

Flux of the Paramyxovirus Hemagglutinin-Neuraminidase Glycoprotein through the Endoplasmic Reticulum Activates Transcription of the *GRP78-BiP* Gene

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The cellular glucose-regulated protein GRP78-BiP is a member of the HSP70 stress family of gene products, and the protein is a resident component of the endoplasmic reticulum, where it is thought to play a role in the folding and oligomerization of secretory and membrane-bound proteins. GRP78-BiP also binds to misfolded proteins, and this may be one mechanism for preventing their intracellular transport. An induction in synthesis of the GRP78-BiP protein occurs in cells infected with paramyxoviruses (R. W. Peluso, R. A. Lamb, and P. W. Choppin, Proc. Natl. Acad. Sci. USA 75:6120-6124, 1978). We have studied the expression and activity of the *GRP78-BiP* gene and synthesis of the GRP78-BiP protein during infection with the paramyxovirus simian virus 5 (SV5). We wished to identify the viral component capable of causing activation of *GRP78-BiP* since GRP78-BiP interacts specifically and transiently with the SV5 hemagglutinin-neuraminidase (HN) glycoprotein during HN folding (D. T. W. Ng, R. E. Randall, and R. A. Lamb, J. Cell Biol. 109:3273-3289, 1989). Expression of cDNAs of the SV5 wild-type HN glycoprotein and a mutant form of HN that is misfolded but not the SV5 F glycoprotein or SV5 cytoplasmic proteins P, V, and M caused increased amounts of GRP78-BiP mRNA to accumulate, as detected by nuclease S1 protection assays. As unfolded or misfolded forms of HN cannot be detected to accumulate during SV5 infection, the data suggest that the flux of HN through the ER in SV5-infected cells can cause activation of *GRP78-BiP* transcription.

Infection of a wide variety of cells with the paramyxovirus simian virus 5 (SV5) causes the induction in synthesis of a group of polypeptides (M_r 98,000, 96,000, and 78,000) referred to as glucose-regulated proteins (44, 45). These cellular polypeptides are the same polypeptides whose synthesis is stimulated in cells transformed by avian sarcoma viruses (25, 52, 54) and in cells deprived of glucose or under conditions in which glycosylation is inhibited (46, 52). Although maintenance of a high concentration of glucose in the medium has been reported to decrease the synthesis of these polypeptides in transformed cells (52), this is not the case in paramyxovirus-infected cells (6, 45), and a recent study suggests that the 78,000- M_r polypeptide is induced in Rous sarcoma virus-transformed cells independent of glucose starvation (53). The glucose-regulatory proteins of M_r 98,000 and 96,000, which are glycosylated and unglycosylated forms of the same polypeptide, and M_r 78,000 are now designated GRP94 and GRP78-BiP, respectively (reviewed in reference 34).

GRP78-BiP is a member of the hsp70 family of stress-related proteins and is a resident and soluble component of the lumen of the endoplasmic reticulum (ER) (17, 35). *GRP78-BiP* is regulated at the transcriptional level (4, 48), and in addition to glucose deprivation, transformation of cells, and paramyxovirus infection, mammalian GRP78-BiP protein synthesis (and mRNA synthesis, where examined) is induced by inhibition of glycosylation (4, 39, 57), treatment of cells with amino acid analogs (22, 27), disruption of intracellular calcium stores (12, 58, 62), low extracellular pH (59), expression of misfolded proteins (28), and increased

expression of secreted proteins (11, 60; reviewed in reference 15). Interestingly, GRP78-BiP protein synthesis is strongly resistant to hypertonic salt concentrations (44), conditions which greatly decrease initiation of cellular protein synthesis (38). GRP78-BiP has been implicated in having a role in the folding and assembly of proteins in the ER (2, 14, 18, 26, 35, 43), to mark aberrantly folded proteins destined for degradation (10, 14, 32) or to aid in solubilizing aggregated proteins during periods of stress (35). Recently it has been shown that the SV5 and Sendai virus hemagglutinin-neuraminidase (HN) glycoprotein and the vesicular stomatitis virus glycoprotein (G) specifically and transiently associate with GRP78-BiP during glycoprotein folding (33, 37, 49). It has been found that GRP78-BiP and HN form a complex which can be detected from the time of HN synthesis up to a point just before oligomerization of the immature HN molecules to form the native tetramer (37). In addition, GRP78-BiP plays a second role in that it becomes more stably associated with altered forms of HN or G that are misfolded (33, 37), as has also been found with misfolded forms of several proteins, including the influenza virus hemagglutinin (HA) (14, 23).

We are interested in the molecular requirements for GRP78-BiP induction after infection of cells with the paramyxovirus SV5. In addition to HN, SV5 encodes the fusion (F) glycoprotein, a small nonglycosylated integral membrane protein (SH), the viral membrane protein (M), the major nucleocapsid protein (NP), and the nucleocapsid-associated proteins L, P, and V (reviewed in reference 30). As most of the cDNA sequences encoding the SV5 virus proteins have been cloned, sequenced, and expressed in eukaryotic cells (20, 21, 40-42, 51, 55), we examined whether the synthesis of particular SV5 proteins is responsible for the induction of GRP78-BiP synthesis, and we find

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that it is due to expression of the HN protein. As unfolded or malformed forms of HN cannot be detected to accumulate in wild-type SV5-infected cells, we conclude that the flux of synthesis of folding-competent HN molecules stimulates GRP78-BiP synthesis.

MATERIALS AND METHODS

Cells and viruses. CV-1 cells were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum. The W3 strain of SV5 was grown in MDBK cells as described before (45). The virus (3×10^8 PFU/ml) was stored in DME containing 1% bovine serum albumin (BSA) at -70°C . UV-irradiated SV5 was prepared by using a 15-W germicidal lamp for 5 min at a distance of 7 cm for 3 ml of undiluted virus spread evenly in a 10-cm dish; residual infectivity under such conditions was $<10^3$ PFU/ml. Simian virus 40 (SV40) stocks were grown in CV-1 cells as previously described (42). For infection of cells with SV5, confluent monolayers of CV-1 cells were washed twice with either phosphate-buffered saline (PBS) or DME and then infected with SV5 (approximately 50 to 100 PFU/cell) for 1 to 2 h at 37°C . Following infection, the virus-containing medium was removed and the cells were maintained in DME plus 2% fetal calf serum. For mock infections or infections with UV-irradiated SV5, CV-1 cell monolayers were washed in parallel with the cells to be infected, incubated in DME plus 1% BSA (mock) or UV-irradiated SV5 for the duration of infection, and then maintained in DME plus 2% fetal calf serum and treated in parallel with the SV5-infected cells. Infections with the SV40 virus stocks were done as described before (42). For mock SV40 infections, cell cultures were incubated in DME alone for the duration of the infection period.

Analysis of mRNA abundance and transcription rates. Cytoplasmic RNA was isolated from virus-infected or mock-infected CV-1 cells as described previously (7). Briefly, cell monolayers were washed with PBS and lysed in buffer containing 0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 1% β -mercaptoethanol. The nuclei were removed by centrifugation, and the mRNA-containing supernatant was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and then precipitated with ethanol. Nuclease S1 protection assays of endogenous *GRP78-BiP* mRNA were done similarly to those described by Wu et al. (61), using a human *GRP78-BiP* gene probe (detailed in the legend to Fig. 4). Following overnight hybridization of the denatured probe to CV-1 cell RNA, the single-stranded nucleic acids were digested with 600 U of nuclease S1 (Boehringer Mannheim Biochemicals, Indianapolis Ind.) for 60 to 90 min at 37°C . Proteins were extracted with phenol-chloroform-isoamyl alcohol (24:24:1), and the nucleic acids were precipitated with ethanol. Nuclease S1-resistant products were separated by electrophoresis on 4% polyacrylamide gels containing 8 M urea, and the gels were subjected to autoradiography. For quantitation, multiple exposures of each gel were scanned by laser densitometry.

In vitro run-on transcription reactions (13) were performed in isolated CV-1 cell nuclei in the presence of [^{32}P]UTP, as described previously (1). Radioactive RNA was hybridized to nitrocellulose filters containing gene-specific plasmids pHG23.1.2 (*GRP78-BiP*), pHA7.6 (P72), pH2.3 (HSP70) (61), and pHF β A-1 (actin) (16). The vector plasmid pGEM2 (Promega Biotech, Madison, Wis.) was also immobilized on the nitrocellulose filter, as a control for

nonspecific hybridization. The hybridizations were carried out in 50% formamide-6 \times SSC (1 \times SSC is 0.15 M sodium chloride and 0.015 M sodium citrate)-5 \times Denhardt's solution (1 \times is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA)-0.1% sodium dodecyl sulfate (SDS)-50 μg of tRNA per ml at 42°C for 65 to 80 h. Following hybridization, filters were washed sequentially in 6 \times SSC-0.2% SDS, 2 \times SSC-0.2% SDS, and 0.2 \times SSC-0.2% SDS for 30 to 60 min each at 65°C . Results were visualized by autoradiography, and the radioactivity was quantitated with a phosphoimaging analyzer (Molecular Dynamics, Sunnyvale, Calif.).

Radioisotopic labeling, immunoblot analysis, and immunoprecipitation of polypeptides. Proteins were labeled metabolically with [^{35}S]methionine in methionine-free DME (short pulse labeling) or in continuous-label medium (methionine-free DME supplemented with 10% complete DME and 2% fetal calf serum) for longer labeling periods. The duration of labeling periods is described in the figure legends. For analysis of whole-cell lysates, cells were washed with PBS, pelleted, and lysed in gel sample buffer (29). Lysates were sonicated, boiled, and stored at -70°C . Proteins were separated by electrophoresis through 10% polyacrylamide-SDS gels (29) or 15% polyacrylamide-SDS gels (44). The gels were processed by fluorography, dried, and exposed to Kodak X-Omat film.

For immunoblot analysis, proteins were electrophoretically transferred from polyacrylamide gels to nitrocellulose filters (3), and the filter was blocked in PBS containing BSA (5 mg/ml) for 1 h at room temperature or overnight at 4°C , incubated with anti-HN serum (36) for 60 to 120 min, and washed two to three times with PBS plus 0.05% Nonidet P-40. Filters were then incubated with ^{125}I -labeled goat anti-rabbit immunoglobulin G (IgG) and washed as described above. Blots were subjected to autoradiography in the presence of an intensifying screen.

For immunoprecipitations, metabolically labeled cells were pelleted and lysed in 1% Nonidet P-40-150 mM NaCl-50 mM Tris-HCl (pH 7.4)-2 mM phenylmethylsulfonyl fluoride for 2 to 5 min on ice. Nuclei were pelleted by centrifugation for 2 to 5 min, and the cytoplasm-containing supernatant was incubated with SV5-specific antiserum or anti-BiP monoclonal antibody (MAb) (2) for 60 to 120 min at 4°C . Protein A-agarose beads (Boehringer Mannheim Biochemicals) were added, and the incubations were continued another 30 to 90 min. Beads were washed three times in wash buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 M NaCl, 50 mM Tris-HCl [pH 7.4]) and once in PBS as described before (2). Proteins were eluted from the beads by boiling in gel sample buffer and analyzed by electrophoresis, as described above.

RESULTS

Induction of GRP78-BiP protein synthesis during SV5 infection. It has been found previously in cells infected with the paramyxovirus SV5 that the synthesis of GRP78-BiP is induced from the earliest time (6 to 9 h postinfection [p.i.]) that viral protein synthesis can be detected (44, 45). To confirm this observation and to provide positive identification of GRP78-BiP, lysates from mock-infected CV-1 cells, mock-infected CV-1 cells treated with tunicamycin (an inhibitor of N-linked glycosylation), and SV5-infected cells were either analyzed on gels directly or immunoprecipitated with the rat anti-BiP MAb (2). As shown in Fig. 1, the amount of GRP78-BiP immunoprecipitated from tunicamycin-treated cells was approximately threefold greater than

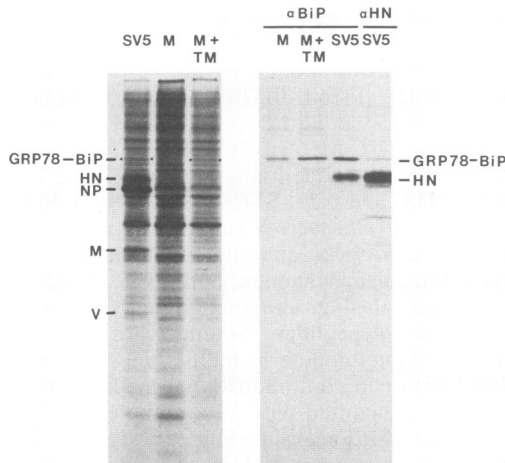


FIG. 1. Synthesis of GRP78-BiP is induced during SV5 infection. Confluent monolayers of CV-1 cells were mock infected or infected with SV5 and maintained in DME plus 2% fetal calf serum. At 14 h p.i., cells were labeled with 50 μ Ci of [35 S]methionine per ml in methionine-free DME for 30 min. Tunicamycin (2 μ g/ml) was added to mock-infected cells 2 h prior to and maintained during radioisotopic labeling. Lysates containing approximately equal cell numbers were analyzed directly or immunoprecipitated with rat anti-BiP MAb or anti-HN IgG. Polypeptides were separated by electrophoresis on 15% polyacrylamide-SDS gels and subjected to autoradiography. Positions of GRP78-BiP (78 kDa) and the viral structural proteins HN (70 kDa), NP (63 kDa), M (35 kDa), and V (22 kDa) are indicated. Lanes: M, mock-infected cells; SV5, SV5-infected cells; TM, tunicamycin-treated cells; BiP, immunoprecipitated with anti-BiP MAb; HN, immunoprecipitated with anti-HN IgG.

that from untreated cells. When SV5-infected cell lysates were immunoprecipitated under ATP-depleting conditions with anti-BiP MAb, a four- to fivefold greater quantity of GRP78-BiP was precipitated than from mock-infected cells and HN was coprecipitated with the BiP MAb (Fig. 1) (37). Previously it has been found under steady-state labeling conditions that the molar ratio of GRP78-BiP to HN in the complex is 0.7:1, which suggests that one or more molecules of GRP78-BiP associate with each newly synthesized HN molecule (37). To confirm further the nature of the associations, lysates from SV5-infected cells were immunoprecipitated with anti-HN antibody (which recognizes both immature and mature forms of HN), and as shown in Fig. 1, GRP78-BiP coimmunoprecipitated with the HN protein. The anti-HN antibody does not precipitate GRP78-BiP from uninfected cells (37).

Transcriptional activation of *GRP78-BiP*. Dactinomycin treatment of SV5-infected cells inhibits the induction of GRP78-BiP protein synthesis, a finding which is suggestive of increased transcription of the gene (45). To determine whether SV5 infection of CV-1 cells stimulates *GRP78-BiP* expression at the transcriptional level, *in vitro* run-off transcription assays with isolated nuclei were performed. In preliminary experiments it was found in mock-infected cells that between 0 and 3 h posttreatment, there was an unexpected but reproducible transient stimulation (fivefold) of *GRP78-BiP* transcription, not observed in uninfected cells, i.e., cells for which medium changes were not made (data not shown). This stimulation of *GRP78-BiP* transcription declined to a basal rate by 6 h posttreatment. Several changes in the mock infection procedure were made in an attempt to identify the perturbant inducing *GRP78-BiP* tran-

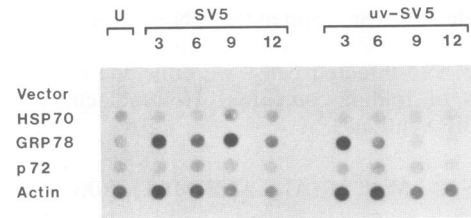


FIG. 2. *GRP78-BiP* is transcriptionally activated during SV5 infection. CV-1 cells were uninfected (U), infected with SV5, or mock infected with UV-irradiated SV5. Nuclei were isolated at the times p.i. indicated (in hours) and were used for run-on transcription assays in the presence of [α - 32 P]UTP. Radiolabeled RNA was hybridized to nitrocellulose filters containing plasmids pHG23.1.2 (*GRP78-BiP*), pHA7.6 (P72), pH203 (HSP70), pHF β A-1 (actin), and pGEM2 (vector). Plasmid pHG23.1.2 contains a 1.0-kb insert of sequences derived from the 5' end of the human *GRP78-BiP* cDNA. Plasmid pHA7.6 contains 600 bp of 5' sequences from the human P72 cDNA (constitutive heat shock gene) (57a). Plasmid pH2.3 contains the human HSP70 gene, and pHF β A-2 contains sequences encoding human actin. Following hybridization, the nitrocellulose filters were washed as described in Materials and Methods, exposed to Kodak X-Omat film for autoradiography, and then quantitated with a phosphoimage analyzer.

scription in mock-infected cells, including changing the medium on the cells 12 h prior to the experiment to supplement glucose levels and washing cells in DME equilibrated in an atmosphere of 5 to 7% CO₂ to eliminate pH fluctuations. However, these procedural changes did not prevent the transient activation of *GRP78-BiP* transcription at 0 to 3 h posttreatment in mock-infected cells. Thus, to examine *GRP78-BiP* transcription induced by SV5 infection at 6 to 9 h p.i., given the susceptibility of *GRP78-BiP* to transcriptional induction, the mock infections were performed with SV5 that had been inactivated by UV irradiation.

Nuclei were isolated from uninfected cells, SV5-infected cells, and UV-inactivated SV5-infected cells at 3, 6, 9, and 12 h p.i. and used for *in vitro* run-off transcription assays. Radiolabeled run-off transcripts were hybridized to nitrocellulose filters containing gene probes for *GRP78-BiP* and, as controls, *hsp70*, the heat-inducible member of the heat shock family; P72, the constitutive member of the heat shock family; and actin, an unrelated control gene probe. The autoradiographic data are shown in Fig. 2, and radioactivity was quantitated with a phosphoimage analyzer. Both SV5 and UV-irradiated SV5 caused a transient fivefold increase in *GRP78-BiP* transcription at 3 h p.i., which declined by 6 h p.i.; this was expected given the results obtained with the mock-infected cells. Most important for the experiments described here, in SV5-infected cells but not in cells infected with UV-irradiated SV5 there was a reproducible fivefold increase in *GRP78-BiP* transcription at 9 h p.i., and this increased transcription rate slowly declined up to 15 h p.i., the last time point measured (Fig. 2 and data not shown). SV5 infection or mock infection with UV-irradiated virus did not cause any change in the transcription rate of HSP70 or P72 at 3, 6, 9, and 12 h p.i. Although the transcription rate of actin was threefold greater in SV5- and mock-infected cells at 3 h p.i. than in cells for which the medium was not changed, its increased transcriptional rate declined equally in both cases over time. No hybridization was detected to vector sequences immobilized on the nitrocellulose filter, indicating that minimal nonspecific hybridization occurred. The increase in the rate of *GRP78-BiP* transcription during

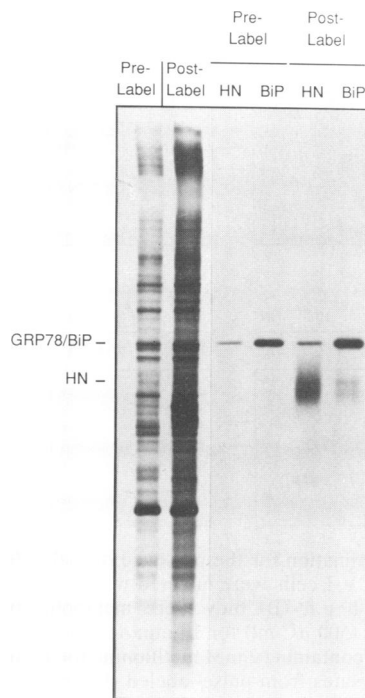


FIG. 3. Previously synthesized and newly synthesized GRP78-BiP are competent to bind HN. CV-1 cells were labeled with [35 S]methionine for 8 h in continuous-label medium, incubated in complete medium for 2 h, infected with SV5, and incubated in complete medium (prelabel). A parallel plate of CV-1 cells was infected with SV5 and labeled with [35 S]methionine in continuous-label medium from 4 to 12 h p.i. (postlabel). At 12 h p.i., cells were washed with PBS, scraped from each set of dishes, and dispersed into three aliquots. One aliquot was lysed in 2% SDS for direct analysis of polypeptides. Two aliquots were prepared for immunoprecipitations with anti-HN IgG (HN) or anti-BiP MAb (BiP). Polypeptides were separated by SDS-PAGE and subjected to autoradiography.

SV5 infection is reflected by an approximately threefold increase in the level of *GRP78-BiP* mRNA accumulation over the levels found in mock-infected cells, as determined by nuclease S1 protection assays (data not shown, but see below for use of this assay).

Association of previously synthesized and newly synthesized GRP78-BiP with HN. Synthesis of HN can be detected between 4 and 6 h p.i. (44), and at all times examined it was found to be associated with GRP78-BiP (data not shown). This suggests that the SV5-induced transcriptional activation of the *GRP78-BiP* gene and synthesis of the new GRP78-BiP protein occur well after complexes between HN and preexisting GRP78-BiP have formed and that new synthesis of GRP78-BiP is not required for complex formation with HN. To examine this further, CV-1 cells were labeled with [35 S]methionine for 8 h, infected with SV5, and then maintained without radioisotope. In a parallel experiment, SV5-infected cells were labeled with [35 S]methionine from 4 to 12 h p.i. At 12 h p.i., lysates were prepared from both sets of cells and incubated with either anti-HN IgG serum or anti-BiP MAb. As expected, the HN antibody coimmunoprecipitated a small fraction of GRP78-BiP synthesized during infection (Fig. 3, postlabel, HN) and the anti-BiP MAb coprecipitated a small amount of HN (Fig. 3, postlabel, BiP) from lysates of cells labeled during SV5 infection.

When lysates from prelabeled cells were immunoprecipitated with either anti-HN IgG serum or anti-BiP MAb, only GRP78-BiP could be detected (Fig. 3, prelabel). These data suggest that GRP78-BiP synthesized under normal growth conditions, prior to SV5 infection, will interact with newly synthesized HN molecules.

GRP78-BiP transcription is induced by HN synthesis. We were interested to determine whether we could detect an increase in *GRP78-BiP* mRNA accumulation caused by expression of individual SV5 polypeptides, particularly the HN glycoprotein. It has been found previously that increased synthesis of secretory proteins in cells causes increased expression of GRP78-BiP (11) and that the presence of an altered form of the influenza virus HA which is malformed, associates with GRP78-BiP, and remains localized in the ER significantly increases the amount of *GRP78-BiP* mRNA accumulation (28). However, synthesis of normal HA did not increase the accumulation of *GRP78-BiP* mRNA (28). The SV5 cDNAs encoding the glycoproteins F and HN and the cytoplasmic proteins M, P, and V were expressed in CV-1 cells by using SV40 recombinant virus vectors as described previously (42, 51, 55). In addition, we expressed a mutant form of HN (HN_{g0}) that lacks all four sites for addition of N-linked glycosylation. HN_{g0} does not fold into a native conformation, associates with GRP78-BiP in a relatively stable manner (half-time, >3 h), fails to be transported intracellularly out of the ER, and turns over very slowly (36). Thus, HN_{g0} would be expected to cause an increase in *GRP78-BiP* mRNA accumulation and serves as a positive control. To monitor the basal level of *GRP78-BiP* RNA accumulation, cells were mock infected, and to control for effects of the vector, cells were infected with wild-type SV40. Cytoplasmic RNA was isolated at various times p.i., and the level of *GRP78-BiP* mRNA was analyzed by nuclease S1 protection, using as a probe a cDNA to human *GRP78-BiP* mRNA. Autoradiograms were quantitated by laser scanning densitometry.

Mock infection did not lead to an accumulation of *GRP78-BiP* mRNA over levels found in uninfected cells, and most important for these experiments, *GRP78-BiP* mRNA did not accumulate in wild-type SV40-infected cells over the levels found in mock-infected cells (Fig. 4A). In addition, there was no change in the level of *GRP78-BiP* mRNA accumulation in cells infected with the SV40 recombinant viruses expressing the F glycoprotein (Fig. 4A) or the cytoplasmic proteins P, V, and M from the levels of *GRP78-BiP* mRNA found in mock-infected cells (data not shown). In contrast, in cells infected with SV40 vectors expressing either the wild-type HN (SV40/HN) or the mutant form of HN (SVHN_{g0}), *GRP78-BiP* mRNA accumulated threefold above the levels found in wild-type SV40- or mock-infected cells by 42 to 48 h p.i. (Fig. 4A). As a control to monitor the levels of RNA used in each of the nuclease S1 protection experiments, the level of HSP70 mRNA was measured, as this gene is not induced by SV5 infection (Fig. 2) or SV40 infection (data not shown). The results of an RNA dot-blot assay showed that there was no change in any recombinant or wild-type SV40 virus infection (data not shown). Thus, the nuclease S1 protection assays for GRP78-BiP are specific.

To correlate the increased accumulation of *GRP78-BiP* mRNA with HN-GRP78-BiP protein complex formation, SV40 recombinant virus-infected cells were labeled with [35 S]methionine and immunoprecipitated with anti-HN serum. As shown in Fig. 4B, HN-GRP78-BiP and HN_{g0}-GRP78-BiP complexes could be isolated. We have not quantitated the expression levels of wild-type HN and HN_{g0}

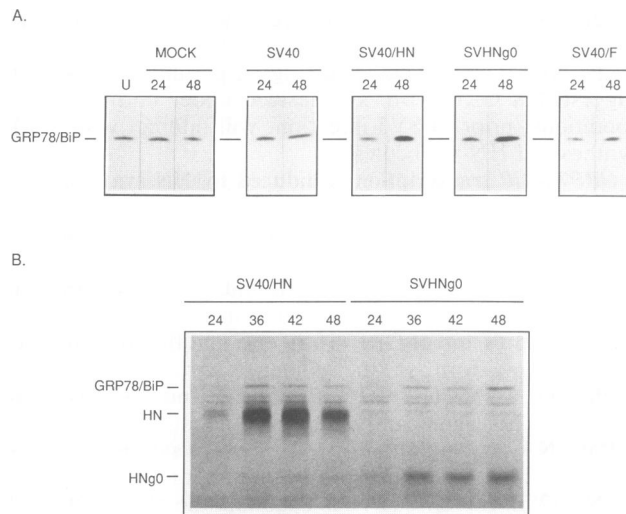


FIG. 4. GRP78-BiP mRNA accumulation increases in response to expression of wild-type or mutant HN molecules. Confluent monolayers of CV-1 cells were infected with SV40 recombinant viruses expressing HN (SV40/HN); HN_{g0}, a mutant form of HN lacking all four N-linked glycosylation sites (SVHN_{g0}); and F (SV40/F). Parallel cultures were uninfected (U), mock infected (mock) or infected with wild-type SV40. (A) At the times p.i. indicated (in hours), cytoplasmic RNA was isolated. The RNA was hybridized to a 650-bp *Bst*EII-*Pvu*II fragment isolated from plasmid pHG26.8 and uniquely 5'-end labeled with ³²P at the *Bst*EII site. Plasmid pHG26.8 contains approximately 800 bp of 5' sequences from the human *GRP78-BiP* cDNA (57a). Human *GRP78-BiP* mRNA protects a 500-bp fragment of this probe (data not shown). RNA from CV-1 cells protects an identically sized fragment, indicating that the human cDNA and monkey mRNA are highly homologous. Protected DNA fragments were analyzed on a 4% polyacrylamide gel containing 8 M urea. The position of the protected *GRP78-BiP* mRNA fragment (500 bp) is indicated. (B) At the times indicated (in hours), HN or HN_{g0} molecules were immunoprecipitated from [³⁵S]methionine-labeled SV40 recombinant virus-infected cell lysates with antiserum raised against SDS-denatured HN (36). Polypeptides were analyzed by electrophoresis on 10% polyacrylamide-SDS gels. GRP78-BiP, HN, and HN_{g0} are indicated.

on a per-cell basis to correlate the relative expression levels with the induction of GRP78-BiP. However, the data shown in Fig. 4B suggest that HN_{g0} is expressed at levels lower than wild-type HN and yet causes an equally large or larger induction of *GRP78-BiP* mRNA and protein accumulation. We expected this result because wild-type HN has only a specific and transient association with GRP78-BiP (half-time, 25 min) during its folding process, whereas the interaction of HN_{g0} with GRP78-BiP (half-time, >3 h) is relatively stable. Thus, the steady-state levels of these species that interact with GRP78-BiP are very different.

HN synthesis and not an accumulation of malformed HN molecules induces GRP78-BiP synthesis. *GRP78-BiP* transcriptional induction and the increased rate of GRP78-BiP protein synthesis during SV5 infection could be in response to GRP78-BiP associating with HN molecules which spontaneously misfold or remain unfolded during maturation. Alternatively, the flux of HN synthesis and transient association between HN and GRP78-BiP as HN folds into its native conformation could require an increased level of GRP78-BiP protein and thus signal *GRP78-BiP* gene activation. In an attempt to distinguish between these two possi-

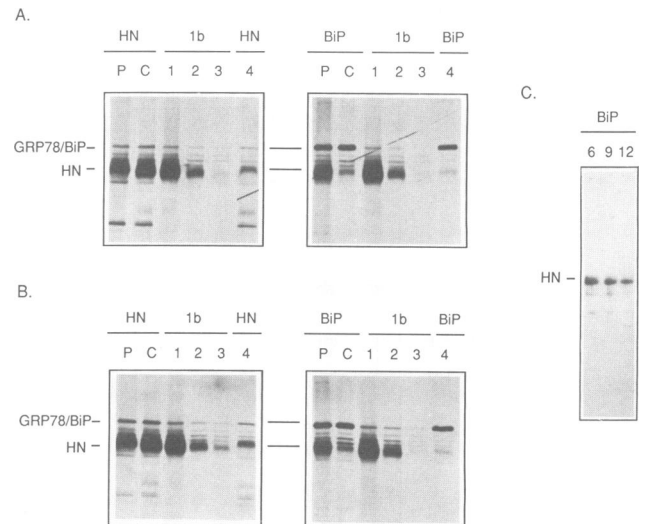


FIG. 5. Examination for the presence of malformed HN in SV5-infected cells. CV-1 cells were infected with SV5, and at either 6 h p.i. (A) or 12 h p.i. (B) they were metabolically labeled with [³⁵S]methionine (500 μ C/ml) for 10 min and incubated in DME-2% fetal calf serum containing 2 mM methionine for 10 min (pulse) or 90 min (chase). Lysates from pulse-labeled (P) or pulse-chase-labeled (C) cells were immunoprecipitated with anti-HN IgG serum (lanes HN, P, and C) or anti-BiP antibody (lanes BiP, P, and C). Parallel lysates from pulse-chase-labeled cells were incubated with MAb HN-1b for three sequential rounds of immunoprecipitation to deplete folded forms of HN present in the lysate (lanes 1b). The depleted lysates were then incubated with anti-HN IgG (lanes HN) or anti-BiP MAb (lanes BiP). (C) SV5-infected CV-1 cell lysates at 6, 9, or 12 h p.i. were immunoprecipitated with anti-BiP MAb, and the polypeptides were subjected to SDS-PAGE, transferred electrophoretically to nitrocellulose, and immunoblotted with antiserum raised against SDS-denatured HN and [¹²⁵I]-labeled goat anti-rabbit IgG.

bilities, we sought evidence for an accumulation of unfolded HN in SV5-infected cells. At 6 or 12 h p.i., SV5-infected cells were metabolically labeled for 10 min with [³⁵S]methionine and either lysed immediately (pulse) or incubated in normal medium for 90 min and then lysed (chase). Aliquots of the lysates were immunoprecipitated with either the anti-HN IgG or the anti-BiP MAb. It was found, as expected, that the HN antibody coprecipitated labeled BiP after both the pulse and chase periods because unlabeled HN molecules synthesized after the pulse-label associate with labeled BiP molecules, whereas the BiP antibody coimmunoprecipitated HN synthesized during the pulse-label but hardly at all after the chase period (Fig. 5A). Another aliquot of the chase lysate was incubated in three serial immunoprecipitations with conformation-specific MAb HN-1b (47) to deplete the lysate of normally folded HN molecules, as described previously (37). The MAb HN-1b recognizes an epitope which forms in HN monomers approximately 3 to 4 min after synthesis and which is retained in HN dimers and tetramers. The serial immunoprecipitations with HN-1b remove all normally folded forms of HN and possibly HN folding intermediates. HN-1b does not recognize unfolded forms of HN (37). The lysates, in duplicate, depleted of normally folded HN protein were then incubated with either the anti-HN IgG serum to immunoprecipitate any unfolded or malformed HN molecules or with the anti-BiP MAb. As shown in Fig. 5, at 6 or 12 h p.i. less than 1% of the HN

synthesized in a 10-min pulse-label is unfolded or malformed after a 90-min chase period and very little of this material remained associated in a complex with GRP78-BiP. Thus, these data suggest that at either 6 or 12 h p.i. there is very little HN in an unfolded or malformed form (Fig. 5A, lane HN/4). The two- to three-polypeptide species that were found in most lanes of immunoprecipitates that migrated between GRP78-BiP and HN correspond in size with two members of the HSP70 family, HSP70 and P72. It is presently unclear whether this is nonspecific immunoprecipitation or represents specific interactions, as has been suggested to be necessary for the translocation and synthesis of membrane-compartmentalized proteins (5, 8).

To examine for an accumulation of malformed HN molecules that might remain in a semistable complex with GRP78-BiP, cell lysates were immunoprecipitated with the BiP MAb at 6, 9, or 12 h p.i., and polypeptides were subjected to polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, and then immunoblotted with anti-HN serum and ^{125}I -labeled goat anti-rabbit IgG. As shown in Fig. 5B, the level of HN molecules that were complexed with GRP78-BiP at each time point was similar and did not dramatically increase as would be expected if malformed HN molecules were accumulating and leading to the induction of GRP78-BiP synthesis. The absence of accumulated malformed forms of HN in cells is mirrored by the absence of malformed forms of G in vesicular stomatitis virus-infected cells (33). However, with some strains of influenza virus, up to 10% of the HA fails to fold to the native conformation (14; reviewed in reference 24). In aggregate, the data reported here support the view that the induction of *GRP78-BiP* mRNA and protein synthesis is due to the increased flux of HN molecules through the ER.

DISCUSSION

Increased transcription of the gene encoding GRP78-BiP has been observed after perturbations to cells that directly or indirectly affect the ability of proteins to fold to their native conformations in the ER. These perturbations include inhibition of glycosylation (4, 28, 57), disruptions of intracellular calcium stores (12), and direct expression of proteins that cannot fold normally (28).

Our analysis of the maturation pathway of the paramyxovirus SV5 HN glycoprotein (37) indicates that HN folding and oligomerization (half-time, ~30 min) is fairly slow compared with the assembly of HA (half-time, 7 to 10 min [14]) or vesicular stomatitis virus G (half-time, 6 to 8 min) (9). Unfolded or immature HN molecules that could not be recognized by conformation-specific antibodies were found to associate with one or more molecules of GRP78-BiP (37). Newly synthesized HN associated and dissociated from GRP78-BiP (half-time, ~20 to 25 min) in an inverse correlation with the gain in reactivity with the HN conformation-specific antibodies, suggesting that the transient association of GRP78-BiP with immature HN is part of the normal HN maturation pathway (37). In addition, GRP78-BiP binds in a semistable manner to altered HN molecules that are unable to gain reactivity with the conformation-specific MAbs, and these mutant HN molecules are retained in the ER as a semistable complex (half-time, >3 h) (36).

It was of considerable interest to investigate further the observation that SV5 infection of cells causes the induction of synthesis of GRP78-BiP (44, 45). Our findings indicate that in SV5-infected cells, at 9 h p.i., just as HN reaches its maximum rate of synthesis (44), there is a fivefold increase in

the rate of *GRP78-BiP* transcription, which leads to an increase in GRP78-BiP protein levels (three- to fourfold, but this value has to be treated conservatively because the anti-BiP antibody does not yield 100% recovery [19]). When the individual SV5 polypeptides were expressed from cloned cDNAs, it was found that synthesis of HN but not the F glycoprotein or the cytoplasmic proteins P, V, and M led to an increased accumulation of *GRP78-BiP* mRNA in cells. As unfolded or malformed forms of HN could not be detected to accumulate in SV5-infected cells, the most straightforward interpretation of the data is that the flux of synthesis of folding-competent HN molecules stimulates GRP78-BiP synthesis, as has been found with increased expression levels of soluble proteins (11, 60).

Earlier studies with wild-type and mutant malformed HA indicated that only the mutant malformed HA induced increased accumulation of *GRP78-BiP* mRNA (28). The simplest explanation for the seeming difference between these data and our data with HN is that HA oligomerizes rapidly (half-time, ~7 to 10 min) (14) and thus the steady-state level of unfolded HA is low and does not require more than the normal level of GRP78-BiP molecules available in the ER, whereas HN oligomerizes more slowly (half-time, ~30 min) (37) and the steady-state level of unfolded HN can be expected to be higher. This is reflected by the comparative ease with which wild-type HN-GRP78-BiP complexes can be detected (37) versus the difficulty of showing a transient association of HA with GRP78-BiP (14, 23). Hence, HN molecules would be expected to utilize the available GRP78-BiP molecules for longer than HA, thus requiring an increase in the pool size of GRP78-BiP molecules. The mutant HA and HN molecules both bind GRP78-BiP relatively stably, and thus both molecules reduce the pool size of GRP78-BiP, requiring an induction of GRP78-BiP synthesis. Similar to the observations made with HA, we have found it difficult to demonstrate a transient interaction of the SV5 F protein with GRP78-BiP, and wild-type F protein expression does not cause an increase in *GRP78-BiP* mRNA accumulation. We thus posit that F, like HA, will oligomerize rapidly, but this remains to be determined.

The data we describe here support the view that if molecules are slower to fold (perhaps because they are more complicated to assemble), their rate of synthesis increases, or they fail to fold due to mutations or other defects, than the number of GRP78-BiP molecules has to be increased (15). As an increase in substrate (unfolded or malformed protein) leads to a decrease in unbound GRP78-BiP and an induction in *GRP78-BiP* transcription, it has been suggested there is a feedback mechanism from the ER to the nucleus, which monitors the activity of the GRP78-BiP protein and modulates transcription of the gene (4, 28, 57). The mechanism of the signalling pathway is not known, but it has been suggested that the sequestering of GRP78-BiP into complexes could bring about a release of calcium that is known to be bound to GRP78-BiP (28; reviewed in reference 50). Alternatively, transduction of the signal across the membrane of the ER could be mediated by binding of the GRP78-BiP-protein complexes to the GRP78-BiP transmembrane receptor protein(s) (31, 56).

ACKNOWLEDGMENTS

We thank our colleagues Reay G. Paterson, Davis T. W. Ng, and Benette Phillips for many discussions and help with the experiments and Rick E. Randall (St. Andrews University, Scotland) and Linda Hendershot (St. Jude Children's Research Hospital, Memphis, Tenn.) for making available antibodies.

This work was supported by N.I.H. research grants AI-23173 to R.A.L. and GM-38109 to R.I.M. and grants from the March of Dimes and American Cancer Society Faculty Research Award to R.I.M. S.S.W was supported by N.R.S.A training grant CA09560.

REFERENCES

- Banerji, S. S., N. G. Theodorakis, and R. I. Morimoto. 1984. Heat shock-induced translational control of HSP70 and globin synthesis in chicken reticulocytes. *Mol. Cell. Biol.* **4**:2437-2448.
- Bole, D. G., L. M. Hendershot, and J. F. Kearney. 1986. Post-translational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol.* **102**:1558-1566.
- Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radiiodinated protein A. *Anal. Biochem.* **112**:195-203.
- Chang, S. C., S. K. Wooden, T. Nakaki, Y. K. Kim, A. Y. Lin, L. Kung, J. W. Attenello, and A. S. Lee. 1987. Rat gene encoding the 78-kDa glucose-regulated protein GRP78: its regulatory sequences and the effect of protein glycosylation on its expression. *Proc. Natl. Acad. Sci. USA* **84**:680-684.
- Chirico, W. J., M. G. Waters, and G. Blobel. 1988. 70kD heat shock related proteins stimulate protein translocation into microsomes. *Nature (London)* **332**:805-810.
- Collins, P. L., and L. E. Hightower. 1982. Newcastle disease virus stimulates the cellular accumulation of stress (heat shock) mRNAs and proteins. *J. Virol.* **44**:703-707.
- Collins, P. L., L. E. Hightower, and L. A. Ball. 1978. Transcription and translation of Newcastle disease virus mRNAs in vitro. *J. Virol.* **28**:324-336.
- Deshaias, 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.
- Doms, R. W., D. S. Keller, A. Helenius, and W. E. Balch. 1987. Role of adenosine triphosphate in regulating the assembly and transport of vesicular stomatitis virus G protein trimers. *J. Cell Biol.* **105**:1957-1969.
- 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.
- Dorner, A. J., L. C. Wasley, and R. J. Kaufman. 1989. Increased synthesis of secreted proteins induces expression of glucose-regulated proteins in butyrate-treated Chinese hamster ovary cells. *J. Biol. Chem.* **264**:20602-20607.
- Drummond, I. A. S., A. S. Lee, E. Rosendez, Jr., and R. A. Steinhardt. 1987. Depletion of intracellular calcium stores by calcium ionophore A23187 induces the genes for glucose-regulated proteins in hamster fibroblasts. *J. Biol. Chem.* **262**:12801-12805.
- Fodor, E. J. B., and P. Doty. 1977. Highly specific transcription of globin sequences in isolated reticulocyte nuclei. *Biochem. Biophys. Res. Commun.* **77**:1478-1485.
- Gething, M.-J., K. McCammon, and J. Sambrook. 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. *Cell* **46**:939-950.
- Gething, M.-J., and J. F. Sambrook. 1990. Transport and assembly processes in the endoplasmic reticulum. *Semin. Cell Biol.* **1**:65-72.
- Gunning, P., P. Ponte, H. Okayama, J. Engel, H. Blair, and L. Kedes. 1983. Isolation and characterization of full-length cDNA clones for human α -, β -, and γ -actin mRNAs: skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. *Mol. Cell. Biol.* **3**:787-793.
- Haas, I. G., and M. Wabl. 1983. Immunoglobulin heavy chain binding protein. *Nature (London)* **306**:387-389.
- Hendershot, L., D. Bole, G. Kohler, and J. F. Kearney. 1987. Assembly and secretion of heavy chains that do not associate post translationally with immunoglobulin heavy chain-binding protein. *J. Cell Biol.* **104**:761-767.
- Hendershot, L. M., J. Ting, and A. S. Lee. 1988. Identity of the immunoglobulin heavy-chain-binding protein with the 78,000-dalton glucose-regulated protein and the role of posttranslational modifications in its binding function. *Mol. Cell. Biol.* **8**:4250-4256.
- Hiebert, S. W., R. G. Paterson, and R. A. Lamb. 1985. Hemagglutinin-neuraminidase protein of the paramyxovirus simian virus 5: nucleotide sequence predicts an N-terminal anchor. *J. Virol.* **54**:1-6.
- Hiebert, S. W., R. G. Paterson, and R. A. Lamb. 1985. Identification and predicted sequence of a previously unrecognized small hydrophobic protein, SH, of the paramyxovirus simian virus 5. *J. Virol.* **55**:744-751.
- Hightower, L. E. 1980. Cultured animal cells exposed to amino acid analogues or puromycin rapidly synthesize several polypeptides. *J. Cell. Physiol.* **102**:407-427.
- Hurtley, S. M., D. G. Bole, H. Hoover-Litty, A. Helenius, and C. S. Copeland. 1989. Interactions of misfolded influenza virus hemagglutinin with binding protein (BiP). *J. Cell Biol.* **108**:2117-2126.
- Hurtley, S. M., and A. Helenius. 1989. Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Cell Biol.* **5**:277-307.
- Isaka, T., M. Yoshida, M. Owada, and K. Toyoshima. 1975. Alterations in membrane polypeptides of chick embryo fibroblasts induced by transformation with avian sarcoma viruses. *Virology* **65**:226-237.
- Kassenbrock, C. K., P. D. Garcia, P. Walter, and R. B. Kelly. 1988. Heavy-chain binding protein recognizes aberrant polypeptides translocated in vitro. *Nature (London)* **333**:90-93.
- Kelley, P. M., and M. J. Schlesinger. 1978. The effect of amino acid analogues and heat shock on gene expression in chicken embryo fibroblasts. *Cell* **15**:1277-1286.
- Kozutsumi, Y., M. Segal, K. Normington, M.-J. Gething, and J. Sambrook. 1988. The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. *Nature (London)* **332**:462-464.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
- Lamb, R. A., and R. G. Paterson. 1991. The non-structural proteins of paramyxoviruses, p. 181-233. *In* D. W. Kingsbury (ed.), *The paramyxoviruses*. Plenum Publishing Corp., New York.
- Lewis, M. J., and H. R. B. Pelham. 1991. A human homologue of the yeast HDEL receptor. *Nature (London)* **348**:162-163.
- Lodish, H. F. 1988. Transport of secretory and membrane glycoproteins from the rough endoplasmic reticulum to the Golgi. *J. Biol. Chem.* **263**:2107-2110.
- Machamer, C. E., R. W. Doms, D. G. Bole, A. Helenius, and J. K. Rose. 1990. Heavy chain binding protein recognizes incompletely disulfide-bonded forms of vesicular stomatitis virus G protein. *J. Biol. Chem.* **265**:6879-6883.
- Morimoto, R. I., A. Tissieres, and C. Georgopoulos. 1990. *Stress proteins in biology and medicine*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Munro, S., and H. R. B. Pelham. 1986. An HSP70-like protein in the ER: identity with the 78-kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell* **46**:291-300.
- Ng, D. T. W., S. W. Hiebert, and R. A. Lamb. 1990. Different roles of individual N-linked oligosaccharide chains in folding, assembly, and transport of the simian virus 5 hemagglutinin-neuraminidase. *Mol. Cell. Biol.* **10**:1989-2001.
- Ng, D. T. W., R. E. Randall, and R. A. Lamb. 1989. Intracellular maturation and transport of the SV5 type II glycoprotein hemagglutinin-neuraminidase: specific and transient association with GRP78-BiP in the endoplasmic reticulum and extensive internalization from the cell surface. *J. Cell Biol.* **109**:3273-3289.
- Nuss, D. L., H. Opperman, and G. Koch. 1975. Selective blockage of initiation of host protein synthesis in RNA virus-infected cells. *Proc. Natl. Acad. Sci. USA* **72**:1258-1262.
- Olden, K., R. M. Pratt, and K. M. Yamada. 1978. Role of carbohydrates in protein secretion and turnover: effects of tunicamycin on the major cell surface glycoprotein of chick

- embryo fibroblasts. *Cell* **13**:461-473.
40. Paterson, R. G., T. J. R. Harris, and R. A. Lamb. 1984. Analysis and gene assignment of mRNAs of a paramyxovirus, simian virus 5. *Virology* **138**:310-323.
 41. Paterson, R. G., T. J. R. Harris, and R. A. Lamb. 1984. Fusion protein of the paramyxovirus simian virus 5: nucleotide sequence of mRNA predicts a highly hydrophobic glycoprotein. *Proc. Natl. Acad. Sci. USA* **81**:6706-6710.
 42. Paterson, R. G., S. W. Hiebert, and R. A. Lamb. 1985. Expression at the cell surface of biologically active fusion and hemagglutinin/neuraminidase proteins of the paramyxovirus simian virus 5 from cloned cDNA. *Proc. Natl. Acad. Sci. USA* **82**:7520-7524.
 43. Pelham, H. R. B. 1986. Speculations on the functions of the major heat shock and glucose regulated proteins. *Cell* **46**:959-961.
 44. Peluso, R. W., R. A. Lamb, and P. W. Choppin. 1977. Polypeptide synthesis in simian virus 5-infected cells. *J. Virol.* **23**:177-187.
 45. Peluso, R. W., R. A. Lamb, and P. W. Choppin. 1978. Infection with paramyxoviruses stimulates synthesis of cellular polypeptides that are also stimulated in cells transformed by Rous sarcoma virus or deprived of glucose. *Proc. Natl. Acad. Sci. USA* **75**:6120-6124.
 46. Poussegur, J., R. P. C. Shiu, and I. Pastan. 1977. Induction of two transformation-sensitive membrane polypeptides in normal fibroblasts by a block in glycoprotein synthesis or glucose deprivation. *Cell* **11**:941-947.
 47. Randall, R. E., D. F. Young, K. K. A. Goswami, and W. C. Russell. 1987. Isolation and characterization of monoclonal antibodies to simian virus 5 and their use in revealing antigenic differences between human, canine and simian isolates. *J. Gen. Virol.* **68**:2769-2780.
 48. Resendez, E., Jr., J. W. Attenello, A. Grafsky, C. S. Chang, and A. S. Lee. 1985. Calcium ionophore A23187 induces expression of glucose-regulated genes and their heterologous fusion genes. *Mol. Cell. Biol.* **5**:1212-1219.
 49. Roux, L. 1990. Selective and transient association of Sendai virus HN glycoprotein with BiP. *Virology* **175**:161-166.
 50. Sambrook, J. K. 1990. The involvement of calcium in transport of secretory proteins from the endoplasmic reticulum. *Cell* **61**:197-199.
 51. Sheshberadaran, H., and R. A. Lamb. 1990. Sequence characterization of the membrane protein of the paramyxovirus simian virus 5. *Virology* **176**:234-243.
 52. Shiu, R. P. C., J. Poussegur, and I. Pastan. 1977. Glucose depletion accounts for the induction of two transformation-sensitive membrane proteins in Rous sarcoma virus-transformed chick embryo fibroblasts. *Proc. Natl. Acad. Sci. USA* **74**:3840-3844.
 53. Stoeckle, M. Y., S. Sugano, A. Hampe, A. Vashistha, D. Pellman, and H. Hanafusa. 1988. 78-Kilodalton glucose-regulated protein is induced in Rous sarcoma virus-transformed cells independently of glucose deprivation. *Mol. Cell. Biol.* **8**:2675-2680.
 54. Stone, K. R., R. E. Smith, and W. K. Joklik. 1974. Changes in membrane polypeptides that occur when chick embryo fibroblasts and NRK cells are transformed with avian sarcoma viruses. *Virology* **58**:86-100.
 55. Thomas, S. M., R. A. Lamb, and R. G. Paterson. 1988. Two mRNAs that differ by two nontemplated nucleotides encode the amino coterminal proteins P and V of the paramyxovirus SV5. *Cell* **54**:891-902.
 56. Vaux, D., J. Tooze, and S. Fuller. 1990. Identification by anti-idiotypic antibodies of an intracellular membrane protein that recognises a mammalian endoplasmic reticulum retention signal. *Nature (London)* **345**:495-502.
 57. Watowich, S. S., and R. I. Morimoto. 1988. Complex regulation of heat shock- and glucose-responsive genes in human cells. *Mol. Cell. Biol.* **8**:393-405.
 - 57a. Watowich, S. S., and R. I. Morimoto. Unpublished data.
 58. Welch, W. J., J. I. Garrels, G. P. Thomas, J. J.-C. Lin, and J. R. Feramisco. 1983. Biochemical characterization of the mammalian stress proteins and identification of two stress proteins as glucose- and Ca²⁺-ionophore-regulated proteins. *J. Biol. Chem.* **258**:7102-7111.
 59. Whelan, S. A., and L. E. Hightower. 1985. Differential induction of glucose-regulated and heat shock proteins: effects of pH and sulfhydryl-reducing agents on chicken embryo cells. *J. Cell. Physiol.* **125**:351-358.
 60. Wiest, D. L., J. K. Burkhardt, S. Hester, M. Hortsch, D. I. Meyer, and Y. Argon. 1990. Membrane biogenesis during B cell differentiation: most endoplasmic reticulum proteins are expressed coordinately. *J. Cell Biol.* **110**:1501-1511.
 61. Wu, B. J., C. Hunt, and R. I. Morimoto. 1985. Structure and expression of the human gene encoding major heat shock protein HSP70. *Mol. Cell. Biol.* **5**:330-341.
 62. Wu, F. S., Y.-C. Park, D. Roufa, and A. Martorosi. 1981. Selective stimulation of the synthesis of an 80,000 dalton protein by calcium ionophores. *J. Biol. Chem.* **256**:5309-5312.