Site-Directed Mutation of the Active Site of Influenza Neuraminidase and Implications for the Catalytic Mechanism†

Michael R. Lentz,‡ Robert G. Webster,§ and Gillian M. Air*,‡

Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294, and St. Jude Children's Research Hospital, Memphis, Tennessee 38101

Received December 2, 1986; Revised Manuscript Received March 4, 1987

ABSTRACT: Different isolates of influenza virus show a high degree of amino acid sequence variation in their surface glycoproteins. Conserved residues located in the substrate-binding pocket of the influenza virus neuraminidase are therefore likely to be involved in substrate binding or enzyme catalysis. In order to study the structure and function of the active site of this protein, a full-length cDNA clone of the neuraminidase gene from influenza A/Tokyo/3/67 was subcloned into an M13 vector and amino acid substitutions were made in selected residues by using the oligonucleotide mismatch technique. The mutant neuraminidase genes were expressed from a recombinant SV40 vector, and the proteins were analyzed for synthesis, transport to the cell surface, and proper three-dimensional folding by internal and surface immunofluorescence. The mutant neuraminidase proteins were then assayed to determine the effect of the amino acid substitution on enzyme activity. Twelve of the 14 mutant proteins were correctly folded and were transported to the cell surface in a manner identical with that of the wild type. Two of these have full enzyme activity, but seven mutants, despite correct three-dimensional structure, have completely lost neuraminidase activity. Two mutants were active at low pH. The properties of the mutant enzymes suggest a possible mechanism of neuraminidase action.

Influenza virus has two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Both proteins function in the immune recognition of the virus by the host, and both undergo antigenic variation, allowing viruses to escape previously established immunity to earlier strains. HA binds to host-cell receptors and initiates penetration by fusing viral and host-cell membranes. NA is an enzyme that catalyzes the cleavage of the α-ketosidic linkage between a terminal sialic acid residue and the adjacent residue on carbohydrate chains (Gottschalk, 1957). Although there is much structural and functional information known about the NA, the role of this enzyme during the course of viral infection is still not clear. It may have a general role of facilitating the movement of the virus within the host, by allowing first a penetration of the mucous layer above the target respiratory epithelial cells (Burnet et al., 1947; Burnet, 1948) and subsequent movement from the infected cell by cleavage of the HA receptor from the cell surface (Seto & Rott, 1966; Webster & Laver, 1967; Palese et al., 1974). NA may also prevent aggregation of progeny viruses (Seto & Rott, 1966) and could unmask the activational cleavage site of HA (Schulman & Palese, 1977) by removing sialic acid from the side chains of the viral glycoproteins (Basak et al., 1985).

The biologically active part of the NA protein is a square boxlike head held above the viral envelope by a long narrow stalk anchored to the membrane at its N-terminus (Blok et al., 1982; Fields et al., 1981). Heads can be released from the virions by digestion with Pronase and retain all the biological properties of the intact protein (Laver, 1978; Laver et al., 1982). The three-dimensional structure of the NA heads from A/Tokyo/3/67 has been deduced from X-ray crystallographic studies. The protein is composed of four identical subunits, each made up of six, four-stranded β-sheets (Varghese et al., 1983). The product of catalysis, sialic acid, is observed to bind in a large pocket on the protein surface (Colman et al., 1983). A large number of amino acid side chains line this pocket, 24 of which are highly conserved in different types and subtypes of NA despite 50–70% variation between any two subtype amino acid sequences. These residues are conserved across N1, N2, N7, N8, and N9 subtypes of influenza A for which sequence data are available as well as in influenza B (Air et al., 1985; Colman & Ward, 1985; Dale et al., 1986). The location and conservation of these residues suggest they are involved in enzyme activity. Inhibition of the function of the surface proteins may be a suitable approach to the control of influenza, and we have therefore undertaken a more detailed study of the active site of the NA protein.

We have used oligonucleotide-directed mutagenesis to make amino acid substitutions in 10 conserved residues and 1 nonconserved residue in a cDNA clone of the NA of the A/Tokyo/3/67 (N2) strain of influenza A. The mutant and wild-type NA proteins were expressed from an SV40 late replacement expression vector and assayed for enzyme activity. In this paper we present the results of these experiments and discuss their implications for the catalytic activity of the NA.

Materials and Methods

DNAs, Viruses, and Cells. The cloning of a full-length cDNA copy of the NA gene of influenza virus A/Tokyo/3/67 has been reported (Lentz et al., 1984) and was the source of NA cDNA. The construction of SV40 vector pQPS used in these experiments has been described (Lentz & Air, 1986).

All recombinant plasmids were propagated in *Escherichia coli* strain RR1. CV-1 African green monkey kidney cells, permissive for SV40 replication, were used for expression of recombinant SV40 viruses. Cells were grown in Dulbecco's

† This work was supported by Grants AI-19084 and AI-08831 from NIAID. M.R.L. was supported by NIH Training Grant AI-07150.

* Author to whom correspondence should be addressed.

‡ University of Alabama at Birmingham.

§ St. Jude Children's Research Hospital.

0006-2960/87/0426-5351$01.50/0

modified Eagle's medium containing 50 units/mL penicillin, 50 μg/mL streptomycin, and 10% fetal calf serum.

Manipulation of DNAs. All manipulations of DNA were performed according to standard protocols (Maniatis et al., 1982). Synthetic oligonucleotides were synthesized by Dr. Jeffrey Engler on an Applied Biosystems DNA synthesizer.

Transfection and Virus Stocks. Recombinant SV40 virus stocks were prepared by transfection as described (Lentz & Air, 1986). High titer virus stocks were used at a dilution that produced complete cell death 5 days postinfection, generally 1:10 or 1:20.

Mutagenesis. The full-length clone of Tokyo/67 NA was subcloned into the *Sal*I site of M13mp18. Recombinant phage and replicative form DNA were grown in *E. coli* JM105 cells. The mutagenesis procedure of Zoller and Smith (1983) was used with modifications as described (Lentz & Air, 1986). Recombinant phage were screened for the mutations by plaque lift hybridization (Zoller & Smith, 1984). At least 200 nucleotides were sequenced around every mutation by using oligonucleotide primers and the dideoxy method (Lentz et al., 1984) to confirm that the expected substitution, and no other change, was present. Two of the mutant genes (His-274 to Asp and Arg-152 to Lys) were sequenced completely (1467 nucleotides). We determined the size of every mutant gene by multiple-restriction enzyme digestion and gel electrophoresis to ensure that no deletions were introduced during the mutagenesis or subcloning procedures.

Immunofluorescence of Infected Cells. Internal and surface indirect fluorescent staining of infected cells was carried out as described (Wills et al., 1984) except that 3.7% formalin in phosphate-buffered saline (PBS) was used instead of ethanol/acetic acid to fix surface-stained cells. Two different anti-NA antibody preparations were used: a polyclonal rabbit antiserum and a pool of monoclonal antibodies that specifically recognize the properly folded three-dimensional structure of Tokyo/67 NA.

Radiolabeling, Immunoprecipitation, and Polyacrylamide Gel Electrophoresis of Proteins. Cells were labeled for 6 h with D-[2-^{3}H]mannose or for 3 h with L-[4,5-^{3}H(N)]leucine 3 days postinfection as described (Wills et al., 1984). Radiolabeled cells were lysed and the NA protein was immunoprecipitated (Hunter et al., 1983) by using an excess of anti-Tokyo/67 NA polyclonal antisera preadsorbed to fixed CV-1 cells. Polyacrylamide gel electrophoresis and fluorography were carried out as described (Lentz & Air, 1986).

Neuraminidase Enzyme Assay. The colorimetric assay of Aymard-Henry et al. (1973) was used. Five days postinfection, when cell death was nearly complete, the remaining cells and cell debris from two 60-mm plates infected with each recombinant vector were pelleted at 30000 rpm for 30 min in an SW41 rotor. The pellet was resuspended in 200 μL of 0.25 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 0.15 M NaCl (CMS), and 100 μL of the suspension was transferred to each of two tubes. Fifty microliters of 40 mg/mL fetuin in 0.2 M sodium phosphate or potassium phosphate, pH 6.0, was added to one set of tubes, and 50 μL of 2 mM *N*-acetylneuraminyllactose in the same buffer was added to the other set. The tubes were incubated at 37 °C for 4 h after which time the *N*-acetylneuraminic acid released by the enzyme was chemically converted to a pink chromophore and extracted into 1.5 mL of 95% butanol/5% acetic acid (v/v), and its absorbance was read at 549 nm. Whole influenza virus (5 μL, 10^4 HA units/mL) was used as a source of enzyme for a positive control.

For enzyme assays over a pH range, cells from eight 60-mm plates infected with each recombinant vector were pelleted in an SW28 rotor for 40 min at 27000 rpm and resuspended in 900 μL of CMS. After 100 μL of the cell suspension was distributed into 9 tubes, 50 μL of 40 mg/mL fetuin in 0.2 M potassium phosphate at the desired pH was added to each tube and the assay carried out as described above.

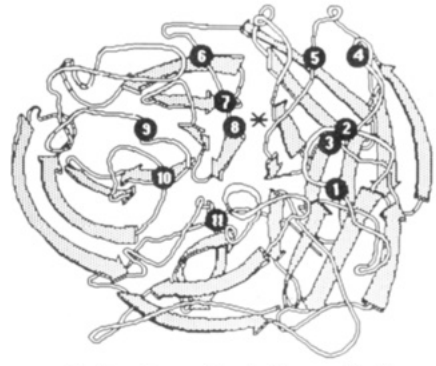

FIGURE 1: Locations of mutations on the three-dimensional structure of NA. Schematic representation of a single monomer viewed from above looking down into the active-site pocket is shown (Varghese et al., 1983). Amino acid substitutions are marked as closed numbered circles.

RESULTS

Mutations of the Neuraminidase Protein. A total of 14 mutations were made, 12 of which are in the conserved residues of the active site. The specific mutations and their locations in the three-dimensional structure (Varghese et al., 1983) are shown in Figure 1 and are described below.

146 Asn to Ser. This is one of two mutated residues that is not located in the active-site pocket. Asn-146 is the site of attachment of one of four N-linked carbohydrate side chains on the head of the Tokyo/67 NA (Ward et al., 1982, 1983), and the mutation abolishes this attachment site. This mutation was made to test the possibility that the WSN (N1) strain of influenza was neurovirulent because the loss of this carbohydrate from the NA altered its enzyme activity. All other strains contain this carbohydrate side chain (Colman & Ward, 1985).

152 Arg to Lys and Ile. This charged residue is located on the top edge of the binding pocket with its side chain pointing directly into the pocket (P. M. Colman and J. N. Varghese, personal communication). Because of its location, it appears to be a good candidate to be involved in binding substrate. Two mutations were made at this position, a conservative change from Arg to Lys, which might provide information on the importance of the positive charge, and a change to Ile, which is much less conservative and alters both the charge and size of the side chain.

178 Trp to Leu. Bachmayer (1972) found that chemical modification of tryptophan in NA inactivated the enzyme, which suggested that Trp might be involved in activity. The only Trp in the vicinity of the substrate-binding pocket is at position 178. A relatively conservative change to Leu was chosen to help maintain local structure possibly dependent on hydrophobic interactions. This mutant was used as template for a second mutagenesis to revert the Leu to Trp.

198 Asp to Asn. The N9 NA from influenza A/tern/Australia/G70C/75 (G70C) is able to hemagglutinate 4 times more efficiently than HA (Laver et al., 1984). This NA has Asn at residue 198 in place of the Asp in most strains (Air et al., 1985). To test the possibility that Asn-198 is responsible for the hemagglutinating and/or increased enzyme activity of the N9 NA, the Asp in the Tokyo/67 strain was mutated to Asn as in G70C.

222 Ile to Val. This residue is well positioned to interact with the substrate and is one of the few hydrophobic residues that are conserved in the binding pocket. A conservative change was chosen to maintain the hydrophobic character of this side chain.

274 His to Tyr and Asn. His is an important residue in the active site of many enzymes, and residue 274 is the only His in the vicinity of the active-site pocket. Since the pH optimum for NA (Mountford et al., 1982) and the pK_a of His are very similar, it seemed possible that this His would be involved in the activity of the NA.

276 Glu to Asp and Gln. These mutations were made because results from the other mutations suggested a catalytic mechanism in which Glu-276 plays a key role. Two mutations at Glu-276 were therefore made, one conserving the negative charge, to confirm the importance of this residue in the model for the catalytic mechanism.

277 Glu to Asp. This charged residue is located near the bottom of the binding pocket with its side chain pointing up into the pocket. A conservative change to Asp was made to preserve the negative charge at this position.

346 Thr to Asn. Thr-346 is not located in the active-site pocket. The same substitution to Asn has been observed to occur in field isolates, and therefore this mutation should not affect the enzyme activity. This mutation was designed to be a control for the complex mutagenesis procedures including subcloning from M13 into the SV40 expression vector, transfection of CV-1 cells, and preparation of SV40 recombinant and helper virus stock used in the expression assays.

371 Arg to Lys. This residue is located directly in the active-site pocket with its side chain directed into the pocket (J. N. Varghese and P. M. Colman, personal communication). A conservative change of Arg to Lys was made to test the importance of this residue in the interaction with the substrate.

406 Tyr to Phe. This residue has recently been added to the list of conserved residues in the active-site pocket (Air et al., 1985). A somewhat conservative change was made that maintains the aromatic nature of the side chain. The mutant residue is much less polar, however, and unable to participate in hydrogen bonding.

Expression of Wild-Type and Mutant Neuraminidase Proteins. NA proteins were expressed in CV-1 African green monkey kidney cells from an SV40 late replacement expression vector (Lentz & Air, 1986). Synthesis and transport of proteins were analyzed by immunoprecipitation of labeled cell lysates and by indirect immunofluorescence of infected cells.

Labeled NA proteins from infected cell lysates were precipitated by using anti-NA rabbit antiserum, separated on 8% urea/phosphate/sodium dodecyl sulfate (SDS) gels and autoradiographed (Figure 2). In this gel system the reduced NA polypeptides migrate with an apparent molecular weight of 70000 daltons and comigrate with NA from disrupted influenza virions. All of the mutant proteins have the same mobility as the wild type with two exceptions. The protein with a mutation at residue 146 (Asn to Ser), which abolishes a carbohydrate attachment site, migrates more quickly (M_r 62000) than the rest of the proteins in accord with the loss of a carbohydrate side chain. Since the length of every mutant gene was checked by restriction enzyme analysis, the altered mobility is not due to a deletion. NA with mutant residue 406 (Tyr to Phe) migrated with an apparent M_r of 76000. The reason for the reduced electrophoretic mobility of this protein is not known. The NA bands from 198 Asp to Asn and the revertant were consistently less intense than those of the rest of the NA proteins, indicating less efficient expression from

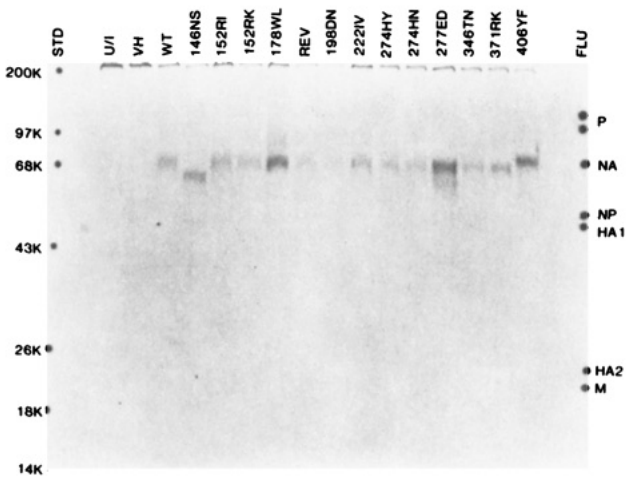

FIGURE 2: Immunoprecipitation of wild-type and mutant NA proteins. Infected cells were labeled with tritiated mannose as described in the text. Anti-Tokyo/67 NA rabbit antiserum was used to precipitate NA from infected cell lysates and then purified by binding to and eluting from *Staphylococcus aureus* protein A. Proteins were run on an 8% polyacrylamide gel, stained, and fluorographed. Whole disrupted $NWS_{HA}/Tokyo_{NA}$ influenza virions (FLU) were used as a marker for the NA protein. Lanes are marked STD for molecular weight markers, U/I for uninfected control cells, VH for cells infected with the vector and helper virus stock with no cloned NA gene, and WT for cells expressing wild-type NA. REV is a revertant of the preceding mutant. Mutants are labeled with the amino acid position number followed by the single letter amino acid codes for the wild-type amino acid and then the amino acid substituted in the mutant.

these two recombinants. SV40 VP1 protein was also less intense on the autoradiograph in these two vectors when L-[4,5-^{3}H(N)]leucine was used as a label (data not shown), suggesting that these virus stocks are less potent.

Indirect immunofluorescence was used to analyze the internal and surface expression of the NA proteins (Figure 3). Infected cells that were fixed and permeabilized prior to adding anti-NA rabbit antiserum stained brightly throughout the cytoplasm with increased intensity in the perinuclear region, suggesting transport of the proteins through the Golgi apparatus. All of the mutants appeared identical with the wild-type NA. Uninfected cells or cells infected with a virus stock of recombinant vector and helper virus with no NA gene (vector/helper, VH) had only background fluorescence.

Surface fluorescence was performed by using two different anti-NA antibody preparations. Rabbit polyclonal antisera recognized both native and denatured NA proteins based on enzyme-linked immunosorbent assay (ELISA) tests on whole native influenza virus and on boiled or detergent-treated virus. A pool of monoclonal antibodies that inhibit NA was only able to recognize NA in the properly folded three-dimensional structure with the same ELISA tests. Surface fluorescence with the rabbit antiserum revealed that the NA proteins were transported to the cell surface. There was no significant difference in the fluorescent staining between wild-type NA and any of the mutants (Figure 3), except that mutants 146 Asn to Ser and 406 Tyr to Phe had a lower percentage of surface fluorescing cells. These same amino acid substitutions, 146 Asn to Ser (loss of carbohydrate) and 406 Tyr to Phe, apparently affected the overall three-dimensional structure of the proteins as they were not recognized by the pool of monoclonal antibodies specific for the properly folded protein. The rest of the mutants appeared identical with wild-type NA with the monoclonal antibodies (Figure 3).

Enzyme Activity of Wild-Type and Mutant Neuraminidase Proteins. Enzyme assays were performed on infected cells 5 days postinfection when cell death was nearly complete. This

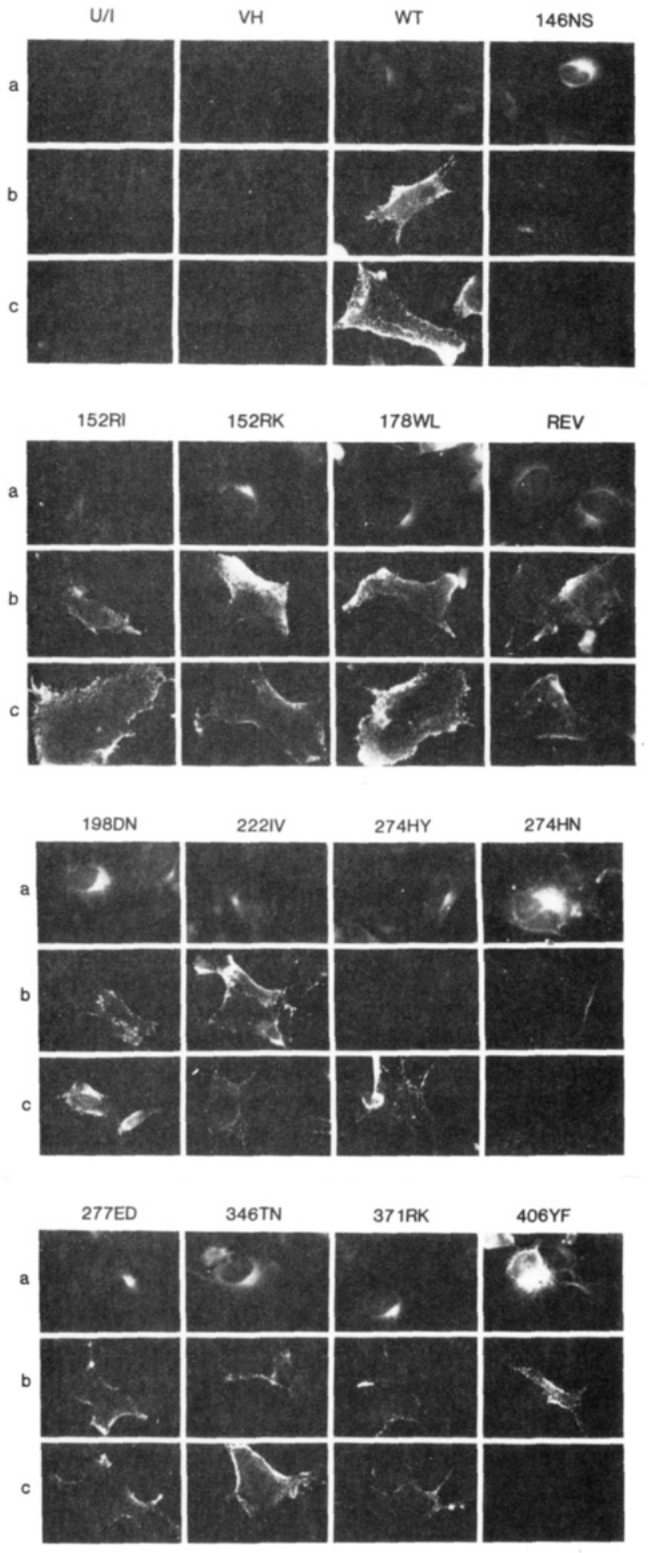

FIGURE 3: Immunofluorescence of cells expressing NA proteins. Infected cells were treated with antibodies as described in the text: (a) internal fluorescence of fixed, permeabilized cells using polyclonal rabbit antiserum as the primary antibody; (b) surface fluorescence of unfixed cells using the same polyclonal antiserum as in (a); (c) surface fluorescence of unfixed cells using a pool of monoclonal antibodies that react exclusively with NA in the properly folded three-dimensional structure; (U/I) uninfected cells; (VH) cells infected with the recombinant SV40 virus stock with no NA gene; (WT) cells expressing wild-type NA; (REV) cells expressing a revertant of the NA with the Trp-to-Leu mutation at residue 178. Mutants are labeled with the amino acid sequence number followed by the one-letter code for the wild-type amino acid and then the mutant amino acid. [178 Trp to Leu was described previously (Lentz & Air, 1986).]

Table I: Properties of Wild-Type and Mutant Enzymes

enzyme	expression[a]	transport[b]	correct folding[c]	neuraminidase activity[d]
wild type	+	+	+	100
346 Thr to Asn	+	+	+	90–100
222 Ile to Val	+	+	+	90–100
revertant	(+)	+	+	70–80
274 His to Tyr	+	+	+	70–80[e]
274 His to Asn	+	+	+	70–80[e]
371 Arg to Lys	+	+	+	5–10
152 Arg to Lys	+	+	+	0
152 Arg to Ile	+	+	+	0
178 Trp to Leu	+	+	+	0
198 Asp to Asn	(+)	+	+	0
276 Glu to Asp	+	+	+	0
276 Glu to Gln	+	+	+	0
277 Glu to Asp	+	+	+	0
146 Asn to Ser	+	(+)	–	0
406 Tyr to Phe	+	(+)	–	0

[a] Expression monitored by immunofluorescence and immunoprecipitation. + indicates normal levels of expression, transport, or folding. (+) indicates intermediate levels of expression or transport. [b] Transport to the cell surface assayed by surface immunofluorescence of unfixed cells. [c] Polypeptide folding determined by binding of monoclonal antibodies specific for native NA. The minus sign indicates incorrect folding. [d] Enzyme activity is expressed as percent as wild-type activity (wild type = 100%). Results shown are using *N*-acetylneuraminyllactose as substrate. [e] Activity with fetuin was 40–60% of wild type.

helped to compensate for slight differences in the concentration of virus stocks. Two substrates were used: *N*-acetylneuraminyllactose (NANL), a trisaccharide, and fetuin, a highly sialated glycoprotein. Fetuin is more likely to mimic the natural substrate for the enzyme, whereas the trisaccharide is expected to have easier access to the active site; several antibodies inhibit activity with fetuin but not with NANL. For all of the mutants we constructed, the enzyme activity with NANL was consistent through many experiments, while activity with fetuin was somewhat variable between different batches of infected cells. One interpretation is that activity with the large substrate (fetuin) is much more dependent on how degraded the membranes are, which we cannot completely control. Although the results with fetuin must therefore be considered semiquantitative, all of the mutants which were active with the small substrate were also active with fetuin, suggesting that none of the mutations are affecting substrate entry into the active site.

Assays were performed at pH 6.0, the optimum for the wild-type enzyme (Mountford et al., 1982). The standard colorimetric assay was used (Aymard-Henry et al., 1973) and the results are shown in Figure 4. Seven of the mutant proteins, with mutations 146 Asn to Ser, 152 Arg to Lys and Ile, 178 Trp to Leu, 198 Asp to Asn, 277 Glu to Asp, and 406 Tyr to Phe, have completely lost enzyme activity for both substrates. The rest of the mutants have activity that ranges from about 4% of wild type (371 Arg to Lys) to fully active (222 Ile to Val and 346 Thr to Asn). Both mutations at residue 274 (His to Tyr and Asn) have about 75% wild-type activity with NANL and 50% wild-type activity with fetuin. When the inactive mutant at residue 178 was reverted to the wild-type sequence, enzyme activity was regained. Activity was not quite as high as wild-type activity (Figure 4) in accord with the somewhat lower expression noted in the immunoprecipitation experiments. The expression and enzyme activity of the mutants are summarized in Table I.

pH Optimum of Wild-Type Neuraminidase and Two Mutants. His-274 is the only histidine in the vicinity of the active-site pocket. Since the pH optimum of the wild-type

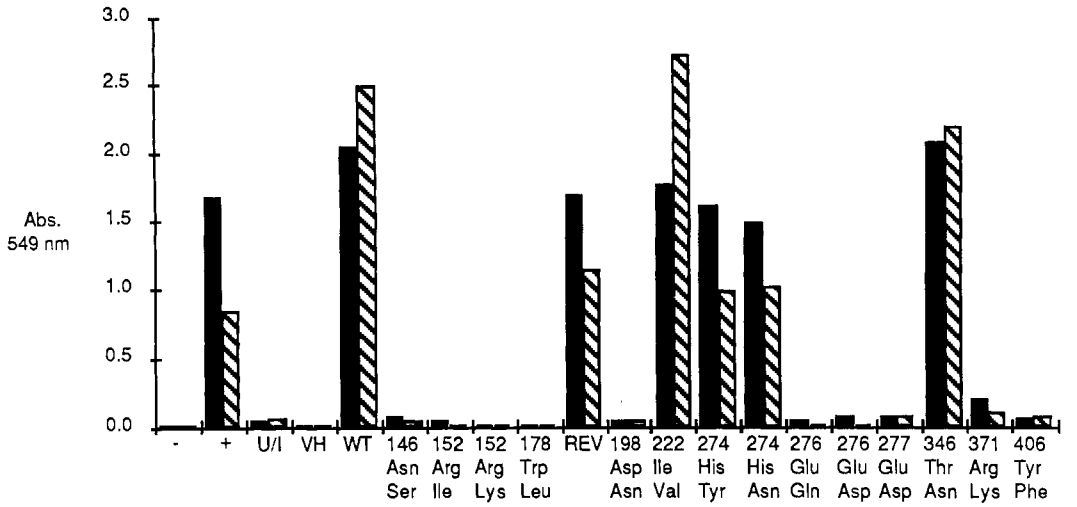

FIGURE 4: Enzyme activity of wild-type and mutant NA proteins. Enzyme activity was quantitated by determining the absorbance of the sample product at 549 nm: (-) reagent blank; (+) positive control using whole influenza virions as the source of NA; (U/I) uninfected cells; (VH) cells infected with recombinant SV40 virus stock with no NA gene; (WT) cells expressing wild-type NA; (REV) cells expressing a revertant of the 178 Trp to Leu mutation. Mutant proteins are labeled with the amino acid position above the wild-type amino acid. Below is the amino acid present at that position in the mutant: (solid bars) *N*-acetylneuraminyllactose; (slashed bars) fetuin. As explained in the text, activity with NANL is consistent between experiments and therefore between mutants; fetuin results are only semiquantitative.

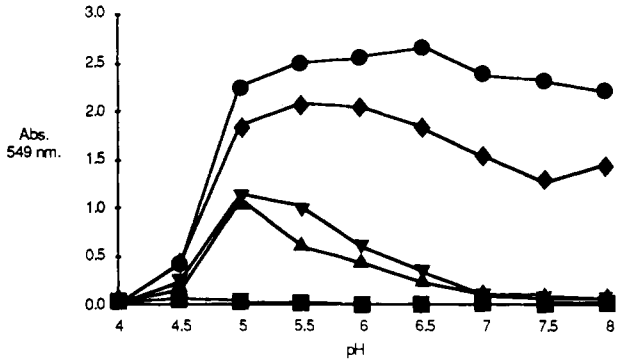

FIGURE 5: Enzyme activity of wild-type and His-274 mutants from pH 4.0 to 8.0. The absorbance at 549 nm corresponds to the level of enzyme activity: (●) positive control using influenza virions as the source of NA; (■) recombinant SV40 vector with no NA gene; (◆) cells expressing wild-type NA; (▲) cells expressing NA with a substitution of Tyr for His at residue 274; (▼) cells expressing NA with a substitution of Asn for His at residue 274.

enzyme is near the pK_a of histidine and two mutations at this position (His to Tyr and His to Asn) reduced the activity of the enzyme by about 25% with NANL and even more with fetuin, we decided to examine these mutations more closely. The enzyme assay was repeated on these mutants and the wild-type protein at pH values from 4.0 to 8.0 with the more natural substrate, fetuin. A single batch of infected cells was used to avoid the variation seen with this substrate between different experiments. The results are graphed in Figure 5. The wild-type enzyme activity rose rapidly from pH 4.5 to a maximum of pH 5.5–6.0 and then gradually fell off to 60–70% of maximum at pH 8.0. Both mutants had their pH optimum shifted to the acidic side, with maximum activity at pH 5.0. This activity was 52% and 54% of wild-type activity for the Tyr and Asn substitutions, respectively. Above the optimum pH, the activity of both mutant enzymes fell off rapidly to nearly zero (less than 10% of the maximum) by pH 8.0. As discussed previously (Lentz & Air, 1986), there is no possibility of obtaining purified mutant proteins from this eukaryotic system for detailed kinetic analysis, and the system is not as controllable as we would like it to be. The difference between purified wild-type NA and the crude SV40-expressed enzyme in Figure 5 emphasizes that we cannot obtain absolute values. Nevertheless, the experiments shown in Figure 5 are reproducible; the difference in pH optimum is always seen in both His-274 mutants.

Mutations at a Catalytically Active Amino Acid Position. The results of the enzyme assays on mutant proteins and in particular the altered pH optimum of the His-274 mutants have led to a model for the catalytic mechanism of neuraminidase, which is described under Discussion. This model implicates Glu-276 as the proton donor for the catalytic reaction. In order to confirm the key role of this amino acid residue, two conservative mutations were made, Glu to Asp and Glu to Gln. When expressed from the SV40 vector, both of these mutants were synthesized and transported to the cell surface as wild-type and were recognized by the monoclonal antibody pool as being properly folded. When immunoprecipitated, both mutants comigrated on polyacrylamide gels with wild-type NA and appeared with similar intensity. Neither mutant had any detectable enzyme activity for either of the two substrates tested (data included in Figure 4).

Discussion

The influenza virus NA provides a unique opportunity to characterize an enzyme active center that is conserved amidst enormous variation in amino acid sequence. There is up to 70% sequence variation between NAs of influenza A and B, and hence those residues that are conserved in all known sequences are likely to be essential for enzyme function. In the structure determined to 2.9-Å resolution (Colman et al., 1983) several such conserved residues line the substrate-binding pocket, and these were obvious targets for site-specific mutagenesis to delineate the mechanism of enzyme action.

Oligonucleotide-directed site-specific mutagenesis has been used to study specific details of a variety of enzymes. The substrate specificity of trypsin has been altered by this technique (Craik et al., 1985), and the role of the complementary hydrogen bonding was analyzed in tyrosyl-tRNA synthetase (Fersht et al., 1985). The specific role of particular amino acids in the catalysis by dihydrofolate reductase (Howell et al., 1986) and carboxypeptidase A (Gardell et al., 1985) have been analyzed by creating specific mutations in these residues.

We have used this technique to study the enzyme active site of the influenza virus neuraminidase protein. Previous studies have described the catalytic properties of wild-type NA including pH optimum, substrate specificity, and kinetics

(Drzeniek, 1972; Mountford et al., 1982), and recent experiments have investigated the effect of antigenic variation on the catalytic activity (Deshairs et al., 1986). However, little information was previously available about the mechanism of enzyme activity. Sequence analysis of ts mutant NA genes yielded only changes that would affect protein folding (Bos & Nayak, 1986), and X-ray diffraction studies have not yet given high-resolution information on the enzyme–substrate interaction. We therefore chose to make mutations in a large number of residues and use the information from all these mutants to deduce a mechanism of catalysis. To minimize the likelihood of altering the folding of the protein, most mutations were designed to be rather conservative. In the absence of enzyme activity, correct folding was monitored by binding of a pool of monoclonal antibodies that we have shown to be specific for native NA.

Fourteen mutations were made, 12 of which are scattered around the pocket known to bind the substrate. The mutant NA genes were then cloned into an SV40 late replacement expression vector and NA expressed in CV-1 cells. The NA polypeptides anchored to the cellular membranes were assayed directly for their NA activity on two substrates, fetuin, a glycoprotein, and *N*-acetylneuraminyllactose, a trisaccharide. A wide range of activity was observed for the different mutants.

Two mutations apparently affected the three-dimensional structure of the protein as the mutant NA proteins were no longer recognized by a pool of monoclonal antibody specific for the properly folded protein. Both of these mutants are enzymatically inactive. Asn-146 mutated to Ser abolishes a carbohydrate attachment site that is present in all NA sequences except the neurovirulent WSN/33. The mutant protein does migrate to the cell surface, but with reduced efficiency. It appears that this carbohydrate site is important for proper folding of the molecule in the N2 subtype; it could have a different role in N1 NA.

The mutation at residue 406 from Tyr to Phe is also not recognized by the structure-specific monoclonal antibodies. This protein is transported to the cell surface with reduced efficiency. The decreased polarity of the Phe side chain in the mutant may interfere with important interactions stabilizing the structure of the protein.

Mutants with substitutions at residues 152, 178, 198, 276, and 277 have also completely lost activity; however, the mutations do not appear to affect the transport or overall structure of these proteins. Residue 152 was mutated from Arg to both Lys and Ile. Neither mutant had any activity, indicating that the function of the Arg in the wild-type is very specific. It should be noted that while Arg to Lys is generally considered a conservative change, the Arg and Lys side chains are unequal in length and an Arg-to-Lys change is sufficient to completely destroy monoclonal antibody binding (Lentz et al., 1984).

As previously reported, Trp-178 mutated to Leu also loses all activity (Lentz & Air, 1986). Trp and Leu are both hydrophobic; however, the indole ring of Trp is both aromatic and able to participate in hydrogen bonding. Chemical modification of Trp in the NA also renders the enzyme inactive (Bachmayer, 1972). Trp-178 may participate in hydrogen bonding or act as a donor in a charge-transfer interaction with the substrate. As a control, this mutant was reverted from Leu at residue 178 to the wild-type Trp, and enzyme activity was regained, indicating that no additional mutations had occurred during the mutagenesis procedure.

Asp-198 was mutated to Asn, and we were surprised to find that the mutant NA is inactive since Asn is found at the corresponding position in the N9 NA of A/tern/Australia/G70C/75 (Air et al., 1985). The three-dimensional structure of N2 NA shows no obvious interactions involving Asp-198. The N9 NA with Asn at this position is able to agglutinate red blood cells; however, the mutant N2 NA with Asn is not. A reciprocal experiment in which the N9 NA of G70C was mutated at this residue to the Asp present in the Tokyo/67 wild-type enzyme was also performed. This mutant NA also completely lost enzyme activity and is unable to agglutinate red cells, suggesting that the local environment surrounding these residues in the N2 and N9 neuraminidases is different.

Another substitution that inactivates the enzyme is 277 Glu to Asp. Both side chains have a negative charge at pH 6, but the side chain in the mutant is shorter than in the wild-type. This charged residue may participate in ionic interactions with the substrate in the wild-type, but it may be unable to make the necessary contacts in the mutant. The Glu side chain might also be involved in stabilizing positively charged reaction intermediates.

Residue 371 was mutated from Arg to Lys, and the mutant NA protein was found to have very low but detectable levels of activity, consistently less than 10% of that of the wild-type enzyme. The precise role of this side chain cannot be predicted without structural details of substrate bound in the active site.

Two mutants have activity that is identical with that of wild-type. One had a change of Ile to Val at residue 222. It seems likely that the Val side chain can substitute for Ile in a hydrophobic interaction, either with substrate or to stabilize the protein structure. The conservation of Ile at this position suggests a selective advantage over Val in viral replication that was not seen in vitro.

His-274 was changed to both Tyr and Asn. Both of the mutants behave identically, having 70–80% wild-type activity with NANL and only 40–60% of wild-type activity with fetuin at pH 6.0. These mutants were further characterized by assaying their activity over a range of pH from 4.0 to 8.0. The pH optimum was shifted from 5.5–6.5 in the wild type to pH 5.0 in both mutants.

Possible Mechanism of Neuraminidase Catalysis. Since the NA is an animal virus glycoprotein containing disulfide bonds, it cannot be expressed in active form from bacterial vectors. Currently, no methods are available to incorporate a mutated gene into the segmented, single-stranded RNA genome of influenza virus. Therefore we have used a relatively efficient mammalian expression vector derived from SV40 (Gething & Sambrook, 1981). Although good levels of NA enzyme activity are found (Lentz & Air, 1986), the amount of protein available is several orders of magnitude down from that obtainable from bacterial systems. Therefore, it is not possible to obtain sufficient quantitites of isolated and purified NA from SV40 or other mammalian expression systems to undertake detailed kinetic analyses as were done for mutants of tyrosyl-tRNA synthetase (Fersht et al., 1985) and other bacterial enzymes. Furthermore, as described above, most of the mutants have completely lost their enzyme activity, making a more detailed analysis of the kinetics of the reaction impossible.

However, the shift in pH optimum to the acidic side when His-274 was altered suggests a possible mechanism for the catalytic activity of the influenza neuraminidase. This has some elements in common with catalysis by lysozyme, an enzyme that also hydrolyzes a glycosidic bond in polysaccharide substrates. The mechanism of lysozyme action (Phillips, 1967; Blake et al., 1967) and the proposed NA mechanism are shown schematically in Figure 6.

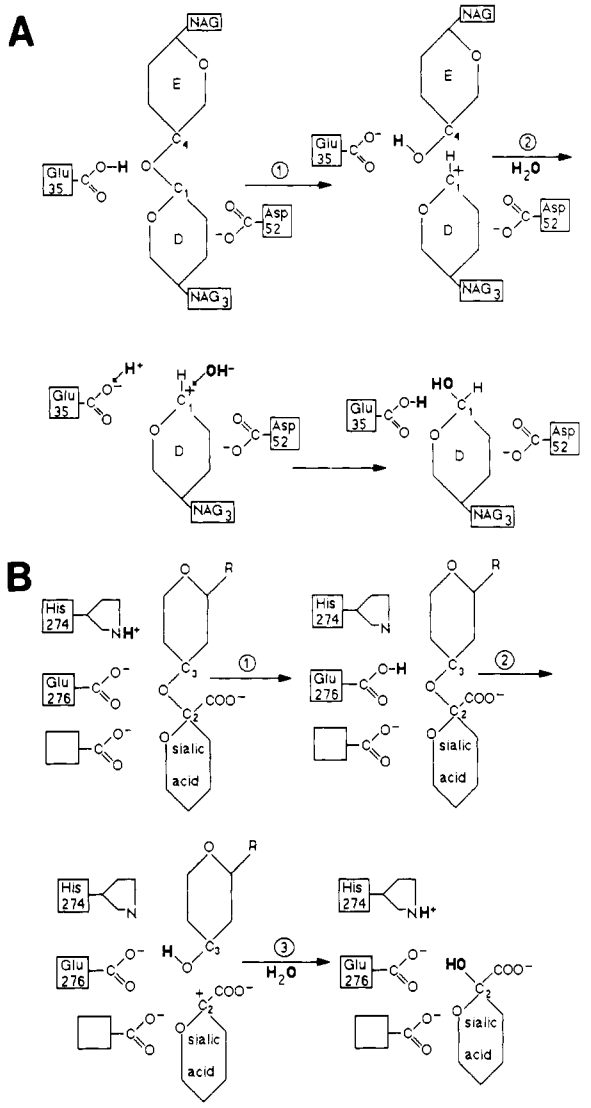

FIGURE 6: Schematic diagrams of the catalytic mechanisms of lysozyme and neuraminidase. (A) Catalytic mechanism of lysozyme. A hexasaccharide [poly(*N*-acetylglucosamine) (NAG) shown here] is bound into subsites A–F of the enzyme. In step 1, the un-ionized carboxyl of Glu-35 donates a proton to the glycosidic bond, thereby breaking this bond. The disaccharide in subsites E and F can diffuse away. At the same time, a carboxonium ion intermediate forms at C_1 of the residue in subsite D, which is stabilized by the ionized carboxyl of Asp-52. In step 2, a water molecule contributes a proton to the carboxyl of Glu-35 and a hydroxyl to the carboxonium ion. After the product diffuses from the active site, the enzyme is ready for another round of catalysis. Adapted from Stryer (1981). (B) Proposed catalytic mechanism of NA. The substrate contains sialic acid linked $\alpha(2,3)$ to any polysaccharide chain (R). After binding to the active site, His-274 donates a proton to the ionized side chain of Glu-276 as shown in step 1. In step 2, Glu-276 uses this proton to break the glycosidic bond, resulting in the release of the polysaccharide minus its terminal sialic acid. An ionized intermediate may form, which would be stabilized by the ionized carboxyl of one of the conserved acidic groups (possibly Glu-277). In the final step, a water molecule reprotonates His-274 and contributes a hydroxyl to the transition-state intermediate, resulting in free sialic acid which can diffuse from the active site. The enzyme is then ready for another round of catalysis.

In lysozyme (Figure 6A), two acidic side chains (Glu-35 and Asp-52) are in close proximity to, but on opposite sides of, the glycosidic bond cleaved in the reaction. Glu-35 is in a hydrophobic environment and has an abnormally high pK_a near 6, leaving this residue protonated at the pH at which the reaction occurs (pH 5). Glu-35 thus can act as a proton donor, passing its proton to the glycosidic oxygen and thereby breaking the bond. Asp-52, which is ionized at pH 5, stabilizes an intermediate carboxonium ion. A water molecule then protonates Glu-35 and provides a hydroxyl for the carboxonium ion to complete the reaction.

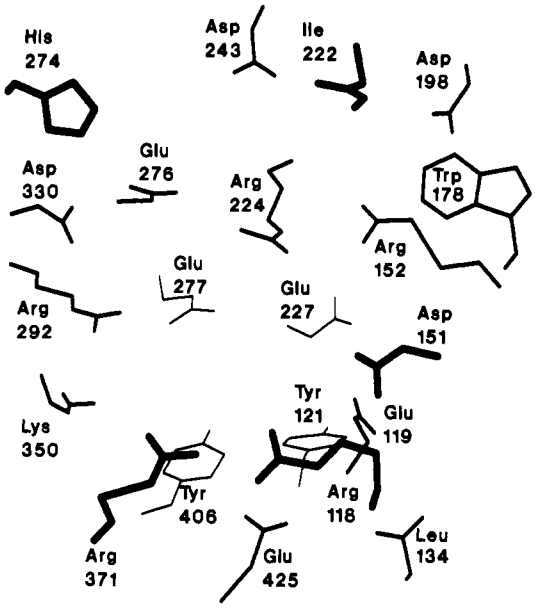

FIGURE 7: Structure of the neuraminidase active site. The conserved residues located in the substrate-binding pocket are shown, the thicker to thinner lines indicating depth into the pocket. Mutations were made in residues 152, 178, 198, 222, 274, 276, 277, 371, and 406. Side chains implicated in the catalytic mechanism are His-274 and Glu-276.

In the NA, it is unlikely that His-274 directly protonates the glycosidic oxygen, because enzyme activity is not abolished by substitution of His-274 to Tyr or Asn. Figure 6B shows a more likely mechanism. We propose that His-274 elevates the pK_a of nearby Glu-276, which would then be the proton donor for the reaction. In the absence of His at residue 274, the pK_a of Glu-276 would resume a more typical value, accounting for the lowered pH required for reaction in the two mutants. Two mutations at residue 276 abolished enzyme activity in accord with the key function proposed for this residue in the catalytic mechanism. The mutation to Asp retains the negative charge but with a reduced pK_a and might have had the same effect as mutating His-274 in reducing the pH optimum of the reaction. The complete abolition of enzyme activity indicates that the shorter side chain is not able to carry out the same function as Glu; the donor proton may be too far away from the bond which is to be cleaved.

The location and orientation of the conserved amino acids in the substrate-binding pocket are diagrammed in Figure 7 (P. M. Colman and J. N. Varghese, personal communication). Which of the several other acid groups in the active site is directly responsible for stabilizing the developing positive charge on a carboxonium ion intermediate is not certain, since it is not known precisely how the substrate is oriented in the active site during the reaction, but Glu-277 is an obvious candidate. Studies are in progress on the interaction of sialic acid with the enzyme (J. N. Varghese and P. M. Colman, personal communication). Sialic acid is only a weak inhibitor of NA (Colman et al., 1983), and we do not know how high the resolution of this structure will be or how closely the interaction with product mimics the binding of substrate. If it is true that the only catalytically active residues are His-274 and Glu-276, with a possible involvement of Glu-277 in stabilizing a charged intermediate, it is likely that the other conserved residues which lost activity (Arg-152, Trp-178, Asp-198, and Arg-371) would be involved in binding the

substrate in the active site (see Figure 7), and studies on the interaction of sialic acid with the enzyme will help confirm this. The crystal structure of an antibody Fab–NA complex (Colman et al., 1987) indicates that inhibition of enzyme activity by the antibody is a result of displacement of Arg-371.

The enzyme activity of the neuraminidase protein is conserved in all the different strains and subtypes of the influenza virus. With detailed information about the activity and structure of the active site of this enzyme, it may be possible to design new inhibitors to effectively control the virus regardless of its antigenic structure.

ACKNOWLEDGMENTS

We are greatly indebted to Dr. Peter Colman, CSIRO, Australia, for sharing unpublished structural information including Figure 7 and for insights into the catalytic mechanism. Dr. Jeffrey Engler, University of Alabama at Birmingham, synthesized the mutagenic oligonucleotides. We thank Lynn Ritchie and Dan Lakeman for expert technical assistance.

REFERENCES

Air, G. M., Ritchie, L. R., Laver, W. G., & Colman, P. M. (1985) *Virology 145*, 117–122.

Aymard-Henry, M., Coleman, M. T., Dowdle, W. R., Laver, W. G., Schild, G. C., & Webster, R. G. (1973) *Bull. W. H. O. 48*, 199–202.

Bachmayer, H. (1972) *FEBS Lett. 23*, 217–219.

Basak, S., Tomana, M., & Compans, R. W. (1985) *Virus Res. 2*, 61–68.

Blake, C. C. F., Johnson, L. N., Mair, G. A., North, A. C. T., Phillips, D. C., & Sarma, V. R. (1967) *Proc. R. Soc. London, B 167*, 378–388.

Blok, J., Air, G. M., Laver, W. G., Ward, C. W., Lilly, G. G., Woods, E. F., Roxburgh, C. M., & Inglis, A. S. (1982) *Virology 119*, 109–121.

Bos, T. J., & Nayak, D. P. (1986) *Virology 154*, 85–96.

Burnet, F. M. (1948) *Aust. J. Exp. Biol. Med. Sci. 26*, 381–387.

Burnet, F. M., McCrea, J. F., & Anderson, S. G. (1947) *Nature (London) 160*, 404–405.

Colman, P. M., & Ward, C. W. (1985) *Curr. Top. Microbiol. Immunol. 114*, 177–255.

Colman, P. M., Varghese, J. N., & Laver, W. G. (1983) *Nature (London) 303*, 41–44.

Colman, P. M., Laver, W. G., Varghese, J. N., Baker, A. T., Tulloch, P. A., Air, G. M., & Webster, R. G. (1987) *Nature (London) 326*, 358–363.

Craik, C. S., Largman, C., Fletcher, T., Roczniak, S., Barr, P. J., Fletterick, R., & Rutter, W. J. (1985) *Science (Washington, D.C.) 228*, 291–297.

Dale, B., Brown, R., Miller, J., White, R. T., Air, G. M., & Cordell, B. (1986) *Virology 155*, 460–468.

Deshairs, C., Kessler, N., Aymard, M., & Quash, G. A. (1986) *J. Gen. Virol. 67*, 409–418.

Drzeniek, R. (1972) *Curr. Top. Microbiol. Immunol. 59*, 35–74.

Fersht, A. R., Shi, J.-P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M. Y., & Winter, G. (1985) *Nature (London) 314*, 235–238.

Fields, S., Winter, G., & Brownlee, G. G. (1981) *Nature (London) 290*, 213–217.

Gardell, S. J., Craik, C. S., Hilvert, D., Urdea, M. S., & Rutter, W. J. (1985) *Nature (London) 317*, 551–555.

Gething, M.-J., & Sambrook, J. (1981) *Nature (London) 293*, 620–625.

Gottschalk, A. (1957) *Biochim. Biophys. Acta 23*, 645–646.

Howell, E. E., Villafranca, J. E., Warren, M. S., Oatley, S. J., & Kraut, J. (1986) *Science (Washington, D.C.) 231*, 1123–1128.

Hunter, E., Hill, E., Hardwick, M., Bhown, A., Schwartz, D. E., & Tizard, R. (1983) *J. Virol. 46*, 920–936.

Laver, W. G. (1978) *Virology 86*, 78–87.

Laver, W. G., Air, G. M., Webster, R. G., & Markoff, L. J. (1982) *Virology 122*, 450–460.

Laver, W. G., Colman, P. M., Webster, R. G., Hinshaw, V. S., & Air, G. M. (1984) *Virology 137*, 314–323.

Lentz, M. R., & Air, G. M. (1986) *Virology 148*, 74–83.

Lentz, M. R., Air, G. M., Laver, W. G., & Webster, R. G. (1984) *Virology 135*, 257–265.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mountford, C. E., Grossman, G., Holmes, K. T., O'Sullivan, W. J., Hampson, A. W., Raison, R. L., & Webster, R. G. (1982) *Mol. Immunol. 19*, 811–816.

Palese, P., Tobita, K., Ueda, M., & Compans, R. W. (1974) *Virology 61*, 397–410.

Phillips, D. C. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57*, 484–495.

Schulman, J. L., & Palese, P. (1977) *J. Virol. 24*, 170–176.

Seto, J. T., & Rott, R. (1966) *Virology 30*, 731–737.

Stryer, L. (1981) *Biochemistry*, 2nd ed., Freeman, San Francisco.

Varghese, J. N., Laver, W. G., & Colman, P. M. (1983) *Nature (London) 303*, 35–40.

Ward, C. W., Elleman, T. C., & Azad, A. A. (1982) *Biochem. J. 207*, 91–95.

Ward, C. W., Murray, J. M., Roxburgh, C. M., & Jackson, D. C. (1983) *Virology 126*, 370–375.

Webster, R. G., & Laver, W. G. (1967) *J. Immunol. 99*, 49–55.

Wills, J. W., Srinivas, R. V., & Hunter, E. (1984) *J. Cell Biol. 99*, 2011–2023.

Zoller, M., & Smith, M. (1983) *Methods Enzymol. 100*, 468–500.

Zoller, M. J., & Smith, M. (1984) *DNA 3*, 479–488.