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The Antigenic Structure of the Influenza Virus A/PR/8/34 Hemagglutinin (H1 Subtype)

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Summary

We have constructed an operational antigenic map of the hemagglutinin of influenza virus A/PR/8/34, which indicates the presence of five immunodominant antigenic regions exhibiting various degrees of operational linkage. These sites have been located by the identification of changed amino acid residues in mutant viruses that are antigenically altered at each site. Comparison of the antigenic features with the three-dimensional structure of the H3 subtype hemagglutinin shows that the antigenic sites correspond to four topographically distinct regions of the surface of the protein. One of the sites is formed when two regions that are widely separated in the hemagglutinin monomer associate in the assembled trimer. The location of the sites relative to those proposed for the H3 subtype hemagglutinin suggests that carbohydrate modulates the antigenicity of specific regions of the hemagglutinin.

Introduction

The hemagglutinin (HA) is the major glycoprotein of influenza virus, and mediates the adsorption and penetration of virus into host cells. The native HA is formed by the association of three HA monomers which, as a precondition of virus infectivity, are cleaved enzymatically into the amino-terminal HA1 (molecular weight 46,000) and carboxy-terminal HA2 (molecular weight 27,000) polypeptides (for review see Ward, 1981). The crystallographic analysis of the HA of influenza virus A/Hong Kong/68 by Wilson et al. (1981) has shown that the HA monomer forms two distinct domains: a distal domain of globular shape, which is made up exclusively by the major portion of the HA1 polypeptide, and a proximal fibrous stem-like structure, which anchors the HA into the viral lipid envelope and is made up by the HA2 and part of the HA1 polypeptides.

It is well established that the HA is the critical viral target structure for the immune defense mechanisms and that influenza virus can escape the existing antiviral immunity of the host population by changing either gradually (antigenic drift) or abruptly (antigenic shift) the antigenicity of the HA (Dowdle et al., 1974; Kilbourne, 1975; Webster and Laver, 1975; Stuart-Harris, 1979). Precise characterization of the antigenicity of the HA is a prerequisite for understanding the mechanism by which antigenic evolution (particularly antigenic drift) occurs in this protein. To this end, two basic approaches have been used. In the first, fragments of the HA that were of different sizes and were produced either synthetically or by cleavage of the intact molecule were tested for their ability to elicit or react with anti-HA antibodies. Most or all antigenic structures recognized by neutralizing anti-HA antibodies are formed by the HA1 polypeptide (Brand and Skehel, 1972; Eckert, 1973; Jackson et al., 1979; Russ et al., 1981). The use of cleavage products to dissect the antigenicity into individual components was less successful, however, since the antigenicity of the HA1 polypeptide was greatly diminished upon reduction and alkylation of the intramolecular disulfide bonds, suggesting that antigenicity was largely dependent on the tertiary protein structure (Jackson et al., 1978). Synthetic oligopeptides used as immunogens have been shown in a few cases to elicit antisera that react with the intact HA (Green et al., 1982; Müller et al., 1982). However, the utility of this approach for characterization of peptides involved in the formation of antigenic structures cannot be determined until the antipeptide antibody specificities can be accurately compared with those elicited by the intact molecule.

The second approach is based on comparison of the amino acid sequences of antigenically distinct HAs, and is responsible for most of the information regarding the antigenicity of the HA. It has been shown that amino acid alterations in HAs derived from antigenic variants of the H3 subtype HA, arising either naturally or by in vitro selection, are clustered into four regions on the three-dimensional structure of the HA (Wiley et al., 1981). This provides evidence for discrete regions in which residue changes induce antigenic alterations in the HA.

We have previously described the construction of an operational antigenic map of the H1 subtype HA of influenza virus A/PR/8/34 (PR8) (Gerhard et al., 1981). Four operationally distinct antigenic sites (designated Sa, Sb, Ca, Cb) could be delineated by comparative antigenic analysis of 34 antigenically unique PR8 mutant viruses with 58 monoclonal anti-HA antibodies. We now report the expansion of the operational antigenic map by inclusion of additional mutant viruses and monoclonal antibodies in the comparative antigenic analysis and the identification of amino acid substitutions that induced the observed antigenic alterations. We discuss the resulting antigenic topography of the PR8 HA, which differs in several aspects from that proposed for the H3 subtype HA.

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Results and Discussion

The Operational Antigenic Map of the HA of Influenza Virus PR8

Influenza virus mutants that exhibit minor antigenic alterations in the HA can readily be selected by incubating cloned influenza virus seed with a monoclonal anti-HA antibody and subsequently expanding the non-neutralized virus fraction in a host system that supports influenza virus replication (Gerhard and Webster, 1978; Yewdell et al., 1979). The antigenic alterations exhibited by these virus mutants can be used as operational markers for distinct antigenic regions of the HA. Certain groups of mutant viruses express antigenic alterations that prevent corresponding groups of monoclonal anti-HA antibodies from binding to the viruses. However, these alterations have no detectable effect on the binding of other groups of antibodies, and on this basis an operational antigenic map can be constructed. Thus a previous antigenic analysis of 34 antigenically unique PR8 virus mutants with 58 monoclonal anti-HA antibodies permitted us to assemble the mutant viruses into four antigenic groups, each presumed to characterize an operational antigenic site on the HA of PR8 (Gerhard et al., 1981). This antigenic map seemed to be incomplete, however, because eight of the anti-HA antibodies reacted well with the parental virus PR8 and any of the 34 mutants derived thereof, suggesting that these mutant viruses lacked antigenic alterations in one or several antigenic regions recognized by BALB/c antibodies.

We have now used five additional antibodies (three tenuously mapped and two not previously mapped) to select five additional PR8 mutant virus sets. Subsequent antigenic analysis of these mutants by radioimmunoassay with the original group of antibodies and 32 additional hybridoma antibodies showed that 17 of these mutants were antigenically unique, and that all but one of the 90 antibodies could be mapped. The resulting antigenic map, based on the reactivity of antibodies used for mutant virus selection, is shown schematically in Table 1. It differs from the previous map in that one mutant virus group (DV-NV-SV, characterizing antigenic site Ca) could be subdivided into two subgroups: SV-WV-ZV and DV-NV-TV, characterizing subsites Ca_1 and Ca_2, respectively. These subsites remained, however, extensively interlinked through the mutant sets SV and TV. In addition, the new mutant sets JV and MV expanded the mutant virus groups characterizing sites Sa and Cb, respectively. However, with both mutant virus sets new linkages became evident between sites Sa and Ca_1 and between Cb and Ca_2. Thus when the number of mutant viruses was expanded, some of the features of these regions as distinct antigenic sites started to break down. Nevertheless, more than 50% of the hybridoma antibodies included in this analysis still mapped exclusively to single sites (Table 2). Consequently, with respect to the majority of anti-HA antibodies, the individual mutant virus groups characterized regions on the HA that underwent antigenic alterations independent from neighboring regions (Urbanski and Margoliash, 1977). Their designation as antigenic sites was therefore maintained. Table 2 also shows the various types of intersite linkages—that is, the number of antibodies that exhibited strongly reduced binding to one or several mutant viruses characterizing different sites. These linkages will be discussed below in conjunction with the location of the corresponding antigenic sites on the HA.

The HA1 Polypeptide Sequence of the Parental PR8 Virus Used for Mutant Virus Selection

Direct sequence analysis of specific regions of the genome RNA was performed by the dideoxy-chain termination sequencing procedure (Sanger et al., 1977), modified for analysis of RNA (see Experimental Procedures). Four synthetic oligonucleotide primers were synthesized which were of complementary sense to virion RNA, and which hybridized to the HA1 region of the HA gene at intervals of 200 to 300 nucleotides (Winter et al., 1981) (see Figure 1). These primers allowed the determination of the nucleotide sequence of the region of the genome encoding the mature (without signal peptide) HA1 polypeptide of influenza virus A/PR/8/34 (Mount Sinai), from which the various mutants were derived (Figure 1). This nucleotide sequence shows 12 differences, including a three-base deletion, when compared with that of PR8 (Cambridge) (Winter et al., 1981), which code for seven different amino acid residues and one amino acid deletion. The deletion of an A_3 triplet—nucleotides 471–473 in PR8 (Cambridge)—results in the absence from PR8 (Mount Sinai) of the lysine residue 130 residues from the amino terminus of the mature HA1 of PR8 (Cambridge). The C to A difference at position 469 means that the potential carbohydrate attachment sequence (Asn-X-Ser or Asn-X-Thr; Neuberger et al., 1972) at amino acid residues 127–129 of PR8 (Cambridge) is absent from PR8 (Mount Sinai). The differences in sequence presumably represent the variation that has occurred during the independent laboratory passage of virus which was originally isolated from the same source. It is clear from this that the description of a prototype sequence as the definitive sequence for any virus subtype, or particularly for any virus strain, must be viewed with caution.

The Location of Altered Amino Acid Residues in Mutant Viruses

PR8 mutant viruses were screened for mutations by analyzing sequence-specific reaction products from different mutants in parallel, which allowed the detec-

Table 1. Construction of the Operational Antigenic Map of PR8 BA

Operational Site:		Sb		Sa				Ca_1			Ca_2			Cb			
Mutant set :		EV	BV	CV	PV	KV	JV	WV	ZV	SV	TV	NV	DV	MV	LV	AV	RV
No of Mutants :		3	6	4	5	2	5	4	1	2	4	3	2	3	3	1	3
Anti-bodies :	E	0	+/0	+	(+)	+	(+)	(+)	+	+	+	+	+	+	+	+	+
	B	0	0	+	+	+	(+)	(+)	+	+	(+)	+	+	+	+	+	+
	C	+	+	0	0	+/0	+/0	+	+	+	+	+	+	+	+	+	+
	P	+	+	+/0	0	0	+/0	+	+	+	+	+	+	+	+	+	+
	K	+	+	+/0	+/0	0	+/0	(+)	+	+	+	+	+	+	+	+	+
	J	+	+	+/0	(+)	(+)	0	+/0	+	+	(+)	+	+	+	+	+	+
	W	(+)	+	+	+	+	+/0	0	0	0	+/0	+	+	+	+	+	+
	Z	+	+	+	+	+	+	+/0	0	+	(+)	+	+	+	+	+	+
	S	(+)	+	+	+	+	+/0	0	0	0	+/0	+	+	+	+	+	+
	T	+	+	+	+	+	+	+	+	+/0	0	+/0	+/0	+	+	+	+
	N	+	+/0	+	+	+	+	(+)	+	(+)	+/0	0	+	+	+	+	+
	D	(+)	+	+	+	+	+	+	+	+/0	+/0	+/0	0	+/0	+	+	+
	M	+	+	+	+	+	+	+	+	+	+	+	+	0	+/0	+	+/0
	L	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	(+)
	A	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0
	R	+	+	+	+	+	+	+	+	+	+	+	+	+/0	+/0	+	0

Sixteen anti-HA antibodies were used to select from parental PR8 virus sixteen corresponding sets of mutant viruses. The number of antigenically unique mutants is indicated by the digit below each mutant virus set. The antibodies were then tested by radioimmunoassay (see Experimental Procedures) for their binding capacity to parental virus and the various mutant viruses.

Plus: Antibody shows equal binding to parental virus and any mutant within the given mutant virus set. Plus in parentheses: antibody has slightly reduced (50%–75%) binding to one or several mutants compared to parental virus. Plus/zero: antibody has strongly reduced (>75%, usually nondetectable) binding to at least one mutant. Zero: antibody has slightly or strongly reduced binding to all mutants within the given mutant virus set. The mutant virus sets that are used as markers for partially independently mutating antigenic regions (Sb, Sa, Ca_1, Ca_2, Cb) are boxed.

tion of nucleotides unique to individual viruses (Figure 2). In the example shown, virus CV5 exhibits a unique U residue at position 453, while virus mutants KV4 and NV2 both contain C residues at this position representative of the wild-type sequence. This indicates that virus CV5 has a C to U change at position 453. Similarly, virus KV4 has a C to A change at position 458, and virus NV2 has a G to A change at position 498. Approximately 18,900 nucleotides were analyzed by this approach, allowing the changed amino acid residues to be detected in 46 mutant PR8 viruses. Table 3 shows the nucleotide changes that were determined; the amino acid changes that can be deduced are also shown in terms of their position relative to the amino terminus of the mature HA1 subunit of both PR8 (Mount Sinai) (H1 subtype) and influenza virus A/Aichi/2/68 (H3 subtype) (Verhoeyen et al., 1980), following alignment of the protein sequences (Winter et al., 1981). For ease of reference to the recently determined structure of the H3 subtype HA molecule (Wilson et al., 1981), all amino acid residue positions will be given in the text as their relative H3 position. The virus mutants are classified according to the groups shown in Table 1, and represent a total of 38 unique viruses as determined by partial nucleotide sequence analysis. Eight mutant viruses (JV2, JV9, PV11, EV6, NV2, LV1, LV4 and MV5) contained mutations that were identical with those found in other mutants. The reasons for these apparent discrepancies between the antigenic and

Table 2. Mapping of Antibodies to Distinct Mutant Virus Groups

No. of Antibodies	Mapping to Mutants Characterizing Sites: Sb	Sa	Ca_1	Ca_2	Cb
16	+	−	−	−	−
12	−	+	−	−	−
5	−	−	+	−	−
7	−	−	−	+	−
10	−	−	−	−	+
12	+	+	−	−	−
1	+	+	+	−	−
1	+	+	+	+	−
4	+	−	−	+	−
1	+	+	−	−	+
1	−	+	+	−	−
10	−	+	+	+	−
1	−	+	+	−	+
4	−	−	+	+	−
3	−	−	+	−	+
1	−	−	−	+	+

The results from radioimmunoassay including 89 antibodies are shown schematically. Plus: antibodies exhibit strongly reduced binding in radioimmunoassay to at least one of the mutant viruses characterizing the indicated site (see Table 1). Minus: none of the mutations exhibited by the corresponding mutant viruses resulted in strongly reduced binding of the given antibodies. One antibody (not included in this table) bound to all mutant viruses.

sequence analyses are not clear. In some cases, they may be due to small variations in the radioimmunoassay. It is also possible that variations in the growth, purification, storage or immunoadsorbent preparation of the various mutant viruses led to secondary modifications in the HA which were detected in the antigenic analysis. Lastly, some of the discrepancies may be due to the presence of undetected mutations.

Several pairs of mutant viruses (JV1 and JV3, KV4 and PV12, BV11 and EV2, BV12 and BV13, SV6 (SV3) and WV15, and WV11 and ZV1) have undergone mutation at the same position, giving rise to different amino acid residues. The ability of antibodies to differentiate within mutant pairs is in most cases not surprising, as the residue changes often involved amino acids that differed considerably in size or charge or both (for example, SV6, with positively charged Arg at position 240, and WV15, with negatively charged Glu at the same position). The only remarkable differentiation is between JV1 and JV3, as Phe and Tyr both have uncharged side chains of similar size. Overall, the substituted amino acids had larger side chains than the parental residue in 22 mutants and approximately equal or smaller side chains in 16 mutants. A change in charge occurred in 20 mutations.

Double amino acid changes were detected in three mutant viruses; SV3 has amino acid changes at residues 240 and 273, WV8 has amino acid changes at residues 182 and 207 and JV18 has a changed residue 167 and a lysine insertion between residues 165 and 166. In addition to these coding mutations, two noncoding changes were detected, in WV8 and WV10. In all cases amino acid changes arose by point mutation, while the lysine insertion in JV18 resulted from the insertion of an A_3 triplet. The wild-type sequence in the region of the insertion is $A_5U_2CU_2$, whereas the sequence of JV18 is A_8U_5; if the C to U transition occurred as an initial event to give the sequence A_5U_5 (as found in JV1 and JV9), then during the replication of the viral RNA this AU-rich sequence may have been incorrectly copied in a slippage or self-copying event to generate the A_3 insertion.

Location of Antigenic Sites in the PR8 HA

The location of the 39 unique amino acid changes in the primary sequence of the HA1 subunit of PR8 (Mount Sinai) is shown in Figure 1, from which it is immediately apparent that the majority of the mutations are clustered toward the middle of the sequence. Two features should be noted regarding the location of the antigenic sites in the sequence. First, in each antigenic site, mutations have been detected in regions widely separated in the primary sequence. Second, residues that were operationally mapped into different antigenic sites can lie very close to each other in the primary sequence, and in some instances overlap (for example, sites Sa and Sb). The tertiary structure of the HA of influenza virus A/Hong Kong/68 (H3N2) has been determined (Wilson et al., 1981). Although the virus used in this study is of a different subtype and shows only a low degree of homology with the H3 subtype HA at the level of primary amino acid sequence (35% in the HA1 subunit; Winter et al., 1981), the strict conservation between subtypes of many structurally important amino acids makes it likely that these functionally related proteins will share many features of their structure as well as a common overall shape (Wilson et al., 1981; Wiley et al., 1981; Winter et al., 1981).

When the changed amino acid residues indicated in Table 3 are located on the three-dimensional structure of the HA (Figure 3), the close relationship between the structures of the HAs of the H1 and H3 subtypes becomes apparent. As would be predicted for amino acid residues involved in antibody recognition, all except one of the changed residues are found in positions at the surface of the HA monomer (the exception is one of the two mutations in WV8, see below). It is also apparent that the residue changes of mutant viruses belonging to the same antigenic group (see Table 1) occupy positions close to each other in the three-dimensional structure. It seems likely, therefore, that an analysis of the antigenic regions of the PR8 HA relative to the three-dimensional structure of

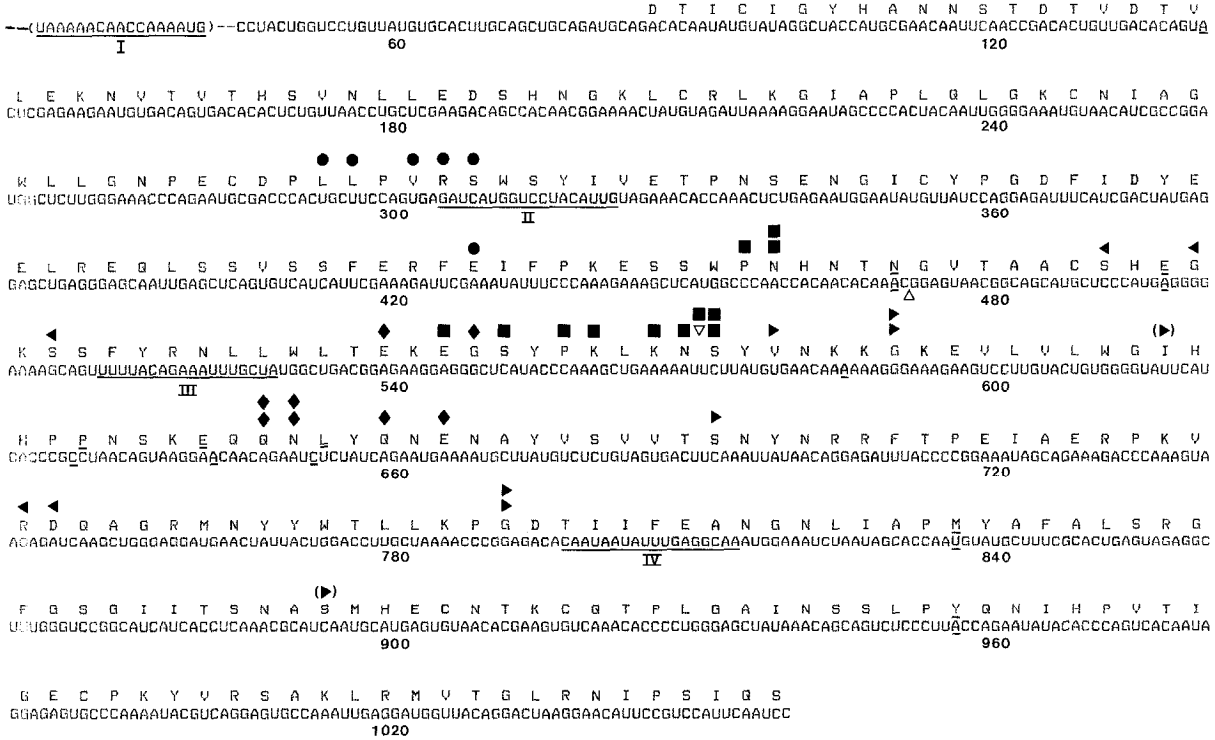

Figure 1. Nucleotide and Deduced Amino Acid Sequence of the Mature HA1 Polypeptide of Influenza Virus A/PR/8/34 (Mount Sinai), and Location of Altered Amino Acid Residues Detected in Mutant PR8 Viruses

The nucleotide sequence is numbered relative to the 5′ end (mRNA sense) of the RNA segment encoding the HA of PR8 (Cambridge) (Winter et al., 1981); those nucleotide and amino acid residues which differ from PR8 (Cambridge) are underlined, and the location of the three-base deletion is indicated by (△). The regions of the RNA to which the sequencing primers hybridized are also underlined. The nucleotide sequence of the region of the RNA to which primer I hybridized was not determined directly, and was assumed to be the same as that found in PR8 (Cambridge). The location and classification of altered amino acid residues detected in mutant viruses (Table 3) are indicated as follows: site Sa (■); site Sb (◆); site Ca_1 (▶); site Ca_2 (◀); site Cb (●). Amino acid residues marked by two symbols have undergone mutation to two different amino acids, and the symbols in brackets represent detected mutations that are probably unimportant (see text). The location of the amino acid insertion found in the mutant JV18 is shown by ( ).

the H3 subtype HA is a permissive approach for determining the location of antigenic sites in the PR8 (and possibly the H3 subtype) HA molecule.

Antigenic Sites Sa and Sb

These sites are defined by residues in the upper part of the globular head of the HA1 subunit (Figure 3). Antigenic analysis divides this region into two operationally distinct areas, identified here as sites Sa and Sb, which are, however, closely linked (Table 2) and cannot accommodate the simultaneous binding of antibodies to each site (Lubeck and Gerhard, 1981). Site Sa comprises the "front" of this region as viewed in Figure 3a, including residues 128 and 129 and residues 158, 160 and 162 to 167 (excluding 164). The Sa site extends down through a region of β structure on the opposite side of the HA monomer to the proposed receptor binding site (Wilson et al., 1981), and in the trimer comes close to the monomer–monomer interface. The site occupies a position relatively near the receptor binding site of the adjacent monomer when the trimer is assembled (see Figure 5).

The antigenic site Sb occupies the "back" of the head region as viewed in Figure 3, comprising the external residues 192, 193 and 196 of a region of α-helix, residue 198, and residues 156 and 159. These residues are along the upper edge of a pocket containing the proposed receptor binding site. Residues 156 and 159 lie in the same region of polypeptide chain as residues 158 and 160 of site Sa. Indeed, it is this polypeptide loop (containing residues 156–160) that separates the operationally distinct antigenic sites Sa and Sb, and residues which lie along this loop map, into one site or the other according to whether they are front-facing (residues 158 and 160, site Sa) or back-facing (residues 156 and 159, site Sb.)

Antigenic Sites Ca (Ca_1, Ca_2) and Cb

Analysis of the location on the HA monomer of the residues that delineate antigenic subsites Ca_1 and Ca_2 shows two widely separated clusters of changed residues. Subsite Ca_1 contains external residues 169, 173, 207 and 240; however, two mutants from this group (WV8 and SV3) exhibit double mutations that may indicate the involvement of residues 182 and 273. Residue 182 seems unlikely to be involved in antibody recognition, since it is buried in the monomer structure, whereas residue 207 (which is also found

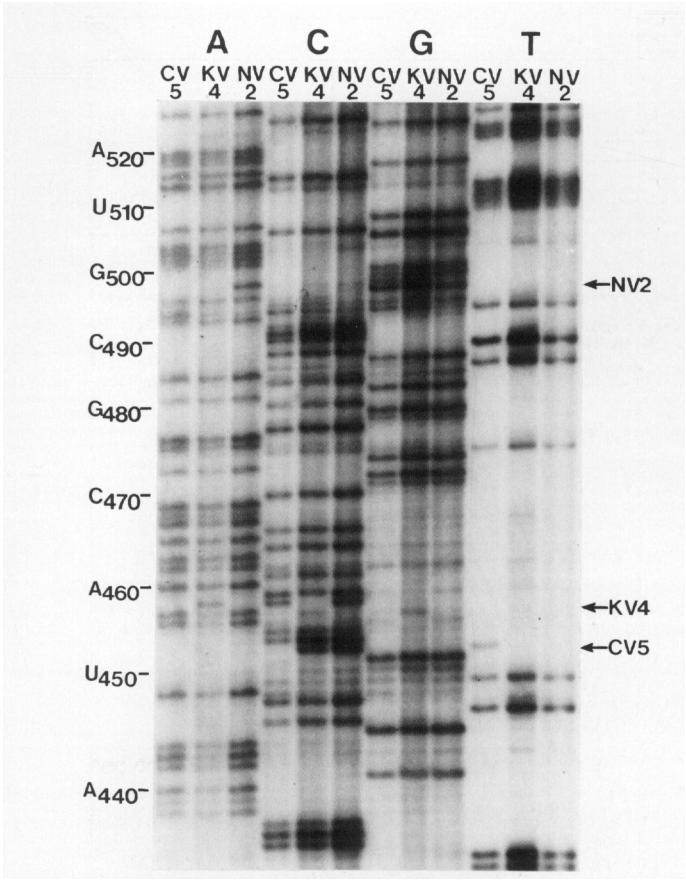

Figure 2. Detection of Altered Nucleotides in Mutant PR8 Viruses

Virion RNAs from mutant viruses CV5, KV4 and NV2 were incubated in sequencing reactions with oligonucleotide II as primer, and the reaction products were separated by electrophoresis as described in the Experimental Procedures. Products from each of the sequence-specific reactions (indicated by A, C, G or T above the lanes, the specificity of the ddNTP in each reaction mixture) were analyzed in parallel, and the altered nucleotides, the locations of which were deduced as described in the text, are shown. The nucleotides are numbered as shown in Figure 1.

in WV8) occupies an external position consistent with other mutants of the same group. Residue 273 is almost certainly not part of this antigenic site, since SV3 and SV6 (the latter contains the same mutation as SV3 at residue 240, but does not contain a mutation at 273) are indistinguishable by radioimmunoassay. The location of antigenic subsite Ca_2 is indicated by mutations in two loops that contain residues 140, 143 and 145 and residues 224 and 225. These loops are close to each other in the three-dimensional structure of the HA monomer, but lie on the opposite side of the monomer to those mapped as belonging to the related subsite Ca_1 (Figure 3). However, these two subsites come into close proximity when the trimer is assembled (Figure 3b), presenting a cleft which spans the monomer–monomer boundary and which we operationally defined as the antigenic site Ca.

The fourth antigenic site, Cb, defines a region near the bottom of the globular head of HA1, distinct from the Ca cleft, as indicated by mutations at residues 78 to 83 (excluding residue 80) and residue 122 (Figure 3). The location of this site is consistent with previous analyses by competition binding of antibodies to sites Cb and Sa, which proved these sites to be topologically distinct (Lubeck and Gerhard, 1981).

Antigenic Properties of the PR8 HA

An overall impression gained from Figure 3 is that many of the external residues of the globular region of the HA1 subunit can affect the ability of antibodies to bind to, and neutralize, the virus. It is only by reference to data defining the antigenic interrelationship of these residues that conclusions can be drawn regarding the boundaries of antigenic sites on the molecule.

In this context one has first to consider how the residue changes might have induced the observed antigenic alterations. In general, the residue changes could have altered the binding avidity of antibodies to the corresponding mutant viruses by inducing conformational changes in the HA or by replacing an amino acid residue that contributed directly (as contact residue) to the interaction between antibody combining site and antigen. In some cases, antibodies might have detected conformational changes induced by the mutations. It is possible also that the few antibodies whose binding avidity was changed by mutations at many different sequence positions in the HA (Table 1) detected such conformational changes. However, the observed changes occurred, with one exception, in surface positions on the HA and clustered, as anticipated from the antigenic analysis, into topographically related regions on the three-dimensional structure of the HA trimer. In addition, the overall topographic relationship between these regions (for example, Sa–Sb, Ca_1–Ca_2, Sa–Ca_1 and/or Ca_2) was consistent with the extent of operational linkages observed in antigenic analyses (Table 2). Taken together, the findings described above are thus compatible with the notion that the changed residues demarcate structures on the PR8 HA which, in general, comprise the actual residues with which the combining sites of anti-HA antibodies interact. A complete and final description of the antigenicity of the PR8 HA would require the knowledge of all amino acid residues with which combining sites of antibodies (or of immune recognition structures in general) interact. However, our antibody panel certainly does not contain all anti-HA antibody specificities (Staudt and Gerhard, 1982), and our mutant virus panel similarly defines probably only a fraction of all possible contact residues on the HA. In particular, the wide spread in the number of residue changes at distinct sequence positions, which altered the binding capacity of individual antibodies (Figure 4), suggests that many epitopes are still only marginally defined by our mutant virus panel.

Nevertheless, on the basis of the present antibody

Table 3. Altered Nucleotide and Amino Acid Residues Detected in PR8 Mutant Viruses

Sa						Sb						Ca_1						Ca_2						Cb					
VIRUS	NUCLEOTIDE		AMINO ACID			VIRUS	NUCLEOTIDE		AMINO ACID			VIRUS	NUCLEOTIDE		AMINO ACID			VIRUS	NUCLEOTIDE		AMINO ACID			VIRUS	NUCLEOTIDE		AMINO ACID		
	pos[n]	mut[n]	mut[n]	position H1	position H3		pos[n]	mut[n]	mut[n]	position H1	position H3		pos[n]	mut[n]	mut[n]	position H1	position H3		pos[n]	mut[n]	mut[n]	position H1	position H3		pos[n]	mut[n]	mut[n]	position H1	position H3
CV1	564	A-G	K-E	161	165	BV1	538	A-G	E-G	152	156	SV3	789	G-A	G-R	236	240	DV4	489	U-C	S-P	136	140	AV1	303	A-G	R-G	74	82
2	556	C-U	P-L	158	162	2	658	A-G	Q-R	192	196	3	891	U-A	S-T	270	273	5	742	G-U	R-I	220	224	LV1	303	A-G	R-G	74	82
5	453	C-U	P-S	124	128	6	547	G-A	G-D	155	159	6	789	G-A	G-R	236	240	NV2	498	G-A	G-R	139	143	4	303	A-G	R-G	74	82
6	569	U-G	N-K	162	166	11	646	A-U	Q-L	188	192	WV8	431	A-U	silent			7	504	A-G	S-G	141	145	7	306	U-C	S-P	75	83
JV1	571	C-U	S-F	163	167	12	649	A-G	N-S	189	193	8	617	U-G	I-M	178	182	TV1	498	G-A	G-R	139	143	MV4	295	U-C	L-P	71	79
2	564	A-G	K-E	161	165	13	650	U-A	N-K	189	193	8	691	C-U	S-L	203	207	26	745	A-G	D-G	221	225	5	306	U-C	S-P	75	83
3	571	C-A	S-Y	163	167	EV2	646	A-G	Q-R	188	192	10	577	U-C	V-A	165	169							9	301	U-A	V-E	73	81
9	571	C-U	S-F	163	167	6	646	A-G	Q-R	188	192	10	710	U-C	silent									RV6	426	G-A	E-K	115	122
18	571	C-U	S-F	163	167	8	664	A-G	E-G	194	198	11	588	G-A	G-R	169	173							7	292	U-C	L-P	70	78
18	567_A	$+A_3$	+K	161_A	165_A							15	790	G-A	G-E	236	240												
KV2	559	A-C	K-T	159	163							ZV1	589	G-U	G-V	169	173												
4	458	C-A	N-K	125	129																								
PV9	550	C-U	S-L	156	160																								
11	550	C-U	S-L	156	160																								
12	456	A-U	N-Y	125	129																								
20	544	A-U	E-V	154	158																								

The changes are shown relative to the parental sequence; thus mutant CV1 has a G at nucleotide 564 rather than the A found at that position in the parental sequence. The corresponding amino acid (at residue 161 in the H1 sequence, or 165 in the H3 sequence, see text) has therefore undergone mutation from lysine to glutamic acid. The mutant viruses are assembled according to the antigenic relationships shown in Table 1.

and mutant virus panels, we suggest that the globular domain of the PR8 HA1 subunit contains five topographically distinct regions or faces. These regions have been designated as antigenic sites because in each case many antibodies have been observed that map to a single site (see Table 2). Relative to these antibodies (which were the majority), these regions fulfilled the operational definition of a discrete antigenic site as an area to which the binding of specific antibodies is not affected by changes occurring in neighboring antigenic sites (Urbanski and Margoliash, 1977). However, antibodies were also isolated that exhibited reduced binding to mutants characterizing different antigenic sites. In general, these linkages are consistent with the location of the corresponding antigenic sites (that is, they were most frequently observed between sites that are in close proximity to each other on the three-dimensional structure, such as Sa and Sb, Ca_1 and Ca_2, Sa and Ca_1 and/or Ca_2). These linkages may further indicate, however, that some amino acids located between the sites defined by the present mutant virus groups may also serve as contact residues for anti-HA antibodies. If so, one might have to visualize the antigenicity of the globular domain of the PR8 HA1 as a large contiguous antigenic area that contains five immunodominant regions which make a major contribution to the epitopes recognized by anti-HA antibodies.

Comparison of Antigenic Sites in the H1 and H3 Subtype HAs

The precision with which the antigenic sites of PR8 correlate with the structure of the H3 subtype HA indicates that the HA molecules of these two subtypes must share many related structural features. The remarkable relationship between antigenic features of the PR8 HA and structural features of the H3 subtype HA argues strongly for the validity of using the H3 structure as a model for the location of the antigenic sites in the PR8 HA. Several studies have previously been undertaken to determine the location of antigenic sites in the H3 subtype HA (Webster and Laver, 1980; Laver et al., 1980, 1981; Both and Sleigh, 1981; Wiley et al., 1981), and a comparison of the location of the proposed antigenic sites in the H1 and H3 subtype HAs is of interest in assessing the general antigenicity of the HA molecule. The analyses of the H3 subtype HA have in general involved the comparison of amino acid changes in natural and laboratory selected variants of the virus to the structure of the HA. However, with the exception of one limited study (Webster and Laver, 1980), the detailed characterization, as described here, of the interaction of a panel of monoclonal antibodies with many antigenically significant amino acid residues has not been performed. Nonetheless, it is generally proposed that the H3 subtype HA contains four antigenic sites, which are located as follows: site A, a loop containing residues 133 and 137 and 140 to 146; site B, defined by residues 155 through 160 and 186 through 197; site C, comprising the bulge in the tertiary structure around the bonded cysteine residues 52 and 277; and site D, which covers the region of interface between monomer subunits (Figure 5) (Webster and Laver, 1980; Laver et al., 1980, 1981; Both and Sleigh, 1981; Wiley et al., 1981). Of these antigenic

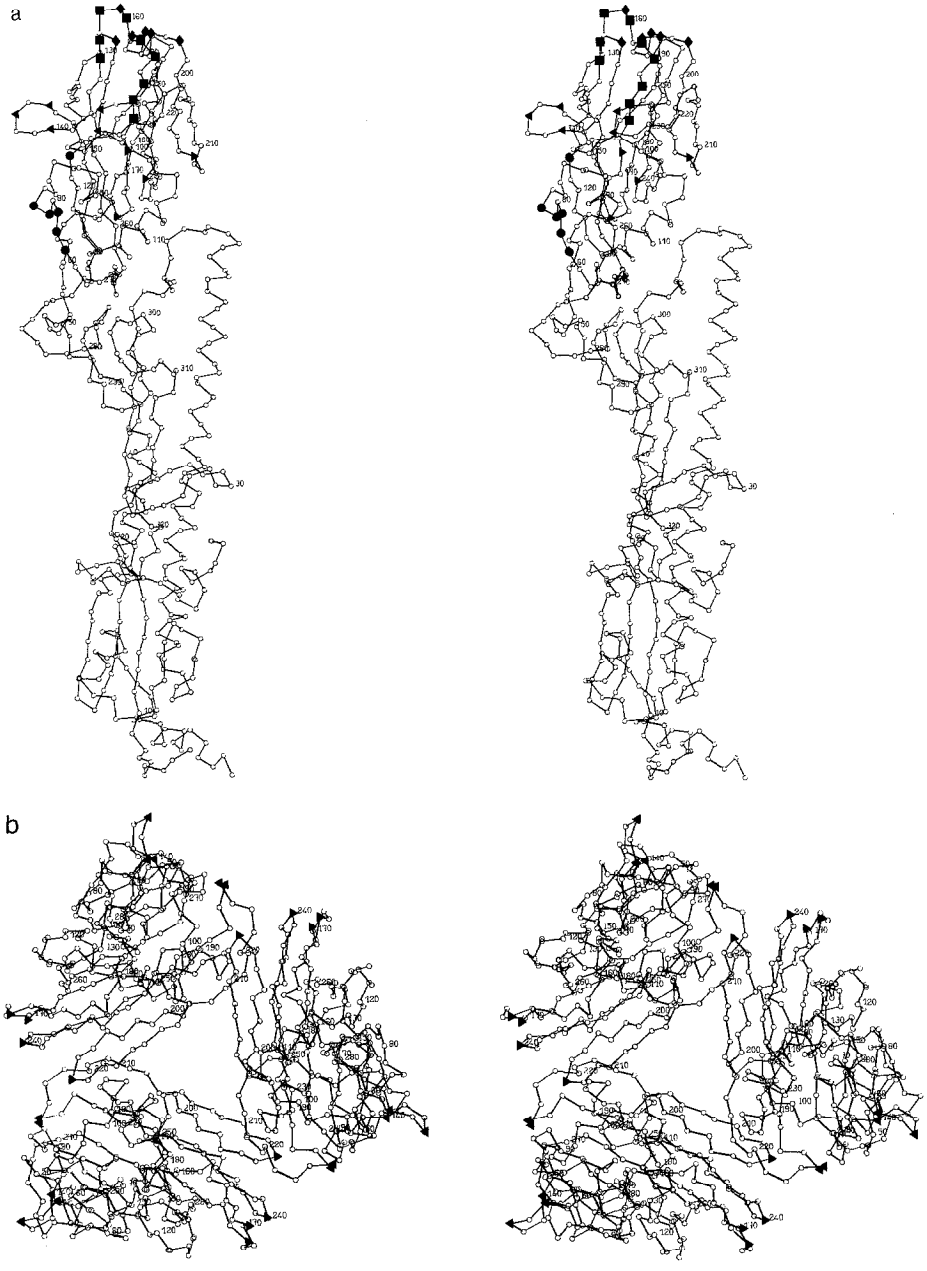

Figure 3. Location of Altered Amino Acid Residues Relative to the Three-Dimensional Structure of the H3 Subtype HA

The altered amino acid residues are marked on stereo drawings of the α-carbon tracing of the complete HA monomer, side view (a), and of residues 48 to 280 of the HA1 polypeptide in the HA trimer, top view (b). For clarity, the HA2 residues are not numbered. The altered residues are classified as follows: site Sa (■); site Sb (◆); site Ca_1 (▶); site Ca_2 (◀); site Cb (●). Only Ca_1-specific and Ca_2-specific residues are shown on the top view (b).

sites, site A is considered to be of the greatest importance, as judged by the epidemiological significance of viruses expressing altered antigenicity at this site, and by the predominance of monoclonal antibodies binding to this site which are generated from mice after immunization with virus (Webster and Laver, 1980).

The location of the antigenic sites suggests the PR8 site Sb to be the equivalent of the H3 subtype site B, although some site B residues (158 and 160; Both and Sleigh, 1981) are mapped here as belonging to the site Sa (Figure 5). We would also define residues 207 (site D in the H3 subtype; Wiley et al., 1981) and 172, and residues 242, 174 and 226, which were proposed previously as possible members of site D in the H3 subtype HA (Wiley et al., 1981), as belonging to site Ca. If a site similar to the PR8 site Ca exists in the H3 subtype HA, it would presumably also be affected by other monomer–monomer interface mutations, such as those previously assigned to the H3

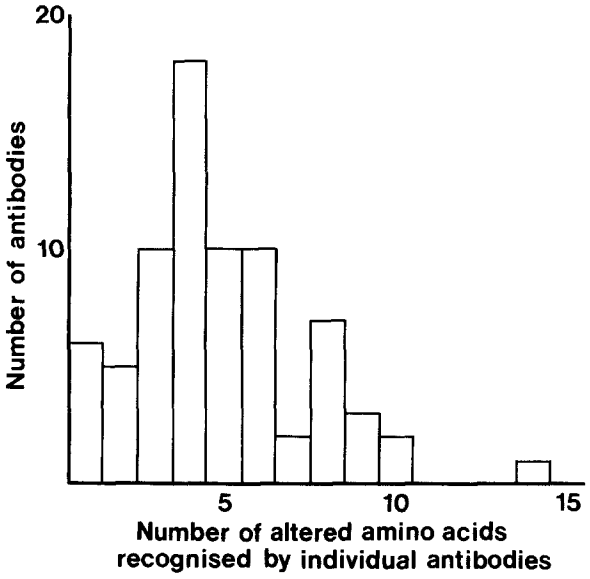

Figure 4. The Number of Altered Amino Acids Recognized by Individual Antibodies

The histogram shows the number of antibodies that exhibit strongly reduced binding to one or more unique mutant viruses. Only antibodies that differ from each other by strongly reduced reactivity with unique mutant viruses are included.

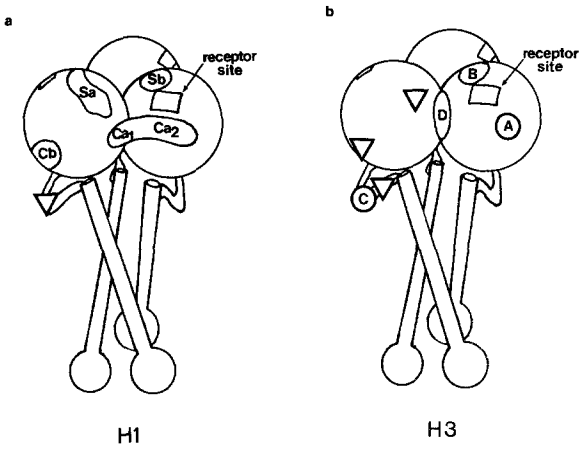

Figure 5. Schematic Representation of the HA Trimer

Shown are the relative arrangements of carbohydrate attachment sites (▽), antigenic sites, and the receptor binding site, for the H1 (a) and H3 (b) subtype HAs. Only the carbohydrate attachment sites in the region of the globular head of the trimer are shown.

subtype site D. It is possible then to assign approximate equivalence to antigenic sites B and Sb, and D and Ca. There are, however, two proposed antigenic sites in the PR8 HA that do not immediately appear to have equivalents in the H3 subtype HA, and vice versa. Although some of the anti-Ca_2 (PR8) monoclonal antibodies map to the structure that forms the H3 subtype site A, the corresponding residues form part of a much larger site (Ca) on the HA of PR8.

It is possible that the inconsistent location of some antigenic sites on the PR8 and H3 subtype HAs is merely an artifact of biased mutant and epidemic variant virus panels studied so far in the two systems. It also may reflect subtle differences in Ir gene control (McDevitt and Benacerraf, 1969) of the immune response to distinct regions of these HAs. Most likely, however, the presence of carbohydrate at the potential attachment sites at residues 81 and 165 of the H3 subtype HA1, and at residue 271 of the PR8 HA1, could result in the observed differences in antigenicity. For instance, site Cb in PR8 and site C in the H3 subtype both occupy positions near the bottom of the globular head of the HA1 subunit (Figure 5). Neither of these sites contains potential carbohydrate attachment sequences. However, such attachment sequences do occur in the region of the H3 subtype HA corresponding precisely to PR8 site Cb, and in the PR8 HA at the region corresponding precisely to the H3 subtype site C. The reciprocal nature of this relationship, wherein antigenic sites in the HA of one subtype do not contain the carbohydrate attachment sequences that are found in the equivalent location of the HA of the other subtype, suggests that the presence of carbohydrate is masking potentially antigenic amino acid residues. Also, antigenic site Sa in the PR8 (Mount Sinai) HA does not contain any carbohy- attachment sequences in this region of the three-dimensional structure (Figure 1), while oligosaccharide is attached at residue 165 of the H3 subtype HA (Wilson et al., 1981). The presence of this carbohydrate is consistent with the absence in the H3 subtype HA of a site corresponding to PR8 site Sa (Figure 5). It is possible that the apparently increased antigenicity of the amino acids of the H3 subtype site A results from carbohydrate masking the amino acid residues of the H3 subtype HA which correspond to the PR8 site Sa. The possibility that oligosaccharide chains may sterically prevent the immune recognition of antigenic sites in the hemagglutinins of different subtypes has been proposed previously, on the basis of considerations of the H3 subtype antigenic sites and the amino acid sequences of fowl plague and A/Japan/305/57 viruses (Wiley et al., 1981). Determining the location of antigenic sites in the HAs of other influenza viruses may provide further insight into the role of carbohydrate in modulating the antigenicity of particular regions of the HA molecule.

Experimental Procedures

Hybridoma Antibodies

The 90 hybridomas included in this analysis have been generated by fusion of lymphocytes from the spleen or mediastinal lymph nodes or both of 15 BALB/c mice following primary, secondary or primary and secondary immunization with PR8 virus or with PR8-virus-infected P815 cells. The fusions were performed as previously described (Gerhard, 1980) with the myelomas P3X63 (Köhler and Milstein, 1976), P3X63Ag8-cl.653 (Kearney et al., 1979) or Sp2/0 (Shulman et al., 1978) as fusion partners. The hybridomas were grown in vitro in Dulbecco's modified Eagles medium supplemented with 7.5% fetal calf serum, 7.5% A-gamma horse serum (both from Flow Laborato-

ries), 2 mM glutamine and 50 μg/ml gentamycin. They were then cloned on soft agar. Hybridomas used for mutant virus selection were grown in the peritoneal cavity of pristane-primed BALB/c mice. The specificity for the HA of PR8 was determined as described previously (Gerhard et al., 1981).

Selection of PR8 Virus Mutants

The PR8 mutants were selected as described (Yewdell et al., 1979) by incubation of plaque-cloned parental PR8 virus seed, A/PR/8/34(H1N1), originally obtained from Mount Sinai Hospital, New York, with an overneutralizing dose of individual monoclonal anti-HA antibodies and growth of the non-neutralized virus fraction at limiting dilution in the allantois on shell culture system. Virus from several positive cultures per selecting antibody was recloned twice by growth at limiting dilution in the absence of antibody, first in the allantois on shell culture system, then in the allantoic cavity of embryonated hens' eggs. The mutant-virus-containing allantoic fluid from individual eggs was aliquoted and stored frozen. From these infectious stocks large quantities of mutant virus were prepared by inoculation of embryonated hens' eggs with approximately 1000 infectious virions and subsequent purification of the virus by pelleting and banding in sucrose gradients for antigenic and RNA sequence analyses.

Antigenic Analysis of Mutant Viruses

Purified virus was quantitated by hemagglutinin assay with chicken red cells as described by Fazekas de St. Groth and Webster (1966). Virus solution (2×10^5 hemagglutinating units per milliliter of phosphate-buffered saline containing 0.4% sodium azide [HAS] was incubated for 30 min at 37°C with Nonidet-P40. This virus solution was then diluted tenfold with HAS and stored as immunoadsorbent stock solution at 4°C. Viral immunoadsorbents were prepared by incubating 25 μl of a 1:100 dilution in HAS of the stock solution in individual wells of polyvinyl microtiter plates (Cooke Engineering) overnight at room temperature. Hybridoma culture fluids, at a standard dilution containing approximately 1 ng antibody/25 μl, were then tested for binding to the various mutant virus immunoadsorbents by radioimmunoassay. In parallel, serial $2^{-0.5}$ dilutions of each antibody's standard dilution were assayed on parental virus immunoadsorbent to generate a standard binding curve for each antibody. The cpm observed in the interaction of the antibody with each mutant virus was then related to the antibody's standard curve and was expressed as the percentage binding relative to the parental virus.

Nucleotide Sequence Analysis of Influenza Virion RNA

Influenza virion RNA was isolated by digestion of purified virus with pronase followed by extraction with phenol and chloroform as described previously (Caton and Robertson, 1979). Four oligonucleotide primers were synthesized by a solid phase phosphotriester method (Duckworth et al., 1981) of which the sequences were primer I d(TAAAAACAACCAAAATG), primer II d(GATCATGGTCCTACATTG) (provided by F. L. Raymond), primer III d(TTTTACAGAAATTTGCTA) and primer IV d(CAATAATATTTGAGGCAA). These oligonucleotides were used to prime the synthesis of DNA complementary to virion RNA in the presence of dideoxynucleoside triphosphates (ddNTPs) (Sanger et al., 1977) as follows. Four reaction mixtures (final volume 2 μl each) were assembled which each contained 50 mM Tris-HCl (pH 8.3), 140 mM KCl, 10 mM $MgCl_2$, 20 mM 2-mercaptoethanol, 2.5 pmole oligonucleotide primer, 0.25 μg unfractionated virion RNA (0.06 pmole template RNA), 0.4 mM dCTP, 0.4 mM dGTP, 0.4 mM dTTP, 17.5 μM α-^{32}P-dATP (spec. act. 230 Ci/mmole) (Amersham), 1.25 U reverse transcriptase (Life Sciences Inc.) and one of the four ddNTPs (approximately 60 μM ddCTP, ddGTP or ddTTP; approximately 3 μM ddATP) (P-L Biochemicals). On some occasions α-^{32}P-dGTP was used as the labeled triphosphate, and the concentrations of the other triphosphates were adjusted accordingly. The reaction mixtures were incubated at 37°C for 30 min in sealed glass capillaries, following which the reactions were termined by addition to 8 μl of 10 mM EDTA. One fifth of each sample (2 μl) was removed and denatured by the addition of 0.75 μl of formamide and boiling in an open tube for 12 min. The reaction products were separated by electrophoresis in ultra-thin 6% acrylamide gels (40 × 20 × 0.017 cm) containing 90 mM tris-borate (pH 8.3), 2.5 mM EDTA and 7 M urea, which were maintained at 70°C (Garoff and Ansorge, 1981). Following electrophoresis, gels were fixed in 10% acetic acid, dried and exposed in direct contact to autoradiographic film at room temperature for between 2 and 14 days.

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