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Antigenic Structure of the Influenza B Virus Hemagglutinin: Nucleotide Sequence Analysis of Antigenic Variants Selected with Monoclonal Antibodies

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We report here the complete nucleotide sequence of the hemagglutinin (HA) gene of influenza B virus B/Oregon/5/80 and, through comparative sequence analysis, identify amino acid substitutions in the HA1 polypeptide responsible for the antigenic alterations in laboratory-selected antigenic variants of this virus. The complete nucleotide sequence of the B/Oregon/5/80 HA gene was established by a combination of chemical sequencing of a full-length cDNA clone and dideoxy sequencing of the virion RNA. The nucleotide sequence is very similar to previously reported influenza B virus HA gene sequences and differs at only nine nucleotide positions from the B/Singapore/222/79 HA gene (Verhoeyen et al., Nucleic Acids Res. 11:4703–4712, 1983). The nucleotide sequences of the HA1 portions of the HA genes of 18 laboratory-selected antigenic variants were determined by the dideoxy method. Comparison of the deduced amino acid sequences of the parental and variant HA1 polypeptides revealed 16 different amino acid substitutions at nine positions. All amino acid substitutions resulted from single-point mutations, and no double mutants were detected, demonstrating that as in the influenza A viruses, single amino acid substitutions are sufficient to alter the antigenicity of the HA molecule. Many of the amino acid substitutions in the variants occurred at positions also observed to change in natural drift strains. The substitutions appear to identify at least two immunodominant regions which correspond to proposed antigenic sites A and B on the influenza A virus H3 HA.

Antigenic variation is a hallmark of the influenza viruses and presents a major obstacle to the effective control of influenza by vaccination. Recent studies of antigenic variation in the influenza viruses have focused on understanding the structural basis for antigenic drift through detailed definition of the antigenic structure of the influenza A virus hemagglutinin (HA). This has been accomplished largely through amino acid sequence comparisons of the HAs of naturally occurring drift strains and by identification of amino acid sequence changes responsible for minor antigenic alterations in the HAs of laboratory-selected antigenic variants (7, 8, 42, 45, 46). The location of these amino acid changes on the three-dimensional structure of the HA has provided evidence for four to five antigenically distinct regions on the influenza A virus HA (8, 47).

Although antigenic variation in the influenza A viruses has been studied extensively, only recently have these types of studies been extended to the influenza B viruses (21, 41, 44). The influenza B virus HA is functionally and structurally analogous to the influenza A virus HA, serving to mediate viral adsorption and penetration into host cells and serving as the major surface antigen of the virus (2). Recent comparison of the primary structures of the influenza A and B virus HAs revealed conservation of the major structural features of the molecule, suggesting a similar three-dimensional structure and a relatively close evolutionary relationship (20). No antigenic shift has been observed in influenza B viruses, perhaps reflecting the lack of an animal reservoir of these viruses (31). Antigenic drift has been demonstrated, however, by analysis with postinfection ferret serum (9, 34) and with panels of monoclonal antibodies specific for the HA (24, 44). In these studies, variation occurred in all epitopes tested, albeit at an apparently slower rate than in influenza A viruses. This observed slower rate of antigenic drift was confirmed recently by comparison of the amino acid sequences of the HAs of several influenza B strains isolated over a 40-year period (21, 41). It was concluded from these studies that antigenic drift in the HA of influenza B viruses occurred, as in influenza A, by the accumulation of amino acid substitutions in the HA1 polypeptide, but at about one-fourth the rate for influenza A viruses. It was also suggested that the amino acid sequence differences among the drift strains occur in regions of the HA molecule which correspond to the proposed antigenic sites on the influenza A virus HA and thus possibly identify corresponding antigenic domains on the influenza B virus HA. It is not known, however, which of these sequence differences are relevant to the antigenic differences and which reflect antigenically silent, random drift. Therefore, the exact number and location of the antigenic sites on the influenza B virus HA remain to be elucidated.

To identify antigenically relevant amino acid substitutions in the influenza B virus HA and thus to define precisely the antigenic structure of this molecule, we have used monoclonal antibodies (24, 44) to select variants of influenza B virus B/Oregon/5/80 with antigenically altered HA molecules. We report here the complete nucleotide sequence of the HA gene of B/Oregon/5/80 and identify the amino acid substitutions in the HA1 polypeptide responsible for the antigenic alterations in the laboratory-selected antigenic variants of this virus. These substitutions appear to define at least two antigenic domains on the influenza B virus HA which correspond to proposed antigenic sites A and B on the influenza A virus H3 HA.

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MATERIALS AND METHODS

Viruses and bacterial strains. B/Oregon/5/80 was originally isolated from a Reye's syndrome patient and was provided by Alan Kendal of the Centers for Disease Control, Atlanta, Ga. This virus is antigenically very similar to the previous vaccine strain, B/Singapore/222/79 (24). Virus was grown in the allantoic cavity of 10-day-old embryonated chicken eggs as described previously and quantitated by hemagglutination of chicken erythrocytes (10). B/Oregon/5/80 was cloned three times by limiting dilution in embryonated chicken eggs, and a seed virus stock was prepared by growth of a single clone in eggs. Viruses were purified by adsorption to and elution from chicken erythrocytes, followed by differential centrifugation and sedimentation through 25 to 70% sucrose gradients in a Beckman SW28 rotor. Virion RNA was isolated by treatment of purified virus with proteinase K and sodium dodecyl sulfate, followed by extraction with phenol-chloroform (3).

Plasmid pAT153/PvuII/8 containing the B/Oregon HA insert was propagated in *Escherichia coli* MC1061 cells as described previously (27).

Monoclonal antibodies. The preparation and characterization of several of the monoclonal antibodies specific for the HA of the B/Oregon/5/80 virus which were used in this study have been described previously (24). Two monoclonal antibodies prepared to the B/HK/8/73 HA (21/6 and 74/1) and shown to be cross-reactive with the B/Oregon/5/80 HA were included in this study and have also been previously described (44).

To expand the panel of monoclonal antibodies and increase the probability of identifying all antigenic regions on the HA, we prepared additional monoclonal antibodies specific for the B/Oregon/5/80 HA by the method of Kohler and Milstein (18). Hybrid cell lines secreting HA-specific antibody were selected after fusion of myeloma cells SP2/0 (35) or P3X63 (18) with immune spleen cells as described previously for influenza virus (19). The culture fluids were screened by enzyme-linked immunosorbent assay, radioimmunoprecipitation, and hemagglutination-inhibition (HI) assays as previously described (17), and positive cells were cloned twice in soft agar. The cloned hybridomas were grown as ascites in the peritoneal cavity of pristane-primed BALB/c mice.

Laboratory selection of variants. Antigenic variants of B/Oregon/5/80 were selected by a single-step neutralization procedure (12). Briefly, monoclonal antibodies to the HA of influenza B (24, 44) were incubated with cloned B/Oregon seed virus at 25°C for 30 min, and the nonneutralized fraction of virus was grown in embryonated eggs. Variants selected in this way were cloned twice by limiting dilution, and the allantoic fluid from individual eggs was stored as sterile stock at −70°C. From these cloned stocks, virus was grown for RNA preparation and serological analysis.

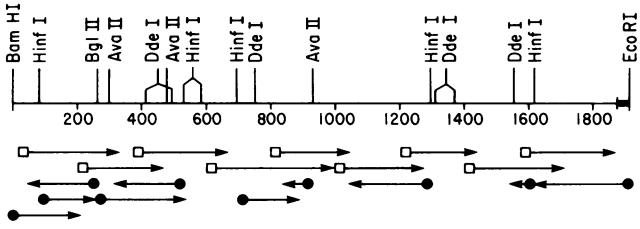

FIG. 1. Restriction endonuclease map and strategy used to sequence the B/Oregon/5/80 HA gene. Arrows with open squares indicate the position and direction of dideoxy sequencing of virion RNA with synthetic oligonucleotide primers. Arrows with closed circles indicate the position and direction (3′ to 5′) of sequence determination by the Maxam and Gilbert method of the cloned full-length cDNA copy of the B/Oregon/5/80 HA gene. The thick line represents plasmid pAT153 sequences.

cDNA synthesis and cloning. Full-length double-stranded cDNA suitable for cloning was synthesized from virion RNA with synthetic oligonucleotide primers to initiate first- and second-strand cDNA synthesis by previously established procedures (16, 27). The primer used in first-strand synthesis contained a G/C ambiguity at position 11 but was otherwise complementary to the 3′ end of the B/Oregon virion RNA segments 4 and 5 as determined by direct chemical sequencing of the virion RNA by the method of Peattie (30; M. T. Berton, unpublished data). One microgram of the first-strand primer (dAGCAGAAGCA$^{G}_{C}$AGCATT) was first phosphorylated by incubation at 37°C for 1 h in a 5-μl reaction volume containing 70 mM Tris-hydrochloride (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM ATP, and 0.5 to 1 U of polynucleotide kinase (New England Biolabs). The phosphorylated primer was mixed with 20 μg of virion RNA, heated at 90°C for 1 min, and quick chilled on ice. The primer-template mixture was then added to a 50-μl reaction mix containing 100 mM Tris-hydrochloride (pH 8.3), 100 mM KCl, 10 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM dGTP, 1 mM dCTP, 1 mM dTTP, 0.5 mM dATP, and 10 μCi of [α-^{32}P]dATP (>3,000 Ci/mmol). Fifty units of reverse transcriptase (Life Sciences) was then added, and the reaction was incubated for 1 h at 42°C. After phenol-chloroform extraction and ethanol precipitation, the cDNAs were denatured at 100°C for 3 min in 98% formamide containing 25 mm EDTA, xylene cynanol, and bromophenol blue, quick chilled on ice, and loaded onto a 3% polyacrylamide gel containing 7 M urea and 1× TBE (90 mM Tris-borate, 2.5 mM EDTA). The gel was electrophoresed for 3 to 4 h at 300 V, and the labeled full-length cDNAs were located by autoradiography for 1 h at room temperature. cDNAs 4 and 5 could not be resolved under these conditions and therefore were coeluted from the gel in 2 M ammonium acetate (16).

Second-strand synthesis was primed with an oligonucleotide (dAGTAGTAACAAG) complementary to the 3′ ends of the full-length cDNAs (2, 21). Briefly, cDNA and phosphorylated second-strand primer were mixed, heated at 100°C for 1 min, and allowed to cool slowly to room temperature. The primer-cDNA mixture was then added to a reaction mix containing 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 6.9), 10 mM $MgCl_2$, 2.5 mM dithiothreitol, 70 mM KCl, and 1 mM of all four deoxynucleotide triphosphates. Five units of the Klenow fragment of *E. coli* DNA polymerase I (New England Biolabs) was added to make a final volume of 50 μl, and the reaction was incubated at 25°C for 2 h. The reaction mixture was phenol-chloroform extracted, ethanol precipitated, and suspended in 10 μl of distilled water. Conversion of single-stranded cDNA to double-stranded cDNA was confirmed on a 4% nondenaturing polyacrylamide gel.

The cDNA (1 μl; ca. 100 ng) was blunt-end ligated to 10 ng of *Pvu*II-cut, phosphatase-treated plasmid pAT153/PvuII/8. The recombinant DNA was transformed into competent *E. coli* MC1061 cells. Positive transformants containing influenza B-specific sequences were identified by a modification of the colony hybridization method (11, 15), using a short-copy cDNA probe (16). To identify HA-specific clones, the transformants were also screened, using as a probe a nick-translated insert isolated from a partial clone of the B/Lee/40 HA gene (pB103/1) provided by Peter Palese, Mt. Sinai

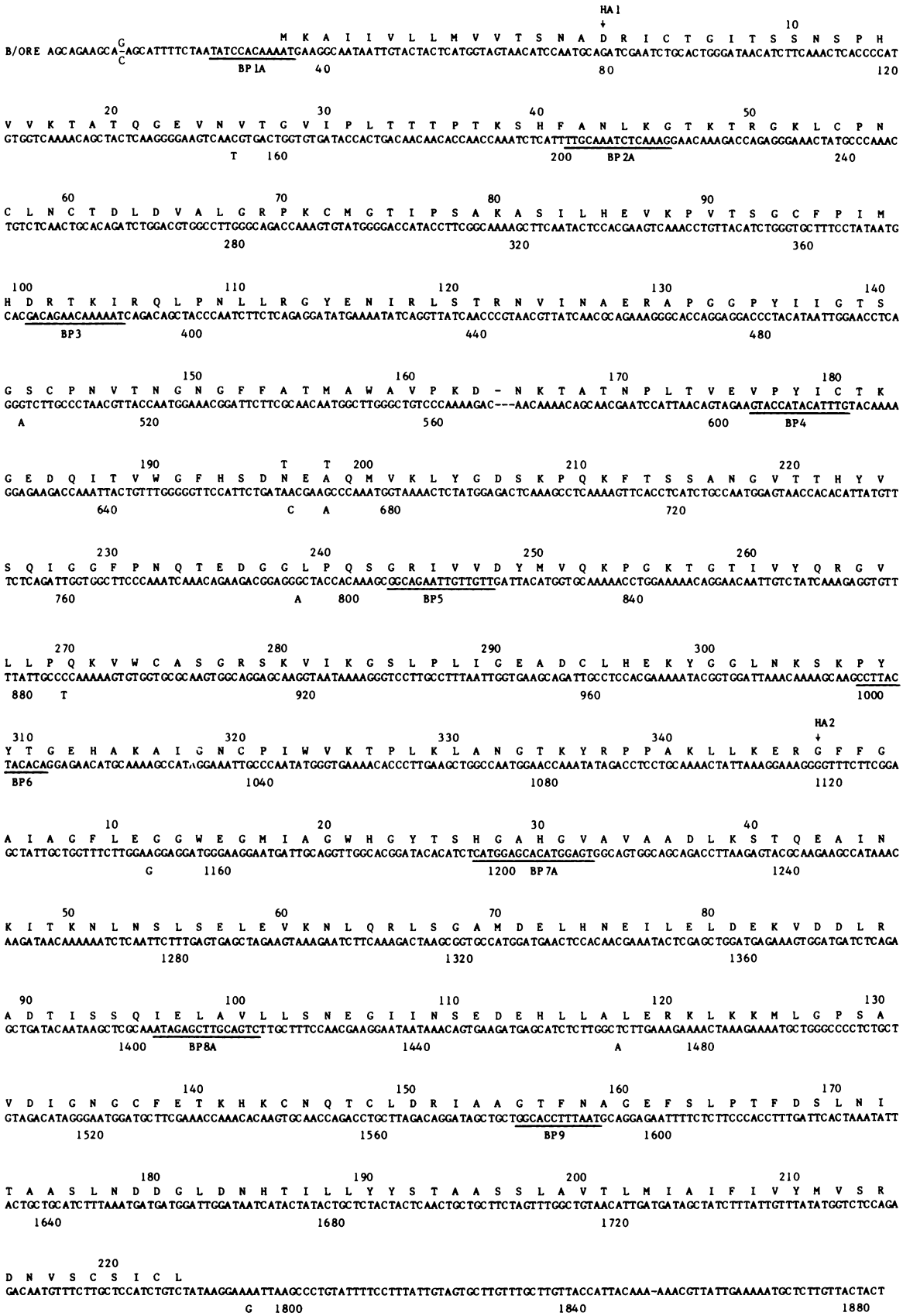

FIG. 2. Complete nucleotide sequence and deduced amino acid sequence of the B/Oregon/5/80 HA gene and comparison with the HA of B/Singapore/222/79. The nucleotide and predicted amino acid sequences are numbered according to the B/Lee/40 HA (21). The numbers above the sequence refer to the amino acid positions, and the numbers below the sequence refer to the nucleotide positions. The primers used in dideoxy sequencing are underlined and numbered as indicated. The dashes represent deletions in the B/Oregon/5/80 sequence relative to the B/Lee/40 HA sequence. Nucleotide sequence differences in the B/Singapore/222/79 HA gene are shown directly below the B/Oregon/5/80 nucleotide sequence, and amino acid differences are shown directly above the predicted amino acid sequence.

School of Medicine, New York, N.Y. The inserts of HA-specific clones were sized on a 1% agrarose mini-gel, and full-length inserts were identified by Maxam and Gilbert (26) sequence analysis of both ends.

Nucleic acid sequencing. Nucleotide sequencing of the cloned HA gene of B/Oregon/5/80 was performed by the method of Maxam and Gilbert (26) except that formic acid was used in the A+G reaction. Radioactive restriction fragments labeled at the 3′ end were prepared by "fill-in" reactions with the Klenow fragment of *E. coli* DNA polymerase I and the appropriate [α-^{32}P]dNTP, followed by secondary restriction enzyme digestion and resolution on a 4% nondenaturing polyacrylamide gel (25, 27).

Nine oligonucleotide primers complementary to the influenza B HA virion RNA at intervals of 200 to 300 nucleotides (see Fig. 1 and 2) were synthesized on an Applied Biosystems model 380A DNA synthesizer by the solid-phase phosphoramidite method. These primers were used to sequence virion RNA by reverse transcription in the presence of dideoxynucleotides as previously described (1). Briefly, 100 to 200 ng of primer and 0.5 μg of unfractionated virion RNA were heated together at 100°C for 1 min in 3.5 μl of distilled water and quick chilled on ice or, in some cases, heated for 3 min at 85°C and then for 1 h at 50°C in Berk-Sharp hybridization buffer (80% formamide, 0.4 M NaCl, 0.04 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.4, 0.001 M EDTA) (4), ethanol precipitated, and dissolved in 3.5 μl of distilled water. Twenty units of Rnasin (Promega Biotech) was then added, and 1 μl of the primer-template was added to each sequencing reaction mixture.

Maxam and Gilbert and dideoxy sequencing reaction products were resolved on 6 and 8% polyacrylamide–7 M urea sequencing gels containing either 1× TBE or a 0.5 to 2.5× TBE gradient (5, 32, 33).

Sequence data were stored, edited, and compiled through the use of the DB system computer programs (38).

RESULTS

Nucleotide sequencing of B/Oregon HA gene. To identify the amino acid substitutions responsible for the antigenic alterations in the laboratory-selected variants of B/Oregon/5/80, it was first necessary to establish the complete nucleotide sequence of the HA gene of this virus. The sequencing strategy and relevant restriction enzyme map for this gene are shown in Fig. 1. The complete nucleotide sequence was determined by a combination of dideoxy sequencing of the virion RNA with synthetic oligonucleotide primers (1, 33) and chemical sequencing of the cloned HA gene (26). The plus-strand sequence was determined by the dideoxy method, with the exception of the first 40 nucleotides, the last 4 nucleotides, and ambiguities at positions 429 and 528 where bands were always seen in all four lanes of the gel. These sequencing artifacts have also been described previously by others (6, 37), and appear to result from the inability of the reverse transcriptase to traverse hairpin structures formed by the sequence in these regions. Approximately 70% of the sequence was determined by the method of Maxam and Gilbert, including the first- and second-strand primer sequences at the 3′ and 5′ ends of the gene. This extended and confirmed the dideoxy sequencing results and resolved the ambiguities at positions 429 and 528. A G/C ambiguity at position 11 represents the sequence of the primer used for first-strand cDNA synthesis.

The complete nucleotide sequence of the B/Oregon/5/80 HA gene is shown in Fig. 2, as well as the deduced amino acid sequence. The entire gene consists of 1,878 nucleotides, with an open reading frame beginning at an AUG at position 34 and ending at a UAA codon at position 1786. The coding strand thus contains a 5′ untranslated region of 33 nucleotides and a 3′ untranslated region of 96 nucleotides. The open reading frame has the coding capacity for a precursor polypeptide of 583 amino acids. As previously reported for the B/Lee/40 and B/Singapore/222/79 HAs (21, 41), the B/Oregon/5/80 HA precursor consists of an N-terminal 15-amino-acid signal peptide, followed by an HA1 region with a maximum of 345 amino acids and an HA2 region of 223 amino acid residues. The cleavage points for the signal peptide and the HA1 and HA2 junction are inferred from N-terminal amino acid sequence data obtained from the B/Lee/40 HA1 and HA2 polypeptides (43). In the absence of carboxyl-terminal amino acid sequence data, the possibility of further processing at the carboxyl terminus of HA1 cannot be ruled out. The nucleotide sequence is very similar to the previously reported influenza B virus HA sequences (21, 41) and differs at only nine nucleotide positions from the B/Singapore/222/79 HA gene (Fig. 2). These nucleotide substitutions relative to the B/Singapore HA gene give rise to only two amino acid substitutions, suggesting that the B/Oregon HA perhaps evolved directly from the B/Singapore HA or at least from a very recent common ancestor.

TABLE 1. Altered reactivity of B/Oregon/5/80 antigenic variants selected with monoclonal antibodies to the HA[a]

Monoclonal antibody	Reactivity of antigenic variant:[b]					
	21/6 V1	114/4 V2	680/1 V1	710/1 V1	152/2 V1	206/2 V2
21/6	−	+	+	+	+	+
114/4	+	−	+	+	+	+
680/1	+	+	−	−	+	+
710/1	+	+	−	−	+	+
152/2	+	+	+	+	−	−
206/2	+	+	−	−	+	−

[a] Reactivity patterns were determined in HI assays.
[b] −, HI titer of monoclonal antibody against variant was at least 16-fold less than titer against parental B/Oregon/5/80 virus; +, HI titer against variant was identical to titer against parental B/Oregon/5/80 virus.

Identification of amino acid changes in HA1 of B/Oregon/5/80 variants. To precisely define the antigenic structure of the influenza B virus HA, we took the approach developed previously for the influenza A virus HA (8, 13, 45). We used a panel of monoclonal antibodies specific for the HAs of B/HK/8/73 and B/Oregon/5/80 (24, 44) in a one-step neutralization procedure to select a corresponding panel of antigenic variants of B/Oregon/5/80 (12). The variants exhibit antigenic alterations in the HA which prevent the binding of the antibody used in their selection as well as other antibodies in the panel. Examples of the antigenic changes seen in several of the variants, as demonstrated by HI assays, are shown in Table 1.

To identify the amino acid changes responsible for the observed antigenic alterations, we sequenced the HA1 coding regions of the HA genes of the antigenic variants for comparison with the parental B/Oregon HA gene sequence. Nucleotide sequence changes were detected by parallel analysis of dideoxy sequencing reaction products (Fig. 3). In this way, 16 different single nucleotide changes were detected, and the amino acid substitutions in a total of 18 variants were identified. Table 2 lists the antigenic variants, their nucleotide sequence changes, and their deduced amino acid changes relative to the B/Oregon/5/80 HA and also to the

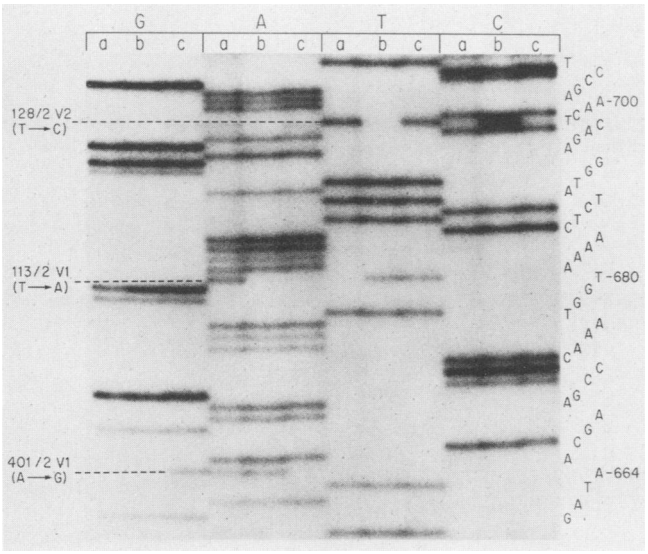

FIG. 3. Detection of nucleotide substitutions in the HA gene of antigenic variants of B/Oregon/5/80. Virion RNAs from antigenic variants 113/2 V1 (lanes a), 128/2 V2 (lanes b), and 401/2 V1 (lanes c) were sequenced by the dideoxy method, using the BP4 primer (Fig. 2). The sequence-specific reaction products (indicated by G, A, T, or C above the lanes) were analyzed in parallel on an 8% sequencing gel, and the nucleotide changes were located as indicated in the autoradiogram.

A/Aichi/2/68 HA of influenza A virus, using the alignment of Krystal et al. (21). The alignment of the B/Oregon/5/80 and A/Aichi/2/68 HA1 polypeptides and the positions of the amino acid changes in the variants are shown in Fig. 4. For the purpose of comparison and for ease of reference to the stucture of the HA of influenza A virus, we will refer to the amino acid positions relative to the H3 HA. In all of the variants, single-point mutations were responsible for the amino acid substitutions, and no double mutants were detected. This is in contrast to previous analyses of antigenic variants of influenza A virus (8, 28) and a recent analysis of antigenic variants of influenza B virus B/HK/8/73 (D. L. Hovanec and G. M. Air, submitted for publication) in which a few variants with multiple mutations, including insertions, were identified. It remains possible, yet unlikely, that additional mutations were not detected due to the sequencing artifacts described above at positions 429 and 528. It also seems unlikely that antigenically relevant amino acid substitutions went undetected in the HA2 region of the variant molecules in view of the relative invariability of this polypeptide in natural and in vitro-selected variants of influenza A and natural variants of influenza B (14, 22, 23, 29, 40, 47).

Most of the amino acid substitutions were nonconservative, resulting in a change in charge, an altered hydrophilicity, or a change in the size of the side chain, such as the Lys to Ile change at position 160A or the Gln to Lys change at position 190. Two pairs of variants (21/6 V1 and 21/6 V2; 680/1 V1 and 710/1 V1) had identical amino acid changes and, as expected, were serologically indistinguishable. In several variants, however, different amino acid substitutions occurred at the same position. For example, the Val at position 192 changed to an Ala in 152/2 V1 and to a Leu in 206/2 V2. Despite the conservative nature of these amino acid changes, these two variants were readily distinguished by the monoclonal antibodies (Table 1).

Location of antigenic sites on influenza B HA. To relate the antigenic sites on the influenza B HA to the three-dimensional structure, we have adopted the A/Aichi/2/68 HA (H3) numbering system for the B/Oregon/5/80 HA amino acid sequence, according to the amino acid sequence alignment of Krystal et al. (21) (Fig. 4). The positions of the amino acid substitutions in the variants are shown in Table 2 relative to the H3 HA. When located on the three-dimensional structure, these changes fall into two to three areas on the surface of the globular head of the HA monomer (Fig. 5). The Asn to Ser substitution at residue 145 in the 21/6 variants results in the creation of a potential carbohydrate attachment site at position 143 directly adjacent to a pre-existent site at position 141. These changes identify regions of the influenza B HA which correspond to antigenic site A on the H3 HA and site Ca2 on the H1 HA (8, 47). The changes at residue 160A (occurring in an insertion of four amino acids relative to the H3 HA) fall into a region corresponding to site B on the H3 HA and site Sa on the H1 HA. Of the 18 variants selected by our panel of monoclonal antibodies, 11 have amino acid substitutions in residues 187 to 196B also corresponding to antigenic site B in the H3 HA and site Sb in the H1 HA. The amino acid substitutions at position 225 occur in a loop at the front of the proposed receptor binding pocket which is equidistant from the loop constituting site A and from the bottom of the α-helix constituting part of site B. It is noteworthy that monoclonal antibody 74/1 selected variants with changes at residue 225 or 160A which are ca. 2.7 nm apart in the predicted three-dimensional structure. It is unclear whether the changes at position 225 identify another antigenic region which overlaps that corresponding to site B or actually fall within site B and reflect differences in the three-dimensional structures of the influenza A and B virus HAs.

Although we have identified two antigenic sites on the influenza B HA which may correspond to sites A and B on the influenza A H3 HA, no substitutions were found in regions corresponding to antigenic sites C, D, or E. Several monoclonal antibodies, however, bind to high titer to all of the variants we have selected, suggesting that the present

TABLE 2. Nucleotide and amino acid sequence changes in the HA1 polypeptide of B/Oregon/5/80 antigenic variants

Antigenic variant	Codon change	Amino acid change	Position in H3 HA
21/6 V1	AAC to AGC (527)[a]	Asn to Ser (150)[b]	145
21/6 V2	AAC to AGC (527)	Asn to Ser (150)	145
74/1 V2	CCA to ACA (796)	Pro to Thr (240)	225
114/4 V2	CCA to CAA (797)	Pro to Gln (240)	225
680/1 V1	AAA to ATA (575)	Lys to Ile (166)	160A
710/1 V1	AAA to ATA (575)	Lys to Ile (166)	160A
74/1 V1	AAA to AAC (576)	Lys to Asn (166)	160A
401/2 V1	AAC to GAC (664)	Asn to Asp (196)	187
146/1 V2	AAC to AAA (666)	Asn to Lys (196)	187
280/2 V1	GAA to AAA (667)	Glu to Lys (197)	188
21/2 V1	CAA to AAA (673)	Gln to Lys (199)	190
206/2 V2	GTA to TTA (679)	Val to Leu (201)	192
113/2 V1	GTA to GAA (680)	Val to Glu (201)	192
152/2 V1	GTA to GCA (680)	Val to Ala (201)	192
238/4 V2	AAA to ACA (683)	Lys to Thr (202)	193
10/4 V2	AAA to AAC (684)	Lys to Asn (202)	193
128/2 V2	TCA to CCA (697)	Ser to Pro (207)	196B
391/1 V2	TCA to TTA (698)	Ser to Leu (207)	196B

[a] Value in parentheses indicates position of nucleotide substitution in B/Oregon HA gene as numbered in Fig. 2.

[b] Value in parentheses indicates position of amino acid substitution in B/Oregon HA1 polypeptide.

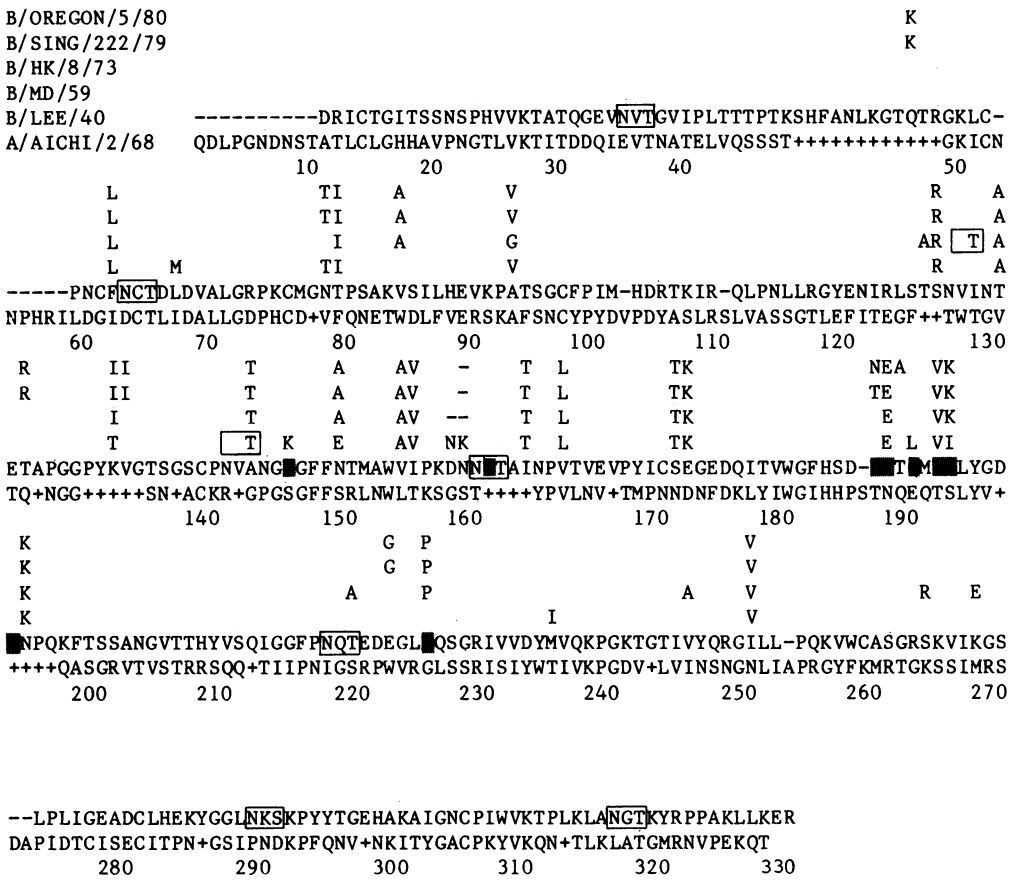

FIG. 4. Comparison of HA1 polypeptides of influenza B viruses and location of the amino acid substitutions in laboratory-selected antigenic variants. The predicted amino acid sequences of the B/Lee/40 and A/Aichi/2/68 HA1 polypeptides are shown numbered according to the A/Aichi sequence for ease of reference to the structure of the H3 HA of influenza A and aligned to maximize amino acid sequence homology (21). Amino acid sequence differences among B/Lee/40, B/MD/59, B/HK/8/73, B/Singapore/222/79, and B/Oregon/5/80 are shown directly above the B/Lee/40 sequence, and the position of the amino acid substitutions detected in the antigenic variants of B/Oregon/5/80 are shaded. Potential carbohydrate attachment sites are enclosed in boxes. Deletions in the B/Lee HA sequence relative to the A/Aichi sequence are indicated by dashes, and deletions in the A/Aichi sequence relative to the B/Lee sequence are indicated by plus signs.

panel of variants lacks changes in at least one antigenic region recognized by the panel of monoclonal antibodies. Interestingly, we have not been able to select antigenic variants with these monoclonal antibodies, suggesting that mutations in this region(s) of the molecule are lethal or occur at a very low frequency.

DISCUSSION

Although great advances have been made in understanding the molecular basis of antigenic variation in the influenza A viruses in the last several years, a comparable understanding of this phenomenon in the influenza B viruses has only recently begun to emerge. The nucleotide sequences of several influenza B virus HA genes have now been established, and comparison of the deduced amino acid sequences has revealed nonconservative amino acid substitutions in five regions of the influenza B virus HA corresponding to the proposed antigenic sites on the H1 and H3 HAs of influenza A virus (21, 41). There is no doubt that certain of these amino acid substitutions are responsible for the observed antigenic differences among these viruses, but it is impossible to know precisely which changes contributed to the antigenic evolution of the influenza B virus HA and thus to define the exact nature and number of antigenic sites on this molecule. In the present study, determination of the complete nucleotide sequence of the HA gene of influenza B virus B/Oregon/5/80 and the nucleotide sequence changes in the HA genes of laboratory-selected antigenic variants of this virus have allowed the location of antigenically relevant amino acid substitutions in the HA and identified at least two possible immunodominant antigenic regions.

Sequence analysis of the antigenic variants has demonstrated that single amino acid substitutions in the HA1 polypeptide are capable of altering the antigenicity of the influenza B virus HA, thus suggesting very similar mechanisms for antigenic drift in the influenza A and B viruses. The amino acid substitutions detected in the antigenic variants also identify regions of the HA molecule which appear from amino acid sequence alignment to correspond to antigenic sites A and B on the H3 HA of influenza A virus, with the majority of the changes falling into site B. In addition, two variants (74/1 V2 and 114/4 V2) had substitutions corresponding to position 225 in the H3 HA, which appears to be situated almost equidistantly between sites A and B and very close to site D (47). Antigenic mapping by Caton et

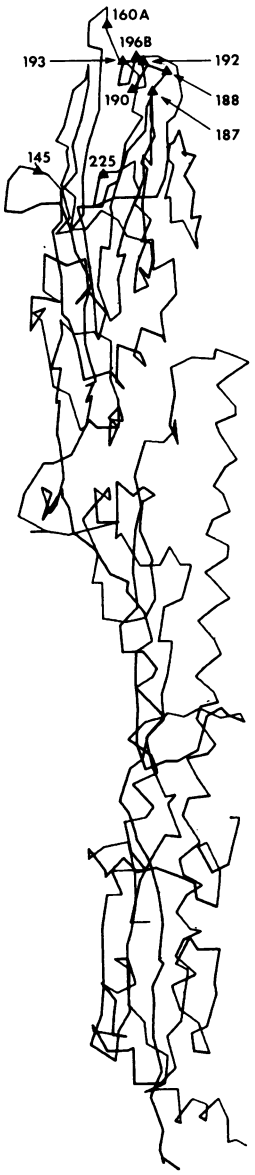

FIG. 5. Location of amino acid substitutions in antigenic variants of B/Oregon/5/80 relative to the three-dimensional structure of influenza A H3 HA. The positions of the amino acid substitutions are marked on the α-carbon tracing of the A/Aichi/2/68 HA monomer (48). Numbering is according to the alignment in Fig. 4.

al. (8) suggested that residues 225 and 145 were involved in forming a single antigenic site (Ca2) on the Hl HA; however, recent evidence demonstrates that substitutions at residue 226 can affect the binding of antibody to site B and site D on the H3 HA molecule (R. G. Webster, unpublished data). The fact that monoclonal antibody 74/1 selected one variant of B/Oregon/5/80 with a substitution corresponding to residue 225 and one with a substitution corresponding to residue 160A suggests that these residues are closer together in the type B HA than the 2.7 nm predicted for the H3 HA and that both contribute to the structure of a single site which may correspond to site B of the H3 HA. It is likely that the amino acid insertions in the influenza B virus HA in this region alter the structure of this molecule relative to the influenza A virus HA and possibly bring these two residues closer together (21). Alternatively, monoclonal antibody 74/1 may possess an unusually large antibody-combining site which recognizes a region spanning both of these residues. In addition, one cannot rule out the possibility that this monoclonal antibody recognizes a site spanning a monomer-monomer boundary which might be sensitive to alterations at position 225, which is very close to the subunit contact region.

Seven amino acid positions observed to change in the variant HAs also appeared to vary among the natural drift strains so far analyzed (Fig. 4). For example, the residue at position 192 appeared to change from Glu to Val from B/Lee/40 to B/MD/59. The Val was conserved through to B/Oregon/5/80 but changed to Glu in a variant selected with monoclonal antibody 113/2 (Table 2). This correlation between variation in the laboratory-selected variants and the naturally occurring drift strains would indicate that these seven amino acid positions are critically involved in the antigenicity of the influenza B HA and may be actual contact residues for antibody binding. No substitutions were detected in this panel of variants in regions of the HA1 polypeptide corresponding to sites C, D, or E, despite the fact that a few differences are seen in these regions when the deduced amino acid sequences of the HAs of the drift strains are compared (Fig. 4) (21, 41). It is possible that the HA specificities defined by our panel of monoclones are not representative of the total repertoire but, rather, consist of a subset restricted to only two regions of the HA molecule. Indeed, Staudt and Gerhard (39) have reported that individual adult BALB/c mice may express distinct antibody repertoires to the H1 HA. On the other hand, it may be that the differences in regions corresponding to sites C, D, and E observed among the HAs of the drift strains are antigenically irrelevant and do not reflect changes in antigenic sites.

It is clear that sequence changes have occurred over a 40-year period in the influenza B HA1 polypeptide and that single amino acid substitutions are capable of altering the antigenic structure of the influenza B virus HA. Thus, antigenic drift probably occurs in influenza B as in influenza A by accumulation of point mutations and selection of antigenically altered viruses by the immune host, although the apparent lower frequency remains unexplained. It is interesting to note that the influenza B virus HA has potential carbohydrate attachment sites in roughly all four regions corresponding to sites A to D on the influenza A virus HA. Since masking of antigenic sites by carbohydrate has been demonstrated as a means of modulating antigenic variation in the influenza A viruses (36), perhaps this mechanism contributes to the lower frequency of variation in the influenza B virus HA. In fact, Krystal et al. (21) recently pointed out that the B/HK/8/73 HA1 acquired two new carbohydrate attachment sites at positions 126 and 141 and lost one at position 217 relative to the B/Lee/40 HA, and in this study, the 21/6 variants of B/Oregon/5/80 gained a potential carbohydrate attachment site at position 143, suggesting a role for carbohydrate in covering and uncovering antigenic sites.

In summary, we have determined the complete nucleotide sequence of the HA gene of influenza B virus B/Oregon/5/80. We have also sequenced the HA1-coding regions of the HA genes of laboratory-selected antigenic variants of this virus and identified single amino acid substitutions which alter the antigenic structure of the influenza B virus HA molecule. These residues are located in regions which in general correspond to antigenic sites A and B of the H3 HA of influenza A virus and thus identify two possible correspond-

ing immunodominant antigenic sites on the influenza B virus HA molecule. Further definition of these antigenic sites through operational antigenic mapping (13) and competitive binding analysis with a large panel of monoclonal antibodies should provide a clearer picture of the antigenic structure of the influenza B virus HA and allow closer comparison with the HA of influenza A virus.

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