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# Expression and Subcellular Localization of Poliovirus VPg-Precursor Protein 3AB in Eukaryotic Cells: Evidence for Glycosylation In Vitro

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**The poliovirus-encoded, membrane-associated VPg-precursor polypeptide 3AB has been implicated in the initiation of viral RNA synthesis. We have expressed 3AB and 3A polypeptides in eukaryotic cells and examined their localization using indirect immunofluorescence and a direct in vitro membrane-binding assay. Results presented here demonstrate that both 3AB and 3A are capable of localizing in the endoplasmic reticulum and the Golgi apparatus in transfected HeLa cells in the absence of any other poliovirus protein. We have also shown that the carboxy-terminal 18 amino acids of 3A that constitute an amphipathic domain are important in membrane binding of 3A and 3AB. Additionally, we demonstrate that a significant fraction of both 3A and 3AB can be glycosylated in a membrane-dependent fashion during in vitro translation in reticulocyte lysate. We demonstrate that 6-diazo-5-oxo-L-norleucine, an inhibitor of glycoprotein synthesis, significantly inhibits poliovirus RNA synthesis in vivo. The implications of glycosylation of 3AB (and 3A) in viral replication are discussed.**

Poliovirus, the prototype member of the *Picornaviridae* family, contains a single-stranded, plus-polarity RNA of approximately 7,500 nucleotides (25, 44). A small virus-encoded protein, VPg, is found to be attached covalently to the 5′-terminal nucleotide of the RNA. Upon infection of human cells, the input viral RNA is translated in the cytoplasm into a long polyprotein which is initially processed into three precursor polyproteins (P1 to -3). Further processing of these precursors by three virally encoded proteases, 2A, 3C, and 3CD, gives rise to mature structural and functional proteins. While the P1 precursor gives rise to capsid proteins, further processing of P2 and P3 precursors results in the generation of functional proteins needed for replication of the viral genome.

Replication of the poliovirus RNA genome proceeds through the formation of a complete negative-strand RNA which serves as the template for the synthesis of progeny virion RNA molecules (14, 15). Among the polypeptides involved in viral RNA synthesis, $3D^{pol}$ is the template- and primer-dependent RNA polymerase which is unable to initiate RNA synthesis but can elongate nascent RNA chains (17, 54). Recently, $3D^{pol}$ has been shown to contain RNA duplex unwinding activity while moving along a template (10). Genetic studies have suggested that at least some functions of 3D can be contributed in *trans* during viral replication (9). 3CD, which is the protease responsible for processing of the P1 precursor, also binds near the 5′ end of poliovirus RNA, and it is believed that it may play a direct role in the initiation of plus-strand RNA synthesis (1, 2, 56). 2C is a membrane-bound viral protein required for RNA replication (8). Although its precise function in RNA synthesis remains obscure, it has recently been shown to contain ATPase and GTPase activities (46). All guanidine-resistant and -requiring poliovirus mutants map in 2C (41, 42). Protein 2C is needed continually for viral RNA synthesis, and its function can be provided in *trans* (28). 2B (or 2BC) is another viral protein required for RNA replication (7, 24). RNA-negative 2B mutants have been shown to be noncomplementable, suggesting that 2B may be required in *cis* during genome replication. Three alleles of 2B that could not be complemented by other mutant viruses were shown to exert dosage-dependent interference with the growth of wild-type virus. Thus, at least some function of 2B can be contributed in *trans*.

Finally, VPg is required for viral RNA replication, and it is believed that the membrane-associated VPg-precursor protein, 3AB, may be used for the delivery of VPg into the 5′ ends of plus- and minus-strand RNAs during RNA replication (47, 48). The mechanism(s) by which this is achieved remains controversial and obscure (22, 30, 31). It has been proposed that a uridylylated form of VPg (or a precursor of VPg) acts as a primer for synthesis of virus-specific RNAs (35, 36, 39, 50–52). Membrane-dependent synthesis of VPg-pU(pU) was demonstrated in a membranous replication complex isolated from HeLa cells infected with poliovirus (50–52). These protein-nucleotidyl complexes can be chased into VPg-linked oligoribonucleotides containing as many as nine or more poliovirus 5′-terminal nucleotides (50–52). Further evidence that VPg is involved in RNA synthesis came from demonstrations that antibodies to VPg immunoprecipitated VPg and VPg-pUpU from infected cells (13) and specifically inhibited in vitro initiation of viral RNA synthesis (4, 35). Other studies that concentrated on minus-strand synthesis in vitro have led to a different model for minus-strand synthesis. In this model, poliovirus $3D^{pol}$ and a host factor (15) catalyze self-priming of minus-strand synthesis on a plus-strand template (3). The hairpin generated at the junction of plus- and minus-strand RNAs is then cleaved by VPg (or a VPg precursor) with concomitant linkage of VPg to the 5′ end of newly made minus-strand RNA (53). Studies with mutant viruses have suggested a *cis* requirement of many poliovirus nonstructural proteins in viral replication. Thus, recent efforts have concentrated on development of RNA replication systems in which translation of viral RNA is coupled to its replication (5, 34). De

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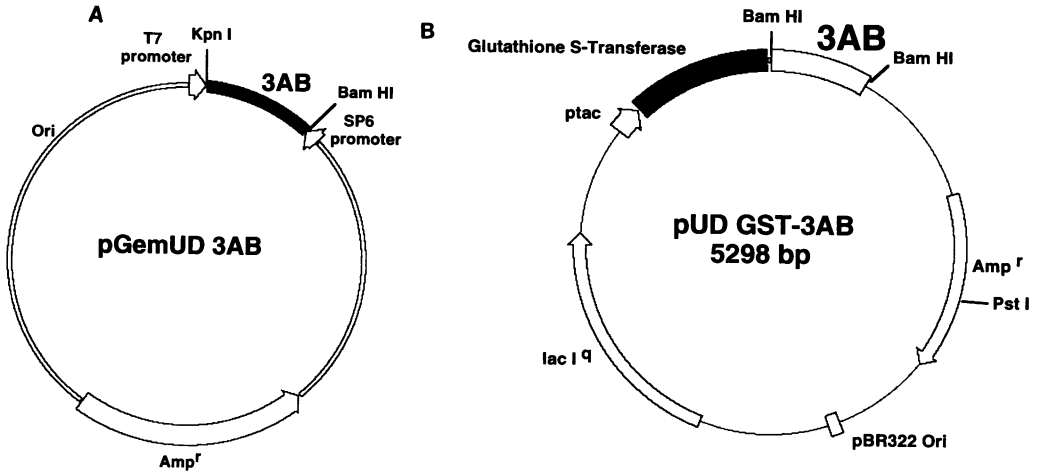


FIG. 1. Construction of recombinant plasmids. Vectors pGem 3 (A) and pGEX-2T (B) were used to construct plasmids for eukaryotic and prokaryotic cell expression of proteins, respectively. (A) pGEM UD 3AB is a recombinant plasmid in which the 3AB sequence is ligated between the *Kpn*I and *Bam*HI sites of the pGEM-3 vector under the control of T7 promoter. (B) Plasmid construction of the GST-3AB fusion protein was performed as described in Materials and Methods.

novo synthesis of infectious poliovirus has been achieved in a HeLa cell-free system (34).

The VPg precursor 3AB is an abundant polypeptide found in poliovirus-infected cells, and the C-terminal 22 amino acids of 3A constitute a hydrophobic domain (48). Because VPg is a highly basic protein, it is believed that it is through the hydrophobic domain of 3A that 3AB is anchored onto cytoplasmic smooth membranes where active RNA replication occurs (20). It should also be noted that this hydrophobic region of 3A is highly conserved in closely related picornaviruses, namely, poliovirus, coxsackievirus, and human rhinovirus (20). Studies with a poliovirus cold-sensitive mutant harboring a defined insertion in 3A of a serine residue at position 15 suggest that the protein is involved in RNA replication (6). Additionally, several mutations introduced into the 3B protein also suggest that covalent attachment of VPg to RNA is absolutely required for RNA synthesis (45). Two recent studies by Giachetti et al. in which site-directed lesions were introduced into the hydrophobic domain of 3AB clearly link this protein to viral RNA synthesis and suggest that the hydrophobicity of the C-terminal region of 3A is necessary to maintain normal viral RNA synthesis (19, 21). A cold-sensitive 3A mutant virus can be complemented in *trans* by the wild-type virus (6). However, a recent study suggests that the function provided by the hydrophobic domain of 3AB cannot be provided in *trans* (19). These apparently conflicting results may indicate multiple functions of protein 3A (or 3AB) in viral replication, and defects in some of these functions may be complementable.

In this study, we have expressed 3AB and 3A proteins in eukaryotic cells and examined their ability to bind cytoplasmic membranes. We demonstrate here that the membrane-binding ability of 3A (or 3AB) is conferred by the C-terminal 18 amino acids of 3A. We also show that a significant portion of 3A and 3AB molecules can be glycosylated during in vitro translation. Finally, we demonstrate that 6-diazo-5-oxo-L-norleucine (DON), an inhibitor of glycoprotein synthesis, inhibits viral RNA synthesis significantly, suggesting a role of protein glycosylation in viral RNA synthesis.

## MATERIALS AND METHODS

**Cells and viruses.** HeLa cells were grown in suspension culture by using S-minimum essential medium (S-MEM) supplemented with 6% newborn calf serum. HeLa monolayers were grown in MEM supplemented with 5% fetal bovine serum. Vaccinia virus stocks were prepared as previously described (18). Vaccinia virus VTF7-3, containing the T7 RNA polymerase gene, was kindly provided by Bernard Moss, National Institutes of Health, Bethesda, Md. (18).

**Construction of recombinant plasmids.** DNA sequences for 3AB (nucleotides 5113 to 5439) and 3A (nucleotides 5113 to 5373) were amplified from the poliovirus clone pT7PV1 (9) by PCR. Sequences containing *Kpn*I and *Bam*HI restriction sites, translational start codon AUG, and stop codon UAG were introduced in the oligonucleotide primers used for PCR. The primers 5′ ATAATAGGTACCATGGGACCACTCCAGTAT AAAGAC 3′ and 5′ ATCCTAGGATCCTCATTGTACCTTT GCTGTCCG 3′ were used for PCR amplification. This amplified 3AB sequence contains the newly introduced *Kpn*I site and translational start codon AUG at the 5′ end, whereas translational stop codon UAG and the *Bam*HI site are present at the 3′ end. Amplified DNAs were cloned in between *Kpn*I and *Bam*HI sites of the vector pGEM-3 (Promega Biotec) under the control of T7 promoter for construction of the pGEM UD 3AB (Fig. 1A) and pGEM UD 3A (data not shown) clones. To construct a 3A deletion mutant, 54 nucleotides corresponding to the 18 amino acids from the C terminus of 3A protein were deleted during PCR amplification. The amplified DNA fragments were ligated with pGEM-3, digested with *Kpn*I and *Bam*HI. To construct the glutathione *S*-transferase (GST)-3AB fusion protein, the pGEX-2T vector (Pharmacia) was digested either with a mixture of *Sma*I and *Pst*I or with a mixture of *Bam*HI and *Pst*I. The larger fragment from the *Sma*I-*Pst*I digest and the smaller fragment from the *Bam*HI-*Pst*I digest were ligated with the PCR-amplified 3AB sequence which was blunt ended by T4 DNA polymerase at the *Kpn*I site to prepare the pGEX-UD-3AB fusion clone (Fig. 1B).

**In vitro transcription and translation.** Standard protocols for transcription and translation were followed (43a). Briefly,

plasmids were linearized by *Bam*HI digestion and used as templates for synthesis of runoff transcripts by T7 RNA polymerase (Promega Biotec) in vitro. The RNAs were then translated in vitro by using either rabbit reticulocyte (Promega Biotec) or HeLa lysates. In vitro-synthesized proteins were labeled with 40 μCi of [$^{35}$S]methionine (specific activity, >1,000 Ci/mmol) or 10 μCi of [$^{14}$C]mannose (specific activity, 295 mCi/mmol) during translation.

**In vitro glycosylation and endo H treatment.** In vitro-transcribed mRNAs were translated in rabbit reticulocyte lysate in the presence of 3.6 equivalents (55) of the canine microsomal membrane (Promega Biotec) in 25-μl reaction mixtures. Translational products were microcentrifuged for 15 min to separate particulate and soluble fractions. For endo-β-*N*-acetylglucosaminidase H (endo H) treatment, the particulate fraction was suspended in 40 μl of 1% sodium dodecyl sulfate (SDS) and heated at 100°C for 3 min. The sample was diluted with water to a final SDS concentration of 0.1%, and the final concentration of sodium acetate, pH 5.5, was adjusted to 50 mM. One half of the sample was treated with 7 mU of endo H (Boehringer Mannheim) for 16 h at 37°C, and the other half was mock treated with buffer containing no endo H.

**Expression and purification of the GST-3AB fusion protein.** *Escherichia coli* cells harboring the pGEX-UD-3AB construct were grown to 0.4 to 0.6 U of optical density at 600 nm at 37°C prior to induction by 0.1 mM isopropyl thiogalactoside (IPTG) (Sigma, St. Louis, Mo.) for 5 h, and harvested cells were resuspended in 1× TSE (50 mM Tris-HCl [pH 8.0]–150 mM NaCl–0.25 mM EDTA) buffer and sonicated mildly (three bursts, 20 s each) on ice. Triton X-100 was then added to 1% final concentration. Cellular debris were removed by centrifugation at 10,000 × *g*. GST fusion protein present in lysate was allowed to bind to preswollen glutathione agarose beads (sulfur linked) for 2 h at 4°C. Beads were separated from lysate by spinning at 2,000 rpm for 5 min in a Sorval SS34 rotor at 4°C, followed by batch washing five times with 50 ml of 1× TSE. Fusion protein was then eluted from agarose beads by using 1 bed volume of 50 mM Tris, pH 8.0, containing 5 mM reduced glutathione (Sigma). Protein preparation was concentrated in a dialysis bag with solid precooled sucrose. Protein concentration was determined by the Bradford assay. Salt concentration in the protein preparation was adjusted to 150 mM, and the sample was quickly frozen on dry ice.

**Preparation of antibody to the fusion proteins.** New Zealand White rabbits were injected first with 166 μg of fusion protein mixed with Freund's complete adjuvant and then with three boosters of 166 μg of protein in Freund's incomplete adjuvant. Finally, a fourth booster of 375 μg of protein was used before final bleeding of the animals.

**Poliovirus infection and in vivo labeling of viral proteins and RNA.** HeLa cells were grown at a concentration of $4 \times 10^5$ cells per ml in suspension culture. Harvested cells were resuspended in S-MEM and infected with poliovirus at a multiplicity of infection of 50. Virus adsorption was for 30 min at room temperature. Infection was carried out at 37°C for 3.5 h in methionine-free S-MEM (GIBCO BRL) containing 8% dialyzed newborn calf serum (GIBCO BRL). Viral proteins were labeled by adding translabel [$^{35}$S]methionine (ICN) at a concentration of 200 μCi/ml and continuing the infection for another 2 h. For viral RNA synthesis, actinomycin D at a concentration of 5 μg/ml was added 15 min postinfection, and different concentrations of DON were added to infected cell cultures. Viral RNA was labeled by adding 25 μCi of $^{32}P_i$ per ml to the infected culture at 1 h postinfection as previously described (14, 15, 30). Virus-infected cells were harvested at 5 h postinfection, and RNA was isolated by phenol extraction and ethanol precipitation. Labeled viral RNA was analyzed by nondenaturing agarose (0.7%) gel electrophoresis.

**DNA transfection.** HeLa monolayers were grown in 1× MEM containing 5% fetal bovine serum to approximately 80% confluency in 60-mm-diameter petri dishes and infected with VTF7-3 recombinant vaccinia virus at a multiplicity of infection of 1 PFU per cell. Virus adsorption was allowed for 1 h at 37°C. Five micrograms of plasmid DNA constructs was then transfected into cells with 30 μg of Lipofectin (Bethesda Research Laboratories) for 16 h. For radiolabeling of proteins, cells were incubated in methionine-free MEM for 40 min at 37°C before addition of labeled methionine. Labeling was usually performed for 1 h in 1 ml of methionine-free MEM containing 10 μCi of [$^{35}$S]methionine translabel (specific activity, >1,000 Ci/mmol) per ml.

**Immunoprecipitation.** In vivo-labeled HeLa cells were washed twice with cold phosphate-buffered saline (PBS). Monolayers were scrapped into cold PBS and collected by centrifugation at 16,000 × *g* for 2 min. The cell pellet was suspended in 1× RIPA buffer (5 mM Tris-HCl [pH 7.9], 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate). Cytoplasmic extracts were prepared, and virus-specific proteins were immunoprecipitated with either rabbit polyclonal antiserum or purified immunoglobulin G (IgG) raised against GST-3AB fusion protein. Proteins were analyzed by electrophoresis in 20% polyacrylamide gels as previously described (12).

**Indirect immunofluorescence.** For analysis of internal antigen distribution, cells were thoroughly washed with PBS$^+$ (PBS containing 0.01% $CaCl_2$-$MgCl_2$) and fixed by incubation in acetone-methanol (1:1) for 7 min at −20°C. Following fixation, cells were washed thoroughly with PBS$^+$ containing 0.2% (wt/vol) gelatin (blocking buffer) for 1 h at 37°C. Primary antibodies were diluted 1:250 with blocking buffer and incubated with cells for 1 h at 37°C. Cells were then washed by three sequential washes of 15 min each in blocking buffer before the addition of secondary fluorescein isothiocyanate-conjugated antibodies at a dilution of 1:100. After staining, cells were further washed in PBS$^+$ containing 0.05% Tween 20 and then PBS$^+$ alone before being mounted with a Gelvatol (Monsanto, Indian Orchard, Mass.)-glycerol solution containing 2.5% 1,4-diazo-bicyclo-[2.2.2]octane (Sigma). Transmission fluorescence microscopy was performed with a Nikon optiphotomicroscope (Nippon Kogaka, Tokyo, Japan).

## RESULTS

**Expression and purification of recombinant 3AB.** The poliovirus 3AB sequence was amplified from the poliovirus infectious clone pT7PV1 (9) by PCR using appropriate primers (see Materials and Methods). Sequences containing *Kpn*I and *Bam*HI sites, translational start codon AUG, and stop codon UAG were introduced in the oligonucleotide primers used for PCR. Amplified DNAs were cloned in the pGEM-3 vector under the control of T7 promoter for construction of the pGEM UD 3AB clone (Fig. 1A). Expression from this clone should yield 3AB containing one extra methionine at the N terminus. When the mRNA transcribed from the clone by T7 RNA polymerase was translated in reticulocyte lysate in the presence of [$^{35}$S]methionine and the labeled protein was immunoprecipitated with an antibody prepared against a VPg nonapeptide (35), a protein of an approximate molecular mass of 12 kDa was clearly detected (Fig. 2B, lane 1). This polypeptide was not precipitated by the preimmune serum (Fig. 2B, lane 2). When the mRNA was translated in a HeLa cell extract and labeled proteins were analyzed directly on an SDS-

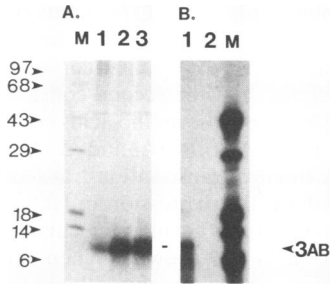


FIG. 2. In vitro translation of cloned 3AB in HeLa (A) and reticulocyte (B) cell extracts. In vitro-transcribed 3AB mRNA was translated in HeLa extract and reticulocyte lysate as described in Materials and Methods. (A) Lane 1, no mRNA; lane 2, 2 μg of 3AB mRNA; lane 3, 2 μg of another preparation of 3AB mRNA. (B) In vitro-translated [$^{35}$S]methionine-labeled products were immunoprecipitated with anti-VPg antibody. Lane 1, immune serum; lane 2, preimmune serum. M, protein molecular weight markers. Molecular weights in thousands are indicated on the left.

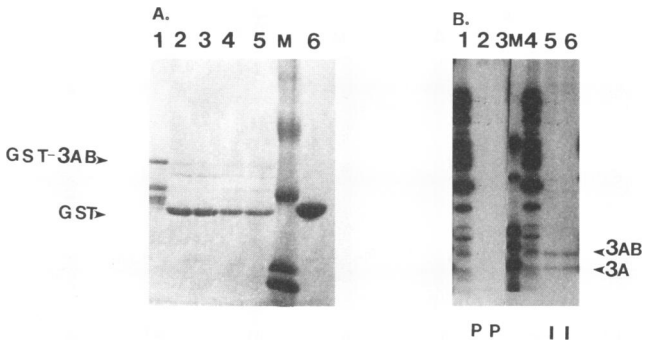


FIG. 3. Expression of 3AB as a fusion protein in *E. coli* and affinity purification of the fusion protein. 3AB was expressed in *E. coli* as a GST fusion protein (GST-3AB) as described in Materials and Methods. (A) Affinity-purified GST-3AB was analyzed by SDS-PAGE followed by staining with Coomassie blue. Lane 1, 3 μg of GST-3AB; lane 6, 5 μg of GST; lanes 2 and 3, GST-3AB digested with 1.5 μg of thrombin per ml for 30 and 60 min, respectively; lanes 4 and 5, GST-3AB digested with 3 μg of thrombin per ml for 30 and 60 min, respectively. (B) [$^{35}$S]methionine-labeled viral proteins were immunoprecipitated with anti-3AB IgG (I) (lanes 5 and 6) or preimmune IgG (P) (lanes 2 and 3). Lanes 1 and 4, labeled viral proteins in virus-infected extracts; lane M, molecular weight markers.

polyacrylamide gel, a similar-molecular-weight protein was synthesized (Fig. 2A, lanes 2 and 3). The reaction mixture containing no 3AB mRNA contained a nonspecific band of similar size; however, this could not be immunoprecipitated by anti-VPg antisera (data not shown). Thus, transcription and translation of the 3AB gene yielded an anti-VPg immunoprecipitable protein with an expected molecular mass of 12 kDa.

The purpose of this study was to study properties of the 3AB protein with particular emphasis on the sequence of the protein needed for membrane binding. For this purpose it was necessary to make a high-titer antibody to this protein. It is well known that in many instances peptide antibodies tend to give high background, thus making it difficult to determine localization of proteins by immunofluorescence assay. For this reason we decided to express 3AB as a fusion protein with GST. Thus another recombinant plasmid (pUDGST-3AB) (Fig. 1B), which is capable of expressing 3AB as a fusion protein, was constructed. The 3AB-GST fusion protein in an *E. coli* extract was bound to glutathione-agarose beads. After thorough washing with buffer, the protein was eluted from the beads by washing with buffer containing reduced glutathione (49). When analyzed by SDS-polyacrylamide gel electrophoresis, a fusion protein having an approximate molecular mass of 38 kDa was clearly detected in the purified preparation (Fig. 3A, lane 1), whereas the plasmid expressing GST alone encoded a protein of an approximate molecular mass of 26 kDa (Fig. 3, lane 6). Some of the fusion proteins detected in the preparation were less than full-length GST-3AB and could presumably be due to degradation of the complete fusion protein (lane 1). The approximate yield of the 3AB-GST protein was 0.1%. When 3AB-GST was digested with thrombin, a protease supposed to cleave the fusion protein such that mature 3AB is released, nonspecific degradation of the fusion protein was detected (lanes 2 to 5). Thus, this method could not be used to generate a purified, mature 3AB protein. Therefore, antibody to 3AB was raised against the fusion protein in rabbits, and IgG was purified by protein A-Sepharose chromatography.

When this antibody was used to immunoprecipitate viral proteins from $^{35}$S-labeled cells infected with poliovirus, two major bands of approximate molecular masses of 12 and 6 kDa that were not observed with preimmune IgG were detected (Fig. 3B, lanes 2, 3, 5, and 6). These bands comigrated with 3AB and 3A in infected cell extracts. VPg (3B) was not detected because it does not contain any methionine. Higher exposure of the autoradiogram showed many other immune IgG-specific bands of molecular masses ranging from 18 to 94 kDa (data not shown). These proteins presumably represent precursors of 3A and 3B.

**Expression of 3AB and 3A in eukaryotic cells.** The VPg precursor 3AB is an abundant protein which is found to localize in the smooth membranes where active RNA replication occurs (48). In order to study localization of 3AB in the cytoplasmic membrane, it was necessary to express 3AB in eukaryotic cells. For this purpose, HeLa monolayer cells were first infected with VTF7-3 recombinant vaccinia virus harboring the T7 RNA polymerase gene. Vaccinia virus-infected cells were then transfected with the 3AB plasmid under control of T7 promoter. Newly synthesized proteins were labeled by addition of [$^{35}$S]methionine. HeLa cell extracts were then prepared, and labeled 3AB was immunoprecipitated with anti-3AB antisera. As shown in Fig. 4A (lanes 2 and 3), a polypeptide having the approximate size of 3AB was readily detected in reaction products immunoprecipitated with anti-3AB, whereas this protein was not present in immunoprecipitates containing preimmune serum (Fig. 4A, lanes 1 and 4). The polypeptide of an apparent molecular mass of 12 kDa that was immunoprecipitated from HeLa cells transfected with the 3AB plasmid comigrated with 3AB immunoprecipitated from HeLa cells infected with poliovirus (Fig. 4C, lanes 2 and 3). Thus, authentic 3AB was expressed in HeLa cells. 3AB was also expressed in CV1 cells; however, the level of expression was not as high as that in HeLa cells (Fig. 4B, lanes 1 and 2). Some higher-molecular-weight proteins were detected in the immunoprecipitate near the top of the autoradiogram (Fig. 4A); these were nonspecific proteins since these bands were also detected in control reactions lacking the 3AB plasmid during transfection (data not shown). In contrast, two labeled proteins migrating close to the molecular mass marker of 29 kDa (second from the top in lane M, Fig. 4B) were specifically associated with the immunoprecipitate from HeLa cells (Fig. 4A, lane 3). One of these proteins was also present in the immunoprecipitate from CV1 cells (Fig. 4B, lane 2, lower

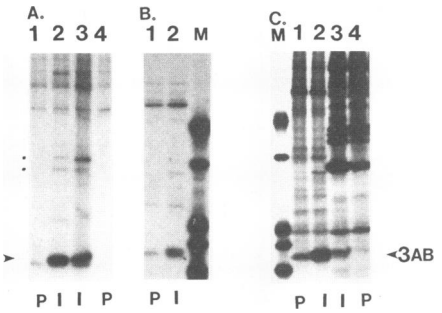


FIG. 4. Expression of 3AB in eukaryotic cells. 3AB was expressed in HeLa (A and C) and CV1 (B) cells by using the vaccinia virus-T7 expression system (18). P and I, immunoprecipitation of expressed 3AB from labeled cell extracts using preimmune IgG and immune IgG, respectively. (A) Lanes 1 and 4, preimmune IgG; lanes 2 and 3, immune IgG. (B) Lane 1, preimmune IgG; lane 2, immune IgG. (C) Lane 1, preimmune IgG; lane 2, immune IgG; lanes 3 and 4, immune IgG and preimmune IgG used to precipitate [$^{35}$S]methionine-labeled viral proteins from infected HeLa extracts. Arrowheads 3AB. Proteins coprecipitating with anti-3AB IgG from cells transfected with the 3AB construct are indicated (dots in panel A).

band). These bands may represent modified forms of 3AB. Alternatively, these polypeptides could be HeLa proteins or vaccinia virus-encoded polypeptides which specifically associated with 3AB.

**Localization of 3AB in HeLa cells by indirect immunofluorescence.** For analysis of distribution of 3AB in transfected HeLa cells, cells expressing 3AB were analyzed by indirect immunofluorescence. Primary antibody (anti-3AB) was incubated with cells, and then secondary fluorescein isothiocyanate-conjugated antibody was added. Transmission fluorescent microscopy was then performed. Cells expressing only vaccinia virus proteins were used as a control (Fig. 5A); very little immunofluorescence was detected in these cells. When cells infected with poliovirus were subjected to immunofluorescence, distinct staining of cells with anti-3AB antibody was observed (Fig. 5B). The fluorescence pattern in infected cells seemed not to be well defined, as many 3AB-related molecules including precursor proteins and VPg-containing RNAs were present in these cells. In sharp contrast to infected cells, 3AB-transfected cells showed two distinct types of immunofluorescence (Fig. 5C). Some transfected cells displayed fluorescence all around the nucleus (perinuclear), suggesting localization of 3AB protein in the endoplasmic reticulum (ER pattern), whereas some other transfected cells clearly showed polarized expression of 3AB on one side of the nucleus. The latter type of fluorescence is typically indicative of Golgi localization (Golgi pattern). Thus, these results suggested that the precursor of poliovirus genome-linked protein, 3AB, is capable of localizing itself on ER and Golgi bodies in the absence of other poliovirus proteins.

**The C-terminal amino acids of 3A are responsible for 3AB membrane binding.** Because VPg is a highly hydrophilic polypeptide, it is believed that it is through the hydrophobic domain of 3A that 3AB is anchored onto cytoplasmic smooth membranes. Figure 6 shows the hydrophilicity pattern of 3AB. Clearly, a highly hydrophobic stretch of amino acids is found between amino acids 60 and 80 of both 3AB and 3A, although smaller stretches of hydrophobic regions are also present between amino acids 21 and 28 and between amino acids 42 and 47 of 3A (Fig. 6). To determine whether (i) 3A itself is able to bind the membrane and (ii) the stretch of 22 mainly hydrophobic amino acids at the C terminus of 3A is responsible for 3AB membrane-binding activity, two deletion mutants of 3AB were constructed. In the first construct, the entire 3B sequence was deleted, and the second construct was a mutant of 3A lacking 18 hydrophobic amino acids from the C terminus of the protein (Δ3A). Both 3A and Δ3A constructs were characterized by in vitro transcription-translation (Fig. 7) and expression in HeLa cells. Expression in HeLa cells from both constructs yielded proteins having molecular weights expected of 3A and Δ3A (data not shown). When the proteins were expressed in HeLa cells and analyzed by indirect immunofluorescence, cellular distribution of 3A was identical to that of 3AB. As seen with 3AB, 3A showed immunofluorescence indicative of Golgi and ER localization (Fig. 5D and E). In sharp contrast to 3A, the Δ3A mutant lacking C-terminal 18 amino acids showed distribution of the protein throughout the cellular cytoplasm (Fig. 5F). Thus, 18 C-terminal amino acids of 3A appeared to be important for Golgi and ER localization of 3A.

To confirm the results obtained by indirect immunofluorescence that C-terminal amino acids of 3A were important for membrane binding, a direct membrane-binding assay was developed. In this assay, mRNAs derived from 3AB, 3A, and Δ3A plasmids were translated in vitro in reticulocyte lysates in the absence and presence of added canine membrane. Following translation, the reaction mixture was microfuged to separate the particulate fraction from the soluble fraction. Labeled proteins (3AB, 3A, and Δ3A) were then immunoprecipitated from particulate and soluble fractions with anti-3AB immune IgG and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). As expected, when translated in the presence of the membrane, most of the in vitro-translated 3AB and 3A were found to be associated with membranes (Fig. 7, lanes 4 and 7). Soluble fractions contained very little of these proteins (lanes 5 and 8). The distribution of the Δ3A protein, however, was significantly different from those of 3AB and 3A. In this case, almost equal amounts of the deleted 3A protein were found to be distributed between the soluble and particulate fractions (lanes 1 and 2). Although not shown here, pelleting of 3AB and 3A with the membrane fraction was completely dependent on added membrane during translation (data not shown). Taken together with the indirect immunofluorescence data, these results suggest that the C-terminal 18 amino acids of 3A play a major role in membrane binding of 3A and presumably of 3AB.

A curious feature of the translation products in the reaction programmed with 3AB mRNA was the presence of a labeled protein migrating at approximately 18 kDa (Fig. 7, lane 7). Also, this 18-kDa band was present exclusively in the membrane fraction (compare lanes 7 and 8). Similarly, an additional protein migrating with the 14-kDa marker was observed in the reaction programmed with 3A mRNA (Fig. 7, lane 4). This band was also absent in the soluble fraction (lane 5). Total unfractionated translation reactions programmed with 3A and 3AB mRNAs also contained these bands (lanes 6 and 9, respectively). The 18-kDa band in the 3AB translation reaction was not detected when translation was performed in the absence of the canine membrane (compare lanes 9 and 10). A similar result was obtained during translation of 3A mRNA in the absence of the membrane (data not shown). These results suggested that the 18- and 14-kDa proteins could be modified forms of 3AB and 3A that are formed in the presence of membranes. In addition, these modified proteins are not cytoplasmic and tend to stick to membranes. It should be pointed out that the 18- and 14-kDa proteins were immuno-

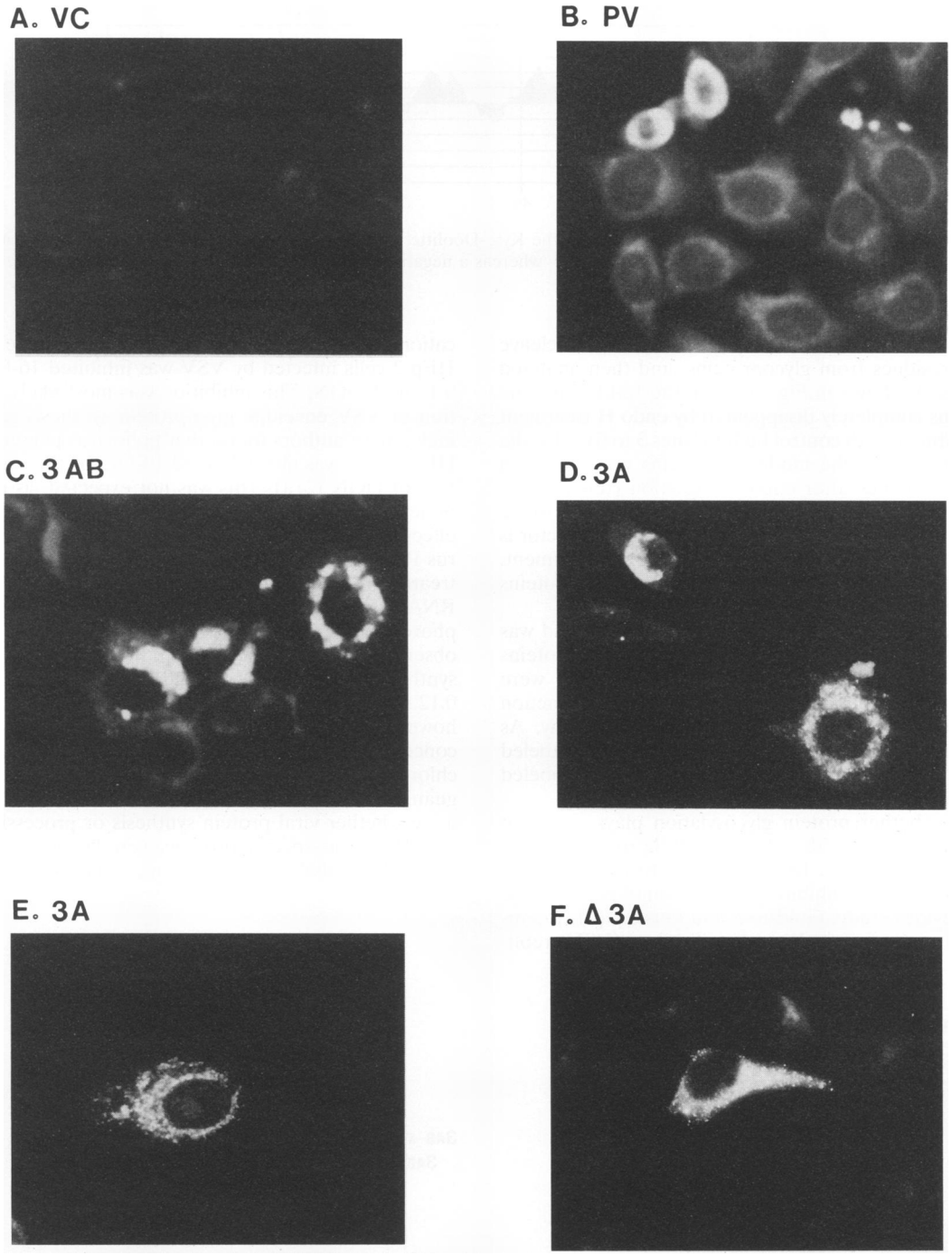


FIG. 5. Analysis of 3AB, 3A, and Δ3A polypeptides expressed in HeLa cells by indirect immunofluorescence. VC, vaccinia virus-infected control; PV, poliovirus-infected cells.

precipitated by anti-3AB and, thus, must be related to 3A and/or 3AB (Fig. 7).

To rule out the possibility that the modified form of 3AB was not generated from the membrane fraction which may be contaminated with unrelated mRNAs, translation reactions were performed with membranes but without 3AB mRNA. Unlike the experiment described in Fig. 7, these reactions were directly analyzed (without immunoprecipitation) by SDS-PAGE. As shown in Fig. 8A, synthesis of the modified, 3AB-related 18-kDa protein was absolutely dependent on 3AB mRNA (lanes 1 and 2). A similar result was also obtained with the 3A-related 14-kDa band, suggesting that its synthesis was also dependent on 3A mRNA (data not shown).

Because membrane-dependent modification of polypeptides (during translation) generally indicates glycosylation of proteins, we wished to determine whether 3AB and 3A were indeed glycosylated in vitro during translation. In this experiment, methionine-labeled 3AB and 3A translation products

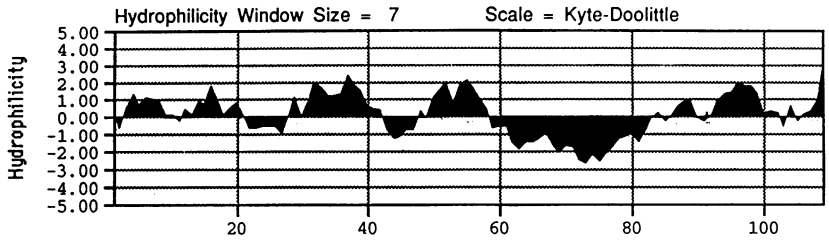


FIG. 6. Hydrophilicity graph of 3AB protein according to the Kyte-Doolitte analysis. Numbers of amino acid residues are indicated on the ordinate. A positive hydrophilicity indicates hydrophilic nature, whereas a negative hydrophilicity indicates hydrophobic nature.

were treated with endo H, an enzyme known to cleave carbohydrate residues from glycoproteins, and then analyzed by SDS-PAGE. As shown in Fig. 8B, both the 18-kDa and the 14-kDa proteins completely disappeared by endo H treatment but not by treatment with control buffer (lanes 3 to 6). Also the radioactivity present in the modified proteins reappeared in the 3AB and 3A bands after endo H digestion (this is more apparent in lanes 6 and 5). Lanes 1 and 2 show a positive control in which the fully glycosylated form of yeast α-factor is converted to a nonglycosylated form by endo H treatment. These results demonstrate that the 18- and 14-kDa proteins are glycosylated forms of 3AB and 3A, respectively.

To further confirm that the 18-kDa 3AB-related band was indeed glycosylated, in vitro translation reactions with proteins labeled with either [$^{35}$S]methionine or [$^{14}$C]mannose were performed. Labeled proteins present in the particulate fraction were analyzed by SDS-PAGE followed by fluorography. As shown in Fig. 8C, only the 18-kDa form of 3AB was labeled with mannose which comigrated with the methionine-labeled 18-kDa protein.

To examine whether protein glycosylation plays a role in poliovirus RNA synthesis, the effect of DON on viral RNA replication was determined. DON is known to interfere with glycoprotein synthesis by inhibiting production of glucosamine (42a). In a previous study, Goldstein and Guskey (21a) demonstrated inhibition of vesicular stomatitis virus (VSV) replication by low concentrations of DON. Plaque formation in HEp-2 cells infected by VSV was inhibited 16-fold by 0.06 to 0.12 mM DON. This inhibition was most likely due to inhibition of VSV envelope glycoprotein synthesis (21a). Interestingly, these authors found that poliovirus plaque formation in HEp-2 cells was also inhibited 10-fold by the same concentration of DON (21a). This was not expected, as poliovirus is a nonenveloped virus. The authors concluded that unlike its effect on VSV replication, DON might interfere with poliovirus RNA synthesis. When poliovirus-infected HeLa cells were treated with 0.06 and 0.12 mM DON and $^{32}$P-labeled viral RNA was analyzed by nondenaturing agarose gel electrophoresis, significant inhibition of viral RNA synthesis was observed (Fig. 9A, lanes 1 to 3). In fact, single-stranded RNA synthesis was reduced to 71 and 21% of the control at 0.06 and 0.12 mM DON, respectively. Double-stranded RNA synthesis, however, was inhibited to 30 and 13% of the control at these concentrations of DON. As a control, 2 mM guanidine hydrochloride was used in a reaction shown in lane 4. As expected, guanidine completely inhibited viral RNA synthesis. To determine whether viral protein synthesis or processing is affected by DON, virus-specific proteins were labeled with [$^{35}$S]methionine in the absence and presence of DON. As can be seen in

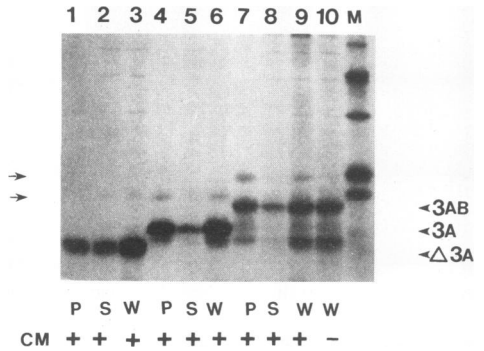


FIG. 7. Distribution of 3AB, 3A, and Δ3A between particulate (P) and soluble (S) fractions during in vitro translation. In vitro-transcribed 3AB, 3A, and Δ3A RNAs were translated in the presence of [$^{35}$S]methionine in rabbit reticulocyte lysate in the absence and presence of the canine microsomal membrane (CM) as described in Materials and Methods. Particulate and soluble fractions were separated by centrifugation and analyzed by SDS-PAGE following immunoprecipitation with anti-3AB IgG. W, whole (unfractionated) reaction mixtures. Lanes 1 to 3, Δ3A RNA; lanes 4 to 6, 3A RNA; lanes 7 to 10, 3AB RNA. Arrows, modified 3AB and 3A (particularly in lanes 4, 6, 7, and 9).

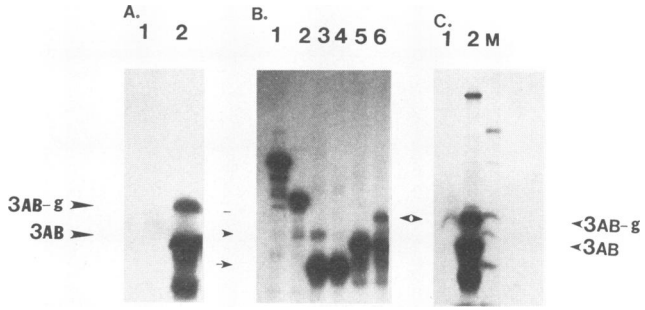


FIG. 8. In vitro glycosylation of 3AB and 3A. In vitro-transcribed 3AB and 3A RNAs (2 μg) were translated in reticulocyte lysate in the presence of [$^{35}$S]methionine (A to C) or [$^{14}$C]mannose (C) with the exogenously added canine membrane and particulate fractions separated by centrifugation and analyzed by SDS-PAGE. Some of the samples were digested with endo H before gel analysis. (A) Lane 1, no 3AB RNA; lane 2, 3AB RNA. (B) Lanes 1 and 2, in vitro-translated yeast α-factor digested with control buffer and endo H, respectively; lanes 3 and 4, 3A RNA-derived translation products digested with control buffer and endo H, respectively; lanes 5 and 6, 3AB translation products digested with endo H and control buffer, respectively. (C) Lane 1, [$^{14}$C]mannose-labeled 3AB translation product; lane 2, [$^{35}$S]methionine-labeled 3AB translation product. 3AB-g, glycosylated form of 3AB. Arrow and arrowhead in panel B, nonglycosylated and glycosylated forms of 3A (lanes 4 and 3), respectively.

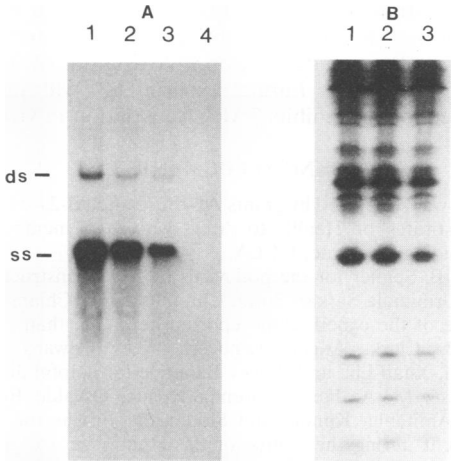


FIG. 9. DON inhibits poliovirus RNA synthesis. (A) Poliovirus RNA synthesis in virus-infected HeLa cells was determined as described in Materials and Methods in the absence (lane 1) or presence of 0.06 mM DON (lane 2), 0.12 mM DON (lane 3), or 2mM guanidine hydrochloride (lane 4). ds and ss, double- and single-stranded RNAs, respectively. (B) Poliovirus-specific proteins were labeled with [$^{35}$S]methionine in the absence (lane 1) or presence of 0.06 mM (lane 2) or 0.12 mM (lane 3) DON.

Fig. 9B, no significant difference in viral protein synthesis or processing was observed in the presence of 0.06 mM DON compared with the control. At 0.12 mM, however, poliovirus protein synthesis was reduced to approximately 55% of the control. This reduction in viral protein translation was likely due to inhibition of viral RNA synthesis. However, synthesis of virus-specific proteins observed at 0.06 and 0.12 mM DON was not inhibited as much as would be expected from the degree of inhibition of RNA synthesis. This is expected, as poliovirus proteins are known to be synthesized in much excess over viral RNA in infected HeLa cells. Thus viral protein synthesis or processing was not grossly affected by DON (lanes 3 to 5). These results suggest that protein glycosylation may play a role in viral RNA synthesis. We also examined the effect of tunicamycin on viral RNA synthesis. Unfortunately, concentrations of dimethyl sulfoxide required to solubilize tunicamycin were found to nonspecifically inhibit viral RNA synthesis. Therefore, whether tunicamycin specifically inhibits viral RNA synthesis could not be determined with certainty (data not shown).

## DISCUSSION

Poliovirus-encoded polypeptide 3AB has been implicated in the initiation of viral RNA synthesis. It is believed that this membrane-bound, VPg-precursor protein donates the VPg (3B) polypeptide to the 5′ ends of newly synthesized RNA (48). How VPg is transferred to viral RNA remains unknown at present. We have expressed 3AB in both prokaryotic and eukaryotic cells. We show here that 3AB specifically localizes in both the ER and the Golgi bodies in transfected HeLa cells. This membrane localization is largely determined by the C-terminal 18 amino acids of the 3A polypeptide. We also show that a significant portion of 3AB can be glycosylated during in vitro translation.

Many of the poliovirus-encoded proteins have been expressed in *E. coli* and insect cells by a number of laboratories (27, 37, 38, 43). Our main interest to express 3AB in HeLa cells was to study its interaction with membranous structures to which it is known to bind during RNA replication. *E. coli* expression of 3AB was performed primarily for preparation of a higher-titer antibody to this protein. Lama and Carrasco reported that expression of 3AB in *E. coli* was highly toxic and that expressed 3AB induced modifications in membrane permeability of these cells (27). In contrast to this report, we found almost no toxic effect of 3AB when the protein is expressed in HeLa cells. This lack of toxicity of 3AB in HeLa cells was not due to a lower level of expression, as the vaccinia virus-T7 system used for protein expression generated relatively large amounts of 3AB in HeLa cells (Fig. 4). Expression in CV1 cells, however, was not as high as in HeLa cells.

Indirect immunofluorescence studies of transfected 3AB clearly demonstrate association of this polypeptide with the ER and the Golgi bodies. Because no other poliovirus protein is present in these cells, we conclude that 3AB itself has the ability to associate with the cytoplasmic membranes. In fact, we have demonstrated that it is the 3A portion of 3AB which allows 3AB to bind to the membrane, as both 3A and 3AB display almost identical patterns of intracellular localization. Both indirect immunofluorescence and in vitro translation studies demonstrate that the amphipathic domain present near the C terminus of 3A is largely responsible for binding of 3A to the cytoplasmic membranous structure (Fig. 5 and 7).

Giachetti et al. first used an in vitro system to study protein-membrane interaction in the poliovirus system (19). We used a similar system to study binding of 3AB, 3A, and Δ3A to membranes during in vitro translation. Although predicted results were obtained in these experiments, in relation to which the domain of 3A was required for membrane binding, the finding that 3AB and 3A can be modified during in vitro translation was extremely surprising. We have presented three lines of evidence which suggest that glycosylation of 3AB and 3A occurs during in vitro translation in the presence of the canine microsomal membrane. First, the modified forms of 3AB and 3A polypeptides appear only when translation is carried out in the presence of the membrane. Their appearance is completely dependent on exogenously added 3A and 3AB mRNA in the system. Second, treatment of in vitro-translated proteins with endo H and endoglycosidase F (data not shown) completely converts the modified forms back to unmodified proteins. Finally, the modified form of 3AB can be labeled with [$^{14}$C]mannose during in vitro translation in the presence of microsomal membranes. Both the modified 3AB and 3A bands are sensitive to protease digestion (data not shown), indicating their protein nature, and these proteins must be related to 3AB (or 3A), as they are specifically recognized by anti-3AB IgG.

Complete glycosylation of in vitro-translated 3AB (or 3A) is never observed. The amount of glycosylated 3AB is found to vary from 10 to 30% in different reactions (Fig. 7 and 8). The reason for this variation is unknown and may be due to differential activity of various membrane preparations used in the experiments reported here. It is interesting that a glycosylation consensus sequence [Asn-X-(Thr/Ser)] (26) is present almost near the middle of the 3A polypeptide (25, 44). The sequence Asn-Ile-Thr (amino acids 45 to 47), present in 3A, could be the site of glycosylation. Future studies will determine the precise site of glycosylation. Although this sequence is present in the mutant polypeptide Δ3A (18), it is not certain whether this polypeptide can be efficiently glycosylated in vitro, as we have detected very little or no glycosylation during in vitro translation of Δ3A (Fig. 7).

The fact that DON, a known protein glycosylation inhibitor (42a), inhibits viral RNA synthesis in vivo suggests that protein glycosylation may play a role in viral replication. Whether viral

RNA synthesis inhibition by DON is due to inhibition of glycosylation of 3AB (or 3A) in infected cells remains to be determined. Incomplete glycosylation of one or more cellular proteins may result in the inhibition of viral RNA synthesis due to a conformational change in the membrane-bound replication complex. It is also possible that a physiological or molecular process other than glycosylation is inhibited by DON. In the experiments described in Fig. 9, DON was added to cells 30 min after infection was initiated. Thus, RNA synthesis inhibition could not be due to interference with virus binding to cellular receptor and/or virus internalization. It is also clear that DON does not inhibit viral protein synthesis or processing (Fig. 9). Thus, DON appears to interfere with viral RNA synthesis. It should be noted that plaque formation by three different enteroviruses in multiple cell lines was inhibited by DON (21a). Thus, the effect of DON is not limited to poliovirus within the enterovirus family. The possibility that DON may inhibit viral RNA synthesis by interference with amino transfer reactions during purine and pyrimidine synthesis appears unlikely for the following reasons. First, Goldstein and Guskey noted only a marginal reversal of viral replication by a combination of ribonucleotides (21a). We found no significant reversal of DON-inhibited RNA synthesis in the presence of a mixture of all four ribonucleotides. It should be noted that in contrast to the experiments reported by Goldstein and Guskey, our infections contained actinomycin D, which, by virtue of its ability to inhibit all three cellular RNA polymerases, may have contributed to an increase in the cellular nucleotide pool. Furthermore, unlike these authors, who exposed cells to DON for 48 to 72 h, we used suspension cultures, and thus, cells were exposed to this agent for only 4 h during infection. The cellular nucleotide pool is not likely to be depleted during this short period, especially in the presence of actinomycin D.

Our initial attempts to determine whether glycosylated 3AB and 3A exist in poliovirus-infected HeLa cells have failed. The main obstacle in detection of modified 3AB and 3A in infected cells appears to be very small amounts of these glycoproteins in infected cells. In addition, immunoprecipitation using anti-3AB antibody is not quantitative. Thus, both the small amounts of the modified proteins and low efficiency of immunoprecipitation contribute to our failure in detection of these molecules in infected cells. It will be necessary to affinity purify glycosylated 3AB (3A) from infected cells before successful immunoprecipitation with anti-3AB antibody can be performed. Microsomal membranes contain only the ER. It is possible that the glycosylated form of 3AB (or 3A) in virus-infected cells may have different mobility than the in vitro glycosylated protein, as passage through the Golgi in intact cells is likely to result in further modification of the protein.

Poliovirus is a nonenveloped virus, and the known posttranslational modifications of poliovirus proteins include proteolytic processing and myristoylation of VP4 (11, 16). A strict requirement for protein glycosylation in poliovirus-infected cells has not been anticipated to date. Two recent reports demonstrate that brefeldin A, a fungal antibiotic that is known to block protein transport from the ER to the Golgi apparatus (29, 33, 40), inhibits poliovirus RNA synthesis (23, 32). Inhibition of poliovirus RNA synthesis by brefeldin A has been proposed to be due to blockade of replication complex formation as a result of inhibition of vesicular transport of poliovirus proteins (23, 32). Is it possible that brefeldin A inhibits glycosylation of 3AB (or 3A) in poliovirus-infected cells? It is worth noting that brefeldin A prevents proper glycosylation of VSV G protein and blocks its transport from ER to the Golgi apparatus (40). It may be that only glycosylated 3AB (or 3A) is able to form proper replication complexes capable of initiating poliovirus RNA synthesis. Another possibility may be that uridylylation of VPg (or 3AB) may depend on glycosylation of 3AB or a larger VPg precursor. Future experiments will determine whether brefeldin A inhibits 3AB glycosylation in vivo.

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