Virology 259, 135–140 (1999)
Article ID viro.1999.9719, available online at http://www.idealibrary.com on IDEAL®

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# High-Yield Reassortant Influenza Vaccine Production Virus Has a Mutation at an HLA-A 2.1-Restricted CD8$^+$ CTL Epitope on the NS1 Protein

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*Received December 22, 1998; returned to author for revision February 15, 1999; accepted March 16, 1999*

Current influenza virus vaccines are prepared using high-yield reassortant virus strains obtained from a mixed infection of the new virus strain and a prototype high-yielding virus strain. The high-titered reassortant virus strain used as vaccine seed virus possesses the recent virus HA and NA and contains the internal genes from the high-growing prototype parent. We established a human CD8$^+$ cytotoxic T cell (CTL) line, 10-2C2, which recognizes an HLA-A2.1-restricted influenza A virus H1, H2, H3 cross-reactive T cell epitope on amino acids 122–130 of the NS1 protein, and unexpectedly we observed that there was decreased lysis of target cells infected with the A/Texas/36/91 (H1N1) vaccine virus strain compared to the lysis of target cells infected with the prototype A/PR/8/34 (H1N1) virus. RT-PCR results showed that the A/Texas vaccine virus strain contained a quasispecies. Approximately 50% of viral RNA of the NS1 gene had a nucleotide substitution that resulted in the N → K amino acid change at the sixth position of the nonamer peptide. Current influenza vaccines are inactivated and do not contain the NS1 protein; however, future influenza vaccines may include live attenuated vaccines and with this mutation a live virus would fail to induce a CD8$^+$ CTL response to this epitope in individuals with HLA-A2.1, a very common allele, and potentially have reduced efficacy. © 1999 Academic Press

## INTRODUCTION

Current influenza vaccines are prepared by inoculation of virus into embryonated hens' eggs, harvesting of fluids, and purification of concentrated virus suspensions, which are then formalin-inactivated and detergent-disrupted. To obtain a seed virus to produce higher virus yields in vaccine production, a reassortant virus that is prepared from a mixed infection of the new virus strain and a prototype high-yielding virus strain may be used. Growth in eggs at high dilution is sought and the selection for progeny virus with the external glycoproteins, HA and NA, of the recent virus strain is performed with antibodies to neutralize progeny virus with prototype HA and NA. The high-yielding reassortant virus strain possesses the recent virus HA and NA and contains the internal genes from the high-growing prototype parent as a result of reassortment of segmented RNA strands during mixed infection. This procedure has been commonly employed since 1968 when it was found to be useful in preparing vaccines to meet the epidemic threat of the Hong Kong (H3N2) influenza A virus (Kilbourne, 1969, 1994). It has become a routine practice helpful for improving virus yields, which are a major problem in the production of inactivated influenza vaccines.

As part of our studies of the human cytotoxic T cell (CTL) responses to influenza A viruses, we established several human CD8$^+$ and CD4$^+$ influenza A-specific CTL lines. We used these CTL lines to find human T cell epitopes including an epitope in the NS1 protein on amino acids (aa) 122–130, recently reported by others (Man *et al.,* 1995). We used this clone as a positive control in the course of performing CTL assays with PBMC of vaccine recipients in a clinical study comparing standard and experimental influenza vaccines designed to be more immunogenic. We wanted to use the same influenza A virus strains in the CTL assays as those that were included in the vaccines in the clinical study to measure the CTL responses to the virus antigens in the vaccine. In the course of preliminary studies using the CD8$^+$ CTL clone that recognizes aa 122–130 on the influenza virus NS1 protein, which is cross-reactive to H1, H2, H3 prototype viruses, we were surprised to observe that there was decreased lysis of target cells infected with the A/Texas/36/91 (H1N1) vaccine virus strain compared to the lysis of target cells infected with the prototype A/PR/8/34 (H1N1) or with the A/Johannesburg/33/94 (H3N2) vaccine virus strains. In repeated experiments lysis of the A/Texas vaccine virus-infected targets was absent or detected at a much lower level than that of these other target cells. We decided to determine whether a mutation had occurred in this epitope on the NS1 protein of this virus, because we expected the

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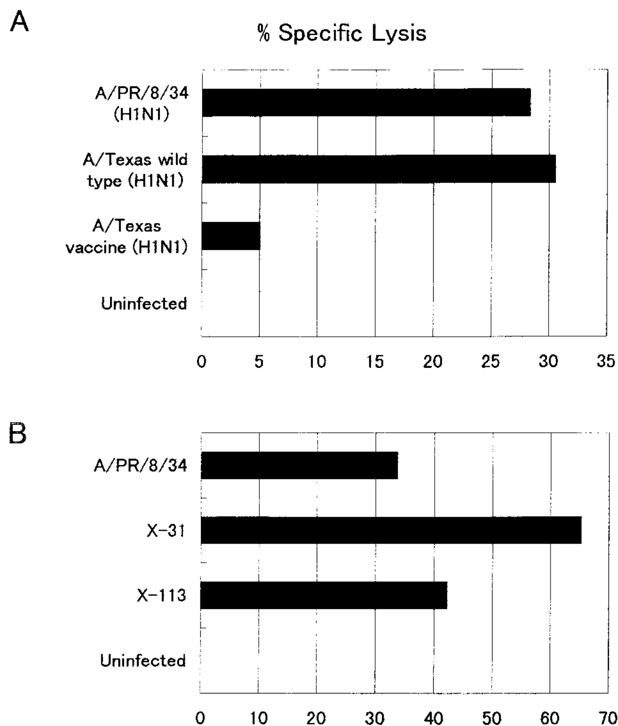



FIG. 1. Percentage specific lysis of target cells, the autologous BLCLs, infected with influenza A viruses by CD8$^+$ T cells clone 10-2C2. The X-113 virus is a reassortant of the A/Texas wild-type strain and the X-31. The A/Texas vaccine strain was derived from the X-113.

sequence to be recognized regardless of whether the NS1 gene in the vaccine virus was derived from either the high-growing parent (A/PR/8/34 (H1N1)) that this CTL clone recognizes or the recently isolated virus that was used to develop a reassortant high-yielding virus that was used to prepare the vaccine for clinical study.

## RESULTS

### Cytotoxic T cell assays using an HLA-A2.1-restricted CD8$^+$ T cell clone 10-2C2 and influenza A viruses

The CD8$^+$ T cell clone 10-2C2 is HLA-A2.1-restricted, recognizes a nonamer peptide on the influenza virus A/PR/8/34 (H1N1) NS1 protein, $^{122}$AIMDKNIIL$^{130}$, and is cross-reactive for prototype strains of the human H1 and H3 subtypes (Jameson *et al.,* 1998; Man *et al.,* 1995). When we examined the cross-reactivity of clone 10-2C2 against recent strains of influenza virus, we found that 10-2C2 could recognize A/Johannesburg/33/94 (H3N2), but not the A/Texas/36/91 (H1N1) vaccine virus strain, although A/Texas belonged to the same subtype as A/PR/8/34 (data not shown). Surprisingly, clone 10-2C2 could recognize the A/Texas/36/91 (H1N1) wild-type strain (Fig. 1A).

The A/Texas vaccine strain was derived from X-113, a high-growing reassortant vaccine seed virus derived from the A/Texas wild-type virus strain (H1N1) and the X-31 (H3N2) virus. X-113 contained genes encoding the surface glycoproteins, H1N1, of the A/Texas parent (E. D. Kilbourne, personal communication) and internal genes of X-31 known to be derived from A/PR/8/34 (H1N1). When we infected target cells with the X-113 or X-31 virus strains, the targets were killed by 10-2C2 (Fig. 1B). To know whether the T cell epitope had changed in the production of the A/Texas vaccine virus strain, viral RNA was isolated from allantoic fluid.

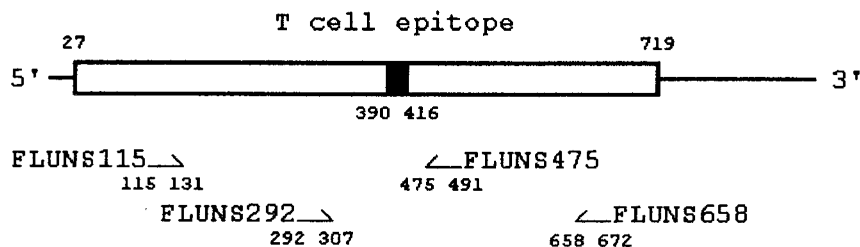
### Nucleotide and amino acid sequence of A/Texas wild-type and vaccine strains

cDNA fragments of the NS1 gene were amplified by RT-PCR and cloned into the pCR2.1 vector. To rule out the possibility that the mutation was introduced simply by nucleotide misincorporation during PCR, two different set of primers were used and more than two plasmid clones were sequenced (Fig. 2). We performed two different PCRs with the A/Texas vaccine strain. Every PCR produced a clear single band of the expected size on 2% agarose gel.

The nucleotide sequences between nucleotides 308 and 474, which code for amino acids 95 to 149, were determined. The nucleotide sequences of the A/Texas wild-type strain fell into two groups, a GTG group and an ATG group (Table 1 and Fig. 2). There were two nucleotide changes between the two groups and both changes resulted in amino acid changes, which were not in the T cell epitope. The deduced amino acid sequences of both groups in the HLA-A2.1-restricted T cell epitope were the same as that of A/PR/8/34 (J02150), AIMDKNIIL. However, the overall homology of both of these groups to A/PR/8/34 was 91% in the nucleotide sequence and 89% in the amino acid sequence. BLAST search data showed that the A/Texas wild-type strain had the highest homology to the sequence of a more recent H1N1 virus, A/Chile/1/83(H1N1) (X15282), in the nucleotide sequence (the GTG group had 97% and the ATG group had 98%).

The nucleotide sequences of the A/Texas vaccine strain also fell into two groups, an AAC group and an AAA group (Table 1 and Fig. 2). Ten of 19 clones from two separate PCR experiments using two different primer sets belonged to the AAA group. Two nucleotide differences were found between the two groups, one of which resulted in an amino acid change within the T cell epitope. In the AAC group the T cell epitope was AIMDKNIIL, which is the same as that of A/PR/8/34, while in the AAA group the T cell epitope was AIMDKKIIL. The other nucleotide difference also resulted in an amino acid change, but it was at aa 101, which is not in the T cell epitope. Both groups were more similar to A/PR/8/34 (the AAC group had 98% and the AAA group had 97% homology in nucleotide sequences) than to the A/Texas wild-type strain (less than 93% homology in nucleotide sequences), probably as a result of the recombination with

# NS1 cDNA and PCR primers




**nucleotide sequences**

```
                          308                                                        367
A/PR/8/34               : TCTTGAGGAAATGTCAAGGGAATGGTCCATGCTCATACCCAAGCAGAAAGTGGCAGGCCC
vaccine, AAA group      : ...................A.C......................................
vaccine, AAC group      : .....................C......................................
wild type, GTG group    : .A....A..G................T.........G.......................
wild type, ATG group    : .A....A..G...........C....T.........G.......................

                          368                                                        427
                        : TCTTTGTATCAGAATGGACCAGGCGATCATGGATAAAAACATCATACTGAAAGCGAACTT
                        : ....................................G..A....................
                        : ....................................G.......................
                        : .......G.....G.............A........G....................T..
                        : .......G...................A........G....................T..

                          428                                           474
                        : CAGTGTGATTTTTGACCGGCTGGAGACTCTAATATTGCTAAGGGCTT
                        : ...............................................
                        : ...............................................
                        : ...................T..........G.C...A..........
                        : ...................T..........G.C...A..........
```

**amino acid sequences**

```
                          95                                                    149
A/PR/8/34               : LEEMSREWSMLIPKQKVAGPLCIRMDQAIMDKNIILKANFSVIFDRLETLILLRA
vaccine, AAA group      : ......N.........................K......................
vaccine, AAC group      : ......D................................................
wild type, GTG group    : I.....E.F..M..........V.V.........................T....
wild type, ATG group    : I.....D.F..M..........V...........................T....
```

FIG. 2. (Top) The localization of HLA-A2.1-restricted T cell epitope and the PCR primers in the NS-1 gene cDNA. (Bottom) Determined nucleotide sequences and deduced amino acid sequences around the T cell epitope (GenBank Accession Nos. AF137362 to AF137365). Nucleotides and amino acids that are the same as the A/PR/8/34 are represented as dots.

X-31, which contained internal genes of A/PR/8/34 (Kilbourne, 1969).

### Cytotoxic T cell assays using synthetic peptide

To confirm that the N → K amino acid change was the reason the clone did not recognize the A/Texas vaccine virus strain, we performed CTL assays using a synthetic peptide based on the sequence of the A/Texas egg-passaged vaccine virus strain. It had been reported that the nonamer peptide on the A/Alaska/6/77 (H3N2) virus strain, AIMEKNIML, was not recognized by CTLs that were able to recognize the nonamer peptide, AIMDKNIIL, on the A/PR/8/34 (Man *et al.,* 1995), so we also tested the synthetic peptides that had the D → E amino acid change and the I → M amino acid change. As expected, 10-2C2 could not recognize AIMDKKIIL, while AIMEKNIML and AIMDKNIML were recognized (see Fig. 3).

## DISCUSSION

We found that the A/Texas vaccine virus strain contained a quasispecies. Nucleotide and amino acid sequences of the NS1 gene were divided into two groups

TABLE 1

Summary of RT-PCR and Sequencing

| | Amino acid sequence in T cell epitope | Total | Primer set used | | | |
|---|---|---|---|---|---|---|
| | | | FLUNS115 and 475 | | FLUNS292 and 658 | |
| | | | Expt. 1 | Expt. 2 | Expt. 1 | Expt. 2 |
| Vaccine, AAA group | AIMDKKIIL | 10 | 3 | 2 | 3 | 2 |
| Vaccine, AAC group | AIMDKNIIL | 9 | 0 | 3 | 3 | 3 |
| Wild-type, GTG group | AIMDKNIIL | 2 | 1 | | 1 | |
| Wild-type, ATG group | AIMDKNIIL | 5 | 1 | | 4 | |

and one had changes in its amino acid sequence in the HLA-A2.1-restricted T cell epitope. The sixth position of the nonamer was changed from asparagine (N), a neutral and polar amino acid, to lysine (K), a basic amino acid. This N → K amino acid change resulted in loss of recognition by the $CD8^+$ T cell clone 10-2C2. When asparagine at the sixth position was replaced by aspartic acid (D), an acidic amino acid, this nonamer was not recognized by 10-2C2 either (Jameson *et al.,* unpublished data). Changes from neutral to charged amino acids at the sixth position may alter the interaction between the nonamer peptide and the HLA-A2.1 molecule or the interaction between the HLA-A2.1 molecule–nonamer peptide complex and the T cell receptor. RT-PCR results showed that approximately 50% of viral RNA of the NS1 gene had a nucleotide substitution, which resulted in the N → K amino acid change. If the RT-PCR reflected actual percentages, 50% of virus particles had the T cell epitope, and target cells were not lysed as well. In some assays with high killing of peptide pulsed or virus-infected target cells there was a lower level killing of the A/Texas vaccine virus infected target cells. The changed T cell epitope may work as an antagonist (Klenerman *et al.,* 1994; Bertoletti *et al.,* 1994).

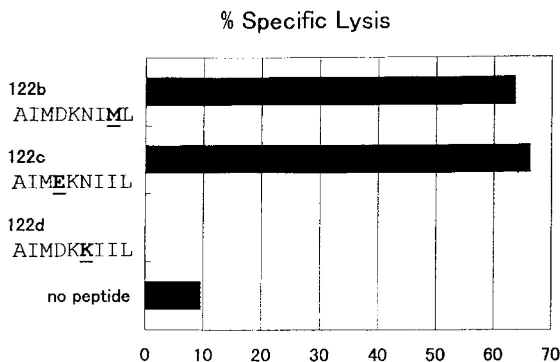



FIG. 3. Percentage specific lysis of target cells, the autologous BLCLs, pulsed with synthetic NS1 peptides (the concentrations of each oligopeptides were 25 $\mu$g/ml) by $CD8^+$ T cell clone 10-2C2. The peptides used are as follows: 122b, AIMDKNIML; 122c, AIMEKNIIL; 122d, AIMDKKIIL [A/Texas vaccine]; Underlined amino acids are different from A/PR/8/34.

Homology search results showed that the NS1 gene of the A/Texas vaccine strain was more similar to the NS1 gene of the A/PR/8/34 than to the recently isolated A/Texas wild-type strain, suggesting that the NS1 gene of the A/Texas vaccine strain was derived from the X-31 parent, not from the wild-type strain. During serial egg passage of the vaccine strain using the X-113 virus, an N → K amino acid change occurred.

Most of the T cell epitopes identified in influenza A virus are on internal proteins (McMichael, 1994; Parker and Gould, 1996; Jameson *et al.,* 1998), which are more conserved among strains than are the surface proteins. Because of this conservation of internal proteins, it seems reasonable to use a high-yielding reassortant virus selected based on its surface proteins, hemagglutinin and neuraminidase, as a vaccine strain (Kilbourne, 1969, 1994). However, in this case a region of the NS1 gene that encodes a T cell epitope underwent a mutation that caused an amino acid substitution, and a HLA-A2.1-restricted $CD8^+$ T cell clone was not able to recognize target cells infected with the vaccine virus.

It is interesting to note that we recently tested this same $CD8^+$ T cell clone against a human influenza A isolate, Hong Kong/156/97 (H5N1). This clone failed to recognize autologous cells infected with the Hong Kong virus (Jameson *et al.,* unpublished data). This virus, isolated from a human with influenza illness, was derived from birds and has an amino acid substitution at the same position in the NS1 HLA-A-2.1-restricted CTL epitope, N → D at amino acid 127 (AF036360). In addition, a recent report documented the same N → K amino acid substitution that we found in the vaccine virus (A/CK/Pennsylvania/1370/83 (U96739) and A/CK/Pennsylvania/21525/83 (U96741), Suarez and Perdue, 1998).

There is no obvious impact of this mutation on the effectiveness of current influenza vaccines. These vaccines contain mixtures of detergent-disrupted, formalin-killed virus that are standardized for hemagglutinin content and are designed to induce and boost protective antibodies to the surface H1N1 glycoproteins. The NS1 protein is not present in the virus particle, and the NS1 gene in the killed virus cannot produce the NS1 protein.

It is useful, however, to learn more about mutations in vaccine viruses because future vaccines, such as live attenuated or inactivated vaccines designed to stimulate $CD8^+$ CTL responses, may utilize such cross-reactive $CD8^+$ CTL epitopes for improved vaccine efficiency. Mutations may occur at $CD8^+$ CTL epitopes on other influenza A proteins. These mutations may offer advantages to the virus for growth in other species or in cell cultures but they would reduce the ability of humans to generate $CD8^+$ CTL responses to the vaccine.

## MATERIALS AND METHODS

### Virus strains and growth

Influenza virus A/PR/8/34 (H1N1), X-31, and A/Johannesburg/33/94 (H3N2) and three strains of A/Texas/36/91 (H1N1) were used in this study. X-31 is a high-yielding reassortant of A/Aichi/2/68 (H3N2) and A/PR/8/34 (H1N1), which has the HA and NA of the H3N2 virus and the internal genes of the A/PR/8 (Kilbourne, 1969). The strains of A/Texas are (1) A/Texas/36/91 (H1N1), wild-type strain; (2) X-113, a high-yielding reassortant of A/Texas and X-31, which has the H1N1 subtype; and (3) A/Texas reassortant vaccine seed virus strain, which was derived from X-113. X-31 and X-113 were kindly supplied by E. D. Kilbourne. The A/PR/8 virus was provided by the Bureau of Biologics FDA, the A/Texas/36/91 (H1N1) virus was kindly supplied by N. Cox of the WHO Influenza Research Center, and the A/Texas vaccine egg-passaged strain was kindly supplied by David Burt of Pasteur Merieux Connaught, Toronto, Canada. Influenza A viruses were propagated in 10-day-old, embryonated chicken eggs. Infected allantoic fluids were harvested 2 days after infection, aliquoted, and stored at $-80°C$ until use.

### Preparation of target cells

The autologous B lymphoblastoid cell line (BLCL) used in this study was established by culturing peripheral blood mononuclear cells with Epstein–Barr virus in 24-well plates as previously described (Green *et al.,* 1993). The HLA alleles of the donor are A2.1, A11, B18, B27, Cw1, Cw7, DR1, DQw1, DQw3, DRw52, and DRw53 (Jameson *et al.,* 1998). BLCL were infected with influenza viruses at an m.o.i. of 15:1 in 1 ml of medium for 12 to 16 h. The infected target cells were labeled with 0.25 mCi of $^{51}Cr$ for 60 min at 37°C. After four washes, the target cells were counted and diluted to $2 \times 10^4$/ml for use in the cytotoxicity assay.

### Cytotoxic T cell assays

CTL assays were performed with 96-well round-bottom plates as previously reported (Bukowski *et al.,* 1989). Briefly, effector cells in 100 $\mu l$ of RPMI 1640 medium containing 10% FBS were added to $2 \times 10^3$ $^{51}Cr$-labeled target cells in 100 $\mu l$ at an effector-to-target (E:T) ratio of 10:1. In cytotoxicity assays using synthetic peptides, peptides were added to target cells at the indicated concentrations and incubated at 37°C for 30 min, after which the effector cells were added. Plates were centrifuged at 200*g* for 5 min and incubated for 4 to 5 h at 37°C. Supernatant fluids were harvested using a supernatant collection system (Skatron Instruments, Sterling, VA), and $^{51}Cr$ content was measured in a gamma counter. Percentage specific $^{51}Cr$ release was calculated with the following formula: (cpm experimental release — cpm spontaneous release)/(cpm maximum release — cpm spontaneous release) $\times$ 100. All assays were performed in triplicate, and the results were calculated from the average of the triplicate wells.

### RNA isolation

RNA was extracted from 600 $\mu l$ of allantoic fluid by the Ultraspec RNA isolation system (Biotecx Laboratories, Inc., Houston, TX) following the manufacturer's recommendations. Extracted RNA was dissolved in 100 $\mu l$ of DEPC-treated water.

### Reverse transcription and PCR

Ten microliters of the extracted RNA was added to 90 $\mu l$ of reverse transcription solution. The final concentrations of each component were as follows: 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM of each dNTP, 1 u/$\mu l$ RNAsin ribonuclease inhibitor (Promega, Madison, WI), 0.001 $A_{260}$u/$\mu l$ pd$(N)_6$ Random Hexamer (Pharmacia Biotech, Uppsala, Sweden), and 0.3 u/$\mu l$ AMV reverse transcriptase (Promega). Reverse transcription was performed at 37°C for 60 min, followed by inactivation at 95°C for 10 min (Terajima *et al.,* 1997). For PCR 1 $\mu l$ of reverse transcription mixture was added to 49 $\mu l$ of PCR solution consisting of 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM of each dNTP, 0.5 pmol/$\mu l$ of each primer, and 1.25 units of Tth DNA Polymerase (Promega). The reaction was carried out at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 48°C for 1 min, and 72°C for 3 min and finally for 1 cycle of 72°C for 8 min 30 s in a DNA Thermal Cycler 480 (Perkin–Elmer, Foster City, CA). The primer pairs used were as follows: FLUNS115 (5′-CCCCATTCCTTGATCGG) and FLUNS475 (5′-TGCTCCCTCTTCGGTGA); FLUNS292 (5′-ACCTAACTGACATGAC) and FLUNS658 (5′-GAGTGAGTGGAGGTC). These primers were designed based on the published nucleotide sequence of NS1 cDNA of the influenza A/Puerto Rico/8/34 (Cambridge) (H1N1) (GenBank Accession No. J02150) by genetic information processing software, GENETYX-WIN (Software Development, Tokyo, Japan). These four regions are conserved in most influenza viruses of the human subtypes H1N1, H2N2, and H3N2. Ten microliters of PCR product was subjected to 2% agarose gel electrophoresis and the gel was stained with ethidium bromide.

## Cloning and sequencing of PCR product

The PCR product was ligated into pCR2.1 vector and the ligated product was used to transform *Escherichia coli* INV$\alpha$F′ by using an Original TA Cloning Kit (Invitrogen, Carlsbad, CA) following the manufacturer's recommendations. Plasmids were prepared by using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA), following the manufacturer's recommendations. Plasmid clones were selected by *Eco*RI (Promega) restriction enzyme digestion and agarose gel electrophoresis (the PCR product was inserted between two *Eco*RI sites in the pCR2.1 vector). The clones whose insert fragments had an appropriate size were sequenced by using a dsDNA Cycle Sequencing System (Life Technologies, Grand Island, N. Y.). The sequencing primers used were T7 Promoter and M13 Reverse primers (Invitrogen).

## Synthetic oligonucleotides and peptides

Synthetic oligonucleotides were purchased from Operon Technologies, Inc. (Alameda, CA). Peptides were synthesized at the Protein Chemistry Core Facility at the University of Massachusetts using an automated Rainin Symphony peptide synthesizer. Peptides were purified by HPLC.

## ACKNOWLEDGMENTS

We thank E. D. Kilbourne for providing reassortant viruses, N. Cox for providing A/Texas/36/91 virus strain, and David Burt for providing several virus strains. This work was partially supported by a research grant from Connaught Laboratories, Toronto, Canada.

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