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In Vitro Phosphorylation and Purification of a Nonstructural Protein of Bluetongue Virus with Affinity for Single-Stranded RNA

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A phosphorylated, nonstructural protein of bluetongue virus, NS2, is synthesized throughout the replication cycle in comparatively large amounts. The protein was detected in both the soluble and particulate fraction of the cytoplasm of infected cells. The particulate NS2 could be solubilized in 0.5 M NaCl. It was found that NS2 in the particulate fraction and immunoprecipitates of NS2 from the soluble protein fraction could be phosphorylated in vitro. It is not known whether the kinase involved is of cellular or viral origin, but after purification of NS2 by affinity chromatography on poly(U)-Sepharose it could still by phosphorylated in vitro without the addition of exogenous protein kinase. The affinity of NS2 for nucleic acid was also investigated. The protein was found to bind to single-stranded RNA. In the presence of purified bluetongue virus mRNA, NS2 formed a complex with an estimated S value of about 22S.

Bluetongue virus (BTV) is the prototype species of the genus *Orbivirus* and a member of the family *Reoviridae* (20). The genome consists of 10 segments of double-stranded RNA (dsRNA), each of which is transcribed into mRNA by a core-associated dsRNA-dependent RNA polymerase (12, 19). At least 10 translation products of the BTV mRNA species have been identified (14; A. A. van Dijk and H. Huismans, submitted for publication). These include the seven structural proteins, P1 to P7, and three distinct nonstructural proteins, NS1, NS2, and NS3. Two NS3 peptides, NS3 and NS3A, with molecular weights of 28,000 and 25,000, respectively, can be distinguished. They are both coded for by segment 10 and have almost identical peptide maps. Furthermore, a fourth possible nonstructural protein, designated NS4, has been identified. This protein has a molecular weight of about 16,000, and indications are that it is coded for by genome segment 9, which also codes for structural protein P6 (van Dijk and Huismans, submitted).

We are interested in relationship between structure and function of the different BTV-specific nonstructural proteins. Very little is known about these proteins. The morphologically best-characterized protein is NS1, which is synthesized in very large amounts and accumulates in BTV-infected cells as tubular structures of various lengths and a side-to-side diameter of 68 nm (9). Nothing is known about the function of NS1, but it has been shown that it is coded for by one of the most highly conserved of the BTV genome segments (8a).

The other major nonstructural protein, protein NS2, is also synthesized in large amounts. Preliminary investigations have indicated a resemblance to σNS of reovirus in that NS2 has an affinity for single-stranded RNA (ssRNA) (8). Such ssRNA-binding nonstructural proteins with a molecular weight of about 40,000 appear to be common to all members of the *Reoviridae* family and have also been demonstrated in the case of rotavirus (2).

In the case of reovirus, σNS binds to reovirus mRNA (10) to form a 19S complex (5). The protein appears to play an early role in reovirus replication (13), and it is possible that it acts in the selection and condensation of the 10 mRNA species. The main distinction between BTV NS2 and the nonstructural protein equivalents in the other members of the *Reoviridae* family is that NS2 appears to be phosphorylated (8).

These preliminary results prompted a more detailed investigation of the phosphorylation of NS2 and the ability of NS2 to bind to ssRNA. We have purified NS2 by affinity chromatography and were able to show that purified NS2 can be phosphorylated in vitro without the addition of an exogenous protein kinase. The isolation of a 22S BTV mRNA-NS2 complex is also described.

MATERIALS AND METHODS

Cells and virus. The BHK-21 cells used in the investigation were grown as monolayers in Roux flasks, by using modified Eagle medium supplemented with 5% bovine serum. An attenuated strain of BTV serotype 10 (designated 10A) was used throughout the study. It was propagated in the BHK cells by using a plaque-purified, low-passage stock virus suspension (7).

Preparation of ^{35}S- and ^{32}P-labeled S100 and P100 fractions from BTV-infected cells. Confluent monolayers of BHK-21 cells in Roux flasks were infected with BTV at a multiplicity of infection of 15 to 30 PFU per cell and incubated at 31°C until the start of the labeling period. After a rinse with methionine-free Eagle medium, the cells were incubated on a rocker platform for 2 h at 37°C in 5 ml of the same medium containing 15 μCi of [^{35}S]methionine per ml. ^{32}P labeling was done the same way, except that the cells were rinsed with phosphate-free Eagle medium and then labeled by using 10 ml of phosphate-free Eagle medium containing 10 μCi of $^{32}P_i$ per ml. The cells were harvested, concentrated by low-speed centrifugation, and then suspended at 5×10^7 cells per ml in 0.01 STE-TX buffer (0.01 M NaCl, 10 mM Tris, 1 mM EDTA [pH 7.4], 0.5% Triton X-100). Nuclei were removed by centrifugation at 1,500 × *g* for 5 min and washed once with half the original volume of 0.01 STE-TX. The supernatants were combined (S10 fraction) and centrifuged for 2 h at 45,000 rpm in an SW50.1 rotor through a 2-ml layer of 40%

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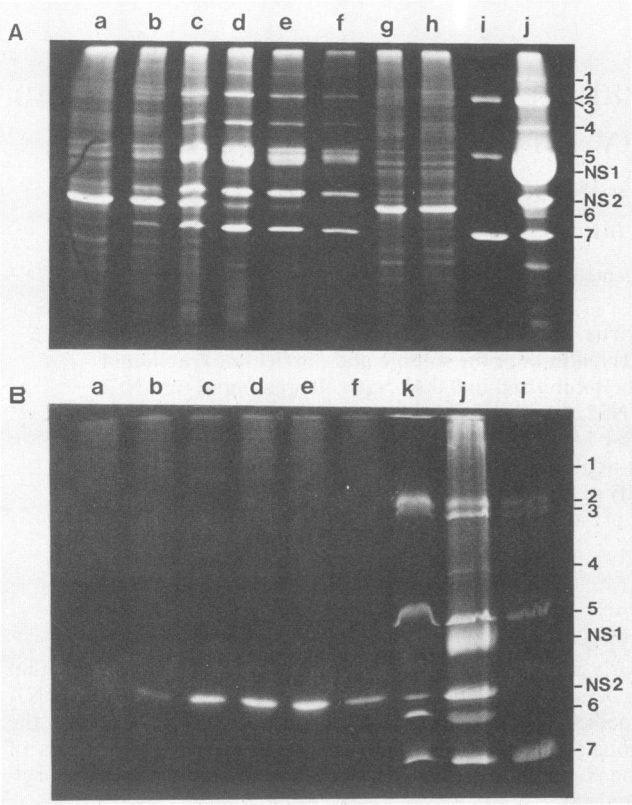

FIG. 1. Autoradiogram of electrophoretically separated proteins in BTV-infected BHK cells labeled with either [^{35}S]methionine or $^{32}P_i$ at different intervals after infection. Monolayer cultures in Roux flasks were infected with 30 PFU per cell and incubated at 31°C. At the following intervals p.i., the cells were labeled with ^{32}P or ^{35}S precursors as indicated under Materials and Methods: 2 to 4 h (lane a); 7 to 9 h (lane b); 11 to 13 h (lane c); 15 to 17 h (lane d); 20 to 22 h (lane e); and 26 to 28 h (lane f). Control cells were labeled at 2 to 4 h (lane g) and 20 to 22 h (lane h) after mock infection. The cells were harvested after the labeling period. The cytoplasmic extracts of the [^{35}S]methionine-labeled cells were analyzed by discontinuous SDS-PAGE (A). The results in panel B show immunoprecipitates of the S100 protein extracts from $^{32}P_i$-labeled cells after immunoprecipitation of 200-μl portions of the different S100 fractions with rabbit serum that contained antibodies against the structural and nonstructural BTV proteins (7). An immunoprecipitation control with ^{35}S-labeled soluble proteins is shown in lane k. Other controls are ^{35}S-labeled BTV proteins in purified virus (lane i) and in the particulate fraction (lane j). 1 to 7, P1 to P7.

sucrose. The supernatant (S100) was divided in small portions and kept at −20°C. The pellet (P100) was suspended in 0.01 STE (STE-TX without Triton X-100) (using 20% of the original S10 volume) and stored at −20°C.

Preparation of SW fractions. For large-scale preparation, a P100 fraction was prepared from 10^9 BTV-infected BHK cells as described above and suspended in 4.5 ml of 0.01 STE. NaCl was added to a final concentration of 0.5 M, and the suspension was centrifuged for 1 h at 100,000 × *g*. The supernatant was divided in smaller portions and frozen at −70°C. For the large-scale purification of NS2, unlabeled salt-wash (SW) fractions were mixed with a suitable amount of a ^{35}S-labeled SW fraction prepared from BTV-infected cells. SW fractions from uninfected cells were prepared the same way as those from infected cells.

Preparation of ^{3}H-labeled ssRNA and dsRNA. ^{3}H-labeled BTV mRNA was prepared by in vitro transcription of CsCl-purified BTV core particles in the presence of [^{3}H]UTP (18). The ^{3}H-labeled BTV dsRNA was obtained by phenol-sodium dodecyl sulfate (SDS) extraction of purified virus (12) that was labeled during the infection cycle with [^{3}H]uridine. ^{3}H-labeled cellular ssRNA was obtained by extraction of uninfected cells labeled during the cellular growth cycle with [^{3}H]uridine. Both RNA preparations were purified by two cycles of precipitation with 2 M LiCl. The precipitates were dissolved in water and stored in small portions at −70°C.

Nitrocellulose-binding assay. A nitrocellulose-binding assay was done as follows. Five-microgram quantities of each of the different ^{3}H-labeled nucleic acids were mixed with increasing amounts of P100 SW fraction from infected or uninfected cells. The mixture was then diluted to a final salt concentration of 0.2 M NaCl in 10 mM Tris hydrochloride, pH 7.4. After 30 min at 4°C, it was further diluted to 1.5 ml with 0.1 STE and filtered through 0.45-μm-pore-size nitrocellulose filter disks (Millipore Corp., Bedford, Mass.). The filters were washed at least four times with 5 ml of 0.1 STE, dried, and counted.

Poly(U)-Sepharose affinity chromatography. A 4-cm poly(U)-Sepharose 4B column in a Pasteur pipette was equilibrated in 0.01 STE buffer containing 10 mM 2-mercaptoethanol. Before being loaded onto the column the salt concentrations of the S100 or SW fractions were adjusted by dilution with 0.01 STE to 0.2 M NaCl. Samples were loaded on the column at a rate of 0.1 ml/min and washed with equilibration buffer until, as measured by optical density and radioactivity, the eluate was reduced to background levels. A linear gradient of 0.15 to 1.15 STE with 10 mM 2-mercaptoethanol was then applied to the column. Peak fractions were pooled as indicated and analyzed by SDS-15% polyacrylamide gel electrophoresis (PAGE) by using the Laemmli buffer system (11). Proteins were concentrated by dialysis and lyophilization or alternatively by potassium chloride-SDS precipitation (11).

In vitro phosphorylation assay. In vitro kinase activity was assayed by using MTD buffer (0.01 M Tris hydrochloride [pH 8.0], 0.01 M $MgCl_2$, 0.01 M dithiothreitol) containing 0.2 μCi of [γ-^{32}P]ATP (1 mCi/ml; 5,200 Ci/mol) as described by Moyer and Summers (15). The final assay mixture was obtained by mixing equal amounts of sample and 2× concentrated MTD buffer. The mixtures were incubated at 37°C for 30 min. ^{32}P incorporation was detected by SDS-PAGE and autoradiography. Phosphoproteins that were labeled with both ^{35}S and ^{32}P were characterized by a method which makes it possible to distinguish by autoradiography between ^{32}P- and ^{35}S-labeled components by using a 0.015-mm-thick sheet of aluminum foil to deflect ^{35}S radiation (4).

RESULTS

Identification of NS2 in infected cells. The synthesis of virus-specific proteins in BTV-infected cells from 2 to 28 h postinfection (p.i.) is shown in Fig. 1A. On discontinuous SDS-polyacrylamide gels in a Tris-glycine buffer system, protein NS2 of BTV migrates in a position between proteins NS1 and P6 of BTV. This is different from the relative electrophoretic mobility of NS2 (formerly called P6A) on phosphate-buffered polyacrylamide gels in the presence of urea. On these gels, NS2 has a higher electrophoretic mobility than P6 and migrates in a position that coincides with that of a major labeled cellular protein (7). This has previously severely handicapped accurate assessment of the time when NS2 is synthesized.

The ^{35}S-methionine pulse-labeling experiment (Fig. 1A) illustrates that at a temperature of 31°C the majority of virus-specified proteins, including NS2, are synthesized between 11 and 22 h p.i. Synthesis of cellular proteins declines very rapidly after 13 h p.i., and from 15 h p.i. almost all protein synthesis is virus specified.

A similar pulse-labeling experiment using $^{32}P_i$ as labeled precursor was also done. Proteins in the soluble (S100) fraction of the cytoplasm were analyzed by immunoprecipitation with a BTV-specific immune serum. The results are shown in Fig. 1B. Protein NS2 is the only ^{32}P-labeled virus protein. The intensity of the labeling at the different intervals after infection appears to coincide with that of the ^{35}S-labeled NS2, indicating that NS2 is phosphorylated throughout the infection cycle.

Partial purification of NS2. ^{32}P-labeled NS2 is also present in the particulate fraction of infected cells (Fig. 2, lane a). This fraction, however, also contains a large number of ^{32}P-labeled cellular proteins as well as ^{32}P-labeled dsRNA. The corresponding ^{35}S-labeled proteins are shown in lane c (Fig. 2). The particulate NS2 was solubilized by treatment with 0.5 M NaCl. This resulted in a partial purification of NS2 after removal of the particulate ^{35}S-labeled viral proteins (Fig. 2, lane e). The majority of the small ^{32}P-labeled cellular proteins and dsRNA was also removed (Fig. 2, lane b), but a number of ^{32}P-labeled contaminants were still present. Partially purified NS2-enriched protein extracts obtained by a high-salt wash of the particulate fraction are referred to as SW fractions.

Binding of components in SW fraction to RNA. SW fractions were prepared from BTV-infected and uninfected cells and then analyzed for their ability to bind BTV mRNA, BTV dsRNA, and cellular ssRNA, respectively (Fig. 3). It is evident that a component in the SW fraction of BTV-infected cells binds both to the cellular ssRNA and to BTV mRNA. In the presence of an excess of SW fraction, most of the BTV mRNA and cellular RNA was bound to the nitrocellulose filters.

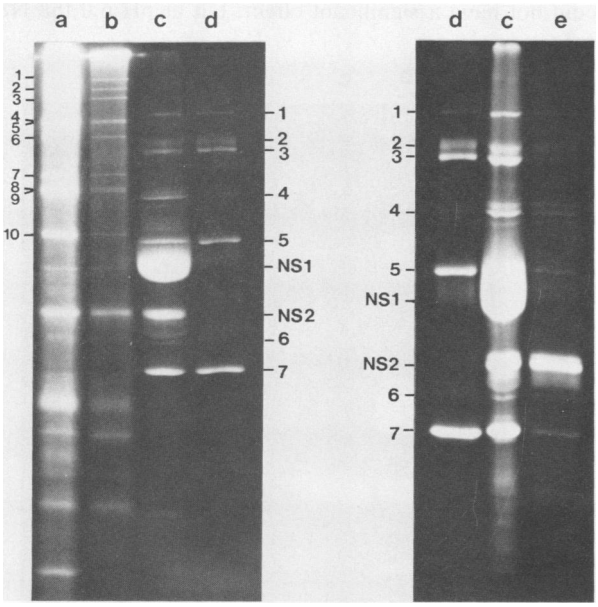

FIG. 2. Autoradiogram of electrophoretically separated BTV proteins solubilized by treatment of the particulate fraction of the cytoplasm of infected cells with 0.5 M NaCl. Cells were labeled with either [^{35}S]methionine or $^{32}P_i$ at 15 to 17 h p.i. as indicated in the legend to Fig. 1. The ^{32}P- and ^{35}S-labeled proteins in the particulate fraction are shown in lane a and both c lanes, respectively. Supernatants of the ^{32}P- and ^{35}S-labeled proteins after high-salt treatment and centrifugation at 100,000 × *g* are shown in lanes b and e, respectively. Purified ^{35}S-labeled BTV proteins are shown in lanes d. The positions of ^{32}P-labeled BTV dsRNA genome segments in lanes a and b are indicated at the left of lane a. 1 to 7, P1 to P7.

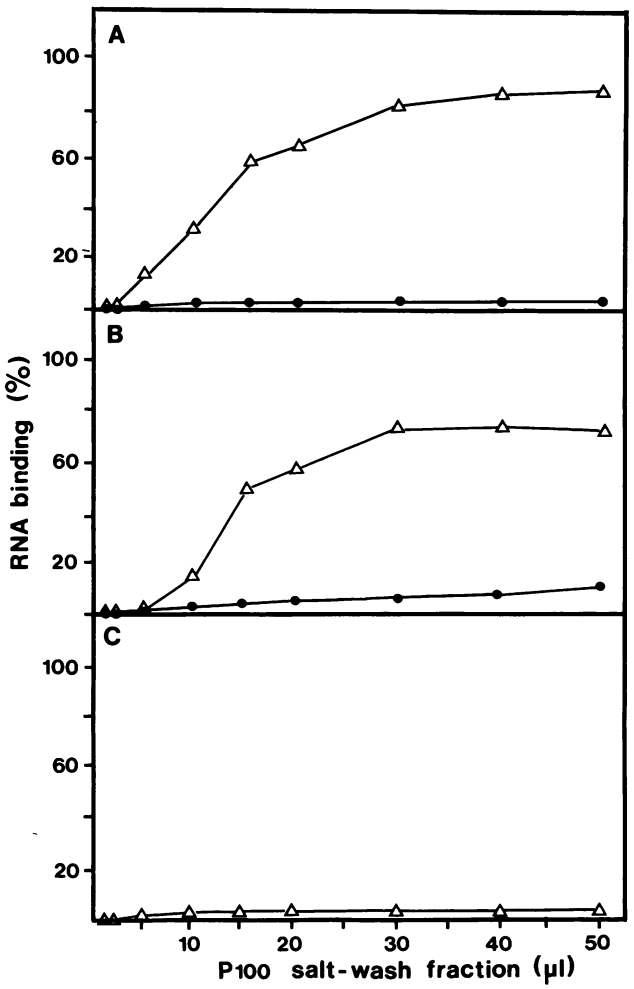

FIG. 3. Nitrocellulose binding assay of ^{3}H-labeled nucleic acid to SW fractions from BTV-infected and uninfected cells. The preparation of [^{3}H]RNA and the binding assay were done as described in Materials and Methods. Quantities (5 μg) of BTV mRNA (A), cellular ssRNA (B), and BTV dsRNA (C) were mixed with different amounts of P100 SW fractions either from BTV-infected cells (△) or from uninfected cells (●), filtered through nitrocellulose filter disks, and the percent RNA bound was calculated from the ^{3}H counts bound to the filter relative to the total counts in each 5-μg sample of RNA.

To identify the binding component, fixed amounts of ^{35}S-labeled SW fractions from BTV-infected cells were mixed with different quantities of ^{3}H-labeled BTV mRNA and analyzed by zonal centrifugation in sucrose gradients under the conditions described in the legend to Fig. 4.

The two amounts of ^{3}H-labeled BTV mRNA used in the experiment were chosen to represent conditions under which the mRNA was either in excess or limiting. The sedimentation profiles indicate that in the absence of RNA all the ^{35}S-labeled proteins sedimented at the top of the gradient. After the addition of a small amount of RNA, a

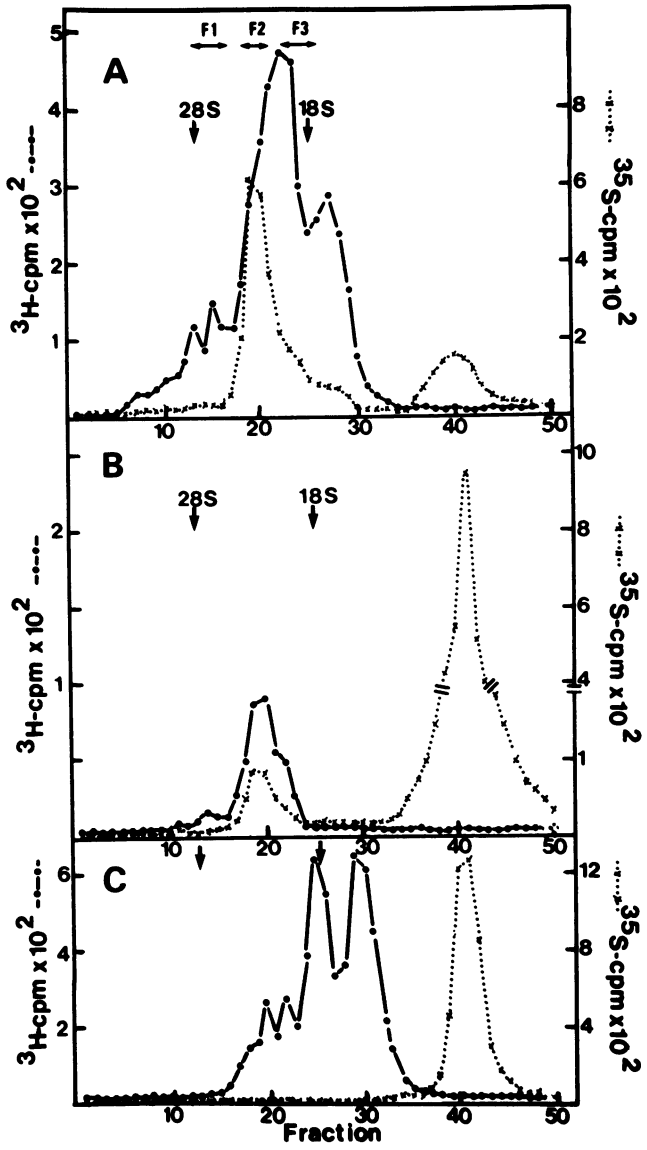

FIG. 4. Zonal centrifugation in a sucrose gradient of ^{3}H-labeled BTV mRNA bound to ^{35}S-labeled proteins in the SW fraction of BTV-infected cells. The ^{35}S-labeled SW fraction and [^{3}H]mRNA were prepared as described in Materials and Methods. Portions (0.1 ml) of the SW fraction were combined with 24 μg (A) and 3 μg (B) of ^{3}H-labeled BTV mRNA. Two control gradients were run, one of the ^{35}S-labeled 0.1-ml SW fraction and another of ^{3}H-labeled 24-μg BTV mRNA. The radioactivity profiles of these gradients were superimposed and depicted in panel C. The final volumes were adjusted to 0.3 ml with 0.1 STE. After 30 min at 4°C the reaction mixtures were centrifuged for 4.5 h at 50,000 rpm in a SW50.1 rotor through a linear sucrose gradient (4 to 40%) in 0.15 STE. Fractions were collected from the bottom, and a 15-μl sample of each fraction was counted. The top of the gradients are to the right. The sucrose gradient fractions in Fig. 4A were pooled as indicated.

small proportion of the ^{35}S-labeled proteins sedimented at a position of about 22S. In the presence of an RNA excess, more of the protein was complexed but the S value remained the same.

Analysis of the proteins in the 22S complex is shown in Fig. 5. The only ^{35}S-labeled protein that was consistently present in the 22S complex is NS2. Trace amounts of polypeptide P1 were, however, also observed in a few cases.

Purification of NS2 by affinity chromatography. The affinity of NS2 for ssRNA suggested the possibility of purifying NS2 by affinity chromatography. Both poly(U)- and poly(A)-Sepharose columns were used. ^{35}S-labeled SW fractions of BTV-infected cells were loaded onto these columns, and after extensive washing the proteins that bound to the column were eluted with a salt gradient as described in Materials and Methods. In the case of the poly(U) column, a peak of ^{35}S-labeled proteins eluted at a salt concentration of 0.52 M NaCl. The proteins in the ascending, top, and descending peak fractions were analyzed by SDS-PAGE. The Serva Blue-stained proteins are shown in Fig. 6A, and the autoradiogram of the same gel is shown in Fig. 6B.

The autoradiogram indicates that the top fraction of the peak (Fig. 6B, lane g) contained NS2 as the only labeled component. Trace amounts of unlabeled high-molecular-weight proteins were sometimes detected in the same fraction on the Serva blue-stained gel. The fraction of the ascending part of the peak (Fig. 6B, lanes e and f) contained a very small amount of labeled P1. In agreement with the result shown in Fig. 5, this could indicate that P1 also has some affinity for ssRNA.

The results obtained with the poly(A) column were similar, except that NS2 appeared to have a weaker affinity for poly(A) than for poly(U). On poly(A) columns, NS2 eluted at a salt concentration of 0.38 M (result not shown), and in contrast to the result with poly(U)-Sepharose (Fig. 6, lane d), the unbound fraction of poly(A) columns still contained a significant amount of unbound NS2.

The effect of an increase or decrease in the pH of the elution buffer was also investigated. An increase in the pH to 8.3 did not have a significant effect, but at pH 6.0 the NS2

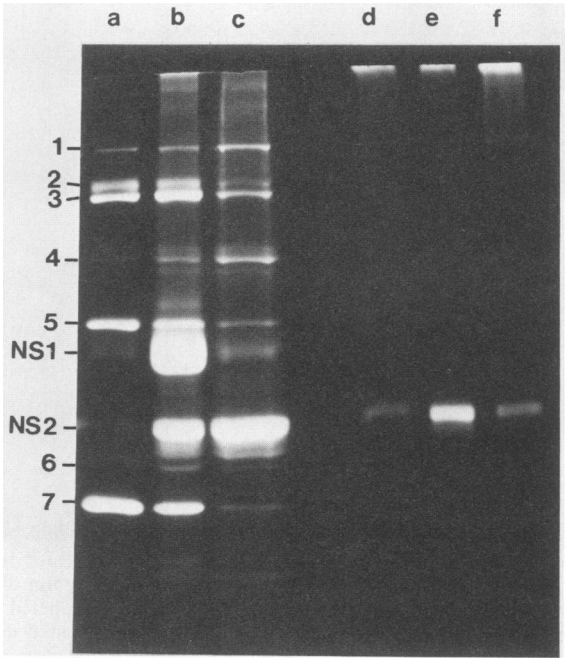

FIG. 5. Autoradiogram of the electrophoretically separated ^{35}S-labeled BTV proteins in pools F1 (lane d), F2 (lane e), and F3 (lane f) of the sucrose gradient shown in Fig. 4A. The controls are ^{35}S-labeled proteins from purified BTV (lane a), a particulate fraction from BTV-infected cells (lane b), and the SW fraction used in the experiment shown in Fig. 4 (lane c). 1 to 7, P1 to P7.

was contaminated with a large number of low-molecular-weight polypeptides. Therefore, a low pH for the elution buffer is not recommended.

The highest yields of NS2 from affinity column purifications were obtained from SW fractions. However, the method was found to be equally applicable to purifying NS2 from soluble fractions of BTV-infected cells as well as to NS2 synthesized in vitro in a cell-free protein synthesis system (results not shown). The method has also been used to purify ^{32}P-labeled NS2.

In vitro phosphorylation of NS2. To characterize the kinase associated with the phosphorylation of NS2, it was investigated whether NS2 could be phosphorylated in vitro as has been described for the vesicular stomatitis virus phosphoprotein NS (1). ^{35}S-labeled particulate (P100) and soluble (S100) protein extracts were prepared from the cytoplasm of uninfected and BTV-infected BHK cells. The soluble proteins in the S100 extracts were concentrated by immunoprecipitation with a BTV-specific serum. The different P100 fractions and immunoprecipitates of the S100 fractions were incubated in a phosphorylation mixture containing [γ-^{32}P]ATP and then analyzed as indicated in the legend to Fig. 7. The result is shown in Fig. 7.

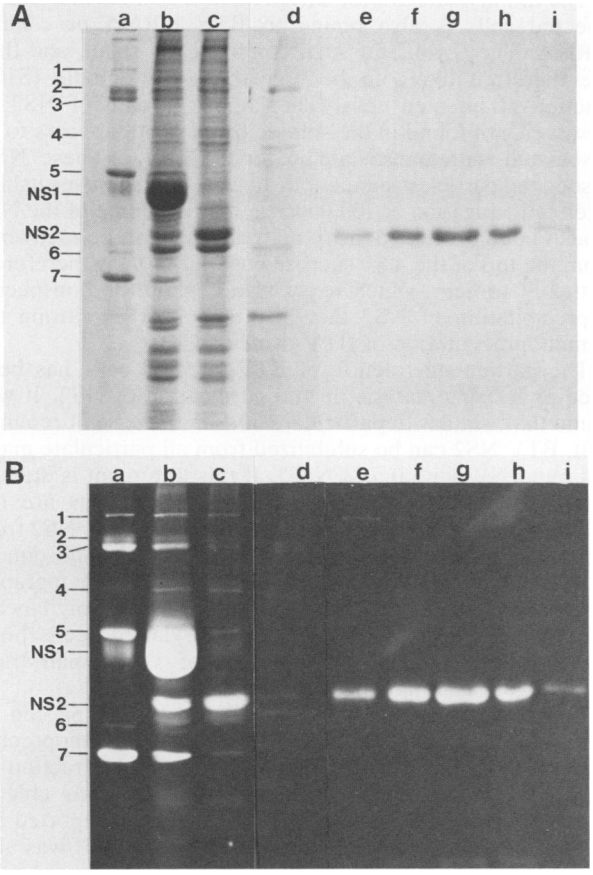

FIG. 6. Electrophoretic separation of the proteins eluted from a poly(U)-Sepharose column during the purification of NS2 from the SW fraction of BTV-infected cells. The ^{35}S-labeled SW fraction was prepared, loaded onto the column, and eluted with a linear 0.15 to 1.15 STE gradient as described. A peak of labeled proteins eluted between fractions 66 and 75 of the gradient at a salt concentration of 0.52 M NaCl. Proteins in the ascending part of the peak (fractions 66 and 69) are analyzed in lanes e and f, the top fraction (fraction 71) is analyzed in lane g, and the descending part of the peak (fractions 73 and 75) is analyzed in lanes h and i. The Serva blue-stained gel is shown in panel A, and the autoradiogram from the same gel is shown in panel B. A fraction from the material that did not bind to the column is shown in lane d. Other lanes: The controls are ^{35}S-labeled proteins from purified BTV (lane a), a P100 fraction from BTV-infected cells (lane b), and the SW fraction loaded onto the column (lane c). 1 to 7, P1 to P7.

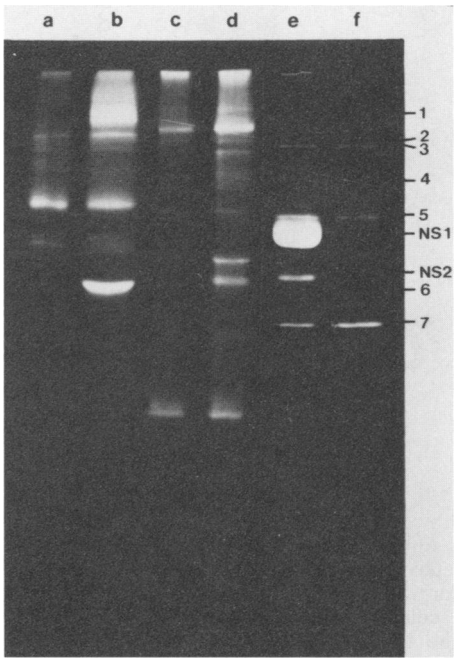

FIG. 7. Autoradiogram showing in vitro phosphorylation of in vivo-synthesized BTV NS2 proteins. Unlabeled S100 and P100 fractions were prepared from the cytoplasm of BTV-infected and mock-infected cells as described in Materials and Methods. Portions of the S100 fractions, each representing approximately 10^6 cells, were immunoprecipitated with a BTV-specific serum as described in the legend to Fig. 1. These immunoprecipitates, as well as equivalent amounts of the P100 fractions, were suspended in 30 μl of 2 mM Tris, pH 8.0, and an equal volume of 2× MTD buffer containing [γ-^{32}P]ATP was added. After 30 min of incubation, the samples were analyzed by means of SDS-PAGE and autoradiography. The results are shown as follows: immunoprecipitated S100 fraction of mock-infected cells (lane a); immunoprecipitated S100 fraction of BTV-infected cells (lane b); uninfected P100 fraction (lane c); and BTV-infected P100 fraction (lane d). Controls are ^{35}S labeled: P100 sample of BTV-infected cells (lane e) and purified BTV (lane f). 1 to 7, P1 to P7.

The results indicate that a protein migrating in the position of NS2 was phosphorylated in both the S100 immunoprecipitate and the P100 fraction of BTV-infected cells (lanes b and d). It is also evident from Fig. 7 that several nonviral proteins were phosphorylated. Since these proteins were observed in both the infected and the uninfected controls, the presence of a cellular protein kinase in the cellular extracts is implicated.

To obtain more information about the kinase involved, NS2 was purified from a ^{35}S-labeled S100 fraction of BTV-infected cells by affinity chromatography as described in the legend to Fig. 6. The ^{35}S-labeled NS2 was concentrated from the high-salt column wash fractions by immunoprecipitation. The immunoprecipitate was assayed for in vitro kinase activity as indicated in the legend to Fig. 8.

Conditions for autoradiography were selected to discriminate between ^{35}S- and ^{32}P-labeled proteins. It is evident

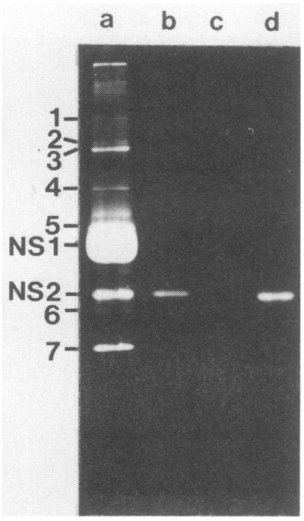

FIG. 8. Autoradiogram showing in vitro kinase activity in a poly(U)-Sepharose-purified NS2 preparation. ^{35}S-labeled NS2 was purified from the S100 fraction of BTV-infected cells by poly(U)-Sepharose column chromatography as described in the legend to Fig. 6. The viral proteins present in the NS2-containing peak fractions that eluted at 0.5 M NaCl were concentrated by immunoprecipitation and assayed for kinase activity as described in the legend to Fig. 7. Lanes c and d were subjected to autoradiography that distinguishes between ^{35}S- and ^{32}P-labeled components (4), whereas lanes a and b were subjected to normal autoradiography. Lanes: a, ^{35}S-labeled P100 fraction of BTV-infected cells; b, ^{35}S-labeled proteins in high-salt eluate from poly(U)-Sepharose column; c, phosphorylation of purified NS2 fraction at time zero, indicating that no ^{35}S label is detected under these conditions; d, phosphorylation of NS2 after 30 min of incubation. 1 to 7, P1 to P7.

from lane b that NS2 was the only ^{35}S-labeled protein recovered from the column. After incubation for 30 min in the phosphorylation mixture, NS2 was found to be labeled with ^{32}P. The column-purified NS2 preparation therefore still contained kinase activity. This kinase activity was therefore either associated with NS2 itself or was caused by the presence of a kinase that copurified with NS2.

DISCUSSION

We have identified a nonstructural protein of BTV, protein NS2, with affinity for ssRNA. The protein is synthesized in comparatively large amounts throughout the infection cycle. Synthesis can first be detected as early as 2 to 4 h p.i., with the largest amounts being synthesized between 16 and 20 h p.i. The protein is characterized by anomalous electrophoretic migration. On phosphate-urea gels it has a higher electrophoretic mobility than P6 (7), whereas on Tris-glycine gels it migrates slower than P6. It has also been observed that on the latter gels NS2 very often separates into two very closely spaced bands. This is suggestive of posttranslational modifications such as phosphorylation or glycosylation. There is no evidence for the glycosylation of NS2, but it has been found that NS2 is the only virus-specified protein that is phosphorylated. The presence of phosphorylated proteins has been demonstrated in many viruses, and in a number of cases the degree of phosphorylation seems to determine the extent of binding to viral nucleic acid (3, 16).

In the case of BTV, it was found that a component in the SW fraction of BTV-infected cells that contained NS2 as the major viral protein could bind to ssRNA but not to dsRNA. A complex with an estimated S value of 22S containing NS2 as the sole protein component could be recovered from mixtures of BTV SW fractions and BTV mRNA. The S value of the complex was found to be independent of the mRNA/NS2 ratio. The narrow size distribution of the complex was rather surprising, in view of the heterogeneous size distribution of BTV mRNA. An equally heterogeneous size distribution of the BTV mRNA-NS2 complex would have been expected if the number of NS2 molecules that are bound to each mRNA were dependent solely on the length of the mRNA species. The possibility that only a limited number of NS2 molecules can bind to each mRNA, as has been found in the case of reovirus σNS (17), needs to be investigated. It is also possible that only certain of the mRNA species bind NS2 effectively. Experiments with individual mRNA species and highly purified NS2 of known specific activity are in progress to investigate this particular aspect in more detail.

The affinity of NS2 for mRNA suggests that the NS2 in infected cells is also bound to BTV mRNA or cellular ssRNA. The S value of such complexes is small, and they are therefore likely to be present in the soluble (S100) fraction of infected cells. The largest amount of NS2 is, however, not found in the soluble fraction but appears to be associated with particulate material. Most of these NS2-associated particles sediment through a 40% sucrose cushion after centrifugation at 100,000 × g for 1 h. Some of the NS2 is also bound to low-density material that can be recovered from the top of the 40% sucrose cushion. NS2 is therefore a "sticky" protein, which might also explain the nonspecific coprecipitation of NS2 that is often observed during the immunoprecipitation of BTV proteins (11).

The particulate fraction of BTV-infected cells has been used as starting material in the purification of NS2. It was found that, similar to particulate σNS in the case of reovirus (10), BTV NS2 can be solubilized from all particulate material by an SW with 0.5 M NaCl. If this treatment is done at a pH of 8.0 or more, the BTV capsid proteins are not solubilized (11) and a significant purification of the NS2 from other viral and cellular proteins is achieved. The subsequent purification by poly(U)-Sepharose affinity chromatography resulted in a fraction that contained NS2 as the only labeled component. Serva blue-stained polyacrylamide gels, however, often indicated the presence of very small trace amounts of contaminating cellular proteins.

This report presents the first evidence that NS2 can be phosphorylated in vitro. It was shown that immunoprecipitates of BTV-specific proteins from the S100 fraction of infected cells contained a protein kinase that was able to phosphorylate NS2. Similar results have been reported for the NS protein of vesicular stomatitis virus (1). It was also found that NS2 in the P100 fraction of BTV-infected cells can be phosphorylated in vitro. However, a number of cellular proteins are also phosphorylated under these conditions. Results presented in this report also indicate that in vivo phosphorylation of NS2 takes place throughout the infection cycle. This implies that the in vitro ^{32}P labeling of already phosphorylated NS2 is caused either by hyperphosphorylation or by some form of phosphate exchange as the result of a dynamic phosphorylation-dephosphorylation reaction. To investigate this aspect, we attempted to phosphorylate NS2 synthesized in an in vitro translation reaction (results not shown). No phosphorylation could be demonstrated. These results are again in agreement with those obtained with vesicular stomatitis virus (6). An experiment was also done in which in vivo-synthesized NS2 was purified

to separate it from the kinase activity. It was, however, found that poly(U)-Sepharose-purified NS2 preparations still contained kinase activity that phosphorylated NS2 in vitro. The most likely explanation is that the kinase is one of the minor cellular proteins that were often found to be associated with purified NS2. Since a very small amount of P1 was also often seen to be present in purified NS2 preparations, the possibility of a viral protein kinase or even autophosphorylation cannot be excluded. This aspect is under investigation.

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