical properties common to other heat shock protein-protein complexes previously described. In addition, the half-life of P1 was increased when bound to HSP70, a property displayed by HSP70-associated p53 in transformed cells (20). Furthermore, HSP70-P1 complexes immunoprecipitated with α-HSP70 antibodies could not be cleaved by the poliovirus protease supplied from poliovirus-infected extracts (21). This suggests that the P1 in the complex is uncleavable by the viral protease, and this is in agreement with the finding that heat shock proteins are associated with unfolded proteins (25).

Although only 1% of the total amount of P1 was observed to be associated with HSP70, pulse-chase experiments revealed that 15% of newly synthesized P1 was associated with HSP70. Thus it appears that HSP70 is preferentially associated with newly synthesized P1. Most probably, a single burst of 1,000 virions per cell requires more than 1% of total P1 present in the cell. However, if HSP70 association with P1 is only transient, the role of the HSP70-P1 interaction may be significant.

Further analysis of the HSP70-P1 complex by sucrose gradient sedimentation showed that it sedimented at 3S to 6S. HSP70 associated specifically with precursor P1 and not with the P1 cleavage products VP0, VP1, and VP3. Therefore, it is unlikely that the interaction between HSP70 and P1 in the complex involves the myristate moiety on the amino terminus of P1 (10, 24), since VP0 is myristylated as well. Moreover, the association of HSP70 and P1 was not distributed by incubation with 1 or 5 mM myristate (21). It may be that HSP70 recognizes the conformation of P1 or a cleavage junction site in P1, conserved in the P1 protein of coxsackievirus B1. It is conceivable that HSP70 can bind to nascent P1 and facilitate its folding into a particular conformation required for productive cleavage and assembly. In this regard, it is generally thought that the proper folding of P1 triggers the cleavages by the viral protease 3CD to yield a 5S promoter particle (1, 6, 13). Such a function for HSP70 would be analogous to that proposed for BiP in facilitating the folding and assembly of immunoglobulin molecules (5, 16) and other secretory proteins (14) in the lumen of the ER. In addition, the role of an HSP70 association with P1 could be analogous to the involvement of HSP70 in the targeting of proteins to the ER or mitochondria in yeasts (9, 12). Thus, HSP70 could not only influence the conformation of P1 as described above, but also facilitate its transport to the site of assembly on the ER vesicle surface.

A potential role of HSP70 in the assembly of adenovirus, a double-stranded DNA virus, has been suggested by detection of HSP70-fiber capsid protein complexes in adenovirus-infected cells (22). Therefore, it is tempting to speculate that HSP70 is playing an antiviral role in virus-infected cells by binding to certain newly synthesized capsid proteins to limit their participation in assembly. Alternatively, HSP70 may have been subverted by the viruses to facilitate assembly of their capsids. Also, it is possible that such an association is due merely to a fortuitous interaction. This is less likely, because the association of HSP70 with poliovirus P1, for example, is highly stable, is specific to the P1 precursor, and is conserved in another enteroviruses. The development of a membrane-dependent in vitro assembly system, the results of further cell fractionation studies, and the use of viral mutants with reduced P1 affinity for HSP70 could be used to test some of these hypotheses and to elucidate the role of HSP70 in poliovirus-infected cells.

ACKNOWLEDGMENTS

We thank Valerie Ray Vaden for technical assistance. We are grateful to Karla Kirkegaard for critical reading of the manuscript. We thank Harley Rotbart (University of Colorado HSC) for providing coxsackievirus B1.

This study was supported by Public Health Service grants AI-25105 (P.S.) and AI-08224 (D.G.M.) from the National Institutes of Health. This work was supported in part by The Council for Tobacco Research, U.S.A., and by a grant from the Lucille P. Markey Charitable Trust.

REFERENCES

1. **Argos, P., G. Kamer, M. J. H. Nicklin, and E. Wimmer.** 1984. Similarity in gene organization and homology between proteins of animal picornaviruses and a plant comovirus suggest common ancestry of these virus families. Nucleic Acids Res. **12**:7251–7267.
2. **Arnold, E., M. Luo, G. Vriend, M. G. Rossman, A. C. Palmenberg, G. D. Parks, M. J. H. Nicklin, and E. Wimmer.** 1987. Implications of the picornavirus capsid structure for polyprotein processing. Proc. Natl. Acad. Sci. USA **84**:21–25.
3. **Bienz, K., D. Egger, and L. Pasamontes.** 1987. Association of polioviral proteins of the P2 genomic region with the viral replication complex and virus-induced membrane synthesis as visualized by electron microscopic immunocytochemistry and autoradiography. Virology **160**:220–226.
4. **Bienz, K., D. Egger, M. Troxler, and L. Pasamontes.** 1990. Structural organization of poliovirus RNA replication is medi-

ated by viral proteins of the P2 genomic region. J. Virol. **64**:1156–1163.
5. **Bole, D., L. Hendershot, and J. F. Kearney.** 1986. Post-translational association of immunoglobulin heavy chains in nonsecreting and secreting hybridomas. J. Cell Biol. **102**:1558–1566.
6. **Burns, C. C., M. A. Lawson, B. L. Semler, and E. Ehrenfeld.** 1989. Effects of mutations in poliovirus $3D^{pol}$ on RNA polymerase activity and on polyprotein cleavage. J. Virol. **63**:4866–4874.
7. **Caliguiri, L. A., and A. G. Mosser.** 1971. Proteins associated with the poliovirus RNA replication complex. Virology **46**:375–386.
8. **Caliguiri, L. A., and I. Tamm.** 1970. Characterization of poliovirus-specific structures associated with cytoplasmic membranes. Virology **42**:112–122.
9. **Chirico, W. J., M. G. Waters, and G. Blobel.** 1988. 70K heat shock related proteins stimulate protein translocation into microsomes. Nature (London) **332**:805–810.
10. **Chow, M., J. F. E. Newman, D. Filman, J. M. Hogle, D. J. Rowlands, and F. Brown.** 1987. Myristylation of picornavirus capsid protein VP4 and its structural significance. Nature (London) **327**:482–486.
11. **Compton, S. R., B. Nelsen, and K. Kirkegaard.** 1990. Temperature-sensitive poliovirus mutant fails to cleave VP0 and accumulates provirions. J. Virol. **64**:4067–4075.
12. **Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman.** 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature (London) **332**:800–805.
13. **Dewalt, P. G., and B. L. Semler.** 1987. Site-directed mutagenesis of proteinase 3C results in a poliovirus deficient in synthesis of viral RNA polymerase. J. Virol. **61**:2162–2170.
14. **Dorner, A. J., D. G. Bole, and R. J. Kaufman.** 1987. The relationship of N-linked glycosylation and heavy chain-binding protein association with the secretion of glycoproteins. J. Cell Biol. **105**:2665–2674.
15. **Estes, P. A., E. J. Suba, J. Lawler-Heavner, D. Elashry-Stowers, L. L. Wei, D. O. Toft, W. P. Sullivan, K. B. Horwitz, and D. P. Edwards.** 1987. Immunologic analysis of human breast cancer progesterone receptors. 1. Immunoaffinity purification of transformed receptors and production of monoclonal antibodies. Biochemistry **26**:6250–6262.
16. **Hendershot, L. M., and J. F. Kearney.** 1988. A role for human heavy chain binding protein in the development regulation of immunoglobulin transport. Mol. Immunol. **25**:585–595.
17. **Iizuka, N., S. Kuge, and A. Nomoto.** 1987. Complete nucleotide sequence of the genome of coxsackievirus B1. Virology **156**:64–73.
18. **Johnson, K. L., and P. Sarnow.** 1991. Three poliovirus 2B mutants exhibit noncomplementable defects in viral RNA amplification and display dosage-dependent dominance over wild-type poliovirus. J. Virol. **65**:4341–4349.
19. **Kang, P.-J., J. Ostermann, J. Shilling, W. Neupert, E. A. Craig, and N. Pfanner.** 1990. Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins. Nature (London) **348**:137–142.
20. **Levine, A. J., J. Momand, and C. A. Finlay.** 1991. The p53 tumour suppressor gene. Nature (London) **351**:453–456.
21. **Macejak, D. G.** Unpublished data.
22. **Macejak, D. G., and R. B. Luftig.** 1991. Association of HSP70 with the adenovirus type 5 fiber protein in infected HEp-2 cells. Virology **180**:120–125.
23. **Palmenberg, A. C.** 1982. In vitro synthesis and assembly of picornaviral capsid intermediate structures. J. Virol. **44**:900–906.
24. **Paul, A. V., A. Schultz, S. E. Pincus, S. Oroszlan, and E. Wimmer.** 1987. Capsid protein VP4 of poliovirus is N-myristoylated. Proc. Natl. Acad. Sci. USA **84**:7827–7831.
25. **Pelham, H. R. B.** 1990. Functions of the hsp70 protein family: an overview, p. 287–299. *In* R. I. Morimoto, A. Tissieres, and C. Georgopoulos (ed.), Stress proteins in biology and medicine. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Perlin, M., and B. A. Philips.** 1973. In vitro assembly of polioviruses. III. Assembly of 14S particles into empty capsids by poliovirus-infected HeLa cell membranes. Virology **53**:107–114.
27. **Putnak, J. R., and B. A. Philips.** 1981. Picornaviral structure and assembly. Microbiol. Rev. **45**:287–315.
28. **Roumiantzeff, M., D. F. Summers, and J. V. Maizel.** 1971. In vitro protein synthetic activity of membrane-bound poliovirus polyribosomes. Virology **44**:249–258.
29. **Rueckert, R. R.** 1990. Picornaviridae and their replication, p. 507–548. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, J. L. Melnick, B. Roizman, and R. E. Shope (ed.), Virology, 2nd ed. Raven Press, New York.
30. **Sarnow, P.** 1989. Role of 3′-end sequences in infectivity of poliovirus transcripts made in vitro. J. Virol. **63**:467–470.
31. **Sarnow, P., Y. S. Ho, J. Williams, and A. J. Levine.** 1982. Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54kd cellular protein in transformed cells. Cell **28**:387–394.
32. **Simoes, E. A. F., and P. Sarnow.** 1991. An RNA hairpin at the extreme 5′ end of the poliovirus RNA genome modulates viral translation in human cells. J. Virol. **65**:913–921.
33. **Tershak, D. R.** 1984. Association of poliovirus proteins with the endoplasmic reticulum. J. Virol. **52**:777–783.
34. **Toyoda, H., M. J. H. Nicklin, M. G. Murray, C. W. Anderson, J. J. Dunn, F. W. Studier, and E. Wimmer.** 1986. A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. Cell **45**:761–770.
35. **Yin, F. H.** 1977. Involvement of viral procapsid in the RNA synthesis and maturation of poliovirus. Virology **82**:299–307.
36. **Ypma-Wong, M. F., P. G. Dewalt, V. H. Johnson, J. G. Lamb, and B. L. Semier.** 1988. Protein 3CD is the major poliovirus proteinase responsible for cleavage of the P1 capsid precursor. Virology **166**:265–270.
37. **Zimmermann, R., M. Sagstetter, M. J. Lewis, and H. R. B. Pelham.** 1988. Seventy-kilodalton heat shock proteins and an additional component from reticulocyte lysate stimulate import of M13 procoat protein into microsomes. EMBO J. **7**:2875–2880.