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Identification of Amino Acid Residues of Influenza Virus Nucleoprotein Essential for RNA Binding

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The influenza virus nucleoprotein (NP) is a single-strand-RNA-binding protein associated with genome and antigenome RNA and is one of the four virus proteins necessary for transcription and replication of viral RNA. To better characterize the mechanism by which NP binds RNA, we undertook a physical and mutational analysis of the polypeptide, with the strategy of identifying first the regions in direct contact with RNA, then the classes of amino acids involved, and finally the crucial residues by mutagenesis. Chemical fragmentation and amino acid sequencing of NP that had been UV cross linked to radiolabelled RNA showed that protein-RNA contacts occur throughout the length of the polypeptide. Chemical modification experiments implicated arginine but not lysine residues as important for RNA binding, while RNA-dependent changes in the intrinsic fluorescence spectrum of NP suggested the involvement of tryptophan residues. Supporting these observations, single-codon mutagenesis identified five tryptophan, one phenylalanine, and two arginine residues as essential for high-affinity RNA binding at physiological temperature. In addition, mutants unable to bind RNA in vitro were unable to support virus gene expression in vivo. The mutationally sensitive residues are not localized to any particular region of NP but instead are distributed throughout the protein. Overall, these data are inconsistent with previous models suggesting that the NP-RNA interaction is mediated by a discrete N-terminal domain. Instead, we propose that high-affinity binding of RNA by NP requires the concerted interaction of multiple regions of the protein with RNA and that the individual protein-RNA contacts are mediated by a combination of electrostatic interactions between positively charged residues and the phosphate backbone and planar interactions between aromatic side chains and bases.

The genome of influenza A virus is composed of eight strands of negative-sense RNA, which in virions and infected cells occurs in the form of ribonucleoprotein (RNP) complexes with the virus-encoded PB1, PB2, and PA proteins and nucleoprotein (NP). The RNP complexes represent the functional templates for influenza virus RNA synthesis, and during infection they contain both negative-sense (vRNA) and positive-sense (cRNA) replicative intermediate RNA but not viral mRNA (reviewed in reference 31). The four RNP-associated proteins are the only viral polypeptides necessary for transcription and replication of a genome segment (24).

PB1, PB2, and PA associate to form a highly regulated RNA-dependent RNA polymerase capable of switching between two modes of transcription to produce either capped and polyadenylated mRNA or replicative copies of the genome segments (31). In contrast, the major function associated with NP is a nonenzymatic single-strand-RNA-binding activity, which has no apparent sequence specificity (3, 27, 43, 49). This RNA-binding activity is reflected in the stoichiometric quantity of the protein in RNPs, from which it has been estimated that one polypeptide interacts with around 20 nucleotides of RNA (11). Although NP encapsidates the RNA, it does not protect it from digestion with RNase (3, 14, 39), suggesting that the RNA is bound on the outside of the structure. NP is apparently responsible for the higher-order structure of RNPs, which occur in the form of helices twisted back into a double-helical hairpin (25, 39), since the structures can persist following removal of the RNA (39, 41). This implies protein-protein contacts between NP monomers (41), which is consistent with the observed cooperative nature of the NP-RNA interaction (49).

Other functions have been postulated for NP; although the polymerase complex can efficiently transcribe short templates, NP is required for synthesis of longer RNAs, leading to the suggestion that it may act as a processivity factor (23). NP may also play a role in the regulation of polymerase activity, since cRNA and vRNA (but not mRNA) synthesis depends on a supply of soluble (i.e., not bound to RNA) NP (4, 45). Intriguingly, NP has recently been shown to make direct protein–protein contacts with the PB1 and PB2 subunits of the polymerase (5, 35), but whether these contacts play a role in regulating polymerase activity is not yet known. NP also interacts with at least two cellular proteins. As expected for a protein that contains nuclear localization signals, it interacts with polypeptides of the importin α family (36, 47), and this proximal interaction is capable of directing the nuclear import of NP-RNA complexes (37). NP also binds filamentous actin, an interaction that influences the cellular localization of NP (13).

A molecular characterization of this multifunctional protein is therefore of some interest, with an analysis of how the polypeptide interacts with RNA being a logical starting point. Two studies have identified an amino-terminal region of NP that is capable of binding RNA (1, 29), but this fragment binds RNA with much lower affinity than the intact polypeptide does, suggesting that other NP sequences are important for high-affinity binding (1). Also, little information is available at the amino acid level on how NP interacts with RNA, and the protein does not contain sequences with homology to previously characterized RNA-binding motifs (1, 9). However, the protein as a whole is very basic (48), and this has led to a widely accepted hypothesis that the positive charges of lysine and arginine residues are involved in contacting the negatively
charged phosphate backbone. Since our long-term aim is to understand the mechanisms by which NP exerts its regulatory effects on polymerase transcription, we decided to further investigate how the protein interacts with RNA. Our strategy was to first identify which regions of the full-length polypeptide make direct contact with RNA, to then test the importance of various classes of amino acid for binding, and to finally employ site-directed mutagenesis to identify the important residues. We show here that direct protein–RNA contacts occur throughout the length of the polypeptide and not just at the N terminus. Intrinsic fluorescence spectroscopy and chemical modification experiments indicate the involvement of tryptophan and arginine residues in binding RNA, and consistent with this, mutational analysis identified two arginine and six aromatic residues essential for RNA binding. These mutationally sensitive residues were not localized to any particular region of the polypeptide but were found throughout the protein. Thus, high-affinity RNA binding by NP requires contributions from multiple regions of the polypeptide.

MATERIALS AND METHODS

Plasmids, RNA, antisera, and viruses. Plasmids capable of directing the expression of authentic NP in eukaryotic cells (pKT5) or fused to maltose-binding protein (MBP) (pMAL-NP) in Escherichia coli have been described previously (13). Certain NP point mutants have been described previously (13), while others were constructed by site-directed mutagenesis of plasmids pKT5 or pMAL-NP by standard procedures. For in vitro RNA-binding assays, a radiolabelled 17-nucleotide synthetic RNA target corresponding to influenza virus A/PR8/34 segment 8, but with nucleotides 84 to 795 (inclusive) deleted, and a C-to-A transition of the penultimate base was transcribed from plasmid pKT5-A3’5’ as previously described (13). Rabbit antisera raised against amino acids 340 to 498 of A/PR8/34 NP (13) have been described previously. Recombinant vaccinia viruses expressing the three influenza virus A/PR8/34 P proteins (46) or bacteriophage T7 RNA polymerase (vTF-7) (18) have been described previously.

Expression and purification of NP. NP fused to MBP-NP was purified from extracts of E. coli TG1 containing plasmid pMAL-NP by affinity chromatography on amylose resin (New England Biolabs) as previously described (13). To remove the MBP moiety, the fusion protein was digested with 0.5% (wt/wt) factor Xa protease (New England Biolabs). After an 18-h incubation at 14°C, the protein was loaded onto a MonoQ ion-exchange column (Pharmacia) equilibrated with 50 mM Tris-Cl (pH 7.6), the column was eluted with a linear gradient of 0 to 2 M NaCl in 50 mM Tris-Cl (pH 7.6). MBP eluted mainly in the flowthrough fractions, while NP eluted as a broad peak at around 800 mM NaCl.

RNA filter-binding assays. Filter-binding assays were performed by incubating a fixed amount of RNA (approximately 1 pmol of RNA per assay cm2) in 25 mM Tris-Cl (pH 7.6)-50 mM NaCl-1 mM MgCl2-0.5 mM dithiothreitol-DTT-5% glycerol at room temperature (unless otherwise specified) for 20 min. The reaction mixtures were passed through nitrocellulose filters equilibrated in 20 mM Tris-Cl (pH 7.6)-50 mM NaCl-0.1 mM EDTA-10% glycerol to stop further reaction and removed unreacted RNA. The remainder of the samples were treated by the addition of 2 volumes of 0.1 M NaH2PO4-1.5 M Na2SO4 (13), and the incorporation of trinitrobenzene was determined by spectrophotometry at 420 nm with a molar absorption coefficient of 1.92 × 104 M−1 cm−1 atm−1 (17) (potential absorption resulting from modification of the polypeptide N terminus was disregarded).

When complete derivatization of primary amine was desired, the protein sample (0.1 to 1 mg/ml) was diluted to 250 µl with water and an equal volume of 4% Na2CO3 followed by 250 µl of 0.1 M HCl and digested for 2-h incubation at 40°C, the reaction was stopped by the addition of 250 µl of 10% SDS and 125 µl of 1 M HCl. The absorption was read at 335 nm, and the number of modified amine groups was determined with respect to a standard curve constructed from a dilution series of bovine serum albumin (22).

To modify primary amine groups with acetyldihydriod, aliquots of MBP-NP were mixed with an equal volume of saturated sodium acetate and treated with two aliquots of acetic anhydride for 30 min each on ice. The samples were passed over a Sephacry G-25 column equilibrated in storage buffer to exchange buffers and remove unreacted acetyldihydriod, and aliquots were reacted with TNBS as described above to quantify remaining unreacted amine groups.

Fluorescence spectroscopy. Intrinsic fluorescence measurements were recorded at 25°C in a Perkin-Elmer LS 50 B luminescence spectrometer with a thermostatically controlled cuvette holder. Wavelength scans were taken from protein samples diluted to 1 µg/ml in 50 mM NaCl-20 mM Tris-Cl (pH 7.6) with an appropriate wavelength of 280-450 nm slit with an emission slit of 5 nm. Scans were averaged from at least five repetitions. Quenching experiments were performed with 5 µg of protein per ml, allowing a 5-min equilibration time after the addition of RNA aliquots. Fluorescence output was then integrated for 1 s, before the addition of further RNA.

Polymerase-binding assays. Xenopus laevis oocytes were maintained and microinjected with synthetic RNAs encoding the influenza virus P proteins (transcribed from plasmids pKT1 to pKT3 as previously described (6, 12)). For polymerase reactions, 2.5 µg of the lysate (corresponding to one-quarter of an oocyte) was incubated with 0.5 µg of fusion protein in 100 µl of IP buffer (100 mM KCl, 50 mM Tris-Cl (pH 7.6), 5 mM MgCl2, 1 mM dithiothreitol, 0.1% Nonidet P-40) for 1 h at room temperature. A 50-µl volume of a 50% (vol/vol) slurry of amylose resin (New England Biolabs) in phosphate-buffered saline was added, and incubation was continued for a further 30 min with gentle mixing. The solid phase was collected by centrifugation and washed three times with 750 µl of IP buffer. Bound material was eluted by boiling in 40 µl of SDS-PAGE sample buffer and analyzed by SDS-PAGE and autoradiography.

RESULTS

Identification of NP sequences in direct contact with RNA. Previously, we have shown that while MBP alone does not detectably bind RNA, a fusion of E. coli MBP and influenza virus NP (MBP-NP) binds RNA with similar affinity to authentic NP purified from influenza virus virions (Kd of 20 nM) (13). To further examine the interaction of NP with RNA, we digested the fusion protein with factor Xa protease to separate the MBP and NP domains and purified the NP to near homogeneity by ion-exchange chromatography (Fig. 1b, lane 1). Purified NP and MBP-NP were then tested for their RNA-binding activity by using a UV cross-linking assay. Protein samples were incubated with a radiolabelled RNA and subjected to UV irradiation to form cross-links between molecules in direct contact. Following removal of free RNA by digestion with...
RNase, polypeptides were separated by SDS-PAGE, visualized by staining with Coomassie blue dye, and examined for bound RNA by autoradiography (Fig. 1a). Both MBP-NP and NP became radiolabelled when subjected to this procedure (lanes 6 and 7, respectively) but did not acquire radiolabel when incubated with RNA in the absence of UV irradiation (lanes 8 and 10). In addition, binding could be competed with unlabelled RNA from viral and cellular sources (data not shown), indicating that the reaction was specific and that the recombinant NP showed similar sequence-independent binding properties to those of authentic NP (3, 19, 49). Moreover, when MBP-NP was irradiated in the presence of RNA and subsequently incubated with factor Xa protease to separate the MBP and NP moieties, only NP was radiolabelled (Fig. 1a, lane 9). Therefore, the RNA-binding activity of MBP-NP depends solely on the NP portion of the fusion protein, and UV cross-linking provides a convenient and specific means of examining this activity.

Previous work has shown that the N-terminal one-third of NP binds RNA, but with substantially lower affinity than the intact protein does (1, 29). Thus, sequences outside of the N terminus contribute to RNA binding, either directly by contacting RNA or indirectly by affecting the folding of the N terminus (1). To distinguish between these possibilities, we chemically digested NP which had been UV cross-linked to a radiolabelled RNA and examined the distribution of radiolabel among the protein fragments. After treatment with 70% formic acid, which induces partial hydrolysis of aspartyl-prolyl bonds (32), five major novel polypeptides were observed (Fig. 1b, compare lanes 1 and 2). The separated fragments were further analyzed by transfer to a polyvinylidene difluoride membrane and immunoblotting with an antiserum directed against the C terminus of NP (α-C) or by amino acid sequencing. Major NP fragments are labelled i to v, and the N-terminal residue (NH₂ residue) is given. (c) Formic acid cleavage map of NP. Numbered arrows indicate the positions of aspartyl-prolyl dipeptides in NP, and the bracket gives the sequences against which anti-NP sera were raised. Thin lines represent the identities of the numbered NP fragments.

**FIG. 1.** RNA-binding activity of recombinant NP. (a) UV cross-linking analysis of RNA binding by MBP-NP and NP. Protein samples were incubated with radiolabelled RNA and subjected to UV irradiation. Excess RNA was removed by RNase digestion, and samples were separated by SDS-PAGE. Radiolabelled proteins were detected by autoradiography (³²P; lanes 6 to 10), and total protein was detected by staining with Coomassie brilliant blue (Coomassie; lanes 1 to 5). Lanes: 1 and 6, MBP-NP; 2 and 7, MBP-NP (samples in lanes 1 and 2 were not irradiated); 3 and 8, MBP-NP; 4 and 9, MBP-NP incubated with factor Xa protease after cross-linking; 5 and 10, NP. Open arrowheads mark the indicated polypeptides. Solid arrowheads mark a contaminant protein in the RNase preparation (top) or factor Xa (bottom). Also shown are the molecular masses (in kilodaltons) of marker proteins. (b) Chemical fragmentation of NP. NP was cross-linked to a radiolabelled RNA and analyzed by SDS-PAGE before (–) or after (+) incubation in 70% formic acid. Total and radiolabelled proteins were detected as in panel a or by Western transfer to a polyvinylidene difluoride membrane and immunoblotting with an antiserum directed against the C terminus of NP (α-C) or by amino acid sequencing. Major NP fragments are labelled i to v, and the N-terminal residue (NH₂ residue) is given. (c) Formic acid cleavage map of NP. Numbered arrows indicate the positions of aspartyl-prolyl dipeptides in NP, and the bracket gives the sequences against which anti-NP sera were raised. Thin lines represent the identities of the numbered NP fragments.
can interact with single-stranded nucleic acids either by polar interactions or by planar stacking with exposed bases (2, 7, 8, 20, 26, 38, 40, 44). To test whether this was the case for NP, we used fluorescence spectroscopy. Tryptophan and to a lesser extent tyrosine residues absorb light at wavelengths around 280 to 300 nm and subsequently emit some of the absorbed energy at an intensity and wavelength dependent on the environment of the fluorescing side chain (42).

Initial experiments revealed that emission spectra obtained from NP by using excitation wavelengths of 280 and 296 nm were superimposable after scaling, suggesting that the majority of the fluorescence arose from tryptophan residues and that there was no significant output from tyrosine residues (data not shown). The peak fluorescence emission ($\lambda_{\text{max}}$) from a 10 nM NP solution was at 334 nm (Fig. 2a), indicating that on balance, the fluorescent tryptophan residues were partially exposed to solvent. In confirmation of this, under conditions where the protein would be expected to be fully denatured (8 M guanidinium chloride), $\lambda_{\text{max}}$ was shifted to 350 nm, along with an 80% decrease in fluorescence intensity (Fig. 2a, data scaled to 100% for comparison). We also measured the emission spectrum of NP extracted from virions and found that it was almost identical to that of the recombinant NP (Fig. 2a), suggesting that the two proteins were of similar overall conformation. Next, we investigated whether the intrinsic fluorescence of NP was altered when the protein was bound to RNA. Although $\lambda_{\text{max}}$ was not significantly altered in the presence of saturating amounts of RNA, the fluorescence intensity was...
reduced by around 35% (Fig. 2b), indicating that the fluorescence of one or more of the tryptophan residues was quenched when the protein was bound to RNA. To investigate this phenomenon further, we titrated a solution of NP with increasing amounts of RNA and measured the fluorescence output (Fig. 2c). Addition of RNA caused an initially sharp but then gradually decreasing fluorescence, which tended toward a plateau when the fluorescence intensity had reached about two-thirds of its initial value, consistent with the drop in fluorescent output seen in Fig. 2b. If the fluorescence quenching was a direct consequence of RNA binding, it would be expected to be reversed under conditions which disrupted protein-RNA contacts. We therefore performed a salt back titration and found that addition of NaCl resulted in an increase in fluorescence intensity, with 50% of the original signal being restored by 500 mM NaCl (Fig. 2d). This value is similar to the salt concentration shown to inhibit RNA binding by 50% (49), indicating that fluorescence quenching directly reflects the NP-RNA interaction. Thus, the environment of tryptophan residues in NP is reversibly altered by binding to RNA.

Chemical modification of NP. NP as a whole is extremely basic (containing 21 lysine and 49 arginine residues [48]), consistent with the hypothesis that positive charges on the protein interact with the negatively charged phosphate backbone of RNA (19). To test this, we chemically modified the protein with reagents specific for arginine or lysine residues. We chose treatments that could be used under relatively mild buffer conditions, since this approach would be more likely to preserve the polypeptide structure (avoiding nonspecific loss of RNA-binding activity) and therefore would preferentially modify surface-exposed residues. Also, to be able to monitor the degree of protein modification, we chose reagents that allowed spectrophotometric quantification of the reaction. However, since these procedures required relatively large quantities of protein for accurate determinations, we used MBP-NP as a substrate since it was easier to prepare the necessary quantities of the fusion protein and since the MBP moiety had no apparent effect on the affinity of the polypeptide for RNA (13).

To investigate the effect of modification of arginine residues on RNA binding, we treated MBP-NP with p-HPG, which reacts with the guanidyl group of arginine (50). Aliquots of MBP-NP were incubated with a 1,000-fold molar excess of p-HPG, and the amount incorporated into protein was determined by spectrophotometry at 340 nm (data not shown). SDS-PAGE analysis revealed that although treatment with p-HPG had not resulted in appreciable degradation of the protein relative to the mock-treated sample, it had slightly altered its electrophoretic mobility (Fig. 3a). Protein recovery was quantified, and in conjunction with the spectrophotometric incorporation data, it was determined that 11% of the arginine residues had been modified (data not shown). MBP-NP contains 55 arginines (49 of which are in NP [15, 21, 48]), so on average, 6 residues per protein had been modified. The MBP-NP samples were tested for their RNA-binding ability in a filter assay, where it was found that the modified protein had lost almost all activity relative to the mock-treated sample (Fig. 3b). In replicate experiments, treatment with p-HPG led to modification of between 11 and 17% (average, 15 ± 3%) of the arginine residues (corresponding to an average of 8 amino acids/protein), and in all cases the modified polypeptides showed very little RNA-binding activity (data not shown). However, the intrinsic fluorescence spectra of the treated polypeptides were not significantly different from those of the mock-treated samples, indicating that the modifications had not affected the overall structure of the polypeptides (data not shown). Therefore, a small number of arginine residues in NP are accessible to modification by p-HPG and are crucial for RNA binding.

To investigate the effect of modification of lysine residues on RNA binding, we treated MBP-NP with TNBS, which introduces trinitrobenzene onto ε-amino groups (17, 22). The reaction is influenced by pH, and so we treated MBP-NP at a range of pH values to generate polypeptides that were modified to various extents. MBP-NP at pH 9.6, 8.8, or 8.0 was incubated with or without TNBS for 5 min. Unreacted TNBS was removed by gel filtration, and the amount incorporated was determined by spectrophotometry. Aliquots were also analyzed by SDS-PAGE and staining with Coomassie blue dye to check for protein recovery and integrity. The extent of ε-amino group modification ranged from 32% (pH 9.6) to 13% (pH 8.0), while there was no apparent degradation of the polypeptides (Fig. 3c). The modified proteins were then tested for their ability to bind RNA as before. MBP-NP samples treated at pH 8.0 or 8.8 were not substantially altered in their ability to retain the radio-labelled RNA compared to a sample mock treated at pH 9.6 (Fig. 3d) or samples mock treated at pH 8.8 or 8.0 (data not shown). However, MBP-NP treated at pH 9.6 showed a reduced (but not abolished) ability to bind RNA, retaining only 40% of the RNA at a dose where unmodified MBP-NP retained over 80% (Fig. 3d). Therefore, modification of the lysine residues of MBP-NP with TNBS did alter the ability of the protein to bind RNA, but only at relatively high values of derivatization.

TNBS modification of lysine replaces the normal positive charge of the amino acid side chain with a bulky and highly negatively charged trinitrobenzene group, which could affect RNA binding by steric effects or charge repulsion even if the modified lysines were not directly involved in contact with RNA. We therefore tested the effect of a smaller, uncharged substitution of amino groups on RNA-binding activity. Accordingly, portions of MBP-NP were treated with acetic anhydride to acetylate ε-amino groups. To quantify the extent of modification, aliquots of each reaction mixture were subsequently treated with TNBS under conditions which would be expected to derivatize all remaining unmodified amino groups (22), to permit spectrophotometric measurement of the number of remaining lysines. Protein samples were also analyzed by SDS-PAGE and staining with Coomassie blue dye to monitor protein integrity and recovery. In the experiment in Fig. 3e, treatment of MBP-NP with approximately 60- and 250-fold molar excesses of acetic anhydride led to acetylation of 50 and 66% of the lysine residues, respectively, and while there was no obvious degradation of the polypeptides, their electrophoretic mobility had decreased slightly compared to that of mock-treated MBP-NP. When the modified polypeptides were tested for their ability to bind RNA, they displayed much reduced but not completely abolished activity compared to mock-treated MBP-NP (Fig. 3f). While 1 pmol of the unmodified sample was sufficient to retain 30% of the input RNA and 10 pmol was sufficient to retain over 90%, the modified polypeptides bound less than 30% of the probe even at a dose of 30 pmol.

Thus, modification of lysines in MBP-NP with either TNBS or acetic anhydride led to a reduction in the RNA-binding ability of the polypeptide, but even the highest levels of derivatization tested (66%; Fig. 3f) did not completely abolish RNA binding. However, MBP contains significant numbers of lysines (36, compared to 21 in NP [15, 21, 48]), unlike arginine residues. Therefore, it was possible that most of the derivatization in the experiments in Fig. 3 occurred in the MBP moiety and that the actual modification levels of NP were significantly lower than is predicted by simple proportion. However, when isolated NP was treated with acetic anhydride, similar levels of
derivatization were obtained, which again resulted in no more than a threefold reduction in RNA-binding activity (data not shown). Overall, the data suggest that lysines do not play as crucial a role as arginines, where modification of a much smaller proportion of the amino acid side chains was sufficient to render the polypeptide almost completely unable to bind RNA (Fig. 3b).

**RNA-binding activity of mutant NP polypeptides.** The experiments described above implicated tryptophan and arginine side chains as being important in RNA binding. However, they did not address the question of which particular residues are involved. Since NP contains only 6 tryptophans (48) (Table 1), we mutated each codon in an attempt to define the residue(s) involved in RNA binding. A similar approach did not seem feasible for arginine residues, since the protein contains 49. However, by examining sequence alignments of NPs from influenza A, B, C, and Dhori orthomyxoviruses (data not shown), we were able to identify several conserved arginine residues at positions 8, 150, 156, 175, 199, 204, 208, 213, 267, 391, and 416 (Table 1). These and the tryptophan residues encoded by pMAL-NP were altered to alanine. In addition, to further probe the importance of aromatic amino acids in binding RNA, we mutated conserved tyrosine and phenylalanine residues at positions 148 and 412, respectively (Table 1).
TABLE 1. Phenotypic summary of NP point mutations

<table>
<thead>
<tr>
<th>Mutant</th>
<th>RNA-binding activity (37°C)</th>
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<tbody>
<tr>
<td>WT</td>
<td>++</td>
</tr>
<tr>
<td>R8→A</td>
<td>++</td>
</tr>
<tr>
<td>W104→A</td>
<td>++</td>
</tr>
<tr>
<td>W120→A</td>
<td>++</td>
</tr>
<tr>
<td>W139→A</td>
<td>++</td>
</tr>
<tr>
<td>Y148→L</td>
<td>++</td>
</tr>
<tr>
<td>R150→A</td>
<td>++</td>
</tr>
<tr>
<td>R156→A</td>
<td>++</td>
</tr>
<tr>
<td>R175→A</td>
<td>++</td>
</tr>
<tr>
<td>R199→A</td>
<td>++</td>
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<tr>
<td>R204→A</td>
<td>++</td>
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<tr>
<td>R208→A</td>
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<td>R213→A</td>
<td>++</td>
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<tr>
<td>R213→A</td>
<td>--</td>
</tr>
<tr>
<td>R267→A</td>
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<tr>
<td>W303→A</td>
<td>--</td>
</tr>
<tr>
<td>W386→A</td>
<td>--</td>
</tr>
<tr>
<td>R391→A</td>
<td>++</td>
</tr>
<tr>
<td>F412→A</td>
<td>--</td>
</tr>
<tr>
<td>R416→A</td>
<td>--</td>
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</table>

* The identities of WT and mutated residues are given in the standard single-letter code.

* RNA-binding activity was assessed by UV cross-linking. ++, essentially WT behavior; +, reduced ability to bind RNA; --, little or no observable RNA-binding activity.

The desired changes were introduced into pMAL-NP by site-directed mutagenesis, and the mutant fusion proteins were expressed in *E. coli* and purified by affinity chromatography on amylose columns as described above. However, many of the mutants accumulated to lower levels than wild-type (WT) MBP-NP did, so that when sample concentrations were adjusted to contain equal amounts of full-length fusion protein (assessed by Coomassie blue staining [Fig. 4b]), they contained larger amounts of contaminating polypeptides, including a family of products of around 50 kDa. These polypeptides reacted with antisera raised against either MBP or the N terminus of NP and therefore most probably represent proteolytic products of the fusion protein (data not shown). Similar presumed degradation products were also observed in some preparations of WT MBP-NP (e.g., Fig. 4b, lane 14). The protein samples were tested for their ability to bind RNA in solution by a UV cross-linking assay. As above, the wild-type MBP-NP fusion protein became radiolabelled when irradiated in the presence of the probe, indicating that it had bound to the added RNA (Fig. 4a, lanes 1, 14, and 15), while no product was seen in the absence of protein (lane 6). The majority of the arginine-to-alanine mutants became radiolabelled to the same intensity as did WT MBP-NP (lanes 2 to 5, 7 to 10, and 12), suggesting that they were not significantly altered in their ability to bind RNA (Table 1). In contrast, the R267 and R416 mutants failed to become detectably radiolabelled (lanes 11 and 13, respectively), indicating that they had lost the ability to bind RNA (Table 1). Of the aromatic mutants, the W104, W207, and Y148-L mutants bound RNA at essentially WT levels (lanes 16, 19, and 22, respectively). However, the W330-A and F412 mutants possessed very little RNA-binding activity (lanes 20 and 23, respectively), while the W120, W139, and W386 mutants showed appreciable but reduced binding (lanes 17, 18, and 21 respectively). The reduced binding of the last three tryptophan mutants was reproducible and could be observed across a range of protein concentrations (data not shown), suggesting that the polypeptides had a reduced affinity for RNA. Thus, consistent with the results of chemical modi-
fication and fluorescence spectroscopy, two arginine, one phenylanine, and four tryptophan residues were identified as being essential for WT RNA-binding activity. In addition, there was no clear correlation between WT RNA-binding activity and the quantity of copurifying MBP-NP “stub” (e.g., compare lanes 21 and 22). Also, the MBP-NP stub possessed very little RNA-binding activity in this assay.

Recently, we have shown that the mutations in the NPs of the temperature-sensitive (ts) viruses A/WSN/33 ts56 and A/FPV/Rostock/34 tsG81 render the RNA-binding activity of the polypeptides ts (35). We therefore tested certain of the point mutants described here for ts RNA-binding. Mutant MBP-NP fusion proteins were prepared as above from E. coli cultures grown at 30°C, and their RNA-binding activities were compared to those of the polypeptides prepared from cultures incubated at 37°C. The abilities of W330-A and W386-A to bind RNA were not appreciably improved by synthesis at 30°C (data not shown). In contrast, when the R267-A, F412-A, and R416-A polypeptides were prepared at 30°C and assayed for their ability to bind RNA at 30°C, they showed much increased activity compared to the same proteins synthesized at 37°C (Fig. 5a, compare lanes 3 and 22). Also, the MBP-NP stub possessed very little RNA-binding activity in this assay.

Interaction of the NP RNA-binding mutants with the influenza virus polymerase. The mutational analysis described above identified specific residues whose substitution reduced RNA-binding activity, potentially through direct alteration of an amino acid side chain normally in contact with RNA. Alternatively, some or all of the alterations may have exerted an indirect effect on RNA binding by perturbing the polypeptide structure, a possibility which seemed especially plausible for the mutations which rendered RNA binding ts. However, defective which caused gross malfolding of the polypeptide struc-
ture would be expected to affect other functions of NP. Previously, we have shown that the W120 and R267 mutations have minimal effect on the ability of NP to bind filamentous actin (13). Similarly, the W120, W139, R267, W330, W386, and F412 mutations have little effect on the ability of the polypeptides to form NP-NP oligomers (16). Recent work has shown that NP also makes direct protein-protein contacts with the PB1 and PB2 subunits of the influenza virus polymerase (5, 35). We therefore tested the ability of the RNA-binding mutants to interact with the polymerase complex. Previously, we have shown that the three influenza virus P proteins assemble into a complex in X. laevis oocytes microinjected with the appropriate mRNAs and that this system provides a convenient source of soluble radiolabelled protein for binding studies (6, 12, 35). Accordingly, oocyte lysates containing the polymerase complex were incubated with MBP or with WT and mutant MBP-NP polypeptides (synthesized at 37°C). After the addition of amylose resin, the solid phase was collected, washed, and examined for bound polypeptides. All three P proteins were precipitated by WT MBP-NP but not by MBP alone (Fig. 6, lanes 2 and 3), indicating a specific association between NP and the polymerase complex. Furthermore, similar quantities of the polymerase complex were precipitated by all the mutant MBP-NPs defective for RNA binding (lanes 4 to 10). Therefore the point mutations which disrupt RNA-binding activity are not associated with the general loss of other NP functions.

**Ability of the NP RNA-binding mutants to support virus gene expression.** NP is known to be essential for virus RNA synthesis (reviewed in reference 31), and an artificial system has been established where NP and the three subunits of the influenza virus RNA polymerase supplied from recombinant vaccinia viruses are sufficient to drive transcription and replication of a synthetic influenza virus genome segment in cells (24). We used an adaptation of this system where NP is supplied from transfected plasmids (13) to test the effects of point mutations disruptive of RNA binding on the ability of NP to support virus gene expression. The mutant NP genes were subcloned into plasmid pKT5 (13), which directs the expression of unfused NP under the control of the bacteriophage T7 RNA polymerase promoter. BHK cells were multiply infected with vaccinia viruses expressing the influenza virus and bacteriophage T7 RNA polymerase subunits (18, 46) and transfected with WT or mutant pKT5 plasmids and the synthetic flu-CAT RNA, and 20 h later the cells were lysed and the accumulation of CAT polypeptide was measured. Figure 7 shows the activities of the NP RNA-binding mutants relative to the WT polypeptide. All four mutants which were unable to bind RNA in vitro (Fig. 7a, R267, W330, F412, and R416) failed to support appreciable levels of virus gene expression. Of the three mutants which displayed reduced RNA-binding activity (RNA +), one also failed to produce significant levels of CAT polypeptide (Fig. 7a, W120) whereas the other two (W139 and W386) supported appreciable but diminished levels of gene expression relative to the WT protein. Western blot analysis of parallel transfections showed that the majority of the NP mutants (with the exception of W120) accumulated in similar quantities to WT NP (Fig. 7b), indicating that their diminished ability to support virus RNA synthesis was not simply the result of poor expression. Thus, the in vitro phenotypes of the mutants correlate with their in vivo function, and as expected, the RNA-binding activity of NP is essential for virus gene expression.
CONCLUSION

Previously, two studies mapped the minimal sequences of NP capable of binding RNA to the N-terminal one-third of the polypeptide (Fig. 8) (1, 29). However, this truncated fragment of NP bound to RNA with lower affinity than did the WT protein, indicating the necessity for other sequences for full binding activity (1). In this study, we show by UV cross-linking and chemical fragmentation that at least two sequences within the C-terminal two-thirds of NP directly contact RNA (Fig. 1b). Furthermore, we found that single-amino-acid replacements throughout much of the length of NP were capable of disrupting the interaction of the polypeptide with RNA (summarized in Fig. 8). The widespread mutational sensitivity of RNA-binding activity is in contrast to the sequence requirements for the characterized protein-protein interactions mediated by NP, in which for any one ligand, disruptive point mutations are generally more localized in distribution (Fig. 8).

The majority of the RNA-binding mutations do not affect other functions of NP, arguing against global malfolding of the polypeptide. None of the lesions described here decrease the ability of NP to interact with the polymerase complex (Fig. 6). With the exception of R416, which also decreases oligomerization of the polypeptide, none of the mutations affecting RNA binding block the ability of the polypeptides to form NP-NP contacts (16). In addition, the W120 and R267 mutations have little effect on the interactions of the proteins with filamentous actin (13). Thus, the majority of the mutations reduce RNA-binding activity specifically. However, some of them do so in a temperature-dependent fashion, affecting the function significantly at 37°C but much less so at 30°C. Thus, for these mutants at least, it seems unlikely that the sequence alterations have removed an amino acid side chain normally in direct contact with RNA. Yet, as discussed above, the mutations are clearly not disrupting the global folding of NP at the elevated temperature, since the polypeptides retain the other activities associated with NP. Furthermore, any effects of the mutations on the overall structure of NP were too subtle to be detected as changes in the resistance of the polypeptides to partial digestion with trypsin or chymotrypsin proteases (data not shown).

Overall, these data are not consistent with the hypothesis that RNA binding by NP is mediated through a discrete N-terminal region of the protein (1, 29). Instead, we propose that high-affinity binding of RNA by NP requires the concerted interaction of multiple regions of the protein with RNA. This hypothesis is consistent with the demonstration of direct protein-RNA contacts outside the 8 region (Fig. 1b) and the identification of inhibitory mutations throughout the length of NP (Fig. 4). This model is also consistent with the proposed wrapping of RNA around NP in a nucleosome-like pattern (25, 49), as well as with the increased thermal stability of WT protein-RNA complexes and the ability of RNA to rescue the binding activity of a ts mutant at elevated temperatures (Fig. 5c). Within this framework, several possibilities exist to explain the ability of widely separated point mutations to ablate RNA binding without affecting other functions of NP. One possibility is that high-affinity binding results from the multiplicative contribution of independent binding sites, so that the loss of any one interaction causes a relatively large decrease in binding affinity resulting from what is effectively a loss of avidity. However, we have recently found evidence from circular dichroism spectroscopy that NP undergoes a conformational change on binding RNA (data not shown). Therefore, an alternative hypothesis is that multiple protein-RNA contacts are required to drive this conformational change necessary for high-affinity binding and that the loss of any one interaction interrupts the process.

Within the model of polyvalent binding, we propose that the individual protein-RNA interactions are mediated by a combination of electrostatic interactions between positively charged residues and the phosphate backbone and planar interactions between aromatic side chains and bases. This is consistent with the sensitivity of the protein to chemical modification of arginine residues (Fig. 3), the changes in the environment of tryptophan residues upon RNA binding (Fig. 2), and the requirement for specific arginine and aromatic residues (Fig. 4). In addition, this mode of single-stranded-nucleic acid binding is well supported by mutational and structural studies of other DNA- and RNA-binding proteins (7, 10, 20, 28, 38, 40, 44). Two studies which probed influenza virus RNP complexes by measuring the protection afforded the RNA from chemical and enzymatic attack found a general protection of RNA phosphates but sensitivity of the bases (3, 28). This was interpreted as indicating that the protein binds to the phosphate backbone and not to the Watson-Crick positions of the bases. However, such findings do not rule out contacts between the protein and other positions of the bases. Indeed, NP binds preferentially to pyrimidine homopolymers, which suggests an interaction between the protein and bases (1). Also, considering that NP is not a sequence-specific RNA-binding protein, RNA modification experiments may well reflect an average state of protection for any one position, rather than a static interaction.

Recently, we have determined that the mutations defined as the ts lesions in the NPs of viruses A/WSN/33 ts56 (S314-N) (33) and A/FPV/Rostock/34 tsG81 (A332-T) (34) specifically induce ts RNA-binding by NP (35). Since these viruses are considered specifically defective for cRNA and vRNA transcription, this finding has implications for the function of NP during replicative transcription. The panel of NP mutants described here should therefore prove useful tools for improving our understanding of the functions of NP in influenza virus transcription.

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