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In Vivo and In Vitro Phosphorylation of Rotavirus NSP5 Correlates with Its Localization in Viroplasms

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NSP5 (NS26), the product of rotavirus gene 11, is a phosphoprotein whose role in the virus replication cycle is unknown. To gain further insight into its function, we obtained monoclonal antibodies against the baculovirus-expressed protein. By immunoprecipitation and immunoblotting experiments, we showed that (i) NSP5 appears in many different phosphorylated forms in rotavirus-infected cells; (ii) immunoprecipitated NSP5 from rotavirus-infected cells can be phosphorylated in vitro by incubation with ATP; (iii) NSP5, produced either by transient transfection of rotavirus gene 11 or by infection by gene 11 recombinant vaccinia virus or baculovirus, can be phosphorylated in vivo and in vitro; (iv) NSP5 expressed in *Escherichia coli* is phosphorylated in vitro, and thus NSP5 is a potential protein kinase; and (v) NSP5 forms dimers and interacts with NSP2. The intracellular localization of NSP5 in the course of rotavirus infection and after transient expression in COS7 cells has also been investigated. In rotavirus-infected cells, NSP5 is localized in viroplasms, but it is widespread throughout the cytoplasm of transfected COS7 cells. NSP5 produced by transfected COS7 cells did not acquire the multiphosphorylated forms observed in rotavirus-infected COS7 cells. Thus, there is a tight correlation between the localization of NSP5 in the viroplasms and its protein kinase activity in vivo or in vitro. Our results suggest that cellular or viral cofactors are indispensable to fully phosphorylate NSP5 and to reach its intracellular localization.

Rotaviruses are members of the *Reoviridae* family, and their genome is composed of 11 molecules of double-stranded RNA (dsRNA), ranging from 3.3 to 0.6 kb in size, that encode six structural proteins and five nonstructural proteins (26). Virus replication occurs in the cytoplasm, but various steps of this process are not precisely known. As the virus enters the cell, viral transcriptase is activated and synthesizes capped nonpolyadenylated mRNAs. These viral mRNAs are translated or used as templates for the synthesis of the genomic dsRNAs. Replication is nonconservative: mRNAs are copied in their negative strand, and the dsRNAs thus formed are encapsidated in new viral particles. Encapsidation and replication are concomitant since dsRNAs are never found free in the cytoplasm but are always associated with viral structures.

The replication of the viral RNA is thought to occur in specialized regions of the cells called viroplasms (32) where structural (VP2 and VP6) proteins as well as some nonstructural proteins (NSP2 and NSP5) are concentrated. Other nonstructural proteins (NSP1 and NSP3) are not localized in the viroplasms but are widespread in the cytoplasm instead and may associate with the cytoskeleton (16, 25). Two glycoproteins, VP7 and NSP4, are localized in the endoplasmic reticulum, which is where rotavirus acquires its external capsid made of VP7 and VP4 (26). The other proteins (VP1 and VP3) have not yet been localized because of the lack of potent preparations of monospecific antibodies against them and/or because of their low level of expression in infected cells. The viral proteins localized in the viroplasms are associated with the dsRNA synthesis (replicase) activity of the rotavirus (2, 14); thus, it is extremely important to understand the mechanisms that lead to the correct intracellular localization of the viral proteins and mRNAs in the infected cells.

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Rotavirus gene 11 encodes a nonstructural phosphoprotein, NSP5 (46), that has a high serine and threonine content (25%) and localizes in the viroplasms (32). With a predicted molecular weight of 22,000 (22K), the protein presents two forms, 26K and 28K, in polyacrylamide gel electrophoresis (PAGE). Gonzalez and Burrone (12) have shown that in addition to being phosphorylated, NSP5 is O-glycosylated through the addition of simple *N*-acetylglucosamine. These authors attributed the 26K-to-28K shift to this rare type of O-glycosylation that occurs on only a few nuclear and cytoplasmic proteins (13). An alternative, out-of-phase open reading frame has been identified in gene 11 and has been shown to be expressed during rotavirus infection (27). This open reading frame shares epitopes with NSP5, as a serum against it reveals NSP5 (27). Homology with the family of guanido kinases found at the C-terminal end of NSP5 and a putative nucleoside triphosphate binding site at the N terminus of the protein have supported the hypothesis that NSP5 is a protein kinase (26). Based on the presence of a cluster of basic amino acids close to the C terminus of the protein, it has also been proposed that NSP5 could be an RNA-binding protein (26).

Phosphorylation of proteins and localization of protein kinases have been shown to be important both in the intracellular localization of proteins and in the regulation of their activities (9, 29). Here, we investigated the in vitro and in vivo phosphorylation of NSP5 and the possible role of phosphorylation in its intracellular localization.

(Part of this work was presented at the 5th International Double Stranded RNA Virus Symposium, Djerba, 1995.)

MATERIALS AND METHODS

Cells, viruses, and recombinants viruses. The RF strain of rotavirus was propagated in MA104 cells in Eagle's minimum essential medium in the presence of trypsin (35). Infections were made at a multiplicity of 5 to 10 PFU/cell. In vivo labeling was performed from 0 to 6 h postinfection with 0.7 MBq/ml of Tran-^{35}S-Label (38 TBq/mmol; ICN) per ml or 0.7 MBq of [^{33}P]phosphoric acid (92.5 TBq/mmol; Amersham) per ml.

SF9 cells and baculovirus were handled as described previously (21). The recombinant baculovirus (a gift from M. Estes [Baylor College of Medicine, Houston, Tex.]) (46) and vaccinia virus recombinant expressing NSP5 of the simian strain SA11 (a gift from B. Coupar and G. Both [Commonwealth Scientific and Industrial Research Organization, North Ride, NSW, Australia]) (30) have been described elsewhere. Vaccinia virus recombinants were grown and titers were determined on MA104 cells as described previously (36). Infections were done at a multiplicity of infection of 10, and immunoprecipitations were performed 6 h postinfection after 5 h of labeling with [^{35}S]methionine or [^{33}P]phosphoric acid.

Transfection of COS7 cells. COS7 cells grown to 70% confluency in 35-mm-diameter plates were transfected by cationic liposomes (Lipofectine) as instructed by the supplier (Bethesda Research Laboratories). Expression of the transfected genes was monitored 36 or 48 h posttransfection.

Serum and monoclonal antibodies. A guinea pig serum raised by hyperimmunization with recombinant NSP5 (SA11 strain) expressed in the baculovirus SF9 expression system was kindly provided by M. Estes. Several hybridoma lines secreting anti-NSP5 antibodies were obtained after immunization of BALB/c mice with a lysate of SF9 cells infected with a recombinant baculovirus expressing NSP5 of the SA11 rotavirus strain and screening by immunofluorescence of rotavirus-infected CV1 cells (38). The monoclonal antibody used during this study was of immunoglobulin G1 (IgG1) (kappa) subclass and designated 158G37. Ascitic fluids obtained in BALB/c mice and supernatant from the culture medium of the clone 158G37 were both used and gave identical results.

Protein analysis. Immunoprecipitated proteins were resolved on 15% polyacrylamide gels after being boiled in loading buffer (10 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 150 mM 2-mercaptoethanol). For electrophoresis under nonreducing conditions, 2-mercaptoethanol was omitted from the loading buffer, and samples were boiled before loading. After electrophoresis, gels were fixed in 20% ethanol–10% acetic acid, dried, and autoradiographed. When ^{35}S- or ^{33}P-labeled proteins were analyzed, the gels were treated with Amplify (Amersham) before drying and autoradiography. To stain the proteins, the gels were directly fixed in 0.25% Coomassie blue (R250)–20% ethanol–10% acetic acid.

Calf intestinal alkaline phosphatase was from Boehringer; lambda phage phosphatase, a prokaryotic protein phosphatase, was from New England Biolabs. Both were used with the buffers provided by the manufacturers.

For immunoblotting, the proteins separated on SDS-polyacrylamide gels were transferred by transverse electrophoresis in 10 mM CAPS (pH 11)–10% methanol buffer to polyvinylidene difluoride membranes. The dried membranes were saturated for 1 h in 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS), washed three times with TBS, incubated for 1 h at 37°C with the hybridoma culture medium (twofold diluted in TBS–3% BSA), washed again three times with TBS, and then incubated for 1 h at 37°C with a 1/500 dilution (in TBS–3% BSA) of goat anti-mouse IgG coupled to the alkaline phosphatase (Biosys, Compiègne, France). The membrane was then washed again three times with TBS, and bands were visualized by incubation in 5-bromo-4-chloro-3-indolylphosphate and 4-nitroblue tetrazolium chloride reagent.

Immunoprecipitation and in vitro kinase assay. Six hours postinfection, MA104 cells in culture plates were washed with 2 ml of cold Eagle's minimum essential medium and lysed in 1 ml of radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl [pH 8], 125 mM NaCl, 30 mM KCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM EDTA, 2 mg of aprotinin per ml). The cell debris were pelleted by centrifugation (100,000 × *g* for 15 min), and supernatants were directly processed for immunoprecipitation or stored at −80°C. To immunoprecipitate the protein, 1 μl of mouse monoclonal ascitic fluid was added to 100 μl of cell lysate supernatant and incubated overnight at 4°C. Then 20 μl of a 50% suspension of protein A-Sepharose (Pharmacia) in RIPA buffer was added, and the incubation proceeded for 1 h at room temperature with end-over-end rotation. Protein A-Sepharose beads were spun down (13,000 × *g* for 10 s), washed three times with RIPA buffer and twice with kinase buffer (50 mM Tris-HCl [pH 8.5], 20 mM $MgCl_2$), and incubated with [γ-^{32}P]ATP (1.85×10^9 MBq/mmol; 7 MBq/assay in 10 μl of kinase buffer) for 30 min at 37°C. Nonincorporated [γ-^{32}P]ATP was removed by two washes with RIPA buffer, and the beads were processed for protein analysis.

UV cross-linking. Six hours postinfection, MA104 cells (in 20-cm^2 culture plates) were washed with 2 ml of cold TBS (50 mM Tris-HCl [pH 8], 137 mM NaCl) and exposed on ice to a germicidal UV lamp for 13 min at a 22-cm distance. The TBS was removed, and the cells were lysed in 1 ml of RIPA buffer. Cell lysate was then centrifuged at 100,000 × *g* for 15 min, and the supernatant was processed for immunoprecipitation.

Plasmid constructions and expression of recombinant NSP5 in baculovirus. A full-length cDNA clone of gene 11 from the RF strain of bovine rotavirus was obtained as described earlier (6). The coding sequence of RF gene 11 was amplified by PCR from the full-length cDNA clone by using appropriate primers and was cloned into pBlueScript (Stratagene). The integrity of the PCR product was confirmed by in vitro transcription-translation in rabbit reticulocyte lysates. The recombinant plasmid pSVL11 was obtained by subcloning this gene in pSVL (Pharmacia). The cDNA encoding for NSP3 was subcloned in the *Bam*HI site of pSVL to give pSVL-7.

Recombinant baculovirus expressing NSP5 from the RF strain was obtained as previously described for NSP3A (37) except that the transfer vector used was pBacPAK1 (Clontech).

Expression in *Escherichia coli*. NSP5 was expressed in *E. coli* by using the expression system developed by Studier (43). Gene 11 of bovine rotavirus RF was cloned in pET14b+ (Clontech) as a *Nco*I-*Bam*HI fragment. Note that in this construction, NSP5 is expressed as a native protein, but the introduction by PCR of a *Nco*I restriction site on the start codon modified the second amino acid of NSP5 (S to N). The resulting plasmid was transfected in *E. coli* BL21/DE3, and the expression of NSP5 was induced by the addition of IPTG (isopropylthio-β-D-galactoside) to a final concentration of 0.5 mM. Three hours later, cells were collected and lysed by adding lyzozyme (0.1 mg/ml) and Triton X-100 (1%). The soluble fraction of NSP5 was recovered in 50 mM Tris-HCl (pH 8)–125 mM NaCl–2 mM EDTA and immunoprecipitated as described above. A clone of the *E. coli* β-galactosidase gene in pET14b+ (Clontech) treated in the same way was used as control.

Indirect immunofluorescence. The cells were grown on clean sterile glass coverslips and fixed with cold 80% ethanol 6 h postinfection or 48 h posttransfection. The cells were washed three times with TBS containing 0.1% Triton X-100 and incubated for 1 h with the monoclonal ascitic fluid diluted to 1/500 in TBS. After three washes with TBS, the cell layer was incubated for 1 h with goat serum anti-mouse IgG coupled to fluorescein, washed again three times, and mounted on glass slide in glycerol. The slides were observed with the 40× immersion objective of a Leitz UV microscope.

Two-hybrid test. The double-hybrid system in the yeast *Saccharomyces cerevisiae* described originally by Fields and Song (10) and later by Bartel and Fields (3) was used to detect the interactions between proteins. RF genes 7, 8, and 11 (encoding NSP3, NSP2, and NSP5, respectively) were subcloned in pGAD424 and pGBT9 (Clontech) (3). The fusion of GAL4 DNA binding or activating domains with rotavirus genes was checked by nucleotide sequencing. The interaction between NSP2 and NSP5 was monitored by cotransfection of DNA-binding and activating plasmids in *S. cerevisiae* HF7c followed by selection on a medium lacking tryptophan and leucine to monitor the cotransfection efficiency and on medium lacking tryptophan, leucine, and histidine to assess the interaction between the two fusion proteins. Negative controls for interaction between NSP5 and murine p53, and other rotaviral proteins were included.

RESULTS

Multiple forms of NSP5. In a first series of experiments (data not shown), we used a polyclonal antiserum against strain SA11 NSP5 described by Welch et al. (46). Multiple bands were observed when a lysate of MA104 cells, infected by SA11 or RF and labeled with [^{35}S]methionine for 6 h postinfection, was immunoprecipitated with this antiserum. In addition to the two major bands, with apparent molecular weights of 26K and 28K, previously described (46), we observed four or five fainter bands with molecular weights ranging from 30K to 35K (see below). Different origins for these bands have been considered: cellular or viral proteins interacting with NSP5, cellular proteins sharing common epitopes with NSP5, the presence of cross-reacting antibodies against cellular proteins in the serum that was obtained by injecting infected SF9 cells (27), or multiple forms of NSP5 not previously observed.

To determine the origin of these extra bands, we made a monoclonal antibody against recombinant NSP5 and used it in similar immunoprecipitations. No differences were observed between the results obtained by immunoprecipitation with monoclonal antibody 158G37 (Fig. 1A) or with the antiserum. Multiple bands were observed with both reagents in lysate from the infected cells, but these were not detected in lysate from uninfected MA104 cells. Furthermore, when the monoclonal antibody was used in Western blotting (Fig. 1B), the same multiple bands were detected only in the rotavirus-infected MA104 cells; they were not observed in mock-infected MA104 or in SF9 cells infected with a recombinant baculovirus expressing NSP5 either from the SA11 or the RF strain. In the case of recombinant baculovirus-infected SF9 cells, only a 26K protein was detected.

The results obtained with the monoclonal antibody showed that the extra bands observed upon immunoprecipitation of MA104 infected cell lysates were not cellular proteins bearing epitopes common to NSP5, since they were not detected in the uninfected cells. Moreover, the extra bands were not cellular

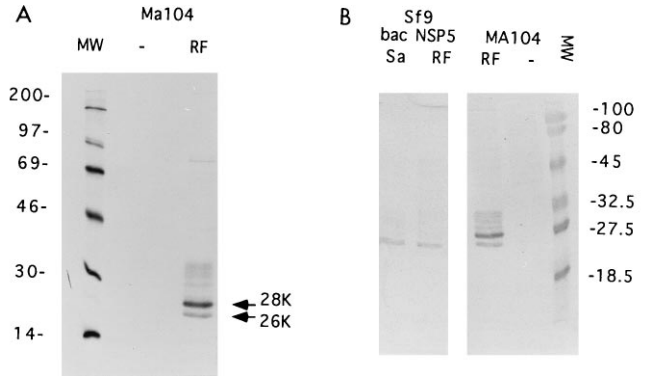

FIG. 1. Multiple forms of NSP5. (A) Lysates from rotavirus-infected (RF) or mock-infected (−) MA104 cells were subjected to immunoprecipitation with the anti-NSP5 monoclonal antibody and separated by SDS-PAGE (15% gel). The 26K and 28K forms of NSP5 are indicated. Sizes of the molecular weight markers (MW) are indicated in kilodaltons. (B) Proteins from lysates of rotavirus-infected (RF) or mock-infected (−) MA104 cells and from SF9 cells infected with baculovirus expressing NSP5 (SA11 [Sa] or RF) were subjected to SDS-PAGE, transferred to a membrane, and probed with the anti-NSP5 monoclonal antibody. Sizes of the standard prestained molecular weight markers (Bio-Rad) (lane MW) are indicated on the right.

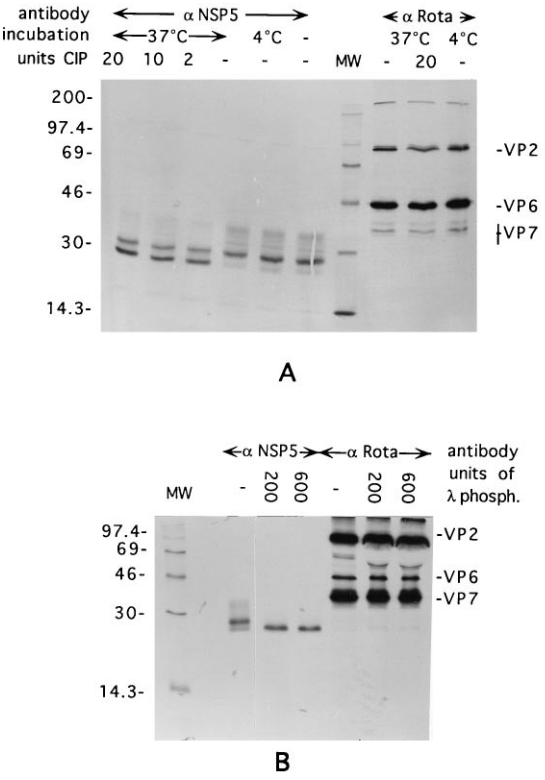

FIG. 2. In vitro dephosphorylation of NSP5. NSP5 immunoprecipitated from rotavirus (RF)-infected MA104 cells labeled with [^{35}S]methionine was treated with calf intestinal phosphatase (CIP) for 2 h at different temperatures (4 or 37°C) and enzyme concentrations (A) or with lambda phosphatase for 2 h at 30°C (B) and then separated by SDS-PAGE. As controls, the same treatment was conducted on viral protein immunoprecipitated with a rabbit hyperimmune serum (α Rota). Positions of the molecular weight standards (MW) (in kilodaltons) and of some viral proteins are indicated.

proteins interacting with NSP5 and coimmunoprecipitated with it, since they were detected by immunoblotting.

The multiple forms of NSP5 are due to phosphorylation. Two kinds of posttranslational modifications of NSP5 have been described: phosphorylation has been observed on the 26K and 28K forms of NSP5 (46), and O-linked glycosylation has been implicated in the 26K-to-28K shift of the apparent molecular weight of NSP5 (12). To test whether phosphorylation could be involved in the multiple forms of NSP5, we treated NSP5 immunoprecipitated from rotavirus-infected MA104 cells with two different phosphatases. Treatment of NSP5 with increasing concentrations of calf intestinal phosphatase (Fig. 2A) decreases the intensity of the high-molecular-weight forms of NSP5 as well as the 28K band and concomitantly increases the intensity of the 26K band. The controls showed that this decrease of the 28K band was not due to (i) protein degradation, since the same electrophoretic mobility was obtained when either NSP5 or rotavirus structural proteins were incubated at 37°C in the phosphatase buffer but in the absence of phosphatase, or (ii) an unspecific effect (deglycosylation), since the migration of the highly glycosylated protein VP7 was not affected by incubation with the enzyme. Furthermore, a complete transformation of NSP5 to the 26K form was obtained when the lambda phosphatase was used in the same test in place of calf intestinal phosphatase (Fig. 2B). Thus, the multiple forms of NSP5, including the 28K form, were all linked to the phosphorylation of the protein.

In vivo phosphorylation of NSP5. To further confirm that the multiple forms of NSP5 were due to phosphorylation of the protein, the NSP5 produced in rotavirus-infected cells was labeled in vivo with [^{33}P]phosphoric acid and immunoprecipitated (Fig. 3). In comparison to NSP5 labeled in vivo with [^{35}S]methionine, the ^{33}P-labeled NSP5 showed an inversion of the relative intensity of the bands: the highest-molecular-weight forms of NSP5 were more intense, and the 26K form was barely visible. These results not only confirmed the phosphatase experiments in indicating that all of the forms of NSP5 were phosphorylated but also suggested that the highest-molecular-weight forms of NSP5 were more heavily phosphorylated than the 26K NSP5. Thus, the different mobilities of NSP5 could be explained by quantitative differences in phosphorylation.

It should be noted that in MA104 cells infected with a recombinant vaccinia virus, only the 26K form of NSP5 was observed, and it was labeled with [^{33}P]phosphoric acid (Fig. 3). Under these conditions, the absence of the higher-molecular-weight forms of NSP5 after both ^{35}S and ^{33}P in vivo labeling revealed an incomplete phosphorylation of NSP5. Thus, it seems that the fully phosphorylated state of NSP5 is acquired only by the protein expressed during rotavirus infection.

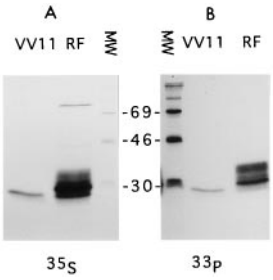

FIG. 3. In vivo phosphorylation of NSP5. (A) MA104 cells infected by the RF strain of rotavirus (RF) or the vaccinia virus recombinant expressing NSP5 (VV11) were labeled with [^{35}S]methionine and then lysed, immunoprecipitated with monoclonal antibody 158G37, electrophoresed on a 15% acrylamide gel, and autoradiographed. (B) Same experiment except that [^{33}P]H_3PO_4 was used for the cell labeling instead of [^{35}S]methionine. Sizes are indicated in kilodaltons.

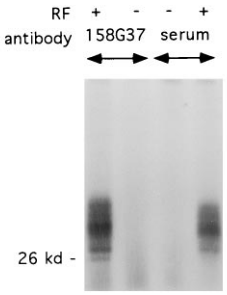

FIG. 4. In vitro phosphorylation of NSP5 synthesized in rotavirus-infected cells. NSP5 from RF rotavirus-infected (+) or mock-infected (−) MA104 cell lysates was immunoprecipitated with an anti-NSP5 monoclonal antibody (158G37) or a guinea pig antiserum against NSP5 (serum). The immune complex was incubated with [γ-^{32}P]ATP for 30 min at 37°C, separated by SDS-PAGE, and autoradiographed.

In vitro phosphorylation of NSP5. Sequence analysis of NSP5 revealed homology of this protein with some kinases (26) and led us to develop an in vitro solid-phase kinase assay. To perform this assay, NSP5 was immunoprecipitated and the complex formed by NSP5, the antibodies, and protein A linked to Sepharose beads, was incubated with [γ-^{32}P]ATP. The labeled proteins were then analyzed by SDS-PAGE. Under these conditions (Fig. 4), NSP5 from rotavirus (RF strain)-infected MA104 cells immunoprecipitated by a monospecific serum or by the monoclonal antibody was labeled in vitro. As for in vivo labeling with ^{33}P (Fig. 3), the highest forms of NSP5 were the most intensively labeled. As we did not detect any kinase activity in the uninfected cell lysate, this result could be due either to an intrinsic kinase activity of NSP5 or to a coimmunoprecipitation of NSP5 with a cellular protein kinase.

NSP5 has protein kinase activity when expressed in *E. coli*. To clarify the origin of the in vitro phosphorylation of NSP5, the protein was first expressed in a prokaryotic system that has different specific protein kinases (7). A high level of expression of NSP5 was obtained upon induction (Fig. 5A), but the high-molecular-weight forms of NSP5 were not observed with the *E. coli*-produced NSP5 (Fig. 5B). When the *E. coli*-expressed NSP5 was used after immunoprecipitation in the in vitro kinase assay, a signal at the molecular weight (26K) of NSP5 was obtained (Fig. 5D). A shorter protein of about 14K and a faint band at 22K that certainly results from a degradation of NSP5 or from an internal initiation were also visible. Shorter products were also recognized by the monoclonal antibody in Western blotting (Fig. 5B). No signal was observed when the experiment was conducted with a lysate of the same strain of *E. coli* but expressing β-galactosidase instead of NSP5. It should be emphasized that the yield of the in vitro phosphorylation of *E. coli* NSP5 was quite low compared to the yield of the in vitro phosphorylation of rotavirus-expressed NSP5. In the latter case, the immunoprecipitated NSP5 did not produce a visible band after Coomassie blue staining of the gel (Fig. 5C) but gave a strong signal in in vitro phosphorylation (Fig. 5D). In contrast, the recombinant NSP5, despite a high efficiency of immunoprecipitation, was poorly labeled. It is possible that NSP5, despite being soluble in *E. coli*, is not correctly folded or that a cellular or rotaviral cofactor is necessary to produce fully phosphorylated and active NSP5 in eukaryotic cells.

Necessity of a cofactor to generate full kinase activity of NSP5. To test the possibility of a misfolding of NSP5 in *E. coli*, we used NSP5 expressed in insect cells from a recombinant baculovirus in the same kinase assay. With this protein (Fig. 6), a heavily labeled band migrating at 26K was observed, clearly

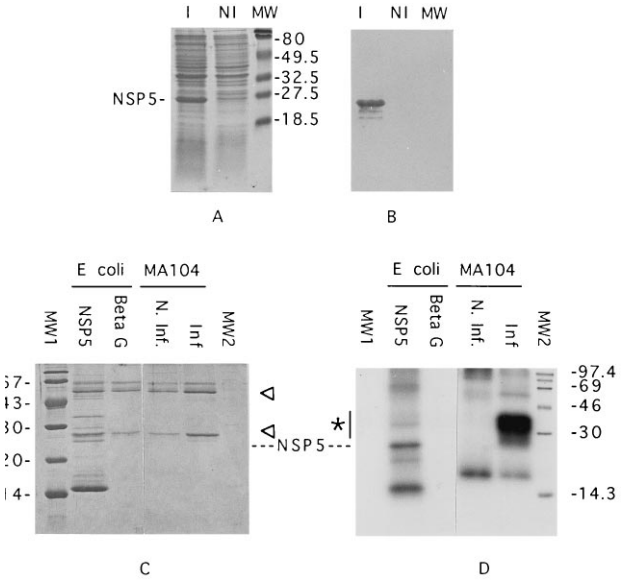

FIG. 5. Expression of NSP5 in *E. coli* and kinase activity. (A and B) Lysates of *E. coli* BL21/DE3 transfected with plasmid pET14B+11 and induced (I) or not induced (NI) by IPTG were electrophoresed on a 15% acrylamide gel and stained with Coomassie blue (A) or transferred to polyvinylidene difluoride membranes and immunodetected by Western blotting with monoclonal antibody 158G37 (B). (C and D) Lysates of *E. coli* transfected with pET14B+11 (NSP5) or pET14B+ expressing β-galactosidase (Beta G) and induced by IPTG were immunoprecipitated with monoclonal antibody 158G37 then subjected to the in vitro kinase assay. The same experiments conducted with lysates of rotavirus-infected (Inf) or not infected (N. Inf.) MA104 cells were used as positive and negative controls. Coomassie blue staining of the gel is shown in panel C (the heavy and light chains of the monoclonal antibody are indicated by open triangles); the autoradiogram of the same gel is shown in panel B. The positions of the 26K forms of NSP5 and of its 26K to 35K in vitro-phosphorylated forms (*) are indicated. Sizes of the molecular weight standards (MW1 and MW2) are indicated in kilodaltons.

indicating the immunoprecipitation of a kinase activity. But side-by-side comparison of this pattern with the pattern obtained with a lysate of rotavirus-infected mammalian cells showed that with the recombinant NSP5, the labeling of the bands between 30K and 35K was very limited and hardly detected. This last point was consistent with the absence of baculovirus-expressed NSP5 having a molecular weight higher than 26K (Fig. 1).

To determine if a complete NSP5 phosphorylation was pos-

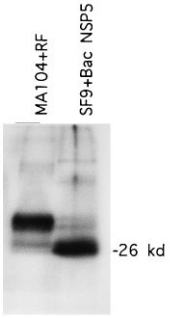

FIG. 6. Comparison of in vitro phosphorylation of NSP5 synthesized in rotavirus-infected MA104 cells and SF9 cells. NSP5 from rotavirus-infected MA104 cells lysate (MA104+RF) or produced by infection of SF9 cells with a recombinant baculovirus (SF9+Bac NSP5) was immunoprecipitated with an anti-NSP5 monoclonal antibody (158G37), incubated with [γ-^{32}P]ATP for 30 min at 37°C, separated by SDS-PAGE, and autoradiographed.

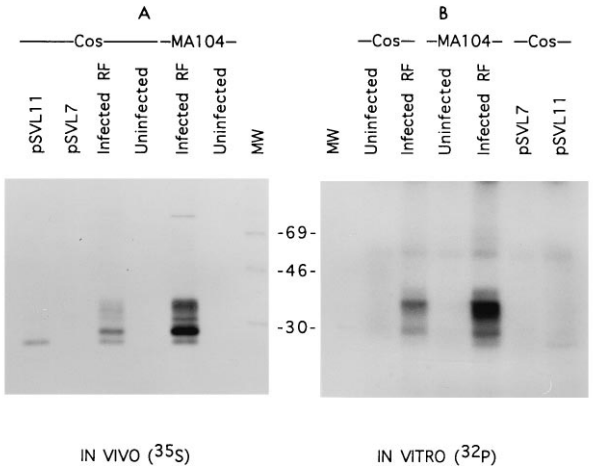

FIG. 7. In vitro phosphorylation of NSP5 synthesized after transfection of gene 11 cDNA in COS7 cells. (A) COS7 cells were either transfected with plasmids allowing the expression of NSP5 (pSVL11) or NSP3 (pSVL7) or infected with the RF strain of rotavirus. At 36 h posttransfection or 1 h postinfection, cells were labeled for 6 h with [^{35}S]methionine and then lysed. NSP5 immunoprecipitated with monoclonal antibody 158G37 was separated by SDS-PAGE (15% gel). As controls, MA104 cells infected with rotavirus or mock infected were treated similarly. (B) Same experiment except that the cells were not labeled and the immune complexes were incubated with [γ-^{32}P]ATP before loading for SDS-PAGE. Sizes of the molecular weight standards (MW) are given in kilodaltons.

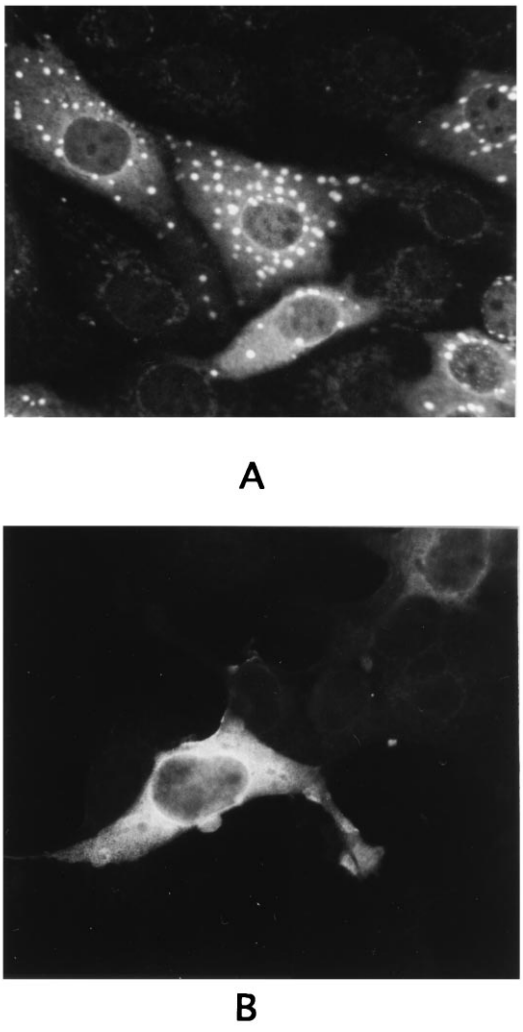

FIG. 8. Localization of NSP5 in cells. Indirect immunofluorescence was conducted with monoclonal antibody 158G37 on MA104 cells infected with the RF strain of rotavirus (6 h postinfection) (A) or on COS7 cells transfected with plasmid pSVL11 (36 h posttransfection) (B).

sible in uninfected mammalian cells, the cDNA of gene 11 was cloned in eukaryotic expression vectors and transfected into COS7 cells. Despite comparable levels of expression of the 26K forms of NSP5 in transfected COS7 cells and rotavirus-infected COS7 cells (Fig. 7), the kinase activity of NSP5 was low and no multiple forms of NSP5 were observed in the transfected COS7 cells. COS7 cells support the replication of rotavirus, and the multiple forms of NSP5 could be observed when COS7 cells were infected by rotavirus (Fig. 7). Furthermore, an in vitro kinase activity similar to the activity associated with NSP5 from MA104 cells could be recovered from rotavirus-infected COS7 cells (Fig. 7).

Altogether, these results showed that despite the existence of an autophosphorylation of NSP5 in different expression systems, none were able to restore the fully phosphorylated state of NSP5, as judged by the presence of high-molecular-weight forms. Thus, the cofactor discussed above should be linked to the infection by a rotavirus.

Cellular localization of NSP5. The inability to recover a fully active NSP5 prompted us to look at the localization of NSP5 in transfected or rotavirus-infected cells. By indirect immunofluorescence, it was shown that 6 h after infection of MA104 cells, NSP5 was clearly localized in viroplasms (Fig. 8A). Despite a viroplasmic distribution of NSP5 in rotavirus-infected COS7 cells (data not shown), NSP5 was widespread in the cytoplasm of COS7 cells transfected with a eukaryotic expression vector allowing the expression of gene 11 (Fig. 8B). Identical results were obtained with a plasmid construct that bore the original coding and 3′ noncoding sequences of gene 11 (data not shown). Thus, NSP5 expressed in COS7 cells by transfection is not able to localize in viroplasms. This result was correlated with the in vitro kinase assay conducted on the same cells: the absence of localization of NSP5 and the absence of the high-molecular-weight forms of NSP5 and of their strong autophosphorylation are linked.

NSP5 forms dimers and interacts with NSP2. NSP5 presents two well-conserved cystein residues in its C-terminal end. We wondered if this protein could multimerize by forming disulfide bonds. When NSP5 was immunoprecipitated under standard conditions and then analyzed under nonreducing conditions (Fig. 9), a smear with an apparent molecular weight between 46 and 69K was observed. That this smear resulted from the dimerization of the different forms of NSP5 was confirmed by performing a western blot assay with the monoclonal antibody on the products of a similar immunoprecipitation (data not shown).

To study the potential RNA-binding properties of NSP5, we used the UV cross-linking assay that we have successfully employed with NSP2 and NSP3 (2, 34). Due to the intrinsic kinase activity of the immunoprecipitated complexes, no differences were observed between cross-linked and non-cross-linked cell lysates (33). However, when ^{35}S-labeled cross-linked and non-cross-linked cell lysates were used for immunoprecipitation of NSP5, we observed a 35K band at the top of the smear formed by NSP5 (Fig. 9). This band was even more visible when NSP5 immunoprecipitated from rotavirus-infected cells was electrophoresed in nonreducing conditions. Since NSP5 is localized in viroplasms together with NSP2, and NSP2 is an RNA-binding

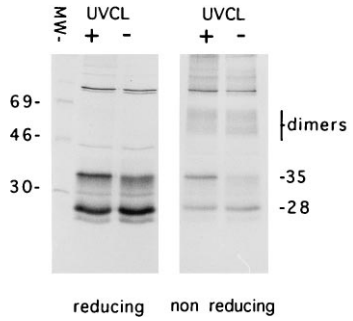

FIG. 9. Interaction of NSP5 with itself and with a 35K viral protein. MA104 cells infected by the RF strain of rotavirus were labeled with [^{35}S]methionine, submitted (+) or not (−) to UV cross-linking (UVCL), immunoprecipitated with monoclonal antibody 158G37, and electrophoresed on a 15% acrylamide gel in reducing (left) or nonreducing (right) conditions. Sizes of the molecular weight standards (MW) are indicated in kilodaltons on the left. Positions of the 28K form of NSP5, of its dimers, and of the 35K (NSP2) protein coimmunoprecipitated are indicated on the right.

protein of 35K (20), we suspected that the 35K protein was indeed NSP2. The interactions of NSP5 and NSP2 with themselves and with each other were tested in the yeast two-hybrid system with the histidine reporter gene. Table 1 shows that in this test, an interaction between NSP5 and NSP2 could be detected with the plasmid pairs NSP2-pGBT9 and NSP5-pGAD424. Note that the well-documented homomultimerization of NSP2 (1, 19) or NSP3 (25) can also be detected in this test. Unfortunately, the dimerization of NSP5 could not be ascertained because NSP5 spontaneously transactivates the reporter genes when it is fused to the DNA binding domain of GAL4.

DISCUSSION

The use of a monoclonal antibody against NSP5 has allowed us to clearly show that NSP5, synthesized in rotavirus-infected cells, is present in several phosphorylated forms having apparent molecular weights ranging from 26K to 35K. Such a high diversity of NSP5 forms has not been described before. In contrast with previous reports (12), we found that the two main forms (26K and 28K) of NSP5 result not from differential glycosylation but from differential phosphorylation. It is possible that dephosphorylation of NSP5 occurred during the alkaline treatment that Gonzalez and Burrone (12) used to remove *N*-acetylglucosamine, since phosphoserine and phosphothreonine are labile in alkali (8). It has also been proposed (13) that cytoplasmic glycosylation might block the sites of phosphorylation and thus serve a regulatory function. It is thus highly probable that NSP5 contains both kinds of posttranslational modifications.

TABLE 1. Interaction of NSP5 with NSP2 tested by double hybrids in yeast

DNA binding domain (pGBT9)	Activating domain (pGAD424)	Growth[a] on selective medium lacking:	
		Leu, Trp	Leu, Trp, His
NSP2	NSP2	+++	++
NSP2	NSP5	+++	+++
NSP2	NSP3	+++	−
NSP5	NSP2	+++	+++
NSP5	NSP3	+++	+++
NSP5		+++	+++
	NSP5	+++	−
P53	NSP5	+++	−
	NSP3	+++	−
NSP3[b]	NSP3	+++	+++

[a] −, no growth; +++, normal; ++, intermediate growth.
[b] Deleted of the first 88 amino acids.

We also showed that NSP5 could be fully phosphorylated in vitro when a lysate of rotavirus-infected cells was immunoprecipitated with anti-NSP5 antibodies and incubated with [γ-^{32}P]ATP. Under such conditions, the highest-molecular-weight forms of NSP5 were the most heavily labeled. This last result was confirmed by metabolic labeling of NSP5 with radioactive phosphate (in rotavirus-infected cells), and it also confirmed the existence of multiple phosphorylated forms of NSP5.

On the one hand, our results suggested that NSP5 has the intrinsic activity of a protein kinase, because (i) the kinase activity was observed with NSP5 expressed in different eukaryotic cells and in *E. coli* and (ii) autophosphorylation is a property frequently encountered in protein kinases (41). However, the products of the in vitro and in vivo phosphorylations were different. Thus, there is a correlation between the in vitro kinase activity and the observation of high-molecular-weight forms of NSP5 in the cells. On the other hand, our results cannot be entirely explained by the coimmunoprecipitation of a cellular protein kinase because (i) vaccinia virus infections were conducted in the same mammalian cell line as rotavirus infections and (ii) transfection experiments were also conducted in rotavirus-susceptible cells. Thus, it can be excluded that the absence of a cellular protein kinase was responsible for the incomplete phosphorylation of the recombinant NSP5 in mammalian cells. Moreover, NSP5 (and other rotavirus proteins) does not possess the canonical motifs borne by most protein kinases (45). The homology noted before (46) occurs with guanido kinase in a region not conserved in this family of proteins (44). Furthermore, the nucleoside triphosphate binding site present on rotavirus group A NSP5 is not conserved in the NSP5 from group C rotavirus despite an overall similarity of 20% (22). It should be noted that cellular (26, 39, 40) and viral (47) serine-threonine protein kinases that do not possess the canonical motifs described have been described. Whether NSP5 can be definitely added to this short list requires further experiments.

It seems that the full phosphorylation of NSP5 which lead to multiple forms of the protein occurs in two steps. A first step of phosphorylation occurs after the synthesis of the 26K protein and is the result of the intrinsic kinase activity of NSP5. This first step can be accomplished by recombinant NSP5. The second step of phosphorylation is observed only when NSP5 is expressed together with all the other viral proteins and possibly after an alteration of the cell physiology induced by rotavirus infection. This second step in phosphorylation is thus the landmark of rotavirus infection and leads to the formation of the more heavily and multiple phosphorylated forms of NSP5. Exactly how this second step is accomplished and how it is specifically induced by rotavirus infection remains to be established. The observation that NSP5 was differently localized in rotavirus-infected cells and in cells expressing NSP5 after transfection of its cDNA could suggest a mechanism. The intracellular localization of NSP5 could require specific processes to deliver the protein to the appropriate site, where it might then be anchored by interactions with its regulatory subunit or with a specific protein kinase. The localization and regulation of the activity of many serine-threonine kinases is the result of such interactions with anchor proteins (4, 9, 29);

thus for NSP5, correct localization and specific phosphorylation could be closely related.

Different mechanisms which are responsible for the correct intracellular localization of proteins exist. The possibility that any kind of signal peptide is present on NSP5 can be ruled out since NSP5 is not correctly localized after the transfection of its encoding gene. NSP5 is O-glycosylated by the addition of monomeric *N*-acetylglucosamine (12). To our knowledge, there is no evidence of this posttranslational modification being linked to intracellular localization. It cannot be excluded that an additional (undetected) posttranslational modification of NSP5 is responsible of its targeting.

A situation similar to that observed here for NSP5 has been reported for NSP2. In the course of rotavirus infection, NSP2 is localized in the viroplasms (32) and, together with structural proteins, is a component of the replicase complex. But NSP2 fails to localize when expressed by vaccinia virus-T7 transfection (19) or by a thermosensitive rotavirus mutant grown at a nonpermissive temperature (24). In most rotavirus proteins, however, the reverse situation has been observed. The expression of VP7 and NSP4 led to the correct localization and posttranslational modifications of the proteins (28, 42). Despite divergent reports on the localization of NSP1 (15, 16), the use of the vaccinia virus-T7 transfection expression system also led to the correct localization of NSP1 (15). NSP3 also seems correctly localized when expressed permanently or transiently in cell lines by using expression plasmids (33). It remains to be established whether structural proteins such as VP6 and VP2 are able by themselves, or in the form of a pseudoparticle, to reach the viroplasms. This will help us determine whether the viroplasmic proteins are localized independently of each other or if they are localized together in the form of a multiprotein complex, composed of structural and nonstructural proteins. It is interesting that in cells infected with a thermosensitive mutant of NSP2, at a nonpermissive temperature VP6 is correctly localized but NSP2 is not (24).

A well-documented mechanism of protein localization involves the preliminary localization of their mRNAs (40) generally by interaction of the 3′ noncoding part of the mRNAs with cellular RNA-binding proteins. In the case of NSP5, its localization in the viroplasm could first require the transport of its encoding mRNAs to the viroplasm where the mRNAs are translated. We have tested this hypothesis by transfecting gene 11 cDNA containing a partial or complete 3′ noncoding sequence. With both constructs, no differences were observed in the localization of NSP5 (33). But the mRNAs synthesized by the expression vectors that we used are polyadenylated, in contrast to the natural rotavirus mRNAs: the 3′ end of each mRNA terminate with the UGACC consensus sequence bound by NSP3 (37). Thus, it is possible that NSP5 failed to localize because of the absence of NSP3 and of a correct 3′ end on NSP5-encoding mRNAs.

The interaction of NSP5 with NSP2 that we observed here is of great importance. Our results raised the question of why we observed this interaction when the cells where treated by UV light and not in normal conditions. Because UV cross-linking between proteins is very inefficient, it can be excluded that we are dealing here with a protein multimer. That NSP5 migrated to 35K when it is cross-linked with an RNA is also very unlikely because we could not find any labeled RNA when we used the 3′- or 5′-end labeling protocols that we previously used with NSP2 and NSP3 (33). A possible explanation is that the interaction between NSP5 and NSP2 is reinforced when the latter is bound to RNA. By cross-linking NSP2 on RNA by UV irradiation, the NSP2-NSP5 complex could withstand the conditions of immunoprecipitation that otherwise dissociate it. It should be emphasized that the immunoprecipitation conditions that we used here are quite harsh and that we could coimmunoprecipitate NSP2 with NSP5 (with other viral or cellular proteins) when the immunoprecipitations were conducted in the absence of SDS (data not shown). The binding of NSP2 to RNA being unspecific (20) and occurring in insects cells (2), it is likely that it also occurs in yeast and thus allows us to detect NSP2 and NSP5 interactions by the two-hybrid test.

The presence of NSP5 in intermediate replication particles or in cell fractions enriched in replicase activity (in the form of VP9 [11] or NS29 [31]) is not clear. We showed previously (2) that in infected cells, NSP2 is associated with replicating viral mRNAs as part of a multiprotein complex which possesses a replicase activity. Due to its interaction with NSP2, it is reasonable to think that NSP5 is also a component of this complex despite the absence of a replicase activity when immunoprecipitation was performed with the anti-NSP5 monoclonal antibody (2). The role of NSP5 in this complex remains a matter of speculation, and numerous phosphoproteins have been implicated in replication of RNA viruses (see, for example, references 5, 17, 18, and 23). Our finding that NSP5 consists of a wide range of isoforms created by differential phosphorylation and dimerization introduces a level of heterogeneity that could be used to distinguish the different viral mRNAs. The diversity of NSP5 forms could make it possible to control the packaging of the right set of genes in the viral particle.

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