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NPI-1, the Human Homolog of SRP-1, Interacts with Influenza Virus Nucleoprotein

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We used the yeast interactive trap system to identify a cellular protein which interacts with the nucleoprotein of influenza A viruses. This protein, nucleoprotein interactor 1 (NPI-1) is the human homolog of the yeast protein SRP1. SRP1 was previously identified as a suppressor of temperature-sensitive RNA polymerase I mutations (R. Yano, M. Oakes, M. Yamaghishi, J. Dodd, and M. Nomura, *Mol. Cell. Biol.* 12, 5640–5651, 1992). A full-length cDNA clone of NPI-1 was generated from HeLa cell poly A+ RNA. The viral nucleoprotein, which had been partially purified from influenza A/PR/8/34 virus-infected embryonated eggs, could be coprecipitated from solution by glutathione agarose beads complexed with a bacterially expressed glutathione-*S*-transferase–NPI-1 fusion protein, confirming the results of the yeast genetic system. Antisera raised against NPI-1 identified a 60-kDa polypeptide from total cellular extracts of both HeLa and MDBK cells. The viral nucleoprotein was coimmunoprecipitated from influenza A/WSN/33 virus-infected MDBK cells by anti-NPI-1 sera, demonstrating an interaction of these two proteins in infected cells. Similarly, NPI-1 was coimmunoprecipitated from MDBK cells by anti-NP sera. These experiments suggest that NPI-1 plays a role during influenza virus replication.

INTRODUCTION

Influenza A virus is a negative strand RNA virus belonging to the orthomyxovirus family. The genome of the virus consists of eight segments and encodes 10 polypeptides. Little is known about host cell functions which contribute to the intracellular replication of influenza viruses, and cellular factors have not been characterized which directly interact with the viral proteins.

Experimental evidence generated in the laboratory of Scholtissek indicates that the nucleoprotein (NP) is a major determinant of species specificity of influenza viruses (Scholtissek *et al.*, 1985). Phylogenetic analysis divides NP genes into two families: one containing NPs predominantly of avian origin, and one containing those of human origin (Bean, 1984; Buckler-White and Murphy, 1986; Gammelin *et al.*, 1989; Scholtissek *et al.*, 1985). The human virus A/HK/1/68 and viruses having genetically related NPs cannot rescue mutants of the avian virus A/FPV/Rostock/1/34 with ts defects in the NP following double infection of chicken embryo fibroblasts (CEF) at 40° (Scholtissek *et al.*, 1985, 1978). However, the human viruses which failed to rescue the ts mutants on CEF cells were able to do so on Madin-Darby canine kidney (MDCK) cells (Scholtissek *et al.*, 1978). Additionally, A/HK/1/68 virus and A/FPV/Rostock/1/34 virus reassortants containing the A/HK/1/68 virus-derived NP replicate in MDBK cells but not in CEFs (Scholtissek *et al.*, 1978). The host-specific rescue of FPV ts mutants and the host restriction of A/HK/1/68 virus reassortants suggest that a factor(s) of host origin, which differs between mammalian and avian cells, is responsible for these phenomena, and that this factor may interact with the influenza A virus NP.

Replication and transcription of influenza virus RNA require four virus-encoded proteins: the NP and the three components of the viral RNA-dependent RNA polymerase, PB1, PB2, and PA (Huang *et al.*, 1990). The NP is the major structural component of the virion which interacts with genomic RNA, and is required for antitermination during RNA synthesis (Beaton and Krug, 1986). NP is also required for elongation of RNA chains (Shapiro and Krug, 1988) but not for initiation (Honda *et al.*, 1988). We used the interactive trap, a yeast-based genetic selection system, to screen a HeLa cell library for proteins which bind to the influenza virus NP. One protein was identified, NPI-1, which is the homolog of the yeast protein SRP1. SRP1 was originally characterized as a suppressor of RNA polymerase I mutations in yeast (Yano *et al.*, 1992, 1994).

MATERIALS AND METHODS

Yeast, bacteria, and plasmids

Yeast strain EGY48 (*Mata trp1 ura3 his3 LEU2::pLEX-Aop6-LEU2*), constructed by E. Golemis, plasmids pEG202, pSH18-34, and pRFHM1 and the HeLa cell cDNA library constructed in pJG4-5 by J. Gyuris were all generously provided by R. Brent (Harvard Medical School). pLexA-NP was constructed by subcloning the

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cDNA of influenza A/PR/8/34 NP as a LexA translational fusion gene into pEG202 (Fig. 1). Yeast strains constructed as part of these studies are described in Table 1. *Escherichia coli* MH3 (*trpC araD lacX hsdR galU galK*) and W31005 were kindly provided by H. Smith (Columbia University, NY) and J. Brosius (Mount Sinai), respectively.

Selection of NP interactors

An interactive trap selection was performed essentially as has been previously described (Gyuris *et al.*, 1993; Zervos *et al.*, 1993). Strain R100 was transformed by the HeLa cDNA library using the lithium acetate method (Ito *et al.*, 1983); 2×10^6 primary yeast transformants were selected on twelve 25×25-cm^2 his^-trp^--glucose plates, pooled, and stored at −70°. Library transformants were selected for leu^+ phenotype on $his^-trp^-leu^-$-galactose plates; the efficiency of plating was approximately 10^{-4} leu^+ colonies per $galactose^+$ colony. Plasmid DNA was isolated from leu^+ library transformants as described by Hoffman and Winston (1987) and introduced into MH3 cells by electroporation. Library plasmids were selected by plating the transformation mix on 1 × A + amp + glucose plates (Miller, 1972).

cDNAs were analyzed by checking specificity of interaction with the NP. Each isolated plasmid was introduced into strains R101 and R102. These strains harbor pSH18-34, a reporter plasmid encoding β-galactosidase with a GAL1 promoter transcriptionally controlled from upstream LexA binding sites. Strain R102 was used as a negative control for NP-specificity of cloned cDNAs. It contains pRFHM1, which encodes LexA fused to a transcriptionally inert fragment of the Drosophila melanogaster bicoid protein. β-Galactosidase activity was assayed on nitrocellulose replicas of the colonies by freeze fracturing the cells and incubating in buffer containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (Miller, 1972). Plasmids which conferred both a leu^+ and β-gal^+ phenotypes in the presence of pLexA-NP but not in the presence of pRFHM1 were saved for further study.

Cloning of the 5′ terminus of NPI-1

The 5′ terminus of NPI-1 was cloned by rapid amplification of cDNA ends (RACE) by the method of Frohman (Frohman, 1990; Frohman *et al.*, 1988). Reverse transcription of 1 μg of poly A+ HeLa cell RNA was performed using the NPI-1 specific oligonucleotide 5′GCAAAGCAGGAGAAACCAC3′. First strand cDNA was tailed with dCTP by terminal transferase. PCR amplification of the reverse transcription product was performed with the nested NPI-1 primer 5′GGGTCCATCTGATAGATATGAGAG3′ and the 5′RACE anchor primer 5′CUACUACUACUAGGCCACGCGTCGACTACTACGGGIIGGGIIGGGIIG3′ (Gibco/BRL). The PCR product was subcloned into pGEM-T (Promega) and was sequenced by standard protocols. 5′RACE products from three independent experiments were cloned and sequenced in order to avoid errors introduced by PCR.

Bacterial expression and purification of GST fusion proteins

The NPI-1 cDNA derived from a HeLa cDNA library was subcloned between the *Eco*RI and *Xho*I restriction endonuclease sites of the glutathione-*S*-transferase fusion vector pGEX-5X-1 (Pharmacia) to generate the plasmid pGST−NPI-1. Protein was induced from bacterial expression plasmids in W31005 cells with isopropyl-β-D-galactopyranoside according to standard protocols (Smith and Johnson, 1988). Bacteria were pelleted 4 hr after induction, washed in ice-cold phosphate-buffered saline (PBS), and resuspended in 1/100 culture volume PBS + 1% Triton X-100. Bacteria were lysed on ice with four 15-sec pulses in a Raytheon sonicator at an output setting of 1 A. Insoluble material was pelleted at 100,000 *g* for 15 min in a Beckman TL-100.3 rotor.

GST−NPI-1 and GST were purified from bacterial lysates on glutathione−agarose beads (Sigma Chemical Co.). Beads were swelled according to the manufacturer's instructions and equilibrated in PBS. Typical binding reactions were done in 500 μl of PBS/0.1% Triton X-100, and included 50 μl bacterial lysate and 10 μl of a 50% slurry of glutathione−agarose beads. Binding reactions were incubated for 5 min at room temperature on a rotating wheel. Beads were collected by centrifugation for 5 sec in a microfuge, and were washed three times in PBS.

NPI-1/NP binding assay

To assay binding of NP to GST−NPI-1/bead complexes typical reactions were performed in 500 μl of ice-cold PBS + 0.05% Nonidet P-40 and contained washed GST−NPI-1/bead complexes and 10 μg partially purified influenza virus polymerase and nucleoprotein preparations (Pol/NP). Virus was prepared from embryonated eggs infected by influenza A/PR/8/34 virus and Pol/NP preparations were purified as previously described (Enami *et al.*, 1990; Parvin *et al.*, 1989). NP was bound for 1 hr at 4° on a rotating wheel. Beads were collected by centrifugation for 5 sec in a microfuge, and were washed three times in PBS + 0.05% NP-40. Washed beads were resuspended in 50 μl SDS sample buffer (Sambrook *et al.*, 1989), boiled for 5 min, and pelleted in a microfuge; 10 μl of each supernatant was separated by electrophoresis on a 12.5% SDS−polyacrylamide gel. Gels were either stained with Coomassie blue or processed for immunoblot analysis. Nucleoprotein was detected by immunoblotting with the monoclonal antibody HT103.

Antisera and immunoblotting

Polyclonal rabbit antisera against NPI-1 was generated by immunization of a female NZY Rabbit (Buckshire

Farms) with 200 μg of purified GST–NPI-1 in complete Freund's adjuvant, followed by two boosts of 100 μg in incomplete Freund's adjuvant at 3-week intervals. The specificity of antisera was demonstrated by immunoblot analysis of GST–NPI-1 in bacterial lysates. Immunoblots were performed by standard methods (Harlow and Lane, 1988). Sera were used at a dilution of 1:1000.

Viruses and cells

Total cell lysates from HeLa and MDBK cells were generated by direct lysing of cells in SDS-sample buffer, followed by shearing of chromosomal DNA by passage through a 25-gauge syringe. Cytoplasmic extracts were generated by lysing cells in ice-cold NP-40 lysis buffer (10 m*M* Tris-Cl, pH 8.0; 100 m*M* NaCl; 1 m*M* EDTA; 1 m*M* DTT; 1% Nonidet P-40; 1 m*M* 4-(2-aminoethyl)benzenesulfonylfluoride-hydrochloride (Pefabloc)). After 10 min on ice nuclei were removed by centrifugation. Proteins were separated by SDS–PAGE, transferred to nitrocellulose, and visualized by immunoblotting.

To generate infected cell lysates containing metabolically labeled proteins 1×10^6 MDBK cells were infected with influenza A/WSN/33 virus at a multiplicity of 10 for 1 hr at 37°. Infection was allowed to proceed in DMEM + 0.1% BSA for 5 hr at which time cells were labeled with 100 μCi ^{35}S-methionine in MEM-met + 0.1% BSA for 1 hr. Label was chased for 1 hr in DMEM + 0.1% BSA at which time total cell lysates were prepared. Extracts prepared by resuspending infected cells in 500 μl ice-cold RIPA buffer (150 m*M* NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 m*M* Pefabloc, 50 m*M* Tris, pH 8.0) were clarified by centrifugation at 100,000 *g* in a Beckman TLA100.3 rotor. Unlabeled cell lysates were prepared from infected cells at 6 hr after infection in a similar manner.

Immune complexes were formed on ice by incubation of 50 μl cell lysates with either 20 μl preimmune or immune rabbit sera or 15 μg of HT103, a mouse monoclonal antibody against NP, in 500 μl RIPA buffer. Immune complexes were adsorbed to agarose-linked protein G beads (Boehringer) for 1 hr. Beads were collected by centrifugation, washed three times in RIPA buffer, and resuspended in SDS-sample buffer. A fraction of precipitated proteins was separated by SDS–PAGE and visualized by autoradiography (labeled proteins) or by immunoblotting (unlabeled proteins).

RESULTS

Isolation of NPI-1

We used the interactive trap to identify proteins which specifically interact with the influenza A virus nucleoprotein (NP). The interactive trap is one of several genetic systems recently developed which use the modular nature of transcription activators to detect protein:protein interactions (Chien *et al.*, 1991; Dalton and Treisman, 1992; Durfee *et al.*, 1993; Gyuris *et al.*, 1993; Vojtek *et al.*, 1993; Zervos *et al.*, 1993). The interactive trap consists of three components: (1) a reporter gene that has no basal transcription; (2) a fusion protein which contains a LexA DNA binding domain that is transcriptionally inert; and (3) proteins encoded by an expression library, which are expressed as fusion proteins containing an activation domain (Fig. 1A). Interaction of the LexA fusion protein and the fusion protein containing the activation domain will constitute a bimolecular transcriptional activator which, in this case, will confer the ability to grow on media lacking leucine (Gyuris *et al.*, 1993; Zervos *et al.*, 1993). In the absence of this interaction the leu2 gene is not transcribed.

The NP gene of influenza A/PR/8/34 virus was subcloned as a translational fusion gene with the LexA gene into pEG202 to generate pLexA-NP (Fig. 1B). Strain R100 (Table 1), which contains pLexA-NP, was transformed with a HeLa cell cDNA library constructed in pJG4-5. pJG4-5 contains an activation domain under control of a GAL1 promoter (Gyuris *et al.*, 1993). Library plasmids were rescued from 100 leu$^+$ colonies. Reproducibility of the interaction of the NP with the encoded library proteins was tested by transforming library plasmids into strain R101. Transformants were screened for galactose-dependent β-galactosidase activity and growth on media lacking leucine. Specificity for NP was analyzed by checking the ability of library plasmids to confer growth on leu$^-$ media and β-galactosidase activity in connection with a different LexA fusion plasmid, pRHMB1, encoding a fragment of the Drosophila melanogaster bicoid protein. Twenty-three library plasmids were confirmed to encode nucleoprotein-interactive proteins. Twelve identical 2.1-kbp clones encoded the carboxy terminal fragment of a protein termed nucleoprotein interactor 1 (NPI-1). Partial DNA sequencing showed that NPI-1 is the human homolog of the yeast SRP1 gene (see below). Other genes encoding proteins which interact with the viral NP will be presented in a future publication once they have been characterized.

Cloning and sequencing of the NPI-1 cDNA

The 2.1-kbp NPI-1 cDNA in pJG4-5 was sequenced by standard protocols. The 5′ cDNA terminus of the NPI-1 gene was cloned by 5′RACE. cDNAs from three independently derived NPI-1 5′RACE products were cloned and sequenced. Nucleotide and derived amino acid sequences of NPI-1 are shown in Fig. 2. The sequence reveals a 2940-bp cDNA which encodes a protein of 538 amino acids with a calculated molecular weight of 60,302 Da and a p*I* = 4.65. The carboxy terminal 276 amino acids were derived from the interactive trap library plas-

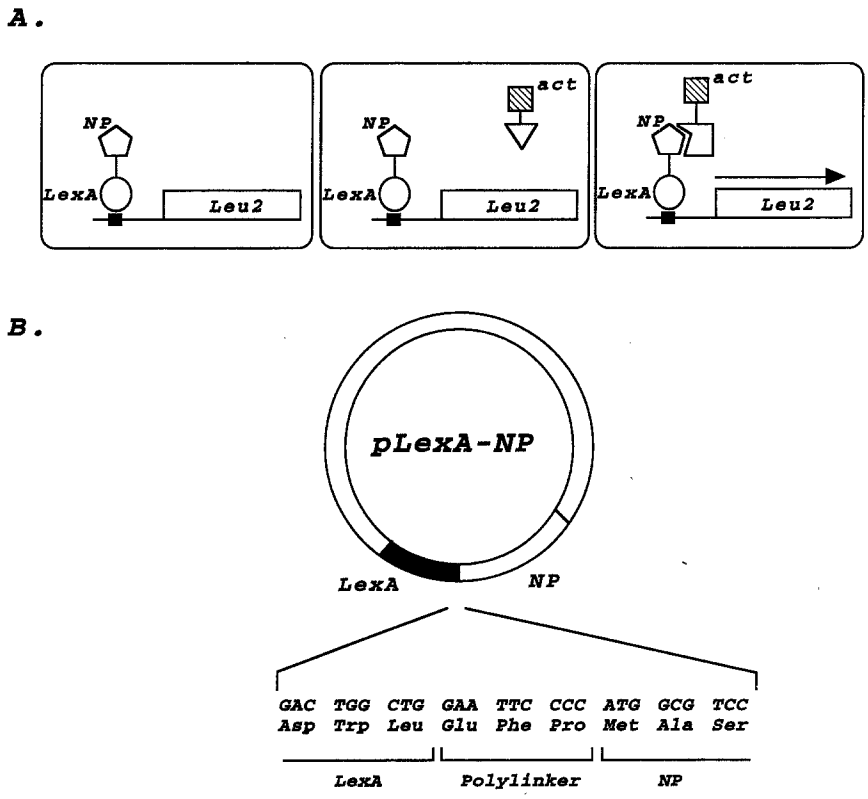

FIG. 1. (A) The interactive trap system. (Left) The R100 yeast strain contains the reporter gene LexAop-LEU2 and a transcriptionally inactive LexA-NP fusion protein. Library proteins are synthesized in R100 transformants in media containing galactose. (Middle) If the library protein does not interact with the LexA-NP fusion protein, then the LEU2 reporter gene is not transcribed. (Right) If the library fusion protein interacts with NP, then the LEU2 gene is transcriptionally active, and the cell forms a colony on leu$^-$ medium. (B) The pLexA-NP bait plasmid used in the interactive trap. The coding region of influenza A/PR/8/34 virus nucleoprotein was subcloned into the *Eco*RI and *Sal*I restriction sites of pEG202. This construction encodes a fusion protein which includes 202 amino acids of LexA and the entire coding region of NP (498 amino acids) separated by 3 amino acids encoded by polylinker sequences derived from the cloning process.

mid. The putative AUG initiator codon is 47 nucleotides from the 5′ terminus; it is in a favorable context for efficient translation initiation since there are A residues at the −3 and +4 position (Kozak, 1987, 1989).

Comparison of the deduced amino acid sequences in the GenBank and EMBL data bases using the FASTA and TFASTA programs (Deveraux *et al.*, 1984) demonstrated that NPI-1 is the human homolog of the *Saccharomyces cerevisiae* protein SRP1 (Yano *et al.*, 1992). SRP1 was cloned as an allele-specific suppressor of ts mutations in the zinc-binding domain of the A190 subunit of RNA polymerase I. The amino acid sequence is highly conserved between NPI-1 and SRP1: 53% identity and 71% similarity at the amino acid level. The amino terminus of NPI-1 has a potential nuclear localization signal (Chelsky *et al.*, 1989); amino acids 25 to 48 are rich in arginine, and contain a stretch of four consecutive arginines at amino acids 25 to 28. NPI-1, like SRP1, contains a series of eight consecutive ARM motifs, which are degenerate 42-amino acid protein subsequences originally identified in the Drosophila armadillo protein (Peifer *et al.*, 1994; Yano *et al.*, 1992) (Fig. 3; see below).

TABLE 1

YEAST STRAINS USED

Strains	Genotype
EGY48	*Mata trp1 ura3 his3 LEU2::pLEXAop6-LEU2*
R100	EGY48, pLexA-NP (TRP1)
R101	EGY48, pLexA-NP, pSH18-34 (HIS3)
R102	EGY48, pRFHM1 (TRP1), pSH18-34

NPI-1 binds to NP *in vitro*

In order to demonstrate that the NPI-1 binds to the viral NP, the NPI-1 cDNA fragment (amino acids 263 to 538) was subcloned into the bacterial expression vector pGEX-5X-1 yielding a glutathione *S*-transferase fusion gene. The expressed fusion protein was purified from bacterial lysates on glutathione–agarose beads. NP, which had been partially purified with the viral polymerase from influenza A/PR/8/34 virus, was specifically precipitated from solution by glutathione–agarose beads complexed with GST–NPI-1 (Fig. 4A, lane 3). NP was not

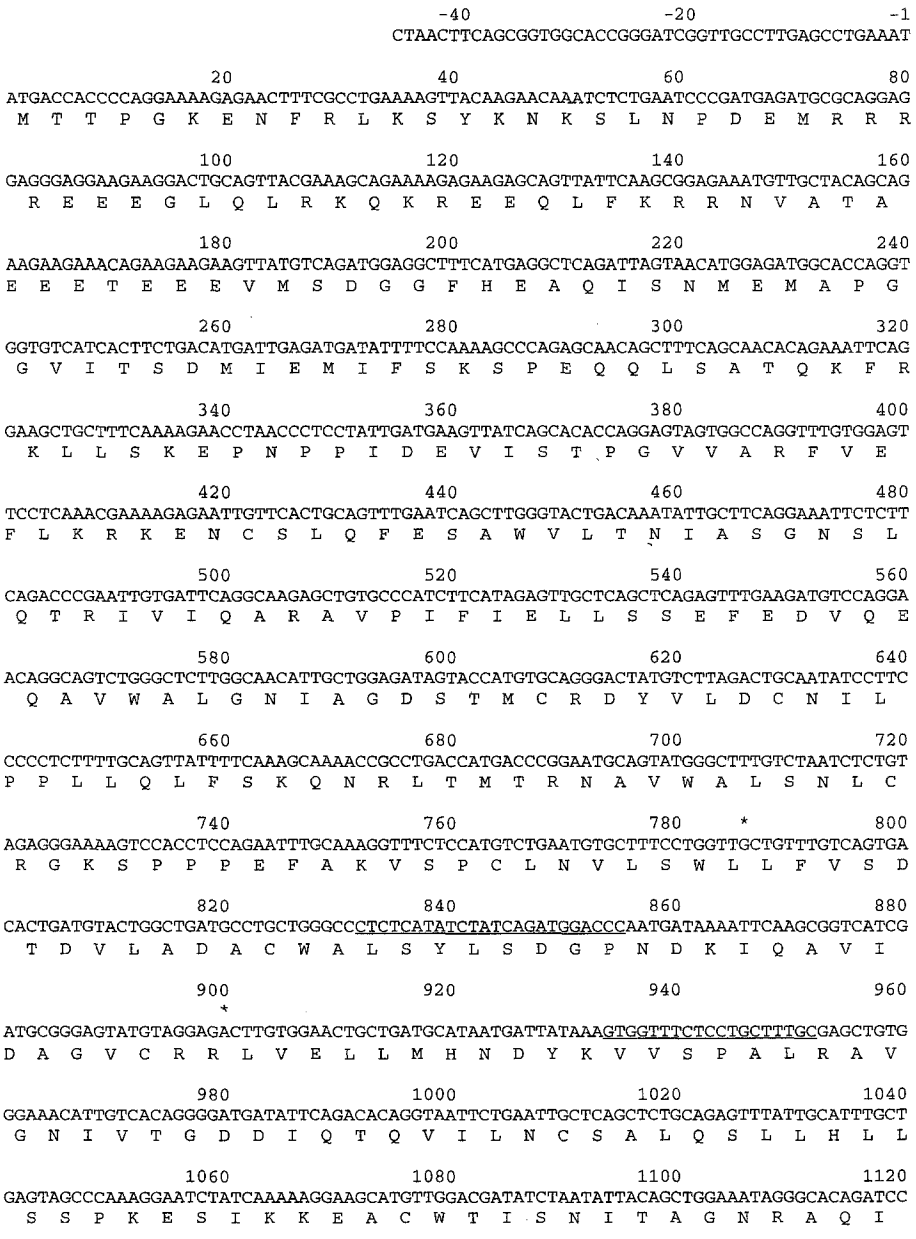

FIG. 2. Nucleotide sequence of NPI-1 cDNA and deduced protein sequence. The coding sequence starts at nucleotide +1 and ends at nucleotide 1614. The 5′ terminus of the library clone is indicated by an asterisk. Regions complementary to nested reverse transcription and 5′RACE primers are underlined.

precipitated from solution by glutathione beads complexed with GST (Fig. 4A, lane 6). The NP band migrates slightly slower than that of the GST–NPI-1 fusion protein; an enlargement of lanes 1 to 4 is shown in Fig. 4B in order to more clearly distinguish NP from NPI-1. The identity of this protein was confirmed by immunoblot analysis using the anti-NP monoclonal antibody HT103 (Fig. 4C, lane 5); in addition, no NP was detected by immunoblot analysis of proteins precipitated by GST alone (Fig. 4C, lane 3). Precipitation of NP by NPI-1 was by direct interaction of these two proteins and not by bridging through one of the viral polymerase proteins or another mammalian peptide since NP expressed in *E. coli* was also coprecipitated as a complex with GST-NPI-1 (data not shown).

Immunodetection of NPI-1 in cell extracts

Rabbit antisera raised against GST–NPI-1 were used to identify a polypeptide from total cellular extracts of both HeLa and MDBK cells with an apparent molecular weight of 60 kDa (Fig. 5). A lower amount of NPI-1 was present in the cytoplasmic fraction generated by lysis of cells in the presence of NP-40 than in the total cellular

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                1140                1160                1180                1200
AGACTGTGATAGATGCCAACATTTTCCCAGCCCTCATTAGTATTTTACAAACTGCTGAATTTCGGACAAGAAAAGAAGCA
Q  T  V  I  D  A  N  I  F  P  A  L  I  S  I  L  Q  T  A  E  F  R  T  R  K  E  A

                1220                1240                1260                1280
GCTTGGGCCATCACAAATGCAACTTCTGGAGGATCAGCTGAACAGATCAAGTACCTAGTAGAACTGGGTTGTATCAAGCC
 A  W  A  I  T  N  A  T  S  G  G  S  A  E  Q  I  K  Y  L  V  E  L  G  C  I  K  P

                1300                1320                1340                1360
GCTCTGTGATCTCCTCACGGTCATGGACTCTAAGATTGTACAGGTTGCCCTAAATGGCTTGGAAAATATCCTGAGGCTTG
  L  C  D  L  L  T  V  M  D  S  K  I  V  Q  V  A  L  N  G  L  E  N  I  L  R  L

                1380                1400                1420                1440
GAGAACAGGAAGCCAAAAGGAACGGCACTGGCATTAACCCTTACTGTGCTTTGATTGAAGAAGCTTATGGTCTGGATAAA
G  E  Q  E  A  K  R  N  G  T  G  I  N  P  Y  C  A  L  I  E  E  A  Y  G  L  D  K

                1460                1480                1500                1520
ATTGAGTTCTTACAGAGTCATGAAAACCAGGAGATCTACCAAAAGGCCTTTGATCTTATTGAGCATTACTTCGGGACCGA
 I  E  F  L  Q  S  H  E  N  Q  E  I  Y  Q  K  A  F  D  L  I  E  H  Y  F  G  T  E

                1540                1560                1580                1600
AGATGAAGACAGCAGCATTGCACCCCAGGTTGACCTTAACCAGCAGCAGTACATCTTCCAACAGTGTGAGGCTCCTATGG
  D  E  D  S  S  I  A  P  Q  V  D  L  N  Q  Q  Q  Y  I  F  Q  Q  C  E  A  P  M

                1620                1640                1660                1680
AAGGTTTCCAGCTTTGAAGCAATACTCTGCTTTCACGTACCTGTGCTCAGACCAGGCTACCCAGTCGAGTCCTCTTGTGG
E  G  F  Q  L

                1700                1720                1740                1760
AGCCCACAGTCCTCATGGAGCTAACTTCTCAAATGTTTTCCATAATACTGTTTGCGCTCATTTGCTTGCCTTGCGCACCT

                1780                1800                1820                1840
GCTCTCTTACACACATCTGGAAAACCTCCGGCTCTCTGTGGTGGGATACCCTTCTAATAAAAGGGTAACCAGAACGGCCC

                1860                1880                1900                1920
ACTCTCTTTTACGGAAAAATCCCTAGGCTTTGGAGATCCGCACTTACATTAGAGTTATGGGAATATACACATATTAATGT

                1940                1960                1980                2000
GGCTCCCTTTTTCTTGTGGGGGAATAAAAGAGGACTCCTCCTCATTCCCTTTAACATGGGGGAAAAAACTGACATTAAAA

                2020                2040                2060                2080
GATGAGACTAAATCTTTATCTTGAATTTTACACAACTACTTACGACAAGGGAGATGTTTAGACCTGTTGGTATACTTCAG

                2100                2120                2140                2160
AGTACTTTTCATGAGTTCTTCCACAGTGAACCCTTGGATTACCTGGTGGCTTTTTCTAGCCAGATTGCATTAATCCTTAC

                2180                2200                2220                2240
TGAGATTGGATGGTTTTCTTTCCTCTATTGGCGCCATTCTTCAGATATTAAAGTTAAACCATCCACTCCCTCACCTTCAG

                2260                2280                2300                2320
CCTTCAGTGAATGTGCTTTCTAGTTGTCAGGAATGCTGAAGAATTAACACTTTGACTCCTAAATGTGATACTGGTGGGTA

                2340                2360                2380                2400
AGAGCAGGGCACATTTAATTTGTTCGCTTTTGCTTCTCTTTGGTCTGGGCACATTTAATTTGTTCGCTTTTGCTTCTCTT

                2420                2440                2460                2480
TGGTCTTTTCGAATACTTAGTAATCGAAAACCATATCCTGTAATTTAATAAAAAAAACTAAGGACGAAAAAACCCCTCCA

                2500                2520                2540                2560
ATTTTCCCAAATGCAATCAGTGTAACTAGGGGCTGTGTTTCTGCATTAAAATAAATGTTTCAGGCTTTGTGGTCCTGATC

                2580                2600                2620                2640
AAGGTCCTCATTAAAAAATTGGAGTTCACCCTAGGCTTTTCCCCTCTGTGACTGGCAGATAACACATACTTTTGAAAGTA

                2660                2680                2700                2720
ACTTTGGGATTTTTTTTCTTAGGTGCAGCTCGATTCTAATCTTTTCATGCTGCACACGATTCCTTTAATCGATAGCATCC

                2740                2760                2780                2800
TTATCTGAAAGAAATAACCATCTTCTCAACATGACCTGCTTAACCCAAATAAGAACAGTGATCTTATAACCTCATTGTTT

                2820                2840                2860                2880
CCTAATCTATTTTATTTCATCTCCTGCTAGTACTGTGCCGCTTCCCCCTCCCCCCACACAAAATAAAAACAGTATCTCGC

TTCTGGCTCATTTT
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FIG. 2 — *Continued*

extract suggesting that most of NPI-1 is located in the nucleus (Fig. 5). This is consistent with results localizing the NPI-1 homolog SRP1 to the nucleus of yeast cells by immunofluorescence (Yano *et al.*, 1992). We have been unable to localize NPI-1 to a particular intracellular compartment by immunofluorescence experiments using our antisera due to the high background fluorescence of these preparations.

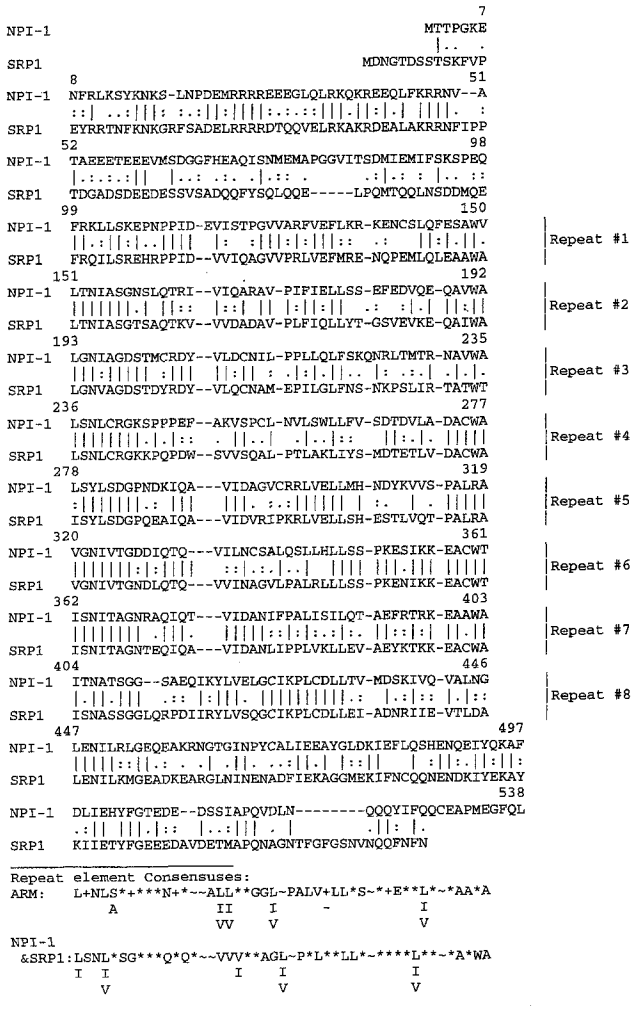

FIG. 3. Comparison of NPI-1 and SRP1 proteins. Vertical lines indicate identity; colons and periods indicate conservative changes. Forty-two amino acid ARM repeats are aligned vertically according to Peifer *et al.* (1994). For a complete comparison of SRP1 to other ARM repeat containing proteins see Peifer *et al.* (1994). The ARM consensus sequence is indicated at the bottom; +, indicates K, R, or H; –, indicates D or E; ~, indicates a gap; *, indicates a non-consensus residue. Since other residues are conserved within the repeats of NPI-1 and SRP, a consensus sequence derived from only these two proteins is also shown.

NPI-1 interacts with NP in infected cells

Since NP formed a complex with NPI-1 *in vitro*, we examined whether NP and NPI-1 form a complex in infected cells. Infected MDBK cells were pulse-labeled with ^{35}S-methionine from 5 to 6 hr after infection; since influenza virus inhibits translation of host mRNA, only viral proteins were labeled (Fig. 6A, lane 1). NP was specifically coimmunoprecipitated from extracts of influenza A/WSN virus-infected MDBK cells by antiserum directed against NPI-1 (Fig. 6A, lane 4). A protein of identical molecular weight was precipitated by a monoclonal antibody directed against NP (Fig. 6A, lane 3). No protein was precipitated by nonimmune rabbit serum (Fig. 6A, lane 2). No labeled polypeptides were precipitated from mock-infected cell extracts by anti-NPI-1 serum (Fig. 6A, lane 8) since insufficient label was incorporated into NPI-1 during the brief labeling period. This demonstrates an interaction of the viral NP and the cellular NPI-1 during influenza A virus infection. The reciprocal precipitation experiment was also performed: NPI-1 was coprecipitated from infected cells by antiserum directed against the NP (Fig. 6B, lane 1).

DISCUSSION

We have cloned a novel human cellular protein (NPI-1) based on its ability to interact with the influenza A

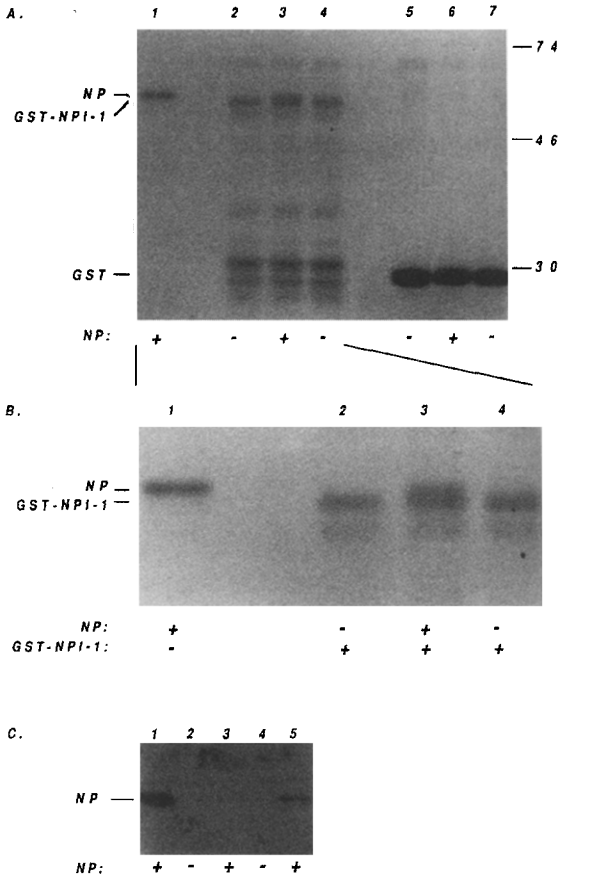

FIG. 4. GST–NPI-1 binds to NP *in vitro*. (A) GST–NPI-1 (lanes 2, 3, 4) and GST (lanes 5, 6, 7) were expressed in bacteria and precipitated from cell lysates on glutathione agarose beads. The complexed beads were then incubated with (lanes 3 and 6) or without (lanes 4 and 7) partially purified influenza virus nucleoprotein and polymerase preparations (2 μg per lane). Precipitated proteins were fractionated on a 12.5% SDS polyacrylamide gel, and stained with Coomassie blue. Unprecipitated influenza A virus NP and polymerase preparation (1 μg) was fractionated in lane 1. (B) Lanes 1 to 4 from (A) have been enlarged in order to emphasize the presence of the NP band in lane 3. (C) Proteins precipitated from solution as in (A) were immunoblotted using the monoclonal antibody HT103 directed against the viral nucleoprotein. Lane 1, unprecipitated nucleoprotein and polymerase preparation. Lanes 2 and 3, GST. Lanes 4 and 5, GST–NPI-1. Incubation of fusion protein/glutathione–agarose bead complexes in the presence or absence of viral NP is indicated below each lane.

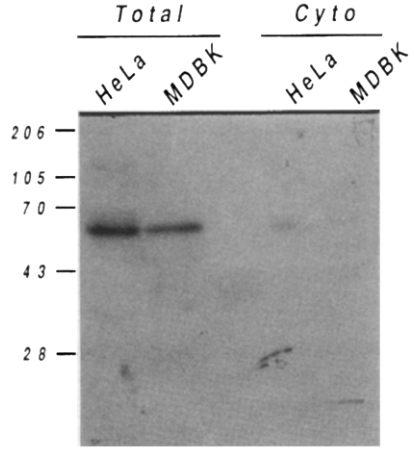

FIG. 5. Immunoblot of total cellular proteins using polyclonal rabbit sera against NPI-1. Total cell lysates and cytoplasmic cell extracts from HeLa and MDBK cell lines were separated by SDS–PAGE, transferred to nitrocellulose, immunoblotted with anti-NPI-1 sera, and developed by ^{125}I-protein A. Each lane contains protein from 1×10^5 cells.

virus nucleoprotein. Interaction of NPI-1 and NP was demonstrated by the yeast interactive trap system, *in vitro* by coprecipitation of the NP with a bacterially expressed NPI-1 protein, and in infected cell extracts by coprecipitation of the NP/NPI-1 complexes both with anti-NPI-1 serum and with anti-NP serum. This suggests that NPI-1 plays a role in the replication of influenza A viruses. NPI-1 is the first cellular protein characterized which interacts with a protein encoded by influenza viruses. In the future, it must be demonstrated at what stage in the replication cycle NPI-1 functions. The NPI-1 could affect any of a number of NP functions which may include: (1) movement of the ribonucleoprotein complex (RNP) to the nucleus during viral entry; (2) vRNA synthesis, including antitermination and elongation; (3) mRNA synthesis, including elongation, polyadenylation, and transport to the cytoplasm; and (4) exit of the RNP from the nucleus during virion assembly.

The fact that both NPI-1 and SRP1 interact with proteins involved in RNA synthesis may imply that there are fundamental similarities between cellular DNA-dependent transcription and influenza viral RNA-dependent RNA synthesis. Cellular factors, like NPI-1, may be shared by the viral and the cellular RNA synthesis machinery performing similar functions. In addition, the NPI-1 may tether the viral RNP to areas of the nuclear matrix where splicing and polyadenylation of mRNA occur (see below). It should be noted that although NPI-1 was isolated from HeLa cells, this cell line is not productively infected by influenza A virus. However, HeLa cells synthesize influenza viral RNA and protein, and have previously been used to examine viral RNA synthesis (Beaton and Krug, 1986).

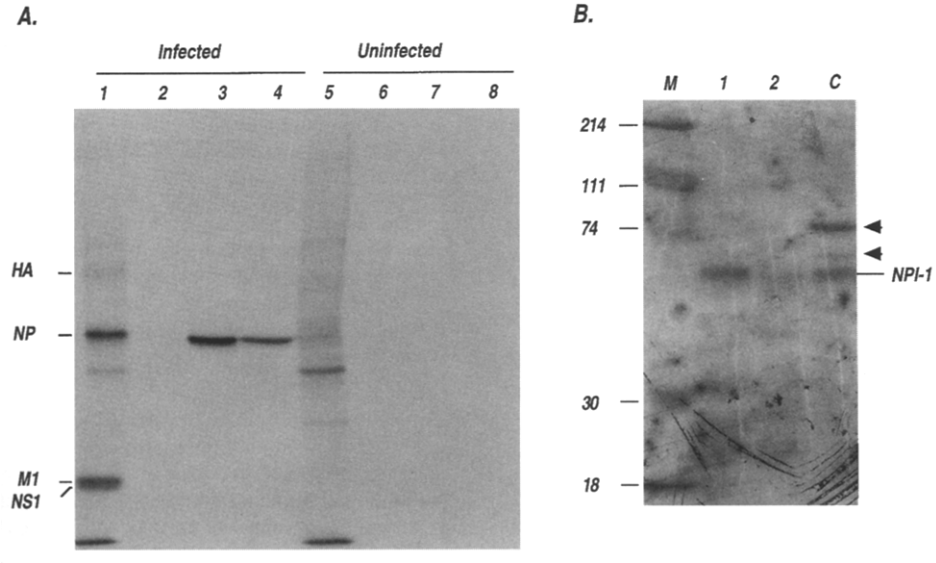

FIG. 6. NP and NPI-1 proteins are coimmunoprecipitated from infected cell extracts as a complex. (A) NP is coimmunoprecipitated from influenza A virus-infected cells by antisera against NPI-1. Infected (lanes 1 to 4) and uninfected (lanes 5 to 8) HeLa cell proteins were labeled with ^{35}S-methionine, and total cell lysates were made in RIPA buffer as described in the text. Complexes of NPI-1 and NP were precipitated using anti-NPI-1 sera. Precipitated proteins were then fractionated by SDS–PAGE and detected by autoradiography. Radiolabeled proteins were isolated from the lysate by immunoprecipitation. Lanes 2 and 6, preimmune rabbit serum; lanes 3 and 7, mouse monoclonal antibody HT103 against NP; lanes 4 and 8, immune rabbit serum against NPI-1. Lanes 1 and 5 are unprecipitated infected and uninfected HeLa cell lysates, respectively; lanes 1 and 5 represent 25% of cell equivalents loaded in other lanes. (B) NPI-1 is coimmunoprecipitated from influenza A virus infected cells by antisera against NP. Infected (lane 1) and uninfected (lane 2) cell lysates were made in RIPA buffer, and were immune-precipitated and fractionated as described under Materials and Methods. For a control (C) an uninfected cell lysate (2×10^5 cells) containing 1 μl of a bacterial extract expressing GST–NPI-1 is shown. The top bands (arrowheads) represent the bacterial GST–NPI-1 species. NPI-1 was detected by immunoblot using an ^{125}I-immune rabbit serum against NPI-1. NPI-1 antibodies were affinity-purified prior to iodination.

The viral NP exists in two forms in the infected cell. One form is associated with ribonucleoprotein complexes (RNP), and the other is a free form (Shapiro and Krug, 1988). Pol/NP preparations used in coprecipitation experiments with NPI-1 were purified over cesium chloride/glycerol gradients (Honda *et al.,* 1988), which dissociate and purify virion proteins away from vRNA. The polymerase proteins were not detected on Coomassie-stained gels (Fig. 4, lane 3); however, coprecipitation of the viral polymerase proteins was not rigorously tested by immunoblot experiments. Further analysis of the NP/NPI-1 interaction will be necessary to identify the form(s) of NP which bind to the cellular NPI-1.

Only one host factor has been assigned a definitive function in the replication process of a negative strand RNA virus. The cellular casein kinase II has been shown to phosphorylate the phosphoprotein P of the vesicular stomatitis virus (VSV) RNA-dependent-RNA polymerase. This is a step which appears to be required in order to activate the viral polymerase (Barik and Banerjee, 1992a,b).

NPI-1 and SRP1 are 53% identical and 71% conserved at the amino acid level. This is a very high degree of conservation between proteins belonging to organisms as distantly related as humans and yeast, and suggests that the NPI-1/SRP1 performs a very basic function in the cell. NPI-1 and SRP1 have eight internal repeats, each of approximately 42 amino acids (Fig. 3). This repeat, termed the ARM motif, was originally identified in the Drosophila segment polarity gene *armadillo* (Riggleman *et al.,* 1989), and it has been identified in a number of other proteins including β-catenin, plakoglobin, p120, APC, and smGDS (Peifer *et al.,* 1994, and references therein). Several ARM proteins are associated with cell adhesion structures. *Armadillo* and its homologs bind to the C-terminal cytoplasmic tail of cadherins, a calcium-dependent class of cell adhesion molecules (CAMs), linking the CAMs to the underlying cytoskeleton at cell–cell junctions (McCrea *et al.,* 1991). In contrast to the *armadillo* protein, SRP1 and NPI-1 appear to be localized to the nucleus. SRP1 is essential for the maintenance of the nucleolar structure and rRNA transcription (Yano *et al.,* 1994). It is also associated with Nup1p1, a yeast nuclear pore complex protein required for nuclear protein import, mRNA export and maintenance of normal nuclear architecture (Belanger *et al.,* 1994). If NPI-1, like SRP1 (Yano *et al.,* 1992), is associated with the nuclear membrane, it is possible that NPI-1 functions to tether viral RNP to the nuclear membranes (Jackson *et al.,* 1982). It should be noted that after this work was submitted for publication, two human cDNA sequences were published which are members of the SRP1 family (Cortes *et al.,* 1994; Cuomo *et al.,* 1994). These proteins, Rch1 and hSRP1, bind to the RAG-1 protein, which is a protein required for the V(D)J genomic rearrangement in developing B and T cells. Both of these genes are 53% identical to SRP1 (Table 2). We assume that the hSRP1 protein is the same as NPI-1 since our sequence differs only at 5 amino acids from the partial sequence of the hSRP1 protein.

TABLE 2

IDENTITY MATRIX OF NPI-1 FAMILY MEMBERS (% AMINO ACID IDENTITY)

	NPI-1	hSRP1[a]	mSRP1	Rch1[b]
NPI1	—			
hSRP1[a]	99	—		
mSRP1	97	98	—	
Rch1[b]	46	46	47	—
SRP1	53	53	53	46

Note. Sequences were obtained from Cortes *et al.* (1994), Cuomo *et al.* (1994), Yano *et al.* (1992), and this work.

[a] hSRP1 represents a partial clone.

[b] Rch1 represents a partial clone.

The carboxy terminal 276 amino acids of the NPI-1, which were sufficient for interaction with the viral NP, contain $4\frac{1}{2}$ ARM repeats. Individual repeats, in general, are approximately 30% identical with the ARM consensus sequence. This is consistent with the degree of conservation in ARM repeats of other proteins (Peifer *et al.,* 1994). We are presently mapping interactive domains of the viral NP and the cellular NPI-1. It will be interesting to determine whether multiple NP-binding domains are present in NPI-1.

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REFERENCES

BARIK, S., and BANERJEE, A. K. (1992a). Phosphorylation by cellular casein kinase II is essential for transcriptional activity of vesicular stomatitis virus phosphoprotein P. *Proc. Natl. Acad. Sci. USA* **89,** 6570–6574.

BARIK, S., and BANERJEE, A. K. (1992b). Sequential phosphorylation of the phosphoprotein of vesicular stamatitis virus by cellular and viral protein kinases is essential for transcription activation. *J. Virol.* **66,** 1109–1118.

BEAN, W. J. (1984). Correlation of influenza A virus nucleoprotein genes with host species. *Virology* **133,** 438–442.

BEATON, A. R., and KRUG, R. M. (1986). Transcription antitermination during influenza viral template RNA synthesis requires the nucleocapsid protein and the absence of a 5′ capped end. *Proc. Natl. Acad. Sci. USA* **83,** 6282–6286.

BELANGER, K. D., KENNA, M. A., WEI, S., and DAVIS, L. I. (1994). Genetic

and physical interactions between Srp1p and nuclear pore complex proteins Nup1p and Nup2p. *J. Cell Biol.* **126,** 619–630.

BUCKLER-WHITE, A. J., and MURPHY, B. R. (1986). Nucleotide sequence analysis of the nucleoprotein gene of an avian and a human influenza virus strain identifies two classes of nucleoproteins. *Virology* **155,** 345–355.

CHELSKY, D., RALPH, R., and JONAK, G. (1989). Sequence requirements for synthetic peptide-mediated translocation to the nucleus. *Mol. Cell. Biol.* **9,** 2487–2492.

CHIEN, C.-T., BARTEL, P. L., STERNGLANZ, R., and FIELDS, S. (1991). The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest. *Proc. Natl. Acad. Sci. USA* **88,** 9578–9582.

CORTES, P., YE, Z., and BALTIMORE, D. (1994). RAG-1 interacts with the repeated amino acid motif of the human homologue of the yeast protein SRP1. *Proc. Natl. Acad. Sci. USA* **91,** 7633–7637.

CUOMO, C. A., KIRCH, S. A., GYURIS, J., BRENT, R., and OETTINGER, M. A. (1994). Rch1, a protein that specifically interacts with the RAG-1 recombination-activating protein. *Proc. Natl. Acad. Sci. USA* **91,** 6156–6560.

DALTON, S., and TREISMAN, R. (1992). Characterization of SAP-1, a protein recruited by serum response factor to the *c-fos* serum response element. *Cell* **68,** 597–612.

DEVERAUX, J., HAEBERLI, P., and SMITHIES, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12,** 387–395.

DURFEE, T., BECHERER, K., CHEN, P.-L., YEH, S.-H., YANG, Y., KILBURN, A. E., LEE, W.-H., and ELLEDGE, S. J. (1993). The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.* **7,** 555–569.

ENAMI, M., LUYTJES, W., KRYSTAL, M., and PALESE, P. (1990). Introduction of site-specific mutations into the genome of influenza virus. *Proc. Natl. Acad. Sci. USA* **87,** 3802–3805.

FROHMAN, M. A. (1990). Race: Rapid amplification of cDNA ends. *In* "PCR Protocols: A Guide to Methods and Applications" (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, Eds.), pp. 228–236. Academic Press, San Diego.

FROHMAN, M. A., DUSH, M. K., and MARTIN, G. R. (1988). Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* **85,** 8998–9002.

GAMMELIN, M., MANDLER, J., and SCHOLTISSEK, C. (1989). Two subtypes of nucleoproteins (NP) of influenza A viruses. *Virology* **170,** 71–80.

GYURIS, J., GOLEMIS, E., CHERTKOV, H., and BRENT, R. (1993). Cdi1, a human G1 and S phase protein phosphatase that associates with cdk2. *Cell* **75,** 791–803.

HARLOW, E., and LANE, D. (1988). "Antibodies: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

HOFFMAN, C. S., and WINSTON, F. (1987). A ten minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *E. coli. Gene* **57,** 267–272.

HONDA, A., UEDA, K., NAGATA, K., and ISHIHAMA, A. (1988). RNA polymerase of influenza virus: Role of NP in RNA chain elongation. *J. Biochem.* **104,** 1021–1026.

HUANG, T.-S., PALESE, P., and KRYSTAL, M. (1990). Determination of influenza virus proteins required for genome replication. *J. Virol.* **64,** 5669–5673.

ITO, H., FUKUDA, Y., MURATA, K., and KIMURA, A. (1983). Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153,** 163–168.

JACKSON, D. A., CATON, A. J., and MCCREADY, S. J. (1982). Influenza virus RNA is synthesized at fixed sites in the nucleus. *Nature* **296,** 366–368.

KOZAK, M. (1987). An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* **15,** 8125–8148.

KOZAK, M. (1989). The scanning model for translation: An update. *J. Cell Biol.* **108,** 229–241.

MCCREA, P. D., TURCK, C. W., and GUMBINER, B. (1991). A Homolog of the *armadillo* protein in *Drosophila* (plakoglobin) associated with E-cadherin. *Science* **254,** 1359–1361.

MILLER, J. H. (1972). "Experiments in Molecular Genetics." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

PARVIN, J. D., PALESE, P., HONDA, A., ISHIHAMA, A., and KRYSTAL, M. (1989). Promoter analysis of the influenza virus RNA polymerase. *J. Virol.* **63,** 5142–5152.

PEIFER, M., BERG, S., and REYNOLDS, A. B. (1994). A repeating amino acid motif shared by proteins with diverse cellular roles. *Cell* **76,** 789–791.

RIGGLEMAN, B., WIESCHAUS, E., and SCHEDL, P. (1989). Molecular analysis of the *armadillo* locus: Uniformly distributed transcripts and a protein with novel internal repeats are associated with a drosophila segment polarity gene. *Genes Dev.* **3,** 96–113.

SAMBROOK, J., FRITSCH, E. F., and MANIATIS, T. (1989). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

SCHOLTISSEK, C., BURGER, H., KISTNER, O., and SHORTRIDGE, K. F. (1985). The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. *Virology* **147,** 287–294.

SCHOLTISSEK, C., KOENNECKE, I., and ROTT, R. (1978). Host range recombinants of fowl plague(influenza A) virus. *Virology* **91,** 79–85.

SHAPIRO, G. I., and KRUG, R. M. (1988). Influenza virus replication *in vitro:* Synthesis of viral template RNAs and virion RNAs in the absence of added primer. *J. Virol.* **62,** 2285–2290.

SMITH, D. B., and JOHNSON, K. S. (1988). Single step purification of polypeptides expressed in Escherichia coli as fusions with glutathione-S-transferase. *Gene* **67,** 31–40.

VOJTEK, A. B., HOLLENBERG, S. M., and COOPER, J. A. (1993). Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* **74,** 205–214.

YANO, R., OAKES, M., YAMAGHISHI, M., DODD, J. A., and NOMURA, M. (1992). Cloning and characterization of SRP1, a suppressor of temperature-sensitive RNA polymerase I mutations in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **12,** 5640–5651.

YANO, R., OAKES, M. L., TABB, M. M., and NOMURA, M. (1994). Yeast Srp1p has homology to armadillo/plakoglobin/b-catenin and participates in apparently multiple nuclear functions including the maintainance of the nuclear structure. *Proc. Natl. Acad. Sci. USA* **91,** 6880–6884.

ZERVOS, A. S., GYURIS, J., and BRENT, R. (1993). Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites. *Cell* **72,** 222–232.