Influenza A viruses cause a highly contagious respiratory disease in humans and are responsible for periodic widespread epidemics with high mortality rates. The influenza A virus NS1 protein (NS1A) plays a key role in countering host antiviral defense and in virulence. The 73-residue N-terminal domain of NS1A (NS1A-(1–73)) forms a symmetric homodimer with a unique six-helical chain fold. It binds canonical A-form double-stranded RNA (dsRNA). Mutational inactivation of this dsRNA binding activity of NS1A highly attenuates virus replication. Here, we have characterized the unique structural features of the dsRNA binding surface of NS1A-(1–73) using NMR methods and describe the 2.1-Å x-ray crystal structure of the corresponding dsRNA binding domain from human influenza B virus NS1B-(15–93). These results identify conserved dsRNA binding surfaces on both NS1A-(1–73) and NS1B-(15–93) that are very different from those indicated in earlier “working models” of the complex between dsRNA and NS1A-(1–73). The combined NMR and crystallographic data reveal highly conserved surface tracks of basic and hydrophilic residues that interact with dsRNA. These tracks are structurally complementary to the polyphosphate backbone conformation of A-form dsRNA and run at an ∼45° angle relative to the axes of helices α2/α2’. At the center of this dsRNA binding epitope, and common to NS1 proteins from influenza A and B viruses, is a deep pocket that includes both hydrophilic and hydrophobic amino acids. This pocket provides a target on the surface of the NS1 protein that is potentially suitable for the development of antiviral drugs targeting both influenza A and B viruses.

Influenza viruses present a major public health problem. Influenza A viruses cause a highly contagious respiratory disease in humans and are responsible for periodic widespread epidemics or pandemics with high mortality rates (1). The most devastating pandemic occurred in 1918, resulting in ∼30 million deaths worldwide (2). The avian H5N1 influenza A viruses currently circulating in birds across Asia, Europe, and Africa are candidates for causing the next pandemic if they acquire efficient human-to-human transmission (3, 4). Although less virulent, human influenza B viruses cause deaths and loss of productivity worldwide each year.

The influenza A virus non-structural protein 1 (NS1A) is a multifunctional dimeric protein that participates in both protein-RNA (5–10) and protein-protein interactions (11–14). NS1A plays a key role in countering host cell antiviral defenses (10, 13, 15, 16) and in viral virulence (3, 10, 12, 17). The NS1 proteins from influenza A (NS1A, 237 residues) and influenza B (NS1B, 281 residues) viruses are homodimeric molecules (7, 9, 18, 19). Although NS1A and NS1B interact with different sets of host proteins (11, 12, 14, 15, 20, 21), they both contain a homologous N-terminal RNA binding domain that binds a wide range of dsRNA sequences with no known sequence specificity (5–8, 22).

Mutation of NS1A surface residue Arg38 to Ala abrogates dsRNA binding in vitro (9), and a recombinant influenza A virus expressing the mutant (R38A)-NS1A protein is attenuated ~1000-fold in replication (10). Analysis of this attenuated influenza A virus (R38A mutant) reveals that the primary role of dsRNA binding by NS1A is to shield the virus against the antiviral state induced by interferon, primarily by inhibiting activation of the 2′−5′ oligo(A) synthetase/ribonuclease L pathway (10). The dsRNA binding activity of the influenza B virus NS1B protein also plays an important role during infection (23, 24). Previous biophysical and structural studies have provided

---

* This work was supported by National Institutes of Health Protein Structure Initiative Grants U54 GM074958 (to G. T. M.), AI49475 (to L. T.), and AI11772 (to R. M. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5 and Table S1.

The atomic coordinates and structure factors (code 1xeq) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

1 These authors contributed equally to this work.

2 A graduate fellow of the Biological, Mathematical, and Physical Sciences (BioMAPS) program at Rutgers University.

3 To whom correspondence may be addressed. E-mail: rkrug@mail.utexas.edu.

4 To whom correspondence may be addressed. E-mail: ltong@columbia.edu.

5 To whom correspondence may be addressed. E-mail: guy@cabm.rutgers.edu.

6 The abbreviations used are: NS1A, NS1 from influenza strain A; NS1, non-structural protein 1 from influenza virus; NS1B, NS1 from influenza strain B; ds, double-stranded.
some insights into the mechanism of dsRNA binding by the NS1A protein. The 73-residue N-terminal domain of NS1A (NS1A-(1–73)) forms a symmetric homodimer with a unique six-helical chain fold (18, 19) and binds double-stranded (ds) A-form RNA (5–8). Sedimentation equilibrium measurements (8) on a complex formed by NS1A-(1–73) and a 16-bp synthetic dsRNA duplex demonstrated a dimer: 1 duplex stoichiometry, with a dissociation constant (Kd) of ~1 μM. Analytical gel filtration and gel shift studies of interactions between NS1A-(1–73) and different double-stranded nucleic acids further demonstrate that NS1A-(1–73) recognizes canonical A-form dsRNA but does not bind dsDNA or dsRNA-DNA hybrids, which have B-type and A/B-type intermediate conformations, respectively (8).

Preliminary NMR chemical shift perturbation studies have implicated surface residues in helices α2/α2' in dsRNA interactions (8). Mutations of surface-exposed basic residues of helices α2/α2' also affect dsRNA binding affinity (9, 14). However, these data alone are not sufficient to provide a reliable mapping of the full set of residues that form the protein-dsRNA interface. Specifically, although a hypothetical “working model” of the complex between dsRNA and NS1A-(1–73) has been illustrated (8), it was not possible in the absence of NMR resonance assignments for the dsRNA-bound protein to define the relative orientation of dsRNA or the extent of the dsRNA binding site on the surface of NS1A.

In this study, we have presented sequence-specific backbone resonance assignments for dsRNA-bound NS1A-(1–73) in this 26.5-kDa complex. These data allow us to properly characterize, for the first time, the details of the dsRNA binding surface of NS1A-(1–73), and the complete set of residues involved in dsRNA binding. These NMR data also define the correct orientation of dsRNA in this complex.

To characterize the phylogenetic distribution of these surface features, we have also determined the 2.1-Å x-ray crystal structure of the corresponding dsRNA binding domain NS1B-(15–93) from human influenza B virus. Comparison of the structures of the conserved dsRNA binding surfaces of NS1A-(1–73) and NS1B-(15–93) reveal that the amino acid residues that interact with dsRNA in both NS1A and NS1B form tracks of basic and hydrophilic functional groups. These tracks are complementary to the polyphosphate backbone conformation of A-form dsRNA, and run at an ~45° angle relative to the axes of helices α2/α2'.

These structural studies identify and characterize surface features of the NS1 proteins of influenza A and B viruses, which underlie their critical dsRNA binding functions. At the center of the common dsRNA binding epitope of both NS1A and NS1B is a deep pocket that may be a suitable target site for drug discovery. The dsRNA binding surface on NS1A-(1–73), determined here using resonance assignments for dsRNA-bound NS1A (1–73), results in very different protein-RNA interactions, and a different orientation of the bound dsRNA, than that illustrated in our earlier working model of the complex (8). These functional surface features of NS1 are strongly conserved across A and B influenza virus strains.

**EXPERIMENTAL PROCEDURES**

Sample Preparation—The N-terminal structural domain corresponding to the first 73 amino acids of the NS1 protein from influenza A/ Udorn/72 virus NS1A-(1–73) was expressed with uniform 13C,15N enrichment and purified according to published procedures (7, 8). The N-terminal structural domain corresponding to the first 103 amino acids of the NS1 protein from influenza B/Lee/40 virus was expressed with a 10-residue N-terminal hexaHis tag (MGHHHHHHHHHSH) and purified, as described elsewhere (25). Single-stranded 16-nucleotide RNAs were chemically synthesized using standard phosphoramidite chemistry (Dharmacon Research, Inc.), annealed, and purified using Superdex 75 gel filtration chromatography, as described previously (8).

NMR Sample Preparation—150-μM samples of 0.2 mM NS1A-(1–73) in 50 mM ammonium acetate, pH 6.0, were slowly added to 150 μM of 0.4 mM dsRNA solutions in the same buffer in 1:2 ratios. NS1A-(1–73)-dsRNA complex formation was confirmed both by NMR and by Superdex 75 gel filtration chromatography, as described previously (8). The resulting samples of NS1A-(1–73)-dsRNA complex used for NMR studies were at a ~0.1 mM complex concentration, 50 mM ammonium acetate, pH 6.0.

NMR Spectroscopy—NMR experiments were collected at 20 °C on a four-channel Varian INOVA 600-MHz spectrometer equipped with a 5-mm triple resonance gradient probe, and Bruker Avance 600 MHz spectrometer equipped with 5 mm triple resonance gradient cryoprobes. 1H, 15N, and 13C resonance assignments for dsRNA-bound NS1A-(1–73) were determined using standard triple resonance NMR pulse sequences, as described elsewhere (26), including two-dimensional [1H,15N]HSQC and three-dimensional HNCO, HNCA, HNCACB, HNCACBCAB NMR data. Data were processed using AutoProc (27), NMRPipe (28), and SPARKY (29) spectral visualization software. Proton chemical shifts were referenced externally to 2,2-dimethyl-2-silapentane-5-sulfonic acid. 13C and 15N resonances were referenced indirectly using gyromagnetic ratios of 13C:1H (0.251449530), and 15N:1H (0.101329188), respectively. These data provided essentially complete assignments for assignable backbone 15N, 1H, 13Cα, and 13Cβ resonances together with 65 of 68 assignable 13Cβ resonances (for a summary, see data summarized in supplemental Fig. S1). As expected, some symmetric sites in the two chains of NS1A (1–73), which have equivalent chemical shifts in the free protein dimer, experienced different chemical environments in the complex because of the proximity of non-identical nucleic acid atoms from the bound heteroduplex RNA molecule. Splitting into resonance pairs with identical intensities was observed in 17 residue pairs of the dimer, indicated by triangles in supplemental Fig. S1. Helical backbone structures were identified by 13Cα and 13Cβ chemical shift values, as summarized in supplemental Fig. S1.

7 Chemical shift data has been deposited for dsRNA-bound NS1A-(1–73) in the BioMagRes Data Bank (BMRB accession number 15117). Coordinates and structure factor files have been deposited for NS1B-(1–103) in the Protein Data Bank (PDB code 1eq).
Influenza NS1 dsRNA Binding Epitope

Chemical shift perturbations were defined for each residue as the sum of the differences in chemical shift (in Hz) between bound and free states for atoms $^1$N, $^1$H, $^{13}$C, $^{13}$C$_a$, and $^{15}$N (where $\Delta \delta = |\Delta \delta_n| + |\Delta \delta_{1HN}| + |\Delta \delta_{1NC}| + |\Delta \delta_{1CC}|$), and $^{13}$C$_{\text{eq}}$, and $|\Delta \delta_{1HH}| + |\Delta \delta_{1HC}| + |\Delta \delta_{1CH}| + |\Delta \delta_{1CC}|)/n$, where $n$ = number of resonances for which such data were available). For split resonances, mean perturbations $\Delta \delta_{\text{avg}} = 0.5(\Delta \delta_n + \Delta \delta_{\text{eq}})$ were used. These chemical shift perturbation data are summarized in supplemental Fig. S1, and tabulated in supplemental Table S-1. The programs HADDOCK and CNS were used for constrained energy minimization, as described elsewhere (30, 31).

Crystallization—NS1B-(1–103) was concentrated to 24 mg/ml in buffer consisting of 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, and 10 mM dithiothreitol. Initial crystal screening was carried out robotically under oil at the High Throughput Crystallization Laboratory at Hauptman Woodward Research Institute in Buffalo, NY. Crystals for the final data collection were grown at 4 °C by the sitting drop vapor diffusion method in 4 M NaBr with 0.1 M citrate buffer at pH 3.9.

Structure Determination—X-ray diffraction data were collected on an ADSC CCD at the X4A beamline (Brookhaven National Laboratory). A single wavelength anomalous diffraction data set to 2.6 Å resolution was collected at the absorption peak of bromide. The crystals belong to the space group P6$_2$2, with cell dimensions of $a = b = 102.0$ Å, $c = 108.8$ Å. Solve/Resolve software (32) was used for locating 20 bromide sites and for phasing and automated model building. Manual model building was performed with the program O (33). Refmac (34) was used for structure refinement against a native dataset to 2.1 Å resolution, also collected at the X4A beamline. Statistics on data processing and structure refinement are summarized in Table 1.7

RESULTS

To completely define the surface of the NS1A involved in binding to dsRNA, we carried out detailed NMR chemical shift perturbation studies on $^{13}$C, $^{15}$N-enriched NS1A-(1–73) in a 1 dimer-1 duplex complex with an unlabeled 16-bp dsRNA duplex consisting of annealed polynucleotides CCAUCCUC and 13C$_n$ where available. For split resonances, mean perturbations $\Delta \delta_{\text{avg}} = 0.5(\Delta \delta_n + \Delta \delta_{\text{eq}})$ were used. These chemical shift perturbation data are summarized in supplemental Fig. S2 and tabulated in supplemental Table S-1. The programs HADDOCK and CNS were used for constrained energy minimization, as described elsewhere (30, 31).

Crystallography—NS1A-(1–73) that were located proximal to dsRNA in the RNA-bound complex, as the RNA duplex consisting of annealed polynucleotides CCAUCCUC and 13C$_n$ was used here did not have internal 2-fold symmetry. As both resonances arising from such asymmetry could be assigned, this information immediately identified some of the residues in NS1A-(1–73) that were located proximal to dsRNA in the complex.

The three helices identified in each chain of dsRNA-bound NS1A-(1–73) (data summarized in supplemental Fig. S1), Ser3-Asp24 (helix a1), Phe2-Leu20 (helix a2), and Ile54-Lys70 (helix a3) are in identical positions in the three-dimensional structure of free NS1A-(1–73) (18, 19), demonstrating that the three $\alpha$-helices of free NS1A-(1–73) remain intact upon complex formation. Far ultraviolet circular dichroism studies of this same NS1A-(1–73)dsRNA complex also demonstrate that the backbone structures of both the six-helical protein domain and the A-form dsRNA do not change significantly in the largely rigid docking process (8).

With resonance assignments for both free (8) and dsRNA-bound NS1A-(1–73) in hand and knowing from circular dichroism studies that there are no significant changes in the overall backbone structure of NS1A-(1–73) upon complex formation, chemical shift differences between free and bound protein were used to identify the dsRNA binding site on the surface of NS1A-(1–73). These chemical shift differences due to complex formation were all localized on the same face of the NS1A-(1–73) homodimer (Fig. 1, left panels), in helices a1/a1’, and a2/a2’, and in residues that were nearby to helices a2/a2’ in the three-dimensional structure. Atoms in residues on the opposite face of NS1A-(1–73) (e.g. in helices a3/a3’) showed negligible chemical shift perturbations (Fig. 1, right panels), demonstrating that the overall three-dimensional fold and core packing of NS1A-(1–73) were preserved in the complex. Symmetrical atomic sites in the protein dimer that are split into two separate resonances (of equal intensity) are highlighted on the three-dimensional structure of NS1A-(1–73) in Fig. 1a, providing a partial mapping of the dsRNA binding epitope. Fig. 1b highlights all residues exhibiting chemical shift perturbations (including residues with split resonances), providing an unambiguous and detailed mapping of the dsRNA binding site on NS1A. Key residues within this binding site include Thr5, Pro31, Asp34, Arg35, Arg38, Lys41, Gly45, Arg46, and Thr49, which are strongly conserved in NS1A proteins of all influenza A viruses.

We next considered the degree to which the dsRNA binding epitope characterized for NS1A-(1–73) is conserved in the NS1B proteins of influenza B viruses. There is ~20% sequence identity between NS1 proteins from influenza strains A and B, and efforts to generate reliable models of the homologous NS1B RNA binding domain from the three-dimensional structures of NS1A-(1–73) have been frustrated by alternative high-scoring sequence alignments, particularly for residues in helix a1 (9). To accurately define equivalent residues in the RNA binding domains of NS1A and NS1B and to properly assess how well the dsRNA binding epitope of NS1A is conserved across all influenza virus strains, we crystallized and solved the three-dimensional structure of the corresponding domain construct, NS1B-(1–103), from human influenza B/Lee/40 virus. The structure was refined using 2.1-Å resolution data to a crystallographic $R$ factor of 0.213 and $R_{free}$ of 0.256 (Table 1). The resulting three-dimensional structure includes residues 15–103 for one polypeptide chain and residues 9–93 for the other chain. Because we only observed electron density for residues 94–103 in one of the two polypeptide chains in which it has an irregular structure with multiple crystal packing contacts, we cannot make any definitive statement about the native structure of this...
portion of NS1B at this point. Similarly, residues 1–8 were not observed for either chain, and residues 9–14 were observed for only one chain. Therefore, we restrict our discussions to the implications of the structure of NS1B-(15–93).

Similar to NS1A-(1–73), NS1B-(15–93) folds as a symmetric six-helical dimer with many positively charged residues on helices /H9251/, /H9251/, /H11032/ (Fig. 2a). One significant difference between the structures is the length of the loop between helices /H9251/ and /H9251/, which is eight residues longer in NS1B than that in NS1A (Fig. 2, b and c). In addition, the two structures differed in the length of the apparently disordered N-terminal sequence preceding helix /H9251/, which is 12 residues longer in NS1B.

This experimental three-dimensional structure of NS1B-(15–93), together with the available NS1A-(1–73) structures (18, 19), provides a structure-based sequence alignment shown in Fig. 2d correctly defining equivalent residues in the two molecules. With this alignment, the backbone root mean square deviations between the crystal structures of NS1A-(1–73) (18) and NS1B-(15–93) is 0.53 Å for the monomer and 1.08 Å for the overall dimer.

Using the correct sequence align-
ment across the influenza A and B families, based on the threedimensional structure alignment, surface features that are conserved across all influenza stains were characterized with the program CONSURF (35). This analysis, plotted on the three-dimensional structures of NS1A-(1–73) and NS1B-(15–93) in Fig. 3, reveals that the majority of strongly conserved residues (20 of 28 residues identical across all NS1A and NS1B sequences) are clustered in or near the dsRNA binding epitope of NS1A-(1–73). These highly conserved residues include (NS1A/NS1B numbering): Thr^5/Thr^{17}, Ser^8/Thr^{20}, Asp^29/Asp^{41}, Pro^{31}/Pro^{43}, Asp^{34}/Asp^{46}, Arg^{35}/Arg^{47}, Arg^{38}/Arg^{50}, Lys^{41}/Arg^{53}, Arg^{46}/Arg^{58}, and Thr^{49}/Thr^{61}. These residues form two conserved surface “tracks” on the dsRNA binding surface (Fig. 3). Among these 20 track residues, 14 are in helices α2/α2’, 4 in helices α1/α1’, and 2 are in the α1/α2 loops. The track pattern on NS1B-(15–93) is strikingly similar to that on

FIGURE 2. Crystal structure of NS1B-(1–103). a, stereo view of NS1B-(1–103) structure showing backbone along with only Arg and Lys side chains. Shown are monomer (b) and dimer (c) structures of NS1B-(1–103) (blue) overlaid onto corresponding crystal structures of NS1A-(1–73) (tan), d, structure-based sequence alignment of NS1B-(1–103) and NS1A-(1–73), highlighting identical residues (blue). The locations of the three α-helices in each sequence are indicated.
provide protein-nucleic acid contacts for surface residues exhibiting significant chemical shift perturbations and to avoid intermolecular contacts with surface residues exhibiting little or no chemical shift perturbation. Thus, the chemical shift data were used together with simple energetic considerations in a rigid body docking so as to provide contacts between nucleic acid hydrophilic groups and conserved hydrophilic groups on the protein surface. These models were further refined by energy minimization with the HADDOCK (31) and CNS (30) molecular mechanics programs. Models were generated for both major groove (Fig. 4) and minor groove (supplemental Fig. S3) binding modes. The models are shown together with the conserved surface track residues (Fig. 4a and supplemental Fig. S3a (stereo images)), the conserved basic edge residues (Fig. 4b), and with the experimental chemical shift perturbation data (Fig. 4c and supplemental Fig. S3b (stereo images)). Similar models are shown for NS1B-dsRNA complexes in supplemental Fig. S4.

In all four models (NS1A and NS1B binding in major and minor groove modes), the NS1A-(1–73) epitope forms a shallow concave surface occupied by the dsRNA (Fig. 4) with the pseudo-2-fold symmetry axis of dsRNA approximately aligned with the symmetry axis of the NS1 dimer, providing approximately symmetric energetics. Key residues identified as important for dsRNA binding by site-directed mutation studies (i.e. residues Arg46 and Lys51 in NS1A (9) and Arg50 and Arg53 in NS1B (14)) are in contact with or close proximity to the phosphate backbone of dsRNA in both binding modes. Other hydrophilic residues in the conserved tracks can interact either directly or via water molecules with the nucleic acid backbone or sugar atoms of dsRNA. For example, in the major groove binding model, residues Arg46 and Thr49 of NS1A-(1–73) have RNA-specific interactions with 2′-OH groups. Extensively bound water networks are identified in these dsRNA binding epitopes of both proteins (18), and such water-mediated interactions may contribute to the energetics of complex formation.

We cannot unambiguously distinguish the two potential binding modes (major versus minor groove) nor rule out the possibility of very rapid interconversion between the two different bound states. Both binding modes are consistent with the fact that dsRNA binding by both NS1A and NS1B is not sequence-specific (5–8, 22). However, the available data best support a single major groove binding mode. Specifically, (i) chemical shift perturbation data for the NS1A-dsRNA complex match the polyphosphate backbone better in the major groove

FIGURE 3. Conserved surface tracks of NS1. CONSURF(35) analysis of the NS1 protein family projected on the surface of NS1A-(1–73) (a and c) and NS1B-(15–93) (b and d). The different orientations are indicated by ribbon diagrams (insets). Residues are colored according to their different degree of conservation across the entire NS1 family, with turquoise indicating sites with the largest variations and burgundy representing the residues without or with very little variation. Separations between the basic edges (−10 Å) and the centers of each “conserved track” (−17 Å) are indicated.

NS1A-(1–73). The conserved tracks on both proteins are oriented at an angle of ∼45° with respect to the axes of helices α2 and α2′ (Fig. 3). At the center of this dsRNA binding surface is a deep pocket with walls formed by antiparallel helices α2 and α2′.

Remarkably, the pattern of conserved tracks in the dsRNA binding epitope of NS1A and NS1B matches the pattern of chemical shift perturbation on this same surface (Figs. 1b and 3). These features of the NS1A-(1–73) surface also complement surface electrostatic features of canonical A-form dsRNA backbone. In both NS1A and NS1B, the inner edges of the conserved tracks, which we refer to as “basic edges,” consist largely of Arg and Lys residues. The spacing between these basic edges is ∼10 Å (Fig. 3), and is similar to the spacing (∼10 Å) between the phosphate backbone groups across the major groove of A-form dsRNA. The spacing between phosphate backbone groups across the minor groove of A-form dsRNA (∼17 Å) is slightly wider.

Although the primary goal of this study was to characterize the surface residues of NS1A and NS1B contributing to the dsRNA binding interface, efforts were also made to use the experimental NMR chemical shift perturbation data to generate models of the dsRNA binding mode. Models of NS1A-dsRNA complexes were produced by manually docking canonical A-form dsRNA into the dsRNA binding epitopes of NS1A and NS1B, aligning the two basic edges of positively charged residues so as to optimally contact the negatively charged phosphate backbone of dsRNA. The modeling was guided by experimental chemical shift perturbation data (Fig. 1b) so as to

Influenza NS1 dsRNA Binding Epitope
binding model (Fig. 4c) than in the minor groove binding model (Supplemental Fig. 2b); (ii) the spacing between basic edges (Fig. 4b; ~10 Å) matches interphosphate distances across the major groove (~10 Å) more closely than across the minor groove (~17 Å); and (iii) the major groove binding model has better interfacial energetics than the minor groove binding model, arising from extensive electrostatic and hydrogen-bonded interactions between polar groups in the dsRNA binding epitope and the polyphosphate backbone, 4’O and 2’OH groups of dsRNA. In either case, however, the data provide unambiguous identification of the surface of NS1A, which functions as the functionally critical dsRNA binding site.

The structural characterization of nonspecific nucleic acid-binding proteins in complexes with DNA or RNA can be complicated by the presence of multiple structures arising from “sliding” of the protein with respect to the nucleic acid or multiple binding modes in dynamic equilibrium. The NMR data on the complex between NS1A-(1–73) and the 16-bp dsRNA provide no indications of extensive interfacial dynamics and are consistent with a single binding mode. In particular, the NMR spectra of NS1A-(1–73) bound to dsRNA are well resolved and exhibit line widths typical of a complex of this size (~30 kDa) (supplemental Fig. S1, c and d). In addition, the chemical shift analysis reveals a pattern of chemical shift perturbations complementary to the polyphosphate backbone of dsRNA bound to a single site on the protein surface. Indeed, the length of A-form 16-bp dsRNA is just sufficient to allow for a single (or in any case a very narrow range) of binding sites for NS1A-(1–73). Even if some amount of sliding is occurring, the data mapping the dsRNA binding epitope presented here provide reliable and detailed information on the nature and location of the conserved surface features of NS1A responsible for its dsRNA binding activity.

To further validate these models, we also compared the surface buried by dsRNA in these dsRNA-NS1A complexes to the surface formed by residues in NS1A-(1–73) that exhibit dsRNA-induced chemical shift perturbations. As shown in supplemental Fig. S5, these surface distributions are remarkably similar, validating the good agreement between the chemical shift perturbation data and our models of NS1A-(1–73)-dsRNA com-
plexes. These complementary patterns of chemical shift perturbation, conserved tracks, and the dsRNA polyphosphate backbone in these complexes are striking and provide clear and unambiguous characterization of the sites on NS1 involved in dsRNA interactions.

DISCUSSION

The combined NMR and crystallographic data presented here provide new insights into structure-function relationships of non-structural protein 1 that are common to influenza viruses A and B. These data identify conserved dsRNA binding surfaces on both NS1A-(1–73) and NS1B-(15–93) that are very different from those indicated in earlier working models of the complex between dsRNA and NS1A-(1–73) (8). The combined NMR and crystallographic data reveal highly conserved tracks of basic and hydrophilic residues complementary to the polyphosphate backbone conformation of A-form dsRNA. The tracks run at a ~45° angle relative to the axes of helices α2/α2’. At the center of this dsRNA binding epitope, and common to NS1 proteins from influenza A and B viruses, is a deep cavity that includes both hydrophilic and hydrophobic amino acids. The pattern of conserved surface residues complements the structure of the polyphosphate backbone on canonical A-form dsRNA and suggests specific interactions that stabilize the complex. Mutations of basic residues within this epitope, including R37A, R38A, K41A, and R44A, reduce the affinity of the complex. Mutations of basic residues within this epitope, and common to NS1B also plays an important role during infection (23, 24). The deep pocket present in both binding sites, illustrated for the crystal structure, complements the dsRNA polyphosphate backbone in dsRNA recognition that are fundamental to the molecular functions of both NS1A and NS1B.

Although the primary means for controlling influenza virus epidemics is vaccination, antivirals provide an important additional line of defense, particularly for a rapidly spreading pandemic (36, 37). Many of the human isolates of H5N1 avian viruses are already resistant to the antivirals amantadine and rimantadine directed against the viral M2 ion channel protein (38), and H5N1 viruses that are partially or completely resistant to the neuraminidase inhibitor oseltamivir (Tamiflu) have recently been reported (39, 40). The emergence of H5N1 viruses resistant to these two classes of antiviral drugs highlights the need for additional antiviral drugs against influenza virus.

Because the dsRNA binding activity of the NS1A protein is required for the inhibition of the interferon-induced antiviral response of the host (10), the dsRNA binding site of NS1A described here is an important target for the development of antiviral drugs. The dsRNA binding site of the NS1B protein is also a target for antivirals, because the dsRNA binding activity of NS1B also plays an important role during infection (23, 24). The deep pocket present in both binding sites, illustrated for NS1A in Fig. 5, is an inviting target for structure-function studies and drug design. Because this binding site is strongly conserved across A and B influenza virus strains, it will likely be difficult for influenza virus to develop resistance to drugs targeting this molecular surface.

REFERENCES

1. Wright, P. F., and Webster, R. G. (2001) Fields Virology, 4th Ed., Lippincott, Williams, and Wilkins, Philadelphia
2. Reid, A. H., Taubenberger, J. K., and Fanning, T. G. (2001) Clin. Microbiol. Infect. 3, 81–87
3. Horimoto, T., and Kawaoka, Y. (2005) Nat. Rev. Microbiol. 3, 591
4. Noah, D. L., and Krug, R. M. (2005) Adv. Virus Res. 65, 121–145
5. Hatada, E., and Fukuda, R. (1992) J. Gen. Virol. 73, 3325–3329
6. Lu, Y., Wambach, M., Katze, M. G., and Krug, R. M. (1995) Virology 214, 222–228
7. Qian, X. Y., Chien, C. Y., Lu, Y., Montelione, G. T., and Krug, R. M. (1995) RNA 1, 948–956
8. Chien, C. Y., Xu, Y., Xiao, R., Aramini, J. M., Sahasrabudhe, P. V., Krug, R. M., and Montelione, G. T. (2004) Biochemistry 43, 1950–1962
9. Wang, W., Riedel, K., Lynch, P., Chien, C. Y., Montelione, G. T., and Krug, R. M. (1999) RNA 5, 195–205
10. Min, I., and Krug, R. M. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 7100–7105
11. Nemeroff, M., Barabino, S. M., Li, Y., Keller, W., and Krug, R. M. (1998) Mol. Cell 1, 991–1000
12. Obenauer, J. C., Denson, J., Mehta, P. K., Su, X., Mukatira, S., Finkelstein, D. B., Xu, X., Wang, J., Ma, J., Fan, Y., Rakewstraw, K. M., Webster, R. G., Hoffmann, E., Krauss, S., Zheng, J., Zhang, Z., and Naeve, C. W. (2006) Science 311, 1576–1580
13. Noah, D. L., Twu, K. Y., and Krug, R. M. (2003) Virology 307, 386–395
14. Yuan, W., Aramini, J. M., Montelione, G. T., and Krug, R. M. (2002) Virology 304, 291–301
15. Krug, R. M., Yuan, W., Noah, D. L., and Latham, A. G. (2003) Virology 309, 181–189
16. Garcia-Sastre, A. (2001) Virology 279, 375–384
17. Donelan, N. R., Basler, C. F., and Garcia-Sastre, A. (2003) J. Virol. 77, 13257–13266
18. Liu, J., Lynch, P. A., Chien, C. Y., Montelione, G. T., Krug, R. M., and Berman, H. M. (1997) Nat. Struct. Biol. 4, 896–899
19. Chien, C. Y., Tejeiro, R., Huang, Y., Zimmerman, D. E., Rios, C. B., Krug, R. M., and Montelione, G. T. (1997) Nat. Struct. Biol. 4, 891–895
20. Yuan, W., and Krug, R. M. (2001) EMBO J. 20, 362–371
21. Chen, Z., Li, Y., and Krug, R. M. (1999) EMBO J. 18, 2273–2283
22. Wang, W., and Krug, R. M. (1996) Virology 223, 41–50
23. Donelan, N. R., Dauber, B., Wang, X., Basler, C. F. M., Wolff, T., and Garcia-Sastre, A. (2004) J. Virol. 78, 11574–11582
24. Dauber, B., Schneider, J., and Wolff, T. (2006) J. Virol. 80, 11667–11677
25. Acton, T. B., Gunsalus, K. C., Xiao, R., Ma, L. C., Aramini, J., Baran, M. C., Chiang, Y.–W., Climent, T., Cooper, B., Denissova, N. G., Douglas, S. M., Everett, J. K., Ho, C. K., Macapagal, D., Paranjek, R. K., Shastry, R., Shih, L. Y., Swapan, G. V. T., Wilson, M., Wu, M., Gerstein, M., Inouye, M., Hunt, J. F., and Montelione, G. T. (2005) Methods Enzymol. 394, 210–243
26. Montelione, G. T., Rios, C. B., Swapan, G. V. T., and Zimmerman, D. E. (1999) in Biological Magnetic Resonance (Krishna, N. R., and Berliner, L. J., eds) pp. 81–130, Kluwer Academic, New York
27. Baran, M. C., Moseley, H. N., Aramini, J. M., Bayro, M. J., Monlecon, D., Locke, J., and Montelione, G. T. (2006) Proteins Struct. Funct. Bioinform. 62, 843–851
28. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) J. Biomol. NMR 6, 277–293
29. Goddard, T., and Kneller, T. (2000) SPARKY NMR Analysis Software, University of California, San Francisco, CA
30. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P.,
Influenza NS1 dsRNA Binding Epitope

31. Dominguez, C., Boelens, R., and Bonvin, A. M. (2003) J. Am. Chem. Soc. 125, 1731–1737
32. Terwilliger, T. C. (2003) Methods Enzymol. 374, 22–37
33. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard (1991) Acta Crystallogr. Sect. A 47, 110–119
34. CCP4 (1994) Acta Crystallogr. Sect. A 50, 760–763
35. Glaser, F., Pupko, T., Paz, I., Bell, R. E., Bechor-Shental, D., Martz, E., and Ben-Tal, N. (2003) Bioinformatics 19, 163–164
36. Longini, I. M., Nizam, A., Xu, S., Ungchusak, K., Hanshaoworakul, W., Cummings, D. A., and Halloran, M. E. (2005) Science 309, 1083–1087
37. Ferguson, N. M., Cummings, D. A., Cauchemez, S., Fraser, C., Riley, S., Meeyai, A., Iamsirithaworn, S., and Burke, D. S. (2005) Nature 437, 209–214
38. Puthavathana, P., Auewarakul, P., Charoenying, P. C., Sangsirivut, K., Pooruk, P., Boonnak, K., Khanyok, R., Thawachsupa, P., Kipphati, R., and Sawanpanyalert, P. (2005) J. Gen. Virol. 86, 423–433
39. de Jong, M. D., Tran, T. T., Truong, H. K., Vo, M. H., Smith, G. J., Nguyen, V. C., Bach, V. C., Phan, T. Q., Do, Q. H., Guan, Y., Peiris, J. S., Tran, T. H., and Farrar, J. (2005) N. Engl. J. Med. 353, 2667–2672
40. Le, Q. M., Kiso, M., Someya, K., Sakai, Y. T., Nguyen, T. H., Nguyen, K. H., Pham, N. D., Nguyen, H. H., Yamada, S., Muramoto, Y., Horimoto, T., Takada, A., Goto, H., Suzuki, T., Suzuki, Y., and Kawauka, Y. (2005) Nature 437, 1108