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Interaction Cloning of NS1-I, a Human Protein That Binds to the Nonstructural NS1 Proteins of Influenza A and B Viruses

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The yeast interaction trap system was used to identify NS1-I (for NS1 interactor), which is a human protein that binds to the nonstructural NS1 protein of the influenza A virus. NS1-I is a human homolog of the porcine 17β-estradiol dehydrogenase precursor protein, to which it is 84% identical. We detected only one NS1-I mRNA species, of about 3.0 kb, in HeLa cells, and the NS1-I cDNA was found to have a coding capacity for a 79.6-kDa protein. However, immunoblot analysis detected predominantly a 55-kDa protein in human cells, suggesting that NS1-I, like the porcine 17β-estradiol dehydrogenase, is posttranslationally processed. Using an in vitro coprecipitation assay, we showed that NS1-I interacts with NS1 proteins from extracts of cells infected with five different influenza A virus strains as well as with the NS1 of an influenza B virus. The fact that influenza A and influenza B virus NS1 proteins bind to NS1-I suggests that this cellular protein plays a role in the influenza virus life cycle.

The virulence and host range of influenza viruses are characterized by complex interactions between host- and virus-specific factors (45). Although much has been learned about the functions of influenza virus proteins, little is known about cellular factors that are involved in viral multiplication. The yeast two-hybrid system (8, 19) has been used to define many biologically significant protein-protein interactions (for a review, see reference 13). We employed this system to search for cellular proteins that are recognized by the influenza A virus NS1 protein and identified the human protein NS1-I (NS1 interactor) on the basis of its binding to NS1.

NS1 is the major nonstructural protein expressed by influenza A and B viruses in infected cells. Although the precise functions of NS1 in infected cells are not clear, several effects of NS1 on both virus-specific processes and host cell functions have been described. Studies of viruses carrying temperature-sensitive NS alleles suggest a regulatory role(s) for NS1 in viral gene expression and/or replication (22, 27, 36, 54). Furthermore, transient expression of NS1 in mammalian cells leads to a retention of poly(A) RNA in the nucleus (14, 42) and inhibition of pre-mRNA splicing (33). These results are also consistent with the preferential nuclear accumulation of NS1 (18). A fraction of NS1 was also detected in association with ribosomes and polysomes in cytoplasmic fractions of infected cells (9, 29). Indeed, recent publications suggest that NS1 functions as a translational enhancer for viral mRNAs (11, 12). Thus, NS1 proteins appear capable of interfering with cellular functions in multiple ways, despite their relatively small size of 14 to 26 kDa.

Some of the effects of NS1 may be mediated by its ability to bind to RNA. In fact, a surprising variety of RNAs has been reported to be complexed by NS1 in vitro, including poly(A) (42), spliceosomal U6 RNA (33, 43), and the conserved 5′- and 3′-terminal ends of influenza virus RNAs (21, 23, 48). A recent report suggests that the NS1 protein prevents the activation of double-stranded-RNA (dsRNA)-activated protein kinase (PKR) by binding to dsRNA (34). Activation of PKR results in downregulation of cellular translation and is believed to be part of an antiviral defense strategy in mammalian cells (16). Influenza A viruses appear to counteract this cellular response, which may in part be mediated by the binding of the NS1 protein to dsRNA (34).

Host proteins interacting with the NS1 protein during infection have yet to be characterized. The identification of such proteins is of great interest, since they may influence the host range and virulence of influenza virus strains. Such a hypothesis is supported by studies of influenza viruses with mutated or heterologous NS alleles which showed altered growth characteristics in different cells (46, 53). Using the yeast interaction trap, we have identified NS1-I by its ability to bind to NS1. NS1-I is homologous to the precursor protein of a porcine 17β-estradiol dehydrogenase (17β-EDH) (31). A nearly identical human protein was cloned independently and shown to encode a functional 17β-hydroxysteroid dehydrogenase (17β-HSD) while the manuscript of this article was in preparation (2). We demonstrated the conservation of the interaction of NS1-I with six different human and avian influenza virus NS1 proteins, suggesting that there is a role for the NS1–NS1-I interaction during the virus life cycle.

MATERIALS AND METHODS

Manipulations of nucleic acids, *Escherichia coli*, and yeast cells followed standard procedures as described elsewhere (3).

Yeast strains, *E. coli* strains, and plasmids. *Saccharomyces cerevisiae* EGY40 (*MAT*α *trp1 ura3 his3*) and EGY48 (*MAT*α *trp1 ura3 his3 LEU2::pLEX-Aop6-LEU2*); plasmids pEG202, pRFHM1, and pSH18-34; and the HeLa cell cDNA library constructed in pJG4-5 were kindly provided by R. Brent (Harvard Medical School) and have been described elsewhere (19, 55). *E. coli* strains used for cloning and expression were MH3 (*trpC araD lacX hsdR galU galK*), DH5α [(F$^-$ Φ*80dlacZ*ΔM15 Δ(*lacZYA-arg*F)*U169 deoR recA1 endA1 hsdR17* ($r_K^- m_K^+$) *supE44* λ$^-$ *thi-1 gyrA96 relA1*], and BL26 [F$^-$ *ompT hsdS$_B$* ($r_B^- m_B^-$) *gal dcm*]. pLexA-NS1 was constructed by subcloning the cDNA of the NS segment of influenza A/PR/8/34 virus downstream of the LexA gene in pEG202. Similarly, pGEX-NS1 was generated by subcloning the NS1 cDNA into the *Bam*HI site of pGEX-5X-1 (Pharmacia). pGEX-NS1-I and pET-NS1-I were constructed by inserting the HeLa cDNA insert of the library plasmid pK5 as an *Eco*RI-*Xho*I fragment into pGEX-5X-1 and pET28a(+) (Novagen), respectively. pcDNA3-NS1-I-AKL was constructed by amplifying the NS1-I open reading frame region of pET-NS1-I by PCR with oligonucleotides K5-AKL and T7 and inserting the resulting cDNA as a blunt-ended *Nco*I-*Sma*I fragment into the expression vector pcDNA3 (Invitrogen). Similarly, pcDNA3-NS1 was generated by amplifying NS1

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cDNA by PCR with oligonucleotides NS-5′ and NS1-3′ as primers and inserting it as an *Eco*RI-*Xho*I fragment into pcDNA3. DNA oligonucleotides were as follows: K5-AKL, 5′-dGCTGGGCCCTCAGTAGTCTTTAAGAATCATC-3′; T7, 5′-dTAATACGACTCACTATAG-3′; NS-5′, 5′-dGCGAATTCATAATG GATCCAAACACTG-3′; NS1-3′; 5-dCGGCTCGAGCTTCAAACTTCTGAC CT-3′.

Identification of NS1-interacting proteins. The interaction trap screen was done by a slightly modified protocol described previously (40). The selection strain was constructed by transforming EGY48 with the bait plasmid pLexA-NS1 and the *lacZ* reporter plasmid pSH18-34. Expression of *lacZ* from pSH18-34 is transcriptionally controlled by a *GAL1* promoter and LexA-dependent operator sites (19). A HeLa cell cDNA library was introduced into the selection strain by the lithium acetate method (25). Primary transformants were selected on Trp$^-$ His$^-$ Ura$^-$ glucose plates. A total of 1×10^6 cells from 3.3×10^5 pooled primary transformants were plated on 150-mm-diameter Trp$^-$ His$^-$ Ura$^-$ Leu$^-$ galactose plates to select for clones expressing NS1-interacting proteins. Viable cells were replica transferred to a nitrocellulose filter and assayed for β-galactosidase activity by using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) as described elsewhere (3). Positive clones were tested for dependence of β-galactosidase activity upon expression of protein from the library plasmid by replica plating onto X-Gal Trp$^-$ His$^-$ Ura$^-$ glucose and X-Gal Trp$^-$ His$^-$ Ura$^-$ galactose plates. Plasmid DNA was isolated from yeast clones expressing β-galactosidase activity on galactose plates and not on glucose plates, and library plasmids were recovered following transformation into *E. coli* MH3 as described elsewhere (40). The specificity of the isolated plasmids was tested by cotransformation with pLexA-NS1 or pRFHM1 into EGY40 harboring pSH18-34. pRFHM1 expresses an unrelated LexA fusion to the *Drosophila* bicoid protein. The resulting strains were assayed for β-galactosidase activity on X-Gal Trp$^-$ His$^-$ Ura$^-$ plates containing glucose or galactose (3). Plasmids that induced β-galactosidase only in the presence of galactose in conjunction with pLexA-NS1 but not pRFHM1 were considered to encode true interacting proteins.

Cloning of NS1-I 5′-end cDNA. Cloning of cDNA derived from the 5′ end of NS1-I mRNA was done by a RACE (Rapid Amplification of cDNA ends) procedure (15) with a 5′RACE kit (GIBCO-BRL). First-strand cDNA was synthesized from 1 μg of HeLa cell poly(A) RNA hybridized to 2.5 pmol of NS1-I-specific oligonucleotide GSP-I by using a modified Moloney murine leukemia virus reverse transcriptase. The cDNA was tailed at the 5′ end with dC by terminal transferase. The product was used as a template for the amplification of the 5′ terminus of NS1-I by PCR using the nested oligonucleotide GSP-II and the PCR anchor primer. The resulting 5′RACE fragment was subcloned into pGEM-T (Promega) to give pGEMK5-5′RACE and sequenced by the standard dideoxy method. A full-length NS1-I cDNA was generated by ligating the *Spe*I-*Spe*I fragment of pGEMK5-5′RACE (corresponding to NS1-I cDNA positions −103 to +937) and the 1.6-kb *Spe*I-*Xho*I fragment of pK5 (positions +938 to +2674). The region of NS1-I cDNA between positions 1 and 2650 was amplified by PCR using oligonucleotides NS1-I-5′ and NS1-I-3′ and subcloned into the *Xba*I and *Xho*I sites of pBS(SK+) to give pBS-NS1-I.

DNA oligonucleotides used were as follows: GSP-I, 5′-dTCCTGATGTTGCT-GTAGACG-3′; GSP-II, 5′-dGCACGACTAGTATGATTTGC-3′; the 5′RACE anchor primer (GIBCO-BRL), 5′-dCUACUACUACUAGGCCACGCGTCGA CTAGTACGGGIIGGGIIGGGIIG-3′; NS1-I-5′, 5′-GCCAGCTGTCTAGAT CTGACCCTCGTCCCG-3′; NS1-I-3′, 5′-GCCAGCTGCTCGAGAAAGGTA TCAAGGCCTTTA-3′.

Viruses, cells, and extracts. Influenza A/WSN/33 (H1N1), A/Berkeley/1/68 (H2N2), A/Beijing/32/92 (H3N2), A/duck/Alberta/76 (H12N5), A/turkey/Oregon/71 (H7N5), and B/Lee/40 viruses were grown in the allantoic cavities of 10-day-old embryonated chicken eggs. Confluent monolayers of Madin-Darby canine kidney (MDCK) cells were infected with influenza viruses at a multiplicity of infection of 10 for 1 h in 35-mm-diameter dishes. Infection was continued at 37°C (influenza A viruses) or 35°C (influenza B/Lee/40) for 5 h in minimal essential medium (MEM) supplemented with 0.1% bovine serum albumin (BSA). Cells were labeled with 100 μCi of [^{35}S]methionine and [^{35}S]cysteine (ICN) per dish for 1 h in Met$^-$ Cys$^-$ MEM. Cells were washed and scraped into ice-cold phosphate-buffered saline (PBS). Cells from one dish were lysed in 500 μl of NET-N buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 150 mM NaCl, 0.05% Nonidet P-40 [NP-40]); to disrupt nuclei, lysates were sonicated for two 30-s pulses in a Raytheon sonicator at a setting of 1 A. Lysates were clarified by centrifugation for 10 min at 20,000 rpm in a TL100.3 rotor, and the supernatants were used for precipitation of proteins. For immunoblot analysis of HeLa, 293, and WI-38 cells, confluent monolayers were lysed directly in sodium dodecyl sulfate (SDS) loading buffer as described elsewhere (20).

Northern (RNA) blot analysis. A 1-μg amount of HeLa cell poly(A) RNA was separated on a 1% agarose–formaldehyde gel, transferred to a nylon membrane (Nytran; Amersham), and UV cross-linked. A ^{32}P-labeled, NS1-I-specific probe corresponding to positions +794 to +1752 was hybridized to the RNA as described elsewhere (3).

Expression of GST–NS1-I fusion protein in *E. coli* and precipitation of viral proteins from cell extracts. NS1-I (amino acids 266 to 736) was expressed in *E. coli* BL26 as a GST (glutathione *S*-transferase)–NS1-I fusion protein with a predicted molecular mass of 77 kDa from pGEX-NS1-I. Production of GST–NS1-I was induced by using isopropyl-β-D-galactopyranoside essentially as described elsewhere (51). GST–NS1-I was adsorbed from bacterial lysates to glutathione-Sepharose beads (Pharmacia) as recommended by the manufacturer. Beads were washed three times with PBS to remove contaminating proteins. A 10-μl volume of glutathione-Sepharose beads coated with GST–NS1-I fusion protein was reacted with 100 μl of extract of virus-infected MDCK cells (see above) in 750 μl of HN-100 buffer (20 mM HEPES [*N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid] [pH 8.0], 100 mM NaCl, 0.5 mM dithiothreitol [DTT]) for 90 min at 4°C. The beads were washed three times with PBS–0.05% NP-40, and precipitated proteins were analyzed by SDS gel electrophoresis and autoradiography. In parallel reactions, viral proteins were immunoprecipitated from 50 μl of infected cell extracts by using 5 μl of anti-NS1 (α-NS1) or α-M1 antiserum and protein A-agarose as described elsewhere (20).

α-NS1-I antiserum and immunoblot analysis. A polyclonal antiserum against NS1-I was raised by immunization of a female rabbit with 300 μg of affinity-purified GST–NS1-I fusion protein in complete Freund's adjuvant followed by two booster injections with 200 μg in incomplete adjuvant at 3-week intervals. The specificity of the antiserum was confirmed by immunoblot analysis of GST–NS1-I expressed in bacterial lysates by standard procedures (20). Typically, the anti-NS1-I serum was used at a dilution of 1:1,000 in immunoblots. Immunofluorescence of NS1-I was performed with affinity-purified antibodies. Anti-GST–NS1-I serum was first depleted of GST-specific antibodies by adsorption to a GST-Sepharose column. NS1-I-specific antibodies were subsequently purified by affinity chromatography by using purified GST–NS1-I fusion protein immobilized on CNBr-activated Sepharose (Pharmacia) (20).

Precipitation of NS1-I proteins by GST-NS1 or α-NS1-I antiserum. GST-NS1 protein was expressed in *E. coli* BL26 transformed with pGEX-NS1 and adsorbed to glutathione-Sepharose (see above). NS1-I proteins were synthesized and labeled with [^{35}S]methionine in coupled 50-μl transcription-translation reactions (Promega-TNT) programmed with 1 μg of pBS-NS1-I or pET-NS1-I. The translation mixture was rotated in 750 μl of NET-100 with 10 μl of GST-NS1-coated glutathione-Sepharose beads for 2 h at 4°C. The beads were washed three times with PBS–0.05% NP-40, and precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. In parallel reactions, 5-μl volumes of α-NS1-I antiserum were incubated with translation reaction mixtures for 1 h at 0°C. Immune complexes were collected on protein A-Sepharose beads in NET-100 buffer as described elsewhere (20) and analyzed by SDS-polyacrylamide gel electrophoresis.

Scanning laser confocal microscopy. 293 cells were grown on glass coverslips to 50% confluency and transfected with the various plasmids by using DOTAP according to the manufacturer's instructions (Boehringer Mannheim). Forty-eight hours after transfection, the cells were fixed and permeabilized for 15 min in PBS containing 2.5% formaldehyde and 0.5% Triton X-100. The cells were stained with affinity-purified NS1-I-specific antibodies (1:100) and/or the NS1-specific monoclonal antibody IA7 (kindly provided by Jonathan Yewdell, National Institutes of Health) at a 1:100 dilution in PBS–3% BSA. Subsequently, the cells were washed and developed by using fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G or Texas red-conjugated goat anti-mouse immunoglobulin G. The samples were mounted with Mowiol 40-88 (Aldrich, Inc.) and analyzed by using a 63× objective and a Leica CLSM confocal imaging system.

Sequence analyses. Sequence comparison through the EMBL and GenBank databases was performed by using the FASTA, TFASTA, and Bestfit programs from the Genetics Computer Group software package (University of Wisconsin, Madison). In addition, the Blast server at the National Center for Biotechnology was used to identify cDNA fragments homologous to NS1-I.

RESULTS

Isolation of NS1-interacting factors. We used the yeast interaction trap system (19, 55) to identify cellular proteins that interact with the nonstructural protein NS1 of the influenza A/PR/8/34 virus. A constitutively expressed LexA-NS1 fusion protein was used as bait to screen a library in which HeLa cell cDNAs were conditionally expressed as translational fusions with an acidic transcription activation domain (19). Colonies were selected, in which both reporter genes, *LEU2* and *lacZ*, were activated by the cDNA-encoded proteins. We used this double selection scheme to increase the stringency for selection of NS1-interacting proteins, because candidates identified in an initial library screen, in which only the *LEU2* reporter gene was used, failed to transcriptionally activate the *lacZ* gene in subsequent genetic tests. The library plasmids were isolated from the selected clones.

The binding specificity of the encoded fusion proteins was tested by assaying the activation of a *lacZ* reporter gene on plasmid pSH18-34. Expression of β-galactosidase from this plasmid is transcriptionally controlled by LexA-specific operator sites. pLexA-NS1 did not activate the reporter when paired

with the acidic activation domain vector used to construct the library (data not shown). The isolated library plasmids were cotransformed with pLexA-NS1 or pRFHM1 into the yeast strain EGY40 harboring pSH18-34. pRFHM1 expresses a LexA-bicoid fusion protein and was used to rule out nonspecific interactions of library proteins with LexA fusion proteins (19). Expression of the acidic domain fusion proteins encoded on the library plasmids is controlled by a *GAL1* promoter and is therefore induced in the presence of galactose but repressed by glucose. The resulting strains were assayed for β-galactosidase activity on X-Gal plates containing galactose and not glucose. After screening 3.3×10^5 independent library transformants, we isolated three plasmids that induced galactose-specific activation of the *lacZ* reporter gene in combination with pLexA-NS1. The three plasmids were derived from different cellular cDNAs, since they did not share a common sequence.

Cloning and sequencing of NS1-I. One of the isolated plasmids, pK5, was analyzed further. It contained a cDNA insert of 1,781 bp with a partial open reading frame of 1,413 nucleotides followed by 368 nucleotides of a potentially untranslated region (Fig. 1). Northern blot analysis of HeLa cell poly(A) RNA by using a pK5-derived probe detected a single transcript of approximately 3.0 kb, suggesting that the plasmid insert represented an incomplete cDNA (Fig. 2). A 5′RACE procedure (15) was employed to obtain the 5′ end of the NS1-I cDNA. Four independent clones that differed only in length at the very 5′ end were sequenced. The longest RACE product extended the human cDNA sequence of pK5 896 nucleotides upstream, resulting in a cDNA of 2,678 bp (Fig. 1). The assembled sequence has one long open reading frame, which can potentially encode a protein of 736 amino acids with a predicted molecular mass of 79.6 kDa. This gene was termed NS1-I (for NS1 interactor). The putative ATG start codon is located 103 nucleotides downstream of the 5′ end and is in the context of a sequence consistent with its being a translational start (28). The cDNA terminated with an oligo(A) tract preceded by a consensus polyadenylation site 16 nucleotides upstream.

Comparison of NS1-I with sequences entered in the EMBL and GenBank databases by using the FASTA and TFASTA analysis programs showed that NS1-I is a human homolog of a 17β-EDH isolated from porcine endometrium (31). The two proteins are 84% identical at the amino acid level. During the preparation of the manuscript of this article, the independent cloning of a human homolog of the porcine 17β-EDH by hybridization to a porcine cDNA probe was reported (2). This homolog can dehydrogenate 17β-estradiol, justifying its designation as a 17β-HSD (2). Since only three amino acids in the NS1-I protein are different from the 17β-HSD sequence, we assume that the two proteins are allelic. In total, we noted 10 nucleotide positions in NS1-I cDNA which are different or missing in the 17β-HSD sequence. In addition, the NS1-I cDNA contains 55 additional nucleotides at the 5′ end in comparison with the 17β-HSD sequence and 3 more nucleotides at the 3′ terminus, which are followed by an oligo(A) tract. NS1-I cDNA also has homology with 22 human cDNA fragments, which were isolated as expressed sequence tags and were identified by a BLAST analysis through the NCBI database (24 November 1995) (fragments are between 134 and 556 bp in length). These cDNAs were generated from different tissues, including fetal liver, spleen, brain, breast, adipose, and adrenal tissues, indicating a broad expression pattern of NS1-I in the human body.

NS1-I protein features two conserved sequence motifs of the short-chain alcohol dehydrogenase family (41). Specifically, amino acids 15 to 22 (TGAGAGLG) are similar to the NAD cofactor-binding site and residues 164 to 168 (YSAAK) correspond to a motif in alcohol dehydrogenases which is suggested to participate in catalysis (7). We also note the presence of the tripeptide AKL at the carboxy terminus. Similar tripeptide motifs have been identified as targeting signals for import into microbodies (for a review, see reference 10). There is a considerable level of sequence identity between NS1-I and two multifunctional proteins isolated from two yeast species, fox-2 of *Saccharomyces cerevisiae* (24) and hydratase-dehydrogenase-epimerase (hde) from *Candida tropicalis* (39). Both yeast enzymes are involved in the β-oxidation of fatty acids. fox-2 and hde are 46 and 45% identical to NS1-I, respectively. Remarkably, there is a contiguous stretch of 20 amino acids in the sequence of hde which are identical to positions 502 to 521 of NS1-I. This motif is also found in the fox-2 protein of *S. cerevisiae*. Specific functions have not been assigned to this region of the two yeast proteins.

NS1-I binds NS1 protein from extracts of influenza virus-infected cells. We employed a coprecipitation assay to confirm the physical interaction between NS1-I protein and NS1 expressed in influenza virus-infected cells. The partial NS1-I cDNA isolated in the interaction trap screen was used to construct a GST–NS1-I fusion gene which was expressed in *E. coli*. GST–NS1-I fusion protein from bacterial lysates was adsorbed to the affinity matrix glutathione-Sepharose and purified from contaminating bacterial proteins. The immobilized fusion protein was used to bind and precipitate ^{35}S-labeled proteins from extracts of MDCK cells infected with human influenza A/WSN/33 virus (Fig. 3). The precipitated proteins were analyzed by SDS gel electrophoresis and visualized by fluorography. The NS1 protein of the influenza A/WSN/33 strain is 98% identical to its A/PR/8/34 counterpart employed in the yeast interaction screen. Aliquots of the same extract were used in parallel reactions to immunoprecipitate influenza virus proteins NS1 and M1 (Fig. 3, lanes α-NS1 and α-M1). Figure 3 demonstrates that GST–NS1-I efficiently precipitated a protein which comigrates with immunoprecipitated NS1 protein from the infected cell extract (compare second and third lanes). This interaction was specific for NS1-I, since no proteins were detected in precipitates with GST alone (Fig. 3, sixth lane). NS1 could not be precipitated from extracts containing SDS (data not shown). Proteins from mock-infected cells were not precipitated by GST–NS1-I (Fig. 3, eighth lane), showing that a virus-encoded protein was recognized by the fusion protein. This experiment confirmed that NS1-I binds specifically to the influenza A virus NS1 protein.

Immunoblot analysis of NS1-I. We generated rabbit antiserum against NS1-I in order to characterize the endogenous cellular NS1-I protein. Extracts of human cell lines derived from lung tissue (WI-38 cells), kidney tissue (293 cells), and cervical epithelium (HeLa cells) were prepared by direct lysis in SDS sample buffer to minimize proteolysis. Total cell proteins were analyzed by immunoblotting with α-NS1-I antiserum. For all three cell lines, the predominant protein band which was detected had an apparent molecular mass of 55 kDa (Fig. 4). This was a surprising result, since the isolated NS1-I cDNA had one long open reading frame with the potential to encode a 79.6-kDa protein and Northern blot analysis of HeLa cell poly(A) RNA had shown the presence of a single corresponding 3-kb NS1-I mRNA species. This finding suggests that NS1-I may be similar to its porcine homolog in that it is posttranslationally processed: the mature 17β-EDH in porcine endometrium is a 32-kDa protein which is derived from the N terminus of an 80-kDa precursor, as suggested by biochemical and genetic evidence (31, 32). The fate of the C-terminal cleavage product has not been reported. By analogy, the 55-

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                                        -103  TCTGACCCTCGTCCCGCCCCCGC  -80

-81  CATTCGCCGCCTCCTCCTGTCCCGCAGTCGGCGTCCAGCGGCTCTGCTTGTTCGTGTGTGTGTCGTTGCAGGCCTTATTC   -1

1    ATGGGCTCACCGCTGAGGTTCGACGGGCGGGTGGTACTGGTCACCGGCGCGGGGGCAGGATTGGGCCGAGCCTATGCCCT   80
      M  G  S  P  L  R  F  D  G  R  V  V  L  V  T  G  A  G  A  G  L  G  R  A  Y  A  L    27

81   GGCTTTTGCAGAAAGAGGAGCGTTAGTTGTTGTGAATGATTTGGGAGGGGACTTCAAAGGAGTTGGTAAAGGCTCCTTAG  160
       A  F  A  E  R  G  A  L  V  V  V  N  D  L  G  G  D  F  K  G  V  G  K  G  S  L    53

161  CTGCTGATAAGGTTGTTGAAGAAATAAGAAGGAGAGGTGGAAAAGCAGTGGCCAACTATGATTCAGTGGAAGAAGGAGAG  240
     A  A  D  K  V  V  E  E  I  R  R  R  G  G  K  A  V  A  N  Y  D  S  V  E  E  G  E    80

241  AAGGTTGTGAAGACAGCCCTGGATGCTTTTGGAAGAATAGATGTTGTGGTCAACAATGCTGGAATTCTGAGGGATCATTC  320
      K  V  V  K  T  A  L  D  A  F  G  R  I  D  V  V  V  N  N  A  G  I  L  R  D  H  S   107

321  CTTTGCTAGGATAAGTGATGAAGACTGGGATATAATCCACAGAGTTCATTTGCGGGGTTCATTCCAAGTGACACGGGCAG  400
       F  A  R  I  S  D  E  D  W  D  I  I  H  R  V  H  L  R  G  S  F  Q  V  T  R  A    133

401  CATGGGAACACATGAAGAAACAGAAGTATGGAAGGATTATTATGACTTCATCAGCTTCAGGAATATATGGCAACTTTGGC  480
     A  W  E  H  M  K  K  Q  K  Y  G  R  I  I  M  T  S  S  A  S  G  I  Y  G  N  F  G   160

481  CAGGCCAATTATAGTGCTGCAAAGTTGGGTCTTCTGGGCCTTGCAAATTCTCTTGCAATTGAAGGCAGGAAAAGCAACAT  560
      Q  A  N  Y  S  A  A  K  L  G  L  L  G  L  A  N  S  L  A  I  E  G  R  K  S  N  I   187

561  TCATTGTAACACCATTGCTCCTAATGCGGGATCACGGATGACTCAGACAGTTATGCCTGAAGATCTTGTGGAAGCCTTGA  640
       H  C  N  T  I  A  P  N  A  G  S  R  M  T  Q  T  V  M  P  E  D  L  V  E  A  L    213

641  AGCCAGAGTATGTGGCACCTCTTGTCCTTTGGCTTTGTCACGAGAGTTGTGAGGAGAATGGTGGCTTGTTTGAGGTTGGA  720
     K  P  E  Y  V  A  P  L  V  L  W  L  C  H  E  S  C  E  E  N  G  G  L  F  E  V  G   240
                                                                              |->
721  GCAGGATGGATTGGAAAATTACGCTGGGAGCGGACTCTTGGAGCTATTGTAAGACAAAAGAATCACCCAATGACTCCTGA  800
      A  G  W  I  G  K  L  R  W  E  R  T  L  G  A  I  V  R  Q  K  N  H  P  M  T  P  E   267

801  GGCAGTCAAGGCTAACTGGAAGAAGATCTGTGACTTTGAGAATGCCAGCAAGCCTCAGAGTATCCAAGAATCAACTGGCA  880
       A  V  K  A  N  W  K  K  I  C  D  F  E  N  A  S  K  P  Q  S  I  Q  E  S  T  G    293

881  GTATAATTGAAGTTCTGAGTAAAATAGATTCAGAAGGAGGAGTTTCAGCAAATCATACTAGTCGTGCAACGTCTACAGCA  960
     S  I  I  E  V  L  S  K  I  D  S  E  G  G  V  S  A  N  H  T  S  R  A  T  S  T  A   320

961  ACATCAGGATTTGCTGGAGCTATTGGCCAGAAACTCCCTCCATTTTCTTATGCTTATACGGAACTGGAAGCTATTATGTA 1040
      T  S  G  F  A  G  A  I  G  Q  K  L  P  P  F  S  Y  A  Y  T  E  L  E  A  I  M  Y   347

1041 TGCCCTTGGAGTGGGAGCGTCAATCAAGGATCCAAAAGATTTGAAATTTATTTATGAAGGAAGTTCTGATTTCTCCTGTT 1120
       A  L  G  V  G  A  S  I  K  D  P  K  D  L  K  F  I  Y  E  G  S  S  D  F  S  C    373

1121 TGCCCACCTTCGGAGTTATCATAGGTCAGAAATCTATGATGGGTGGAGGATTAGCAGAAATTCCTGGACTTTCAATCAAC 1200
     L  P  T  F  G  V  I  I  G  Q  K  S  M  M  G  G  G  L  A  E  I  P  G  L  S  I  N   400

1201 TTTGCAAAGGTTCTTCATGGAGAGCAGTACTTAGAGTTATATAAACCACTTCCCAGAGCAGGAAAATTAAAATGTGAAGC 1280
      F  A  K  V  L  H  G  E  Q  Y  L  E  L  Y  K  P  L  P  R  A  G  K  L  K  C  E  A   427

1281 AGTTGTTGCTGATGTCCTAGATAAAGGATCCGGTGTAGTGATTATTATGGATGTCTATTCTTATTCTGAGAAGGAACTTA 1360
       V  V  A  D  V  L  D  K  G  S  G  V  V  I  I  M  D  V  Y  S  Y  S  E  K  E  L    453

1361 TATGCCACAATCAGTTCTCTCTCTTTCTTGTTGGCTCTGGAGGCTTTGGTGGAAAACGGACATCAGACAAAGTCAAGGTA 1440
     I  C  H  N  Q  F  S  L  F  L  V  G  S  G  G  F  G  G  K  R  T  S  D  K  V  K  V   480

1441 GCTGTAGCCATACCTAATAGACCTCCTGATGCTGTACTTACAGATACCACCTCTCTTAATCAGGCTGCTTTGTACCGCCT 1520
      A  V  A  I  P  N  R  P  P  D  A  V  L  T  D  T  T  S  L  N  Q  A  A  L  Y  R  L   507

1521 CAGTGGAGACCGGAATCCCTTACACATTGATCCTAACTTTGCTAGTCTAGCAGGTTTTGACAAGCCCATATTACATGGAT 1600
       S  G  D  R  N  P  L  H  I  D  P  N  F  A  S  L  A  G  F  D  K  P  I  L  H  G    533

1601 TATGTACATTTGGATTTTCTGCCAGGCGTGTGTTACAGCAGTTTGCAGATAATGATGTGTCAAGATTCAAGGCAGTTAAG 1680
     L  C  T  F  G  F  S  A  R  R  V  L  Q  Q  F  A  D  N  D  V  S  R  F  K  A  V  K   560

1681 GCTCGTTTTGCAAAACCAGTATATCCAGGACAAACTCTACAAACTGAGATGTGGAAGGAAGGAAACAGAATTCATTTTCA 1760
      A  R  F  A  K  P  V  Y  P  G  Q  T  L  Q  T  E  M  W  K  E  G  N  R  I  H  F  Q   587

1761 AACCAAGGTCCAAGAAACTGGAGACATTGTCATTTCAAATGCATATGTGGATCTTGCACCAACATCTGGTACTTCAGCTA 1840
       T  K  V  Q  E  T  G  D  I  V  I  S  N  A  Y  V  D  L  A  P  T  S  G  T  S  A    613

1841 AGACACCCTCTGAGGGCGGGAAGCTTCAGAGTACCTTTGTATTTGAGGAAATAGGACGCCGCCTAAAGGATATTGGGCCT 1920
     K  T  P  S  E  G  G  K  L  Q  S  T  F  V  F  E  E  I  G  R  R  L  K  D  I  G  P   640

1941 GAGGTGGTGAAGAAAGTAAATGCTGTATTTGAGTGGCATATAACCAAAGGCGGAAATATTGGGGCTAAGTGGACTATTGA 2000
      E  V  V  K  K  V  N  A  V  F  E  W  H  I  T  K  G  G  N  I  G  A  K  W  T  I  D   667

2001 CCTGAAAAGTGGTTCTGGAAAAGTGTACCAAGGCCCTGCAAAAGGTGCTGCTGATACAACAATCATACTTTCAGATGAAG 2080
       L  K  S  G  S  G  K  V  Y  Q  G  P  A  K  G  A  A  D  T  T  I  I  L  S  D  E    693

2081 ATTTCATGGAGGTGGTCCTGGGCAAGCTTGACCCTCAGAAGGCATTCTTTAGTGGCAGGCTGAAGGCCAGAGGGAACATC 2160
     D  F  M  E  V  V  L  G  K  L  D  P  Q  K  A  F  F  S  G  R  L  K  A  R  G  N  I   720

2161 ATGCTGAGCCAGAAACTTCAGATGATTCTTAAAGACTACGCCAAGCTCTGAAGGGCACACTACACTATTAATAAAAATGG 2240
      M  L  S  Q  K  L  Q  M  I  L  K  D  Y  A  K  L  *                                 736

2241 AATCATTAAATACTCTCTTCACCCAAATATGCTTGATTATTCTGCAAAAGTGATTAGAACTAAGATGCAGGGGAAATTGC 2320

2321 TTAACATTTTCAGATATCAGATAACTGCAGATTTTCATTTTCTACTAATTTTTCATGTATCATTATTTTTACAAGGAACT 2400

2401 ATATATAAGCTAGCACATAATTATCCTTCTGTTCTTAGATCTGTATCTTCATAATAAAAAAATTTGCCCAAGTCCTGTT 2480

2481 TCCTTAGAATTTGTGATAGCATTGATAAGTTGAAAGGAAAATTAAATCAATAAAGGCCTTTGATACCTTTAAAAAAAAAA 2560

2561 AAAAAAAAAAAAAAA                                                                  2575
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kDa protein detected by our antiserum may also result from processing of a precursor protein. However, the size of the 55-kDa NS1-I is very different from that of its porcine homolog.

A mutant NS1-I protein alters the intracellular localization of the viral NS1 protein. The immunofluorescence analysis of the cellular NS1-I protein in human 293, HeLa, and WI-38 cells showed a punctate staining pattern in the cytoplasm (data not shown). Similar patterns have previously been observed for peroxisomal proteins (44). Since NS1-I carries a C-terminal AKL tripeptide that resembles a peroxisomal targeting signal, it is likely that NS1-I is associated with peroxisomes. Removal of the AKL tripeptide caused NS1-I to localize to the entire cytoplasm (Fig. 5A, large arrows). The influenza virus NS1 protein localized to the nucleus of transfected cells (Fig. 5B, small arrows). Coexpression of the transfected mutant NS1-I protein resulted in the relocalization of NS1 to the cytoplasm (large arrows in Fig. 5B); in these cells, cytoplasmic NS1 colocalized with the mutant NS1-I protein as determined by confocal microscopy. This experiment suggests that the two proteins can interact in human cells.

Coprecipitation of NS1-I by GST-NS1. We attempted to further analyze the interaction of NS1-I with NS1 proteins in influenza virus-infected cells by coimmunoprecipitation. Using a variety of detergent conditions, we were unable to solubilize NS1-I from human 293 cells and coimmunoprecipitate NS1. We believe that conditions which solubilize NS1-I either denature the proteins or disrupt the NS1-I–NS1 complex. We therefore decided to employ an in vitro approach to demonstrate the binding of NS1-I to NS1. Coupled transcription-translation reactions were used as a source for soluble NS1-I. Two plasmids were constructed as templates to program the rabbit reticulocyte lysates with NS1-I-specific RNAs synthesized by T7 RNA polymerase: pBS-NS1-I, which encodes a full-length NS1-I protein of 79.6 kDa, and pET-NS1-I, which expresses a protein in which amino acids 266 to 736 of NS1-I are fused to 35 plasmid-encoded amino acids, resulting in a product with an expected molecular mass of 55 kDa. This truncated NS1-I protein lacks the conserved dehydrogenase motifs close to the N terminus. NS1 was expressed as a GST fusion protein in *E. coli* and immobilized on glutathione-Sepharose. As a control, we loaded glutathione-Sepharose beads with GST. The GST- or GST-NS1-coated beads were used to precipitate NS1-I proteins synthesized in the reticulocyte lysates. The precipitated proteins were analyzed by gel electrophoresis (Fig. 6). We consistently observed the production of a 48-kDa protein band by the reticulocyte lysate that was not related to the NS1-I-encoding plasmids (Fig. 6, lanes T).

The truncated pET-NS1-I-derived protein was efficiently precipitated by GST-NS1 and by α-NS1-I antiserum bound to protein A-Sepharose (Fig. 6, pET-NS1-I, lanes GST-NS1 and α-NS1-I). GST alone did not precipitate either form of NS1-I (Fig. 6, lanes GST); this demonstrates specificity of the interaction for the NS1-I peptide. We found that full-length NS1-I also was precipitated by GST-NS1, although at a lower efficiency compared with that for the truncated version of NS1-I (Fig. 6, pBS-NS1-I, lane GST-NS1).

NS1-I binds NS1 proteins from divergent influenza A and B viruses. Mutations accumulate in the NS1 genes of human influenza A viruses at a steady rate over time (6). Thus, the length of time between the isolations of two human strains is directly proportional to the number of amino acid differences in their respective NS1 proteins (6). If the NS1–NS1-I interaction is important for the life cycle of influenza viruses, then it is expected that all NS1 proteins retain the ability to bind to this protein. Consequently, the binding of NS1-I to NS1 proteins from other influenza A viruses should be detectable despite the considerable variation in the NS1 alleles (4, 35). We therefore examined the NS1–NS1-I interaction by a coprecipitation assay using extracts from cells infected with different influenza A virus strains.

We first examined the ability of GST–NS1-I fusion protein to bind to NS1 proteins from two recently isolated human influenza A virus strains, A/Berkeley/1/68 and A/Beijing/32/92. Figures 7A and B show that NS1 proteins from both strains were specifically precipitated (Fig. 7A and B, lanes GST–NS1-I). We reproducibly observed a low immunoprecipitation efficiency for NS1 protein from the Beijing strain with our α-NS1 antiserum. The NS1 proteins of the influenza A/Berkeley/1/68 and A/WSN/33 viruses are 90.8% identical, but the NS1 sequence of the A/Beijing/32/92 virus is not known. We then examined the question of whether the more divergent NS1 proteins of the avian influenza virus strains A/duck/Alberta/76 and A/turkey/Oregon/71 are also recognized by GST–NS1-I fusion protein. The NS1 proteins of these strains are 66.5 and 63.6% identical to that of the A/WSN/33 virus. Significantly, the NS1 of A/turkey/Oregon/71 is only 124 amino acids long and lacks most of the carboxy-terminal half of the other NS1 proteins; these proteins are 202 to 237 amino acids in length (38). Nevertheless, precipitation of a protein band comigrating with the NS1 protein from both strains was observed (Fig. 7C and D, lanes GST–NS1-I). The NS1 and M1 proteins of A/duck/Alberta/76 could not be separated by the gel system used. We reproducibly detected significant amounts of nucleoprotein in the GST–NS1-I precipitates of these avian strains. The reason for this finding is not known. Finally, the coprecipitation assay was used to test the interaction of NS1 from the human influenza B/Lee/40 virus with GST–NS1-I fusion protein. Surprisingly, GST–NS1-I specifically precipitated the influenza B virus NS1 protein, although it has only 20.6% amino acid sequence identity with the NS1 of the A/WSN/33 virus (Fig. 7E, lane GST–NS1-I).

DISCUSSION

We have identified a human protein, NS1-I, using the influenza A virus NS1 protein as bait in the yeast interaction trap system. The specificity of NS1-I binding to NS1 was confirmed in two independent assays. First, we showed that NS1-I activated a LexA-dependent *lacZ* reporter gene in the yeast two-hybrid system in conjunction with a LexA-NS1 fusion protein, but not with an unrelated LexA-bicoid fusion protein. This finding rules out the possibility that NS1-I interacts with the DNA-binding domain of LexA (5). Secondly, we demonstrated the specific interaction of NS1-I as a GST fusion protein with

FIG. 1. Nucleotide sequence of NS1-I cDNA and derived amino acid sequence of the encoded protein. The sequence of 2,678 bp was determined by dideoxy sequencing of two overlapping clones. The first one, pK5, contained the HeLa cell cDNA from the isolated yeast library plasmid from nucleotide position +794 (marked by an arrow) to +2575. The second clone, pGEM-K5-5′RACE, resulted from the 5′RACE procedure used to obtain cDNA derived from the 5′ terminus of NS1-I and contains positions −103 to +947. The open reading frame encodes 736 amino acids. The putative polyadenylation signal at nucleotides +2529 to +2534 is marked by a line.

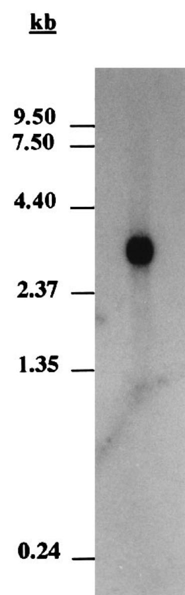

FIG. 2. Northern blot analysis of HeLa cell poly(A) RNA with an NS1-I-specific probe. A 1-μg amount of HeLa cell poly(A) RNA was separated on a formaldehyde-agarose gel and transferred to a nylon membrane. A ^{32}P-labeled probe derived from the cDNA insert of the library plasmid pK5 was used to detect NS1-I mRNA. The positions of RNA size markers are indicated.

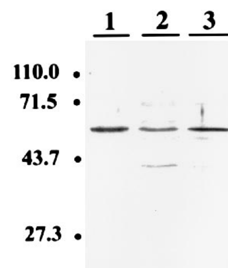

FIG. 4. Immunoblot analysis of NS1-I. Confluent monolayers of WI-38 (lane 1), HeLa (lane 2), and 293 (lane 3) cells were lysed directly in SDS sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with antiserum raised against affinity-purified GST–NS1-I protein. Numbers on the left indicate molecular mass in kilodaltons.

the NS1 protein expressed in influenza virus-infected cells using a glutathione-Sepharose-based coprecipitation assay. The reciprocal precipitation of soluble NS1-I protein synthesized in vitro by GST-NS1 fusion protein was also shown. Finally, a mutant NS1-I protein caused the redistribution of NS1 from the nucleus to the cytoplasm, suggesting that these proteins interact in vivo.

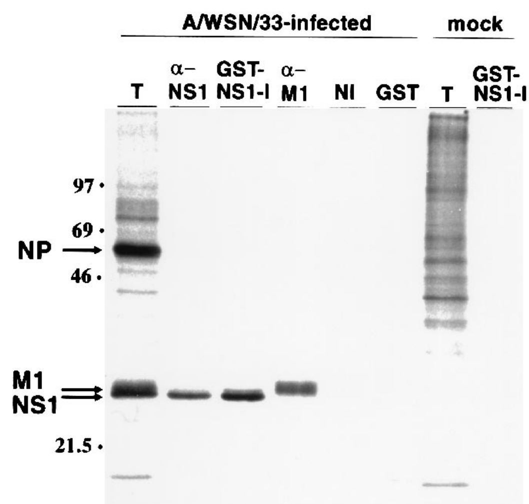

FIG. 3. Coprecipitation of NS1 protein from extracts of influenza A virus-infected MDCK cells by GST–NS1-I. Monolayers of MDCK cells were infected with influenza A/WSN/33 virus at a multiplicity of infection of 10 or were mock infected. Cells were labeled with [^{35}S]methionine and [^{35}S]cysteine from 5 to 6 h postinfection. Proteins were extracted and used for binding to glutathione-Sepharose beads coated with GST–NS1-I or GST protein (lanes GST–NS1-I and GST). As controls, extracts were used in immunoprecipitations with α-NS1, α-M1, or nonimmune serum (lanes α-NS1, α-M1, and NI). Proteins were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Aliquots of the total extracts corresponding to 10% used for the glutathione-agarose precipitations are shown (T). The positions of virus proteins and molecular weight markers are indicated on the left.

NS1-I appears to be allelic or identical to a human homolog of the precursor protein of porcine 17β-EDH (31). This human protein was independently cloned by the use of a porcine cDNA probe and shown to be a functional 17β-HSD since it can convert 17β-estradiol to estrone (2). It has yet to be determined if this is the main reaction catalyzed in vivo and whether 17β-estradiol is the normal physiological substrate of this protein. The NS1-I protein is 84% identical to porcine 17β-EDH at the amino acid level. NS1-I also has homology to two multifunctional yeast enzymes, fox-2 of *S. cerevisiae* and hde from *C. tropicalis*, which catalyze reactions of the peroxisomal β-oxidation pathway of fatty acids (24, 39). Thus, it is likely that NS1-I has hydroxysteroid dehydrogenase activity, but additional functions of NS1-I or its cleavage products (see below) cannot be excluded.

NS1-I appears to be processed in human cells. NS1-I cDNA encodes a 79.6-kDa protein, and only one mRNA species of 3.0 kb is detectable by Northern blot analysis in HeLa cells. However, we find that NS1-I exists predominantly as a protein with an apparent molecular mass of 55 kDa in human cell lines derived from lung tissue (WI-38), kidney tissue (293), and cervical epithelium (HeLa). This suggests that NS1-I is similar to its porcine homolog in that it is posttranslationally processed. Extensive biochemical and genetic analyses have indicated that the mature 17β-EDH isolated from porcine endometrium is a 32-kDa protein derived from the N terminus of an 80-kDa precursor protein (1, 31, 32). The Ala-Ala-Pro sequence at positions 320 to 322 of the porcine 17β-EDH precursor has been proposed to be a proteolytic processing site since it corresponds to a conserved cleavage motif within mammalian peroxisomal proteins (32). Both the 80- and the 32-kDa forms of the 17β-EDH can be detected in porcine extracts by polyclonal and monoclonal antibodies (1). The fate and possible function of the cleavage product corresponding to the C-terminal part of the precursor protein have not been reported. Significantly, the NS1-I protein does not contain the putative processing site of the porcine 17β-EDH precursor. Thus, it is likely that processing of the human NS1-I follows a different pathway.

Our rabbit antiserum that reacts with the 55-kDa protein was raised against the C-terminal amino acids 266 to 736 of NS1-I. Therefore, the 55-kDa protein detected by the NS1-I-specific antibodies can in principle be derived from either the N or the C terminus of the full-length NS1-I protein. The precipitation results suggest that the first 265 amino acids at

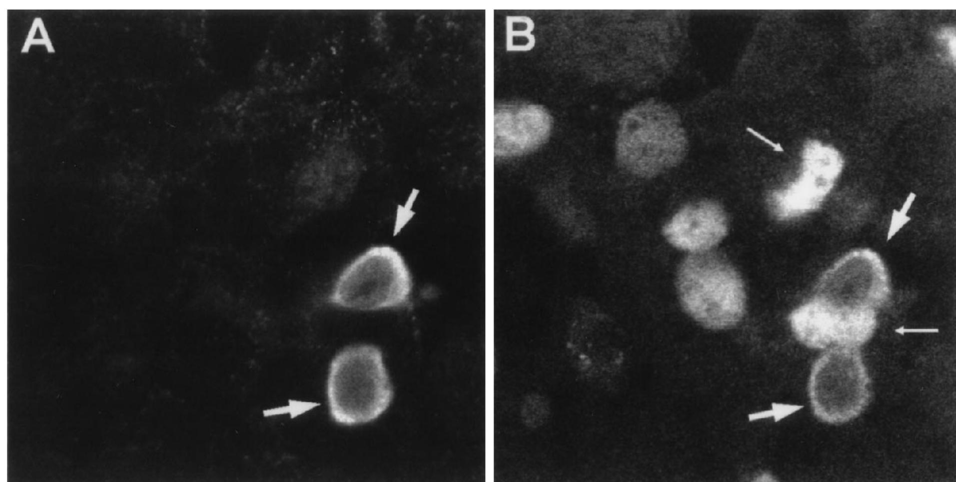

FIG. 5. Intracellular colocalization of NS1 and mutant NS1-I proteins by confocal microscopy. 293 cells were transfected with the expression vectors pcDNA3-NS1-I-AKL and pcDNA3-NS1. 48 h after transfection, the cells were analyzed for expression of NS1-I and NS1 by confocal microscopy with anti-NS1-I (A) or anti-NS1 (B) antibodies. Panels A and B show the same field representing NS1-I- and NS1-specific staining of cells. Large arrows indicate cells which coexpress NS1 and mutant NS1-I proteins. Small arrows indicate cells expressing the NS1 protein alone.

the N terminus of NS1-I are not required for binding to NS1. Thus, the NS1-binding domain in NS1-I appears to be located outside the conserved dehydrogenase motifs close to the N terminus. However, at this time it is not clear whether NS1 binds to NS1-I proteins (cleavage products) that retain the hydroxysteroid dehydrogenase function.

Highly divergent NS1 proteins can bind to NS1-I. In general, the evolution of proteins is constrained by their functions. NS1 proteins of influenza A viruses display extensive sequence variations (35). Yet it is likely that protein function is conserved among NS1 proteins which differ greatly in their amino acid sequences. Thus, we asked whether NS1 proteins derived from different influenza viruses had retained the ability to interact with NS1-I. By coprecipitation analysis of extracts from influenza virus-infected cells, we found that NS1-I binds to the NS1 proteins of the five different virulent influenza A virus strains tested. This finding indicates that an interactive domain is present on all the NS1 proteins, although they are more than 30% divergent. Significantly, the short NS1 protein of the influenza A/turkey/Oregon/71 virus also bound to the NS1-I protein. The NS1 protein of this avian virus strain is only 124 amino acids long and is truncated at its C terminus relative to other NS1 proteins (38). Thus, the NS1-I-binding domain appears to be located within the N-terminal half of the NS1 proteins. A deletion mutation in the corresponding part of the NS1 gene has been found to confer a host range-dependent temperature-sensitive phenotype (52). Experiments to determine if this phenotype is caused by a defect in binding to a cellular protein(s) are under way.

Although there is only a low level of identity between the amino acid sequences of the NS1 proteins of influenza A and B viruses (approximately 20%), we also demonstrated an interaction between NS1-I and the NS1 protein of an influenza B virus. A comparison of the influenza A and B virus NS1 proteins does not reveal a highly conserved stretch of amino acids (data not shown). Although specific experiments addressing the functional role of NS1 proteins of influenza B viruses are lacking, these NS1 proteins are considered to have functions similar to those of their influenza A virus counterparts (30). We suggest that the ability of the NS1-I protein to bind to the divergent NS1 proteins of human influenza A and B viruses reflects the fact that this cellular protein plays an important role in the viral life cycle.

What is the role of the NS1–NS1-I interaction during influenza virus infections? The precise role of NS1-I and its interaction with NS1 proteins during influenza virus infection clearly remain to be determined. The NS1 protein has been shown to interfere with several cellular pathways, including mRNA splicing (33), nuclear-cytoplasmic transport of poly(A) RNA (14), and translation (11, 34). In principle, NS1-I could therefore be involved in any of these pathways, or other steps of cell metabolism. The binding of NS1 proteins to NS1-I in infected cells might interfere with normal NS1-I function and

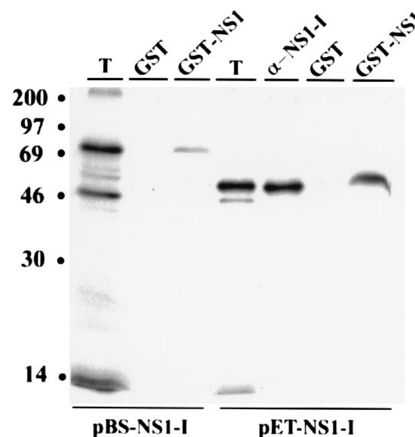

FIG. 6. Precipitation of radiolabeled NS1-I proteins by GST-NS1. Full-length NS1-I protein or an N-terminally truncated version containing amino acids 266 to 736 was synthesized in coupled transcription-translation reactions programmed with plasmid pBS-NS1-I or pET-NS1-I, respectively. Aliquots of the translation reaction mixtures corresponding to 50% (pBS-NS1-I, lane T) or 20% (pET-NS1-I, lane T) of the input used for the precipitations were separated in parallel. The NS1-I proteins were precipitated with glutathione-Sepharose beads coated with GST-NS1 or GST (lanes GST-NS1 and GST) and analyzed by SDS-polyacrylamide gel electrophoresis. For a control, protein encoded by pET-NS1-I was immunoprecipitated by using α-NS1-I antibodies and protein A-Sepharose. The positions and sizes (in kilodaltons) of marker proteins are indicated on the left.

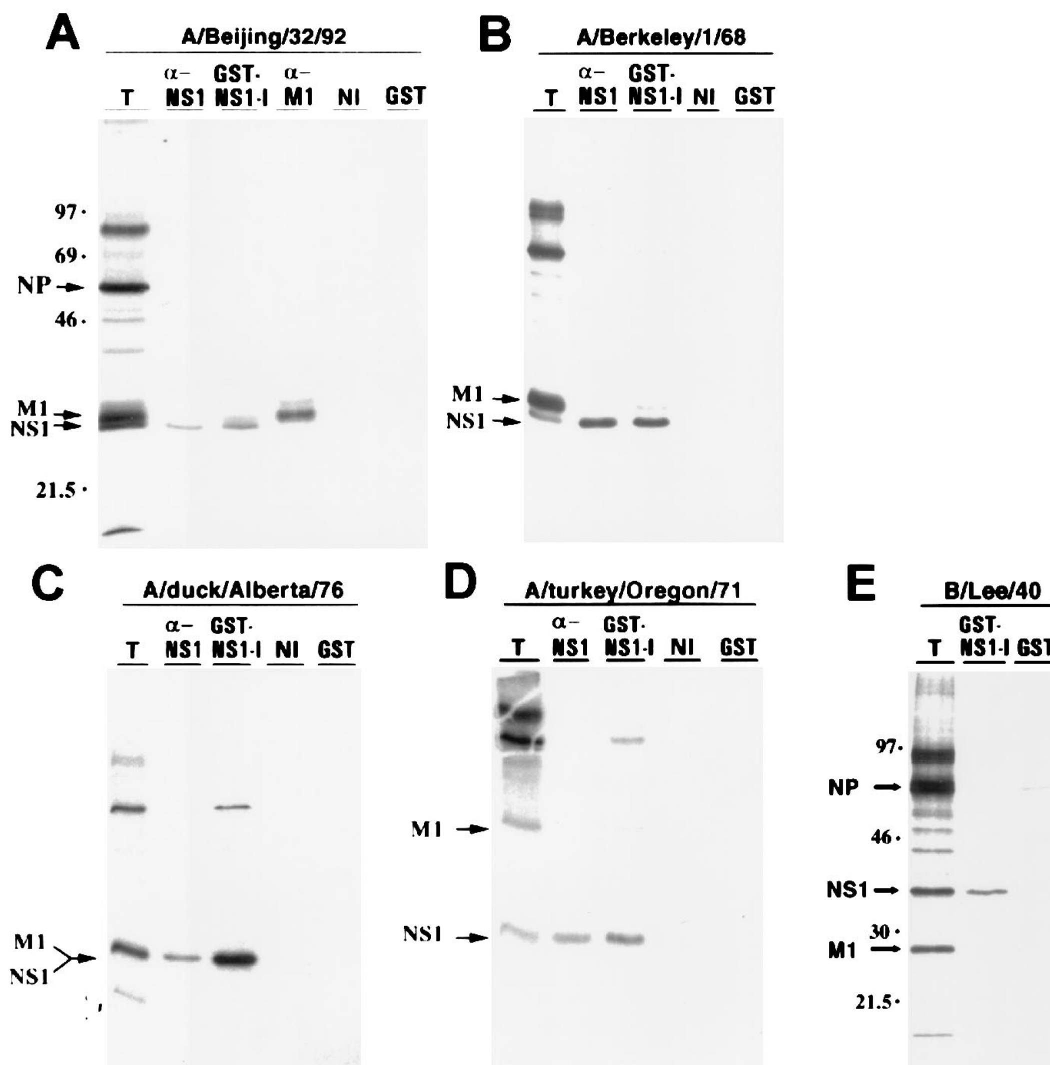

FIG. 7. GST–NS1-I coprecipitates NS1 proteins of influenza A and B virus strains. Extracts of ^{35}S-labeled MDCK cells infected with the influenza A/Beijing/32/92 (A), A/Berkeley/1/68 (B), A/duck/Alberta/76 (C), A/turkey/Oregon/71 (D), and B/Lee/40 (E) viruses were prepared and used in precipitations of viral proteins by glutathione-Sepharose beads coated with GST–NS1-I (lane GST-NS1-I) or GST protein (lane GST) as described in the legend to Fig. 3. In addition, viral proteins were immunoprecipitated with α-NS1, α-M1, or nonimmune serum (lanes α-NS1, α-M1, and NI). Analysis was done by SDS gel electrophoresis and fluorography. Aliquots of the total extracts corresponding to 10% (A and E) and 6.7% (B, C, and D) are shown (T). The positions of viral proteins are indicated on the left. Numbers on the left indicate molecular mass in kilodaltons.

thereby contribute to the changes in cellular gene expression observed during virus infection. The addition of GST–NS1-I fusion protein to nuclear extracts of HeLa cells did not change the splicing of a synthetic pre-mRNA and did not relieve the block of splicing imposed by added NS1 protein (53a). These results suggest that NS1-I participates in a process(es) different from RNA splicing. Since NS1-I appears to be localized to the cytoplasm, it may rather participate as a cofactor of cytoplasmic functions of NS1.

NS1-I appears to be allelic or identical to a functional 17β-HSD. In this context, an interesting finding involving possible roles of steroid dehydrogenases during viral infections is noteworthy: the vaccinia virus SalF7L gene has homology to the human 3β-HSD–Δ^5-Δ^4 isomerase (3β-HSD) (17) and was shown to contribute to the virulence of vaccinia virus strains in mice (37). 3β-HSD is a key cellular enzyme required for the synthesis of steroid hormones. Genes with homology to 3β-HSD have also been found to be conserved in other poxviruses, the fowlpox virus (47) and molluscum contagiosum (37a). The precise mechanism by which the SalF7L gene increases the virulence of vaccinia virus strains is not known. However, the SalF7L gene product encodes a functional 3β-HSD (37), which suggests that the alteration of intra- or extracellular steroid hormone levels may be favorable for viral multiplication in vivo. One interpretation of these results involves a possible impact of steroid hormones on the immune response of infected animals. For vaccinia virus, it has been proposed that steroid hormones synthesized through the expression of the SalF7L gene in infected cells may act to suppress antiviral functions of the immune system and thereby allow extended viral replication (37).

The influenza viruses encode many fewer proteins (10) than do poxviruses (approximately 200). Therefore, influenza viruses may have developed mechanisms which differ from those used by poxviruses to regulate hormone levels during viral infection. Instead of encoding biosynthetic enzymes, the influ-

enza viruses may use their small repertoire of proteins to alter the activity or intracellular location of cellular enzymes to achieve the same effects. Indeed, corticosteroids were shown under certain conditions to enhance virus yields (26, 49, 50). One speculation would be that influenza A and B viruses control steroid hormone levels via the interaction between the viral NS1 and the cellular NS1-I in infected cells.

We also examined the expression of viral proteins in influenza virus-infected cells transiently overexpressing a truncated NS1-I protein to determine whether high levels of NS1-I would affect the normal course of infection. Preliminary results indicate that overexpression of NS1-I in 293 cells reduces expression of influenza A virus proteins (data not shown). The establishment of cell lines stably overexpressing NS1-I should definitely help in future studies to characterize the function of the NS1–NS1-I interaction during influenza virus infection. In addition, a detailed mapping of the interacting domains of NS1 and NS1-I should reveal whether one or more of the temperature-sensitive mutations identified in the NS1 protein (36) can be correlated with a reduced ability to bind NS1-I or NS1-I cleavage products. We also plan to introduce into the NS1 gene of influenza A virus mutations which disrupt or weaken the NS1–NS1-I interaction to determine the consequences on viral multiplication and pathogenesis.

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