Expression of Influenza Virus NS2 Nonstructural Protein in Bacteria and Localization of NS2 in Infected Eucaryotic Cells

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The nonstructural NS2 protein of influenza A/PR/8/34 virus was efficiently expressed in bacteria, and monospecific antisera were prepared against the bacterially synthesized polypeptide. These antisera were cross-reactive among the NS2 proteins of various influenza A viruses. However, they did not react with the NS2 of influenza B/Lee/40 virus nor with other proteins of influenza A viruses such as NS1. Antisera against NS2 were used to determine that the NS2 protein is localized in the cell nucleus during influenza virus infection, as shown by immunofluorescence microscopy. Cells infected with simian virus 40 recombinants containing the influenza virus NS gene revealed that both the NS1 and NS2 proteins appeared in the nucleus, even in the absence of expression of other influenza virus-specific components.

The complete sequence of one influenza A virus (A/PR/8/34) is known, along with the nucleotide sequences of individual genes from numerous influenza A virus variants (reviewed in reference 11). Thus, much information is available on the structural organization of the genomes of influenza viruses, but less is known about the precise function(s) of the products encoded by these various genes. In particular, the function of the two nonstructural proteins NS1 and NS2, coded for by the shortest RNA segment (7, 12, 20), remains undefined. In this paper, we describe attempts to learn more about the smaller of these nonstructural proteins. The NS2 polypeptide was expressed in bacterial cells to generate large quantities suitable for producing antisera. The NS2 protein was partially purified, and monospecific antibodies against NS2 were prepared in rabbits. This reagent was then used to demonstrate that NS2 localizes in the nucleus of influenza virus-infected cells.

MATERIALS AND METHODS

Plasmids. Plasmid pAPR801, a pBR322 derivative containing a double-stranded cDNA of the A/PR/8/34 influenza virus NS gene, and plasmid pAS1/PR8, a pBR322 lambda phage chimeric also having the A/PR/8/34 NS gene, have been described previously (2, 22). Plasmid pA11-SVL2, a eucaryotic expression vector based on the simian virus 40 (SV40) late promoter, was obtained from George Khoury and has been previously described (6). pSVr-INS7, which contains an intact SV40 late region, was obtained from Philip Sharp (15). Plasmids pA11-SVL2 and its derivatives and pAPR801 were grown in *Escherichia coli* C600 cells. Plasmids pAS1/PR8 and pAS1/PR8-NS2 were replicated in *E. coli* N99 and N5151 cells. DNA manipulations, including restriction enzyme digestion, isolation and ligation of fragments, and transformation of *E. coli* with plasmid DNA, were done by standard procedures (17).

Plasmid pAPR801 was digested with *Bst*NI, and the resulting seven fragments were end-filled with the Klenow fragment of DNA polymerase I. *Hind*III linkers (GCAAGCTTGC) (P-L Biochemicals, Inc., Milwaukee, Wis.) were ligated to the fragments which were then digested with *Hind*III. The fragment coding for the portion of the NS2 gene, including nucleotides 527 to 890, was isolated from a 4% polyacrylamide gel. pAS1/PR8 was also digested with *Hind*III. This enzyme cuts the plasmid in two places. The fragments were then treated with bacterial alkaline phosphatase and used in a ligation reaction containing the isolated fragment of the NS2 gene. This preparation was used to transform *E. coli* (N99) cells, and ampicillin-resistant colonies were isolated. DNAs from several colonies were screened with various restriction enzymes, and several plasmids possessing the NS2-coding sequence in the correct orientation relative to the promoter were further screened for the ability to produce NS2 protein. Construction of the resulting plasmid pAS1/PR8-NS2 is diagrammed in Fig. 1.

NS2 production and partial purification. *E. coli* N5151 cells were transformed with pAS1/PR8-NS2 and grown to an optical density of 0.65 at 32°C. To induce the NS2 protein the temperature was quickly raised to 42°C (21). A 1-liter culture was then grown at 42°C for 2 h. The bacteria were pelleted (20 min at 12,000 × g), suspended in phosphate-buffered saline (PBS), and centrifuged again. The bacterial pellet was then stored at 70°C overnight. The NS2 protein was partially purified by a modification of the procedure used for NS1 protein (22). The bacterial pellet was thawed and resuspended in 40 ml of buffer A containing 50 mM Tris-hydrochloride (pH 8), 2 mM EDTA, 0.1 mM dithiothreitol, and 5% (wt/vol) glycerol, to which lysozyme was added at a final concentration of 0.2 mg/ml. The suspension was incubated on ice for 20 min and then centrifuged for 30 min at 12,000 × g. The pellet was resuspended in 10 ml of buffer A, and sodium deoxycholate was added to a final concentration of 0.05%. The mixture was then Dounce homogenized (20 strokes) and centrifuged for 45 min at 12,000 × g. The pellet was resuspended in 20 ml of buffer A and mixed with an equal volume of a buffer containing 10 mM Tris (pH 7.2), 1% Nonidet P-40, 5% sodium deoxycholate, 0.1 M NaCl, and 1

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FIG. 1. The pAS1/PR8-NS2 plasmid was constructed to express only NS2-coding sequences in the absence of the complete NS1 sequence. The NS2 sequences 3' to the splicing junction (nucleotides 27 to 56) were derived from pAPR801 by *Bst*N1 and *Hind*III digestion (2). The sequences 5' to the splice junction (nucleotides 529 to 890) were derived from plasmid pAS1/PR8, which was used previously to express NS1 protein (21). The final construct, pAS1/PR8-NS2, contains a complete copy of the A/PR/8/34 NS2 gene except for a single nucleotide change that was introduced by the commercially prepared *Hind*III linkers. This resulted in an amino acid change in position 9 from phenylalanine to leucine, as shown in the lower portion of the figure. With this exception, the gene product expressed in bacteria has the same amino acid sequence as that predicted for NS2 in virus-infected cells. The numbering system of the nucleotides is based on the previously obtained sequence of the A/PR/8/34 NS gene (2).

mM EDTA. This suspension was incubated on ice for 15 min, Dounce homogenized (20 strokes), and pelleted at $16,000 \times g$ for 30 min. The extraction with sodium deoxycholate and Nonidet P-40 was repeated, and the pellet was resuspended in 6 M guanidinium chloride-1% 2-mercaptoethanol and centrifuged for 1 h at $16,000 \times g$. The resulting supernatant contains partially purified NS2. This preparation was at least 50% pure NS2 as judged by gel electropho-

resis, followed by Coomassie blue R-250 staining, and recovery of NS2 from the induced bacteria was at least 90%.

Antiserum production. Before immunization, the guanidinium chloride was removed from the partially purified NS2 preparation by dialysis against two changes of distilled water. This resulted in the formation of a precipitate which was pelleted at $8,000 \times g$ for 15 min. Both the supernatant and pellet contained NS2 protein. The pellet was used for

subcutaneous inoculation, and the supernatant was used for intravenous inoculation. A sample of each fraction was run on a 7 to 14% polyacrylamide gradient gel. These gels were stained with Coomassie blue R-250, and the amount of NS2 present was quantitated by comparison with known amounts of protein standards run on the same gel. Approximately 500 μ g of NS2 protein in suspension was combined with an equal volume of Freund complete adjuvant. This was injected subcutaneously into a New Zealand White rabbit at eight sites along its back, and 4 weeks later this was repeated except that incomplete adjuvant was used. Finally, the animal was given an intravenous boost of ca. 15 μ g of the NS2 protein (supernatant fraction; see above) 2 weeks after the last subcutaneous injection. The animal was bled 1 week after the final boost. In addition to this serum, an antiserum was raised against NS2 isolated by preparative gel electrophoresis of the pellet after Nonidet P-40 and sodium deoxycholate extraction. An immunization protocol similar to the one described was used to generate this antiserum.

Construction of SV-NS vectors and expression of NS1 and NS2 proteins in CV-1 cells. SV40 recombinants containing the influenza virus NS gene were obtained by insertion of a complete NS gene into the *Bam*HI site of pA11-SVL2 (7). For this purpose, plasmid pAPR801 was digested with *Eco*RI; the fragments were end-filled, ligated with *Bgl*II linkers (CAGATCTG) (New England Biolabs, Beverly, Mass.), and digested with *Bgl*II. The cDNA containing the NS gene sequences was purified by polyacrylamide gel electrophoresis. pA11-SVL2 was digested with *Bam*HI, which cleaves at a single site in the late region near the agnoprotein initiation codon of SV40 DNA (6). The digested pA11-SVL2 and the NS gene fragment were ligated and used to transform competent *E. coli* C600 cells. Colonies containing the NS gene were identified by filter hybridization and screened with restriction enzymes to determine the orientation of the NS gene relative to the SV40 promoter.

The recombinant pA11-SVL2 vectors containing the NS gene (10 μ g of DNA) were digested with *Xba*I to remove pBR322 sequences. After phenol extraction and ethanol precipitation, the mixture was religated without further purification. The ligated DNA was mixed with 10 μ g of pSVr-INS7 (15) which had been digested with *Eco*RI and religated to eliminate pBR322 sequences. pSVr-INS7 DNA is added to provide the late SV40 functions in cotransfected cells. The mixture was transfected into CV-1 cells with a modification (18) of the calcium phosphate precipitation procedure. Two weeks later, cells and medium were frozen and thawed three times and used as virus stocks.

Immunoprecipitation. Confluent MDCK cell monolayers grown in 35-mm dishes were infected with 0.1 ml of egg-grown influenza viruses (A/PR/8/34, A/Japan/305/57, A/Udorn/72) for 1 h at 37°C. At 6 h postinfection, the cells were labeled with [³⁵S]methionine (200 μ Ci/ml) for 1 h as reported previously (19). Confluent CV-1 cell monolayers in 35-mm dishes were infected with 0.1 ml of SV40 recombinant virus stocks. At 2 days postinfection, the cells were labeled with [³⁵S]methionine for 2.5 h. After the labeling period, cells were lysed in 200 μ l of buffer containing 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.01 M Tris-hydrochloride (pH 7.4), and 1 mM phenylmethylsulfonyl fluoride. Samples (25 μ l) of labeled cell extracts were incubated overnight with rabbit sera at 4°C. Formalin-fixed *Staphylococcus aureus* cells (100 μ l; Bethesda Research Laboratories, Rockville, Md.) were added, and the mixture was then incubated for 30 min at 4°C. Samples were pelleted (2 min at 15,000 $\times$ g) at 4°C and

FIG. 2. Biosynthesis and stability of NS2 protein in *E. coli*. Bacterial cell cultures containing pAS1/PR8-NS2 were induced for 30 min at 42°C and then pulse-labeled with [³⁵S]methionine (100 μ Ci/ml) for 5 min. The [³⁵S]methionine was removed, and cells were maintained at 42°C in medium containing unlabeled methionine for the times indicated. At the end of the incubation, cell extracts were electrophoresed on 7 to 14% polyacrylamide gels containing 6 M urea. Gels were dried and autoradiographed. Lane 1, Uninduced bacterial cell culture; lane 2, induced bacterial cell culture (chase time = 0 min); lane 3, induced bacterial cell culture (chase time = 30 min); lane 4, induced bacterial cell culture (chase time = 60 min); lane 5, induced bacterial cell culture (chase time = 90 min); and lane 6, influenza A/PR/8/34 virus-infected MDCK cells (pulse-labeled for 60 min with [³⁵S]methionine 7 h postinfection [see the text]).

suspended in 250 μ l of 500 mM LiCl-100 mM Tris-hydrochloride (pH 8.5). They were then pelleted again and washed once with distilled water. Finally, samples were resuspended in loading buffer and electrophoresed on 7 to 14% polyacrylamide gradient gels containing 6 M urea as described previously (19).

Immunofluorescence. For the experiment shown in Fig. 5, CV-1 cells were grown on eight-well multitest slides (Flow Laboratories, Inc., McLean, Va.) and were infected with influenza A/PR/8/34 and B/Lee/40 viruses at a multiplicity of infection of ca. 3. Cells were fixed with 3% formaldehyde in PBS (pH 7.4) and permeabilized with 0.05% Triton X-100. Slides were then incubated with rabbit anti-NS2 serum diluted 1:40 or 1:125 in PBS containing 5% goat serum, washed, and in the case of A/PR/8/34 virus infections, incubated with the mouse monoclonal anti-hemagglutinin antibody H28-E23 (1), likewise diluted into PBS with goat serum. Cells were then washed and exposed to a mixture of rhodamine-conjugated goat anti-rabbit immunoglobulin G (IgG) F(ab)₂ and fluorescein-conjugated goat anti-mouse IgG F(ab)₂ antibodies (Cappel Laboratories, Cochranville, Pa.) which were also diluted into PBS containing 5% normal goat serum. Cells were then washed, mounted, and observed and photographed in a Reichert-Jung Polyvar microscope equipped with epifluorescence and differential interference contrast optics.

For other experiments (see Fig. 6 and 9; Table 1), cells

FIG. 3. Expression of influenza virus NS2 protein in *E. coli*. (A) *E. coli* cells (N5151) transformed with either pAS1/PR8-NS2 or pAS1/PR8 (22) were grown at 32°C for 30 min; the temperature was then shifted to 42°C for induction. The equivalent of 1 ml of bacterial cell culture was separated on a 7 to 14% polyacrylamide gel containing 6 M urea and stained with Coomassie blue R-250. Lane 1, Low-molecular-mass markers (14.4 to 92.5 kilodaltons); lane 2, uninduced bacterial cell culture; lane 3, bacterial cell culture containing pAS1/PR8-NS2 induced for 2 h; and lane 4, bacterial cell culture containing pAS1/PR8 induced for 2 h. (B) NS2 was purified from a bacterial cell culture containing pAS1/PR8-NS2 induced for 2 h. The purified NS2 was dialyzed to remove guanidinium chloride. The resulting precipitate was pelleted and suspended in a volume of buffer equal to that of the supernatant. Equivalent amounts of supernatant and pellet were separated on a 7 to 14% polyacrylamide gel and stained with Coomassie blue R-250. Lane 1, Low-molecular-mass markers [same as in (A)]; lane 2, purified NS2 pellet fraction; and lane 3, purified NS2 supernatant fraction.

were seeded onto 13-mm cover slips at ca. 10^5 cells per cover slip. The next day these cells were infected and fixed with 1% formaldehyde for 1 h at room temperature, followed by incubation with 0.1% Triton X-100 for 3 min. Cover slips were treated with 100 μ l of NS2 antiserum at 1:125 dilution or 1:50 dilution of NS1 antiserum (22) for 30 min at 37°C, washed, and stained with fluoresceinated goat anti-rabbit IgG (Cappel) at a 1:200 dilution for 30 min at 37°C. Cells were viewed on a Leitz fluorescence microscope.

Absorption of antisera. Antisera were absorbed by a solid-phase procedure. Purified NS1 or NS2 (50 μ l at a concentration of 100 μ g/ml in PBS) isolated from bacteria was adsorbed to wells of polyvinyl chloride microtiter plates for 1.5 h at 37°C. Fifty microliters of borate buffer (0.02 M [pH 8]; 0.9% NaCl, 0.05% NaN₃) with 1% bovine serum albumin was added and incubated an additional 1.5 h at

37°C. Plates were washed three times with PBS. Antisera (50 μ l of a 1:50 dilution in PBS plus 0.1% bovine serum albumin) were added to the wells and incubated 1.5 h at 37°C. This step was repeated twice. The sera were diluted with 50 μ l of 10% goat serum and used for immune fluorescence as described above.

RESULTS

Construction of a plasmid containing the NS2 gene. The plasmid pAS1/PR8-NS2 was constructed to express the influenza A/PR/8/34 virus NS2 protein in bacteria. For this purpose, we modified plasmid pAS1/PR8 (22) which was previously used to express the gene for the NS1 protein. The NS2 mRNA in influenza virus-infected cells differs from the NS1 mRNA by the absence of nucleotides 57 to 528, which are spliced out (11, 12). These sequences were removed

from pAS1/PR8 by digestion of the plasmid with *Hind*III which retains nucleotides 5' to the splice site and religation with a DNA fragment containing the sequence of the NS2 mRNA 3' to the splice site (Fig. 1). This construction resulted in an open reading frame colinear with the deduced coding sequence of NS2, with the exception of the ninth codon, which underwent a change from phenylalanine to leucine (see lower part of Fig. 1).

Expression of NS2 protein. The pAS1/PR8-NS2 plasmid was used to transform N5151 *E. coli* cells. The cells were shifted to 42°C to induce the synthesis of NS2 protein. Cells were pulse-labeled for 5 min with [³⁵S]methionine and chased in unlabeled medium for varying amounts of time. Analysis of labeled cells before and after temperature shift by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography is shown in Fig. 2. A new protein band appears in cells shifted to 42°C (lane 2) that is not present in cells grown at 32°C (lane 1). The new protein with a molecular weight of 12,000 is similar in size to the influenza virus NS2 protein. The amount of labeled NS2 protein after a 90-min chase (lane 5) is similar to the amount of label after a 5-min pulse (lane 2). Thus, the NS2 protein is synthesized at high levels in induced *E. coli* cells, and very little degradation of NS2 is observed over a 90-min time course.

Figure 3 shows a Coomassie blue-stained gel of transformed *E. coli* cells before a temperature shift (lane 2) and after a 2-h incubation at 42°C (lane 3). For comparison, cells transformed with pAS1/PR8 and incubated at 42°C to induce synthesis of NS1 are shown in lane 4. These results show that NS2 protein can be produced in large quantities in bacteria, similar to the production of NS1 (22). From data shown in Fig. 3, we estimate the NS2 protein to be approximately 5 to 10% of total cellular protein.

Partial purification of NS2. The NS2 produced in *E. coli* during a 2-h incubation at 42°C was partially purified to raise antisera against NS2. Cells from a 1-liter culture were treated with lysozyme and then lysed with deoxycholate. The NS2 was not solubilized by the detergent and was pelleted at 12,000 × *g*. After further extraction of the pellet with deoxycholate and Nonidet P-40 to remove bacterial contaminants, the NS2 was then solubilized with guanidinium chloride. At this stage, the NS2 is ca. 50% of the total protein, and the yield (2 to 4 mg from a 1-liter culture) is nearly quantitative. When the guanidinium chloride was removed by dialysis, a precipitate formed. Both the precipitate and the resulting supernatant contained NS2 (Fig. 3B, lanes 2 and 3). Both fractions were used for immunization of rabbits. Usually the NS2 remaining in the supernatant showed a further degree of purification after removal of the precipitate (lane 3).

Reactivity of antisera against NS2. The specificity of antisera raised against NS2 isolated from *E. coli* was tested in immunoprecipitation experiments. Influenza virus-infected cells were labeled with [³⁵S]methionine, solubilized with detergents, and reacted with antisera against either NS1 or NS2 purified from *E. coli*. The immune complexes were precipitated with fixed *S. aureus* cells and analyzed by sodium dodecyl sulfate-gel electrophoresis and autoradiography (Fig. 4). The anti-NS2 serum specifically precipitated NS2 and not NS1 from cells infected with A/PR/8/34 virus (Fig. 4A, lane 4), whereas the anti-NS1 serum specifically precipitated NS1 (lane 3) as shown previously (22). The cross-reactivity of antiserum against NS2 among influenza viruses of different subtypes is shown in Fig. 4B. The antiserum raised against the NS2 of A/PR/8/34 virus expressed in *E. coli* precipitates the NS2 proteins of an H2

FIG. 4. Specificity of antiserum made to bacterially synthesized NS2. (A) Influenza A/PR/8/34 virus was used to infect MDCK cells. Six hours postinfection, the cells were labeled with [³⁵S]methionine for 1 h, lysed, and used for immunoprecipitation. The experiment shown was performed with antiserum against NS2 purified by preparative gel electrophoresis. The same result was obtained with antiserum against NS2 purified by detergent extraction. Lane 1, Virus-infected cell extract; lane 2, virus-infected cell extract immunoprecipitated with normal rabbit serum; lanes 3 and 5, virus-infected cell extract immunoprecipitated with anti-NS1 antiserum; and lane 4, virus-infected cell extract immunoprecipitated with anti-NS2 antiserum. (B) Influenza A/Japan/305/57 (H2N2) and A/Udorn/72 (H3N2) viruses were used to infect MDCK cells. Cells were labeled, extracted, and used for immunoprecipitation. Lane 1, Influenza A/Japan/305/57 virus-infected cell extract; lane 2, influenza A/Japan/305/57 virus-infected cell extract immunoprecipitated with normal rabbit serum; lane 3, influenza A/Japan/305/57 virus-infected cell extract immunoprecipitated with anti-NS2 antiserum; lane 4, influenza A/Udorn/72 virus-infected cell extract; lane 5, influenza A/Udorn/72 virus-infected cell extract immunoprecipitated with normal rabbit serum; and lane 6, influenza A/Udorn/72 virus-infected cell extract immunoprecipitated with anti-NS2 antiserum.

influenza virus (A/Japan/305/57; lane 3) and of an H3 virus (A/Udorn/72; lane 6). In further immunoprecipitation experiments, the antiserum against NS2 was also observed to react with the NS2 of A/duck/Alberta/60/76 (H12) virus but not with that of an influenza B virus (B/Lee/40 virus; data not shown). Thus, the NS2 proteins of influenza A viruses belonging to different subtypes are antigenically related, whereas the NS2 protein of an influenza B virus is antigenically distinct.

Immunofluorescent localization of the NS2 protein. The availability of specific antisera allowed us to investigate the

FIG. 5. Intracellular localization of the NS2 protein. CV-1 cells were infected with influenza A/PR/8/34 or influenza B/Lee/40 virus, incubated at 37°C, and then fixed and processed for double immunofluorescence as indicated in the text. (a to d) Cells labeled with rabbit anti-NS2 antisera and rhodamine-conjugated goat anti-rabbit IgG antibodies. Cells were fixed after infection at the times indicated. (e to h) Same cells as in (a to d), labeled with mouse monoclonal antibody to hemagglutinin and fluorescein-conjugated goat anti-mouse IgG. In (i to m), the differential interference contrast images of the same fields are shown. (n and o) Cells infected with influenza B/Lee/40 virus, incubated for 6 h, and labeled as in (a to d). Arrowheads in (b), (f), and (k) show that a cell not expressing hemagglutinin lacks NS2-specific immunofluorescence. Arrows in (d) and (h) show occasional perinuclear NS2-specific immunofluorescence which colocalized with that of hemagglutinin. Small arrowhead in (d) shows NS2-specific fluorescence along some sites of the plasma membrane.

intracellular localization of the NS2 protein during influenza virus infection. This was investigated with indirect immunofluorescence microscopy. CV-1 cells were infected with influenza A/PR/8/34 virus at a low enough multiplicity so that infected cells as well as uninfected cells serving as internal controls were present in the same microscopic field. The infected cells were identified by a monoclonal antibody against the hemagglutinin. Cells incubated for various periods of time after infection were labeled with rabbit anti-NS2 serum and rhodamine-labeled anti-rabbit IgG F(ab)₂ antibody. The viral hemagglutinin was labeled with a mouse monoclonal antibody and fluorescein-labeled anti-mouse IgG F(ab)₂ antibody. The results of these double-immunofluorescent labeling experiments are shown in Fig. 5. The fluorescein and rhodamine fluorescence and differential interference contrast image of the same field are shown for each

TABLE 1. Immunofluorescence with antisera immunoabsorbed with purified NS1 or NS2 protein^a

Treatment	Immunofluorescence reaction with:	
	Anti-NS1 serum	Anti-NS2 serum
None	+	+
NS1 absorption	—	+
NS2 absorption	+	—

^a CV-1 cells infected with influenza A/PR/8/34 virus were fixed 6 h postinfection and used for immunofluorescence as described in the text. Fluorescence with treated sera was considered positive when the fluorescence intensity was not significantly different from that obtained with unabsorbed serum, as determined by the time required to expose ASA 200 film (automatically determined) with the light meter centered over the nucleus of an infected cell (45 ± 15 s). Fluorescence intensity was considered negative when it was similar to that obtained with mock-infected cells.

FIG. 6. Localization of NS2 protein in influenza A/CAM/46 virus-infected CV-1 cells and in influenza A/PR/8/34 virus-infected MDCK cells. Virus-infected cells were fixed 8 h postinfection and used for immunofluorescence as described in the text. (A) A/PR/8/34 virus-infected CV-1 cells. (B) A/CAM/46 virus-infected CV-1 cells. (C) Mock-infected CV-1 cells. (D) MDCK cells infected with A/PR/8/34 virus. (E) Mock-infected MDCK cells. Fixation of MDCK cells with Formalin and Triton X-100 was followed by treatment with acetone at -20°C for 5 min.

time point. Before infection, cells showed background immunofluorescence. With increasing time after infection, NS2-specific immunofluorescence (Fig. 5a to d) became visible in those cells which also expressed the hemagglutinin (Fig. 5e to h). Cells devoid of hemagglutinin staining showed no NS2 fluorescence (large arrowheads), and only occasionally a cell with little hemagglutinin immunofluorescence showed an appreciable intensity of NS2 immunofluorescence (Fig. 5d and h). The predominant NS2-specific immunofluorescence was observed in the nucleus of infected cells but apparently not in the nucleoli. In some infected cells, particularly in later stages of infection, the NS2 immunofluorescence was also found in the perinuclear area of the cytoplasm (Fig. 5d, arrows) and along the plasma membrane (Fig. 5d, small arrowhead) at similar sites in which the hemagglutinin was localized (Fig. 5h, arrows). Cells infected with influenza

B/Lee/40 virus show no NS2-specific immunofluorescence (Fig. 5n and o).

Immunoabsorption of antisera. To further characterize the specificity of the antisera in immunofluorescence experiments, the sera were subjected to immunoabsorption with purified NS1 and NS2 protein preparations. The results are shown in Table 1. The reactivity of anti-NS1 serum (22) with influenza A/PR/8/34 virus-infected CV-1 cells was removed by absorption with NS1 but not by absorption with NS2. Conversely, the reactivity of anti-NS2 serum was not altered by absorption with NS1 but could be absorbed with NS2. This indicates that the immunofluorescence observed with anti-NS2 serum is not due to cross-reactivity of the anti-NS2 serum with NS1.

NS2-specific immunofluorescence in A/CAM/46 virus-infected CV-1 cells and A/PR/8/34 virus-infected MDCK cells.

FIG. 7. Structure of the SV40 NS recombinants. The NS gene was inserted into the late region of the SV40 vector pA11SVL2 (6) as described in the text. The hatched regions represent the A/PR/8/34 NS gene. ATG indicates the position of the start codon for NS1 and NS2. The arrows indicate the direction of SV40-promoted transcription. It should be noted that these DNAs originally contained pBR322 sequences inserted at the indicated *Xba*I site. For transfection of CV-1 cells, these pBR322 sequences were removed by *Xba*I digestion followed by religation of the SV40 recombinant DNA.

An intranuclear localization of NS2 was also observed in CV-1 cells infected with another virus, A/CAM/46 (Fig. 6). Infected cells were incubated with antiserum against NS2 and stained with fluorescein-labeled anti-rabbit IgG antibody. The intense labeling of the nuclei of influenza A/CAM/46 virus-infected cells was comparable to that of A/PR/8/34 virus-infected cells. MDCK cells infected with A/PR/8/34 virus also showed NS2-specific immunofluorescence in their nuclei (Fig. 6d). When infected MDCK cells were treated with Formalin and Triton X-100 alone (under conditions used for CV-1 cells), not all cells showed nuclear labeling (data not shown). However, when the treatment with Formalin and Triton X-100 was followed by incubation with cold acetone, nuclear fluorescence comparable to that of infected CV-1 cells was also seen in infected MDCK cells (Fig. 6d).

Expression and localization of NS1 and NS2 proteins in cells infected with recombinant SV40 viruses containing the NS gene. The SV40 vectors used to express the NS1 and NS2 proteins in CV-1 cells are shown in Fig. 7. SVL2-801M and SVL2-801V contain a single copy of the NS gene, whereas SVL2-801MM contains two tandem copies of the gene in a head-to-tail orientation. SVL2-801M and SVL2-801MM have the NS protein-coding region oriented in the mRNA sense

with respect to the SV40 late promoter; SVL2-801V contains the NS gene in the opposite orientation.

CV-1 cells were infected with the three SV40 virus stocks and were labeled with [^{35} S]methionine 2 days postinfection, and the labeled proteins were analyzed by sodium dodecyl sulfate-gel electrophoresis and autoradiography (Fig. 8A). The SVL2-801M (lane 3) and SVL2-801MM (lane 4) viruses synthesized proteins with the same apparent molecular weights as NS1 and NS2 from influenza A/PR/8/34 virus-infected cells (lane 5). These protein bands were not observed in SVL2-801V (lane 2) virus-infected cells or in mock-infected cells (lane 1). These bands were identified as NS1 and NS2 by immunoprecipitation with specific antisera. Anti-NS1 serum (Fig. 8B) precipitated NS1 from SVL2-801M and SVL2-801MM virus-infected cells (lanes 4 and 5) but not from cells infected with SVL2-801V (lane 3). Likewise, anti-NS2 serum precipitated NS2 from SVL2-801M and SVL2-801MM virus-infected cells (Fig. 8C, lanes 4 and 5) but not from cells which were infected with SVL2-801V virus containing the NS gene in opposite direction to the SV40 promoter (lane 3). Precipitation of labeled NP from extracts of A/PR/8/34 virus-infected cells (Fig. 8C, lane 6) was not a consistent observation (cf. Fig. 8C with Fig. 4 and 8B).

The localization of NS1 and NS2 expressed by SV40 recombinant viruses was examined by immunofluorescence microscopy (Fig. 9). The prominent nuclear fluorescence observed with influenza virus-infected cells labeled with anti-NS1 serum or anti-NS2 serum is also observed in cells infected with SVL2-801M virus (Fig. 9A and B). Similar results were obtained with cells infected with SVL2-801MM virus (data not shown). In contrast, SV42-801V recombinant virus-infected cells showed background immunofluorescence with the anti-NS2 antiserum (Fig. 9C).

DISCUSSION

Construction of pAS1/PR8-NS2 permitted efficient expression in bacteria of a polypeptide homologous to the NS2 of influenza virus-infected cells. This polypeptide remained insoluble after disruption in bacteria and was partially purified by subsequent detergent extractions. Rabbit antisera made against these NS2 preparations were shown by immunoprecipitation to be monospecific for NS2 proteins, but they broadly cross-reacted with NS2 polypeptides induced by a variety of influenza A viruses. NS2 polypeptides from H1, H2, H3, or H12 subtype virus-infected cells reacted with these antisera. This is not surprising since the most distantly related NS2 proteins (H1 and H12) in this series have a greater than 80% amino acid sequence homology (3). On the other hand, the NS2 polypeptide of an influenza B virus, which lacks extensive sequence homologies with the corresponding A virus polypeptides (5), is not immunoprecipitated by these monospecific antisera. Although the bacterially expressed NS1 and NS2 polypeptides share eight amino acids at the amino terminal, the respective antisera made against these proteins do not cross-react in immunoprecipitation experiments with influenza virus-infected cell extracts and cell extracts of bacteria induced to synthesize NS1 (22) or NS2. Furthermore, immunoabsorption experiments revealed that the reactivity of anti-NS2 serum was not removed by absorption with a purified NS1 preparation.

The availability of an NS2-specific antiserum allowed us to reexamine the question of intracellular localization of the NS2 polypeptide in influenza virus-infected cells. Previous results were based on subcellular fractionation experiments which had produced conflicting data. Early work had sug-

FIG. 8. Expression of NS1 and NS2 proteins in CV-1 cells with SV40 vectors. (A) Linear 7 to 14% polyacrylamide gel electrophoresis of infected CV-1 cells labeled for 3 h with [^{35} S]methionine at 2 days postinfection. Lane 1, Mock-infected CV-1 cells; lane 2, SVL2-801V-infected CV-1 cells; lane 3, SVL2-801M-infected CV-1 cells; lane 4, SVL2-801MM-infected CV-1 cells; and lane 5, influenza virus A/PR/8/34-infected CV-1 cells labeled for 1 h with [^{35} S]methionine at 6 h postinfection. The VP1 protein of SV40 virus and the NS1 and NS2 protein are indicated. (B) Immunoprecipitation with anti-NS1 antiserum. Lanes 1 and 7, A/PR/8/34 virus-infected CV-1 cell extract; lane 2, immunoprecipitation of mock-infected CV-1 cells; lane 3, immunoprecipitation of SVL2-801V-infected CV-1 cells; lane 4, immunoprecipitation of SVL2-801M-infected CV-1 cells; lane 5, immunoprecipitation of SVL2-801MM-infected CV-1 cells; and lane 6, immunoprecipitation of influenza virus A/PR/8/34-infected CV-1 cells. (C) Immunoprecipitation with anti-NS2 antiserum. Lane 1 and 7, A/PR/8/34 virus-infected CV-1 cell extract; lane 2, immunoprecipitation of mock-infected CV-1 cells; lane 3, immunoprecipitation of SVL2-801V-infected CV-1 cells; lane 4, immunoprecipitation of SVL2-801M-infected CV-1 cells; lane 5, immunoprecipitation of SVL2-801MM-infected CV-1 cells; and lane 6, immunoprecipitation of influenza virus A/PR/8/34-infected CV-1 cells.

gested an intranuclear localization of a small nonstructural protein (9). More recently, however, support was provided for the idea that the NS2 polypeptide of influenza virus-infected cells is associated with the cytoplasmic fraction throughout the replication cycle (4, 16). Our immunofluorescence data would seem to indicate a nuclear localization of the NS2. Differences in strains used in the studies may provide an explanation for the observed differences in NS2 localization. Alternatively, the cell fractionation techniques used may contribute to the complications observed in these experiments. Specifically, the NS2 polypeptide is a small molecule (ca. 120 amino acids) which could be removed from the nuclear fraction by some isolation procedures. Since in our immunofluorescence experiments the NS2 was predominantly localized in the nucleus regardless of cell type and influenza virus strain employed, it is likely that

conflicting data in the literature are the result of differences in the isolation techniques used by various investigators.

Analysis of cells infected with SV40 recombinants carrying the NS gene of influenza A/PR/8/34 virus confirmed earlier results (13) that splicing of NS-specific mRNAs also occurs with DNA-directed RNA transcription and that it is not dependent on the expression of the other influenza virus genes. The present study has not addressed the question of regulation of NS1-NS2 splicing, and no efforts were made to quantitate the expression of NS1-NS2 transcripts or their translation products. It should be noted, however, that comparable amounts of NS1 and NS2 were immunoprecipitated after pulse-labeling of SV40 recombinant virus-infected cells. No differences in the pattern of NS1-NS2 expression were observed whether the SV40 recombinants containing one or two full-length NS genes were used for infection.

FIG. 9. Immunofluorescence of SV-NS-infected CV-1 cells. Cells were fixed 2 days postinfection as described in the text. (A) SVL2-801M virus-infected cells incubated with anti-NS2 serum. (B) SVL2-801M virus-infected cells incubated with anti-NS1 serum. (C) SVL2-801V virus-infected cells incubated with anti-NS2 serum.

However, as expected, the SV40 recombinant possessing an NS gene that was not under the SV40 promoter control did not direct the synthesis of either NS1 or NS2 proteins. Expression of the SV40 recombinants also allowed us to study the specific intranuclear localization of NS1 and NS2 in the absence of influenza virus replication. Since these SV40 recombinant viruses express only one influenza virus gene, the possibility can be ruled out that nuclear localization of NS1 and NS2 is the result of a tight association of these proteins with the nuclear ribonucleoprotein complex of influenza viruses. (The ribonucleoprotein complex includes the nucleoprotein and some or all of the P proteins.) It would appear that the NS1 and the NS2 polypeptides, either alone or in conjunction with each other, carry the information for migration to the nucleus of infected cells. Recent studies have shown that the SV40 T antigen contains a sequence of five basic amino acids at positions 127 to 131 that can influence whether T antigen is localized in the nucleus or the cytoplasm (8, 14). Similar sequences are present in NS1 and NS2. For instance, four of five amino acids at positions 84 to 88 in the NS2 sequence and positions 17 to 21 in the NS1 sequence of influenza A/PR/8/34 virus are basic amino acids (10). Another sequence that is rich in basic amino acids extends from positions 35 to 46 in the NS1

sequence. Thus, it is possible that these sequences are involved in the nuclear localization of the NS proteins analogous to the SV40 T antigen. Efforts are currently underway to construct SV40 vectors which express NS1 or NS2 polypeptides separately. Such vectors may be helpful in exploring the question of whether NS1 and NS2 proteins of influenza viruses possess "signal" sequences specific for nuclear compartmentalization.

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