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Translation of glucose-regulated protein 78/immunoglobulin heavy-chain binding protein mRNA is increased in poliovirus-infected cells at a time when cap-dependent translation of cellular mRNAs is inhibited

(cap-independent translation/poliovirus mutants/heat shock-like proteins)

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ABSTRACT **All cellular cytoplasmic mRNAs carry a 7-methylguanylate cap attached to their 5′ ends. This cap structure is recognized by cap-binding proteins that then direct the binding of ribosomal subunits to this 5′-end complex. Poliovirus, a plus-stranded RNA virus, interferes with this cellular translation process by proteolytically inactivating the cap-binding protein complex. Subsequently the viral mRNA can be translated by an initiation process in which ribosomes bind internally to the mRNA [Pelletier, J. & Sonenberg, N. (1988) *Nature (London)* 334, 320–325], obviating cap-dependent translation. At least one cellular mRNA, encoding a heat shock-like protein, glucose-regulated protein 78/immunoglobulin heavy-chain binding protein, has been discovered to be translated at an increased rate in poliovirus-infected cells at a time when the translation of other cellular mRNAs is inhibited. The glucose-regulated protein 78/immunoglobulin heavy-chain binding protein mRNA thus exemplifies a cellular mRNA that is translated at a specifically enhanced rate by an as-yet-unresolved cap-independent initiation process in cells when the cap-binding protein complex is not functional.**

Due to the extensive interaction of many DNA and RNA viruses with their host cells during the viral life cycles, viruses and viral mutants have frequently been used as specific probes to examine the biochemical and genetic phenomena that occur in host macromolecular pathways such as transcription or translation (1–4). Poliovirus, a member of the picornavirus family, is an example of a virus that has extensive interactions with its host cell during its growth, including virally induced inhibition of cellular transcription and translation (5). The life cycle of poliovirus proceeds rapidly in tissue culture and ends with the death of the host cell 6 hr after infection. Therefore, it has been difficult to characterize very early events in infected cells, such as the translation of the infecting RNA molecules and the synthesis of the first negative strands. With the discovery that a cloned cDNA copy of the viral genome is infectious upon transfection into mammalian cells (6, 7), it was possible to construct defined viral mutants that have defects in viral RNA amplification (8–12) or in the inhibition of cellular protein biosynthesis (13, 14).

I have attempted to analyze early events in virally infected cells using two defined poliovirus mutants, 3NC202 (8) and 2B201 (15). Mutant 3NC202 bears an 8-nucleotide (nt) insertion in the 3′ noncoding region and is temperature-sensitive for the initiation of negative-strand synthesis. Mutant 2B201 contains a 6-nt insertion in the region encoding polypeptide 2B and also fails to synthesize normal amounts of viral RNA. Because the 3NC202 and 2B201 viruses synthesize negligible levels of viral RNA, they can be useful tools to study the function of input RNA molecules and their translation products in infected cells and thus to analyze the prereplicative events that occur during poliovirus infection.

I report here that an early event in poliovirus-infected cells is the increased translation of mRNA encoding the glucose-regulated protein 78/immunoglobulin heavy-chain binding protein (in B lymphocytes) (GRP78/BiP) (16, 17). Surprisingly, translation of GRP78/BiP mRNA occurs at a time when cap-dependent translation of other cellular mRNAs is greatly reduced from inactivation of the cap-binding protein eIF-4F (18, 19). This finding provides an example of a cellular mRNA that can be translated cap-independently like the viral RNA, possibly by internal ribosome binding (20, 21).

MATERIALS AND METHODS

Cells and Viruses. HeLa cells were maintained either in suspension culture in Joklik's modification of minimal essential medium supplemented with 7% horse serum (GIBCO) or grown on Petri dishes in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (GIBCO). Wild-type poliovirus was isolated from a single plaque derived from HeLa cells transfected with a pSV2-poliovirus plasmid as described (13). Mutant viruses 3NC202 and 2B201 were isolated and propagated as recently described (8, 15). Protocols for preparation of virus stocks and viral infections have been delineated (13).

Labeling and Preparation of Cell Extracts. Cells were infected with wild-type and mutant polioviruses at a multiplicity of infection of 100 and incubated for 3 hr at 39.5°C or 3.5 hr at 37°C. Infected and uninfected cells were labeled by replacing the medium with methionine-free medium containing [^{35}S]methionine (New England Nuclear) at 100 μCi/ml (1 Ci = 37 GBq) and incubating for an additional 20 min at the appropriate temperature. Cells were harvested as described (22). Briefly, cells were washed three times with ice-cold phosphate-buffered saline and collected by low-speed centrifugation (800 × *g* for 10 min). Cell pellets were either used immediately or stored at −20°C at this point. Upon thawing, 1 ml of lysis buffer (50 mM Tris·HCl, pH 8/5 mM EDTA/150 mM NaCl/0.5% Nonidet P-40/0.1 mM phenylmethylsulfonyl fluoride) was added to each pellet, and samples were incubated on ice for 20 min. The extracts were clarified by centrifugation (12,000 × *g* for 10 min), and the supernatant was used as a source of soluble proteins.

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Abbreviations: GRP78, glucose-regulated protein 78; BiP, immunoglobulin heavy-chain binding protein (in B lymphocytes); nt, nucleotide(s); ER, endoplasmic reticulum; CBP, cap binding protein.

Immunoprecipitations were done as described (22).

Gel Electrophoresis and Partial-Proteolysis Peptide Maps. One-dimensional SDS/PAGE was done according to the method of Laemmli (23). Two-dimensional gel electrophoresis was done as described (24). The partial-proteolysis peptide-mapping procedure of Cleveland *et al.* (25) was used in a modified form (22).

Glucose-Starvation of Tissue Culture Cells. Cells were incubated in Dulbecco's modified Eagle's medium/10% dialyzed fetal calf serum without glucose for 20 hr at 37°C. Labeling of starved cells was initiated by replacing the medium with fresh glucose-free medium supplemented with [^{35}S]methionine at 100 μCi/ml and incubating for 30 min. Cells were harvested as described above.

RESULTS

Synthesis of an 80-kDa Cellular Protein Is Not Inhibited in Cells Infected with Poliovirus Mutants. One early function of input viral RNA is to act as mRNA for translation into the viral polyprotein (26). Were these first translation products processed in the same way as the polypeptides produced later in the infectious cycle, after negative strands have been made? To answer this question, HeLa suspension cells were infected with wild-type poliovirus, mutant 2B201 virus at 37°C, or mutant 3NC202 virus at 39.5°C (the nonpermissive condition for viral replication of mutant 3NC202). Extracts were prepared, and labeled proteins were analyzed by SDS/gel electrophoresis. Extracts obtained from wild-type infected cells displayed a typical pattern of viral protein precursors and end products (Fig. 1, lane 1) and efficiently inhibited host-protein translation. Mutant 3NC202- and 2B201-infected cells (Fig. 1, lanes 2 and 3) displayed inhibition of host-cell translation as well, even without any viral replication. In addition to viral protein precursors, an 80-kDa protein could be detected in mutant-derived extracts; this protein could also be seen in uninfected cell extracts (Fig. 1, lane 4) but not in wild-type extracts.

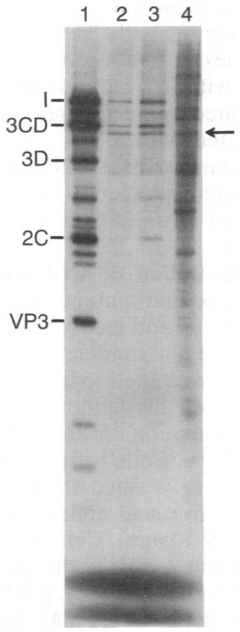

FIG. 1. Detection of an 80-kDa protein in cells infected with different poliovirus mutants. HeLa cells in suspension culture were infected and labeled with [^{35}S]methionine as described. Soluble extracts were analyzed by SDS/PAGE; an autoradiograph is shown. Extracts were obtained from wild-type- (lane 1), mutant 3NC202- (lane 2), mutant 2B201- (lane 3), and mock-infected cells (lane 4). Individual viral proteins are indicated on the left. Arrow denotes the 80-kDa protein.

Conceivably, the 80-kDa protein seen in mutant-infected cell extracts might be an aberrant cleavage product of the viral polyprotein, due to low concentrations of the viral proteases responsible for efficient and accurate processing of viral precursors (27, 28). However, comparisons of the 80-kDa protein with viral precursors by immunoprecipitations and comparison of peptides obtained by partial proteolysis failed to show any identities (data not shown). Subsequently, the 80-kDa protein obtained from mutant-infected extracts (Fig. 2, lanes 1 to 4) was compared with the 80-kDa protein obtained from uninfected cell extracts (Fig. 2, lanes 5–8) by partial proteolysis. Fig. 2 shows that the peptide patterns of the two 80-kDa proteins are very similar or identical, suggesting that the 80-kDa protein is derived from a cellular mRNA translation that is not shut off at a time when host-protein synthesis is selectively inhibited (Fig. 1, lanes 2 and 3).

Next, the fate of p220, a component of the cap binding protein (CBP) complex, that is cleaved in cells infected with wild-type poliovirus, was tested. As expected, given the effective inhibition of host-cell translation in mutant 2B201- and mutant 3NC202-infected cells, p220 was found to be cleaved in extracts from both mutant- and wild-type infected cells (H. D. Bernstein and P.S., unpublished observation). Thus, the translation of 80-kDa protein from its cellular mRNA does not require substantial amounts of functional p220.

Synthesis of the 80-kDa Cellular Protein Is Increased in Poliovirus-Infected Cells. To measure the rate of synthesis of the 80-kDa protein, cells were infected with wild-type virus or mutant 3NC202 virus and pulse-labeled with [^{35}S]methionine at different times after infection. Soluble extracts were prepared and analyzed by SDS/PAGE (Fig. 3). Extracts from 3NC202-infected cells (Fig. 3*A*) showed an increase in the rate of 80-kDa protein synthesis from 2 to 4 hr after infection (lanes 2–4) when the rate of synthesis of other cellular proteins had started to decline. The maximum rate of 80-kDa and virus-specific protein synthesis was reached 4 hr after infection with

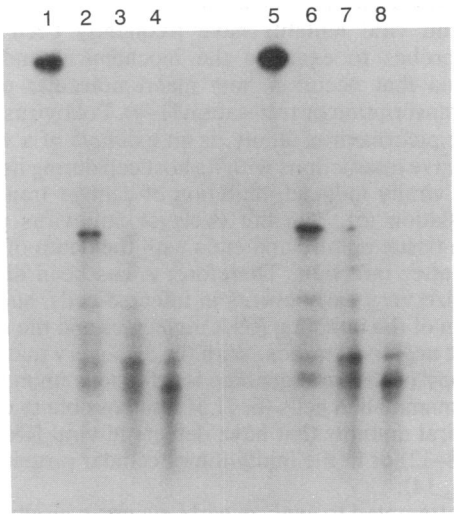

FIG. 2. Limited-proteolysis peptide map of the 80-kDa proteins from uninfected and mutant 3NC202-infected HeLa cells. Gel slices containing the [^{35}S]methionine-labeled 80-kDa proteins were cut out of the gel and loaded into the wells of a second SDS/polyacrylamide gel. Individual wells were treated with increasing concentrations of *Staphylococcus aureus* V8 protease (0, 0.1, 1, and 10 μg of protease, lanes 1–4 and lanes 5–8). After electrophoresis an autoradiograph was obtained. Lanes 1–4 contain 80-kDa protein from mutant 3NC202-infected cells and lanes 5–8 contain protein from uninfected cells.

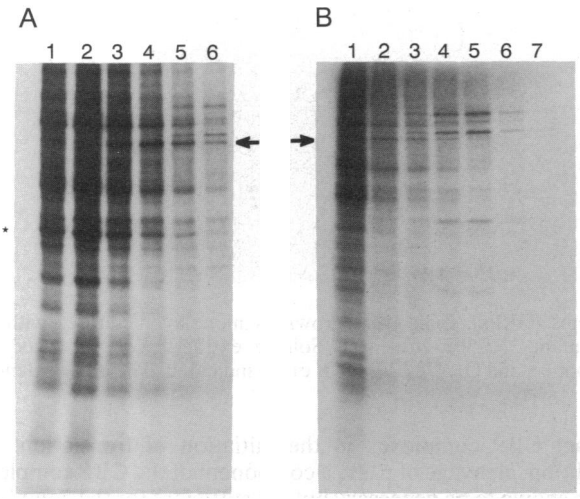

FIG. 3. Time course of 80-kDa protein synthesis in poliovirus-infected cells. Cells grown as monolayer cultures were infected with mutant 3NC202 (*A*) or wild-type poliovirus (*B*). Cells were pulse-labeled with [^{35}S]methionine each hour after infection (lanes 1–7) for 10 min, and soluble extracts were prepared and analyzed by SDS/PAGE. Arrows denote the 80-kDa protein; the star identifies actin.

3NC202 virus (lane 4), and a slow decline of total protein synthesis was seen from 5–6 hr after infection (lanes 5 and 6). A similar pattern of 80-kDa protein synthesis could be seen in extracts from 2B201-infected cells grown at 37°C (data not shown), demonstrating that the time course of synthesis is not due to the growth of mutants at higher temperature. Extracts from wild-type infected cells (Fig. 3*B*) showed a similar but abbreviated pattern; the 80-kDa protein synthesis could be seen from 2 to 4 hr after infection (lanes 2–4, respectively). These findings showed that synthesis of the mRNA encoding the 80-kDa protein is actually enhanced in virally infected cells early in infection.

The 80-kDa Cellular Protein Is the Glucose-Regulated Protein GRP78/BiP. Curiously, the 80-kDa protein was detected in uninfected HeLa cells grown in suspension culture (Fig. 1) but was not detected in cells grown as monolayer cultures unless they were infected with poliovirus (Fig. 3 *A* and *B*, lane 1; Fig. 4; Fig. 5). Comparison of the ingredients in the different media used to grow suspension cells and monolayer culture revealed that cell suspensions were grown in medium with five times less glucose. Concomitantly, a report describing the glucose-regulated protein GRP78 (16) was studied, and the partial-proteolysis map of GRP78, a protein induced during glucose-starvation of mammalian cells, looked very similar to that of the 80-kDa protein.

To test whether the host 80-kDa protein is GRP78, HeLa cells grown as monolayer cultures were deprived of glucose and metabolically labeled with [^{35}S]methionine 20 hr after starvation. As control, HeLa cells grown with glucose were also labeled. Polypeptides were analyzed by SDS/PAGE (Fig. 4*A*). Cells deprived of glucose (Fig. 4*A*, lane 2) displayed a decreased rate of protein synthesis compared with cells grown with glucose (Fig. 4*A*, lane 1). However, the induction of two proteins with molecular masses of 80 and 100 kDa could be seen in starved cells (Fig. 4*A*, lane 2), whereas these proteins were not significantly detectable in cells grown with glucose (lane 1).

The 80-kDa protein seen in 3NC202-infected cells (Fig. 4*B*, lane 1) displayed an electrophoretic mobility identical with that of the 80-kDa protein in glucose-starved cells (Fig. 4*A*, lane 2). Partial-proteolysis maps (data not shown) of the 80-kDa proteins obtained from mutant-infected and glucose-starved cells are also very similar or identical. Immunoprecipitations of the proteins obtained from 3NC202-infected

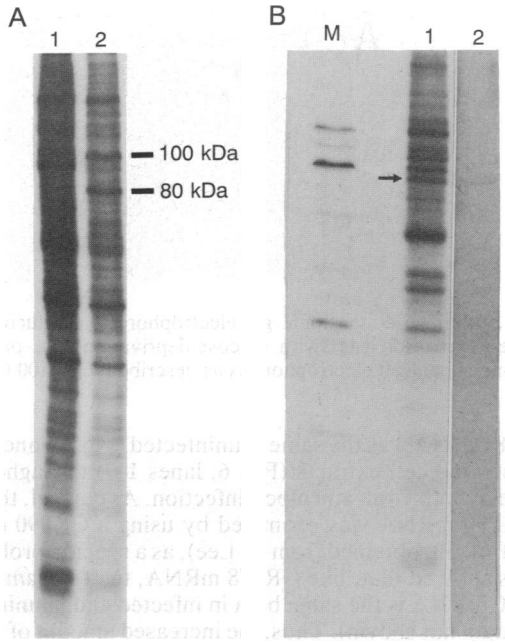

FIG. 4. (*A*) Autoradiographic analysis of polypeptides synthesized during glucose starvation. Cells were grown as monolayer cultures and deprived of glucose as described. Soluble extracts were prepared from untreated cells (lane 1) and glucose-deprived cells (lane 2) and separated by SDS/PAGE. (*B*) Immunoprecipitation of mutant 3NC202-infected lysates with a monoclonal antibody directed against GRP78/BiP. Labeled extracts obtained from 3NC202-infected cells were analyzed by SDS/PAGE either directly (lane 1) or after immunoprecipitation with a monoclonal antibody directed against GRP78/BiP (29) (lane 2); an autoradiograph is shown. Lane M contains viral marker proteins.

cells with a monoclonal antibody directed against GRP78/BiP (29) further verified the identity of the 80-kDa protein with GRP78/BiP (Fig. 4*B*, lane 2).

GRP78 and another glucose-regulated protein, GRP90, have been reported always to be coordinately expressed at the transcriptional level during glucose starvation (30). To examine the levels of the different glucose-regulated proteins in infected cells, the polypeptides were analyzed by two-dimensional gel electrophoresis (Fig. 5). Glucose-deprived cells (Fig. 5*B*) expressed GRP78 (spot c), GRP90 (spot b), and GRP100 (spot a) (20), whereas cells grown with glucose (Fig. 5*A*) expressed these proteins only in small amounts. Levels of these proteins can be compared with that of actin in both glucose-starved cells (*B*) and glucose-supplemented cells (*A*). In cells infected with 3NC202 virus (Fig. 5*C*), the 80-kDa protein comigrated with spot c, indicating that it has the same pI as GRP78. Interestingly, the shape of spot c in *C* is different from that of *A* and *B*, suggesting a posttranslational modification of GRP78. The rate of GRP78 synthesis in mutant virus-infected cells is again evidently higher than in uninfected cells (Fig. 5 *C* and *A*; a polioviral protein can be seen comigrating with actin). However, no synthesis of GRP90 or GRP100 is seen in 3NC202-infected cells (Fig. 5*C*), suggesting that the regulated expression of these proteins can be uncoupled.

The Steady-State Level of GRP78/BiP mRNA Is the Same in Uninfected and Infected Cells. The next question resolved was whether or not the increased level of GRP78 protein was from an enhanced level of GRP78 mRNA in infected cells. Total cytoplasmic RNA, obtained from uninfected and wild-type poliovirus-infected cells, was compared by Northern (RNA) analysis (31) using a GRP78 cDNA, p3C5 (30) (gift from A. Lee, University of Southern California), as a specific probe to detect GRP78 mRNA molecules. The steady-state level of

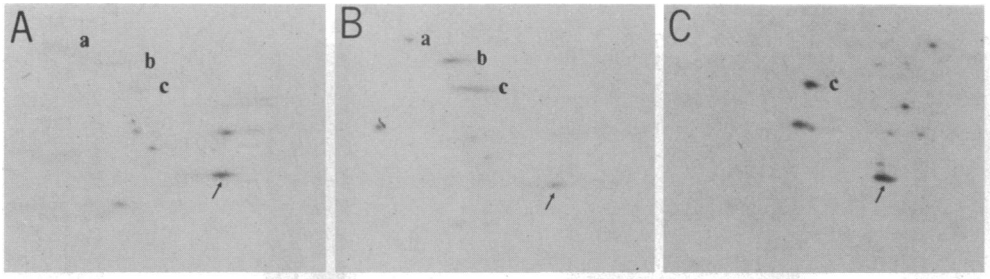

FIG. 5. Two-dimensional gel electrophoresis of glucose-regulated proteins (GRPs). Cells were grown as monolayer cultures without glucose-deprivation (*A*), with glucose-deprivation (*B*), or infected with mutant 3NC202 virus (*C*). Soluble extracts were analyzed by two-dimensional gel electrophoresis as described. GRP100 (spot a), GRP90 (spot b), and GRP78/BiP (spot c) are indicated. The arrows denote actin.

GRP78 mRNA was the same in uninfected (Fig. 6, lanes 8–14) and infected cell extracts (Fig. 6, lanes 1–7) throughout the course of both viral- and mock-infection. As control, the level of GRP90 mRNA was examined by using a GRP90 cDNA, p4A3 (ref. 30; obtained from A. Lee), as a specific probe. The results showed that, like GRP78 mRNA, the total amount of GRP90 mRNA is the same both in infected and in uninfected cells (data not shown). Thus, the increased amount of GRP78 protein in infected cells is due to an increased rate of translation of GRP78 mRNA rather than an increased level of mRNA.

DISCUSSION

Several eukaryotic capped mRNAs have been shown to have a reduced requirement for an intact CBP complex during the initiation of translation: (*i*) capped mRNAs with reduced secondary structure can form initiation complexes with ribosomes from poliovirus-infected cells (32); (*ii*) heat shock protein 70 mRNA is translated more efficiently than the bulk of cellular mRNA in poliovirus-infected cells after heat shock (33); and (*iii*) late adenovirus mRNAs containing the tripartite leader are translated in poliovirus-infected cells (14). These studies have suggested that mRNAs with less RNA secondary structure near their 5′ ends may have less requirement for intact CBP complexes in the initiation of translation. In addition, cleavage of p220, a component of the CBP complex, was shown to be necessary but not sufficient for the complete inhibition of cellular translation in poliovirus-infected cells (34), implying that a second virally induced function may be needed.

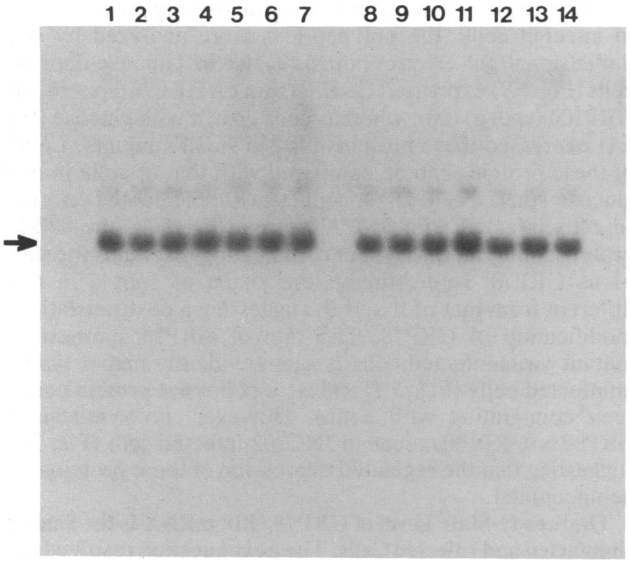

FIG. 6. Autoradiographic analysis of GRP78/BiP RNA in infected and uninfected cells. Monolayer cultures were either infected with wild-type virus (lanes 1–7) or mock-infected (lanes 8–14). At each hour after infection total cytoplasmic RNA was prepared, separated on an agarose/formaldehyde gel, transferred to nitrocellulose, and hybridized with a radiolabeled GRP78/BiP cDNA (p3C5) probe. Arrow denotes the 2700-nt GRP78/BiP mRNA.

The translation of GRP78 not only bypasses the requirements for an intact CBP complex but is actually enhanced in poliovirus-infected cells at a time when the translation of other cellular mRNAs is inhibited. However, it is yet unknown whether GRP78 mRNA is capped or uncapped in infected cells and whether it is translated by the same "cap-independent" mechanism as the viral RNA. It is tempting to speculate that a virally induced function promotes the selective translation of both viral and GRP78 mRNA. This hypothesis is substantiated by a previous report showing that the poliovirus mutant 2A-1 (13, 15), deficient in the cleavage of p220, could be complemented in cell lines persistently infected with 3NC202 mutant virus (8). Host-protein synthesis was normal in the persistently infected cell line, and p220 was cleaved at only low levels. Thus, the persistently infected cell lines functioned to allow translation of the mutant 2A-1 RNA to occur.

The mechanism of cap-independent translation in picornavirus-infected cells is currently under intense scrutiny. Pelletier and Sonenberg (20) have reported that a 490-nt sequence within the 747-nt 5′ noncoding region of the poliovirus genome contains an internal ribosome binding site. Similarly, Jang and colleagues (21) have observed that a segment of the 5′ noncoding region of encephalomyocarditis virus RNA can direct internal entry of ribosomes. These internal ribosome binding sites eliminate the requirement for an intact CBP complex. Comparison of the 221-nt sequence of the 5′ noncoding region of human GRP78 RNA (35) and the poliovirus internal ribosome binding site has not revealed significant sequence homologies (personal observation). However, higher order RNA structures rather than primary nucleotide sequences may be essential to produce a functional ribosome binding site. We are currently constructing hybrid genes containing the 5′ noncoding regions of poliovirus or GRP78 to examine their mechanism of translation *in vitro* and *in vivo*.

GRP78 is located in the endoplasmic reticulum (ER) (36), shares partial amino acid sequence homology with the 70-kDa heat shock protein HSP70 and was recently identified to be identical to BiP (17). GRP78/BiP is induced at the transcriptional level during stress-inducing situations such as glucose-deprivation, calcium ionophore treatment, and accumulation of aberrant proteins in the cell. It has been postulated that GRP78/BiP binds such malfolded or improperly glycosylated proteins, thereby preventing their transport through the ER (37, 38). So far, GRP78/BiP has been reported to be induced at the transcriptional level during glucose starvation (30), in paramyxovirus-infected cells (39, 40), and in Rous sarcoma virus-transformed chicken embryo fibroblasts (41). In con-

trast, GRP78/BiP is induced at the translational level in poliovirus-infected cells.

Whether the increased translation of GRP78/BiP is an unnecessary byproduct of the viral infection or has a function in the viral life cycle is yet unclear. Lytically infected cells, like transformed cells, display dramatic rearrangements of the skeletal framework (42). In poliovirus-infected cells, a proliferation of vesicles generated from ER membranes is also seen (43). Under these circumstances, it might be efficient to translate GRP78/BiP mRNA without the CBP complex to accumulate GRP78/BiP rapidly in the ER, where it may be needed for this membrane rearrangement to occur.

Alternatively, GRP78/BiP may participate directly in the assembly of membrane-bound viral replication complexes by a transient association with viral polypeptides in infected cells. Such a role of GRP78/BiP is plausible because GRP78/BiP has been seen to associate in an ATP-sensitive manner with immunoglobulin heavy chains (29, 38) and to bind to incorrectly folded proteins (37, 38, 44), but GRP78/BiP does not associate with polypeptides when they have gained their native folded state.

In any case, understanding the means by which GRP78/BiP mRNA escapes the fate of other cellular mRNAs in poliovirus-infected cells may shed light on the mechanism of translation in eukaryotic cells. Exploring the potential interactions of the GRP78/BiP protein with other polypeptides in infected and uninfected cells may help reveal the role of GRP78/BiP in mammalian cells.

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