JOURNAL OF VIROLOGY, Jan. 1992, p. 399–404
0022-538X/92/010399-06$02.00/0

Vol. 66, No. 1

Influenza A Virus Transfectants with Chimeric Hemagglutinins Containing Epitopes from Different Subtypes

SHENGQIANG LI, JEROME L. SCHULMAN, THOMAS MORAN, CONSTANTIN BONA, AND PETER PALESE*

Department of Microbiology, Mt. Sinai School of Medicine, 1 Gustave L. Levy Place, New York, New York 10029

Received 22 July 1991/Accepted 12 October 1991

Influenza virus transfectants with chimeric hemagglutinins were constructed by using a ribonucleoprotein transfection method. Transfectants W(H1)-H2 and W(H1)-H3 contained A/WSN/33(H1N1) (WSN) hemagglutinins in which the six-amino-acid loop (contained in antigenic site B) was replaced by the corresponding structures of influenza viruses A/Japan/57(H2N2) and A/Hong Kong/8/68(H3N2) (HK), respectively. Serological analysis indicated that the W(H1)-H3 transfectant virus reacted with antibodies against both the WSN and HK viruses in hemagglutination inhibition and plaque neutralization assays. Furthermore, mice immunized with W(H1)-H3 transfectant virus produced antibodies to the WSN and HK viruses. The results demonstrate that influenza virus transfectants can be engineered to express epitopes of different subtypes on their hemagglutinins.

Influenza virus hemagglutinin (HA) is a glycoprotein which is essential for viral attachment and entry. It is also the major antigenic component on the surface of the virus, and 14 different HA subtypes have been identified (11, 16). The three-dimensional structure of HA (H3 subtype) has been determined by X-ray crystallography (19), and location of amino acid substitutions in laboratory-derived and natural variants has permitted identification of five B-cell antigenic regions on the HA molecule of subtype H3 viruses (17, 18). Studies of subtype H1 HAs has indicated that the structures and locations of antigenic regions are similar to those of the H3 subtype antigenic regions (3, 7, 8), but the overall amino acid sequence conservation between subtype H1 and H3 HAs is less than 50%.

Expression of foreign epitopes on viral surfaces has been reported for several systems, including that of polioviruses (2), but unlike positive-strand RNA viruses, negative-strand RNA viruses have been refractory to direct genetic manipulation. Only recently have we succeeded in transfecting a biologically active ribonucleoprotein (RNP) complex into helper virus-infected cells, which resulted in rescue of transfectant influenza virus containing cDNA-derived RNA segments (4, 13). Using an improved method, Enami and Palese then demonstrated the rescue of infectious virus particles containing cDNA-derived nonstructural protein, neuraminidase, and HA genes (5).

In the present study, we used this system to introduce site-specific mutations into an antigenic domain of influenza virus HA, which resulted in viruses that express chimeric HAs on their surfaces. Chimeric virus W(H1)-H3 contains an HA which induces antibodies to both H1 and H3 subtype viruses. Use of these methods to construct chimeric viruses may help in developing a new generation of effective virus vaccines.

MATERIALS AND METHODS

Viruses and cells. Influenza viruses A/WSN/33(H1N1) (WSN virus) and HK-WSN(H3N1) were grown in Madin-Darby bovine kidney (MDBK) cells. Influenza viruses A/Japan/57(H2N2) (Japan virus) and A/Hong Kong/8/68(H3N2) (HK virus) were grown in embryonated eggs. HK-WSN(H3N1) virus is a recombinant virus which derived its HA gene from HK virus and its remaining genes from WSN virus (14). MDBK cells were used for RNP transfection and selection of transfectant viruses. For virus neutralization and immune staining assays, Madin-Darby canine kidney (MDCK) cells were used. MDBK cells were used for transfection because they have been found to be much more efficient for these purposes than MDCK cells. However, MDCK cells were used for neutralization assays because they are more permissive for a wider range of viruses in plaque assay than MDBK cells.

Construction of plasmids. Plasmid pT3/WSN-HA was previously described (5). Plasmid pT3/WSN-HAm contains a silent mutation in position 563 of the HA gene (G to T) made to create a new *Hin*dIII restriction enzyme site. It was constructed as follows. Two polymerase chain reaction products were obtained by using pT3/WSN-HA as the template and the two primer pairs (i) 5′-GCGCGCAAGCTTC TCTTCGAGCAAAAGCAGGGGAAAATAAAAAC-3′ and 5′-CTTTGGGTATGAATCCCCCTT-3′ and (ii) 5′-CTTACC AATTCCTATGTGAAC-3′ and 5′-GCGCGCTCTAGAAA TTAACCCTCACTAAAAGTAGAAACAAGGGTGTTTT TCC-3′. The first primer contains nucleotides which are complementary to the 3′ end of vRNA (positions 1 to 25), and the second primer includes nucleotides complementary to positions 560 to 540 of cRNA. The third primer contains nucleotides corresponding to positions 561 to 581 of cRNA, and the fourth primer includes nucleotides complementary to the 3′ end of cRNA (positions 1775 to 1753). The two DNAs were digested with *Xba*I and *Hin*dIII and inserted in a trimolecular ligation reaction into *Xba*I- and *Hin*dIII-digested plasmid pUC18. Plasmids pW(H1)-H2 and pW(H1)-H3 were constructed by replacing the *Hin*dIII fragment of pT3/WSN-HAm. The polymerase chain reaction product for pW(H1)-H2 was obtained by using pT3/WSN-HA as the template and the primer pair 5′-GCGCGCA AGCTTCTCTTCGAGCAAAAGCAGGGGAAAATAAAA AC-3′ (as described above) and 5′GAATTGGTAAGCTTC GGATAATCTGATCCTTCCTTCGTCAGCCATAGCAAA TTTCTG-3′. The latter primer contains nucleotides complementary to cRNA at positions 536 to 515. For construction of pW(H1)-H3, the primer pair 5′-GCGCGCAAGCTTC TCTTCGAGCAAAAGCAGGGGAAAATAAAAAC-3′ (as described above) and 5′-GAATTGGTAAGCTTTGGATAT

* Corresponding author.

GTGCTTCCTGATTTCGTCAGCCATAGCAAATTTCTG-3′ was used. The latter primer contains nucleotides complementary to cRNA at positions 536 to 515. It should be noted that these primers were selected to create cDNA clones with amino acid sequences corresponding to the loop structure contained in antigenic site B of H2 and H3 HAs, respectively.

Rescue of transfectant virus. Influenza virus nucleoprotein and three polymerase proteins were purified from virus A/PR8/34 as described previously (5). The RNP complex was prepared as described by Enami and Palese (5), with modifications to increase transfection efficiency. Briefly, 0.5 μg of *Ksp*632I-digested plasmid DNA, 10 μl of purified influenza virus nucleoprotein and three polymerase proteins (1 μg of total protein), and 2 μl of T3 RNA polymerase (50 U/μl; Stratagene) were incubated at 37°C for 20 min in the presence of 40 mM Tris hydrochloride (pH 8.0)–0.5 mM nucleoside triphosphate–10 mM dithiothreitol–8 mM $MgCl_2$–2 mM spermidine in a total volume of 50 μl. The plasmid DNA in the reaction mixture was then digested with 2 μl of RQ_1-DNase (1 U/μl; Promega Corp.) at 37°C for 5 min. The total reaction mixture was diluted with 75 μl of phosphate-buffered saline (PBS) containing 0.1% gelatin immediately before transfection.

MDBK cells on a 35-mm-diameter dish (10^6 cells) were infected with HK-WSN(H3N1) virus at a multiplicity of infection of 1 for 1 h at room temperature and then washed with PBS, treated with 1 ml of dimethyl sulfoxide-DEAE-dextran-gelatin–PBS solution (12) for 30 min at room temperature. After removal of the supernatant, the RNP preparation was added to the cells and they were incubated for 1 h at room temperature. The cells then were overlaid with 2 ml of reinforced Eagle's medium (GIBCO) containing 0.2% bovine serum albumin.

At 18 h posttransfection, the supernatant was collected and used at different dilutions to infect fresh MDBK cells. After virus adsorption, polyclonal rabbit antiserum raised against HK virus was added to the reinforced Eagle's medium at a final concentration of 0.05%. At 48 h later, the supernatant was used for a plaque assay in the presence of the same antiserum at a final concentration of 0.01%. It should be noted that dilute polyclonal anti-H3 serum was used to select for transfectant virus because the epitope specificities of monoclonal anti-HK HA antibodies available in the laboratory had not been characterized. A concentration of polyclonal anti-H3 serum was chosen which was capable of suppressing the helper virus but still permitted replication of transfectant viruses.

RNA sequencing. Virus purification and RNA extraction were done as previously described (4). The nucleotide sequence that encodes the amino acids which correspond to antigenic site B was determined by direct RNA sequencing using reverse transcriptase and the primer 5′-GTTCATG GCCCAACCACACA-3′ (positions 445 to 464 in the HA gene) (10).

Hemagglutination inhibition (HI) analysis and virus neutralization assay. HI analysis was done as previously described (15). Monoclonal antibodies H15-C12m (raised against WSN virus HA) and Fc125 (raised against Japan virus HA) were kindly provided by Walter Gerhard and Thomas Braciale, respectively. Anti-HK monoclonal antibodies and anti-WSN virus polyclonal rabbit serum were generated by standard procedures. Receptor-destroying enzyme treatment of sera from immunized mice was done as previously described (15). For neutralization assays, MDCK cells in 30-mm-diameter dishes were inoculated with approximately 100 PFU of virus which had been preincubated with different antibody dilutions at 37°C for 30 min. Following a 1-h adsorption period, the virus-antibody mixtures were removed prior to addition of an agar overlay. The cell monolayers were stained with 0.1% crystal violet at 72 h postinfection.

Immune staining. MDCK cells on microplates (Nunclon) were infected with influenza virus at a multiplicity of infection of 1. At 8 h postinfection, the medium was removed and the cells were washed with PBS and then fixed in 1% paraformaldehyde (in PBS) for 10 min. Ten micrograms of monoclonal antibody in 100 μl of PBS containing 0.2% bovine serum albumin was added to the fixed cells, and they were incubated for 1 h at room temperature. After two washes with PBS, 50 μl of a 1/500-diluted stock solution of rabbit anti-mouse antibody conjugated with horseradish peroxidase (DAKO Corp.) was added to the cells and they were incubated for 1 h. The cells then were incubated with AEC substrate (DAKO Corp.) by following the manufacturer's protocol.

Immunization. Ten micrograms of purified virus was injected intraperitoneally into BALB/c mice. Equal doses were administered intraperitoneally for all booster immunizations.

RESULTS

Rescue of transfectant viruses. On the basis of the X-ray crystallographic structure of subtype 3 HA, we chose antigenic site B for expression in the chimeric HA molecules. This site contains an antigenic loop which is most distal to the membrane on the HA spikes and is thought to be highly immunogenic. To replace the antigenic loop in the HA of WSN (H1) virus, we created a new *Hin*dIII restriction enzyme site in pT3/WSN-HA (Fig. 1A). This mutated HA gene was cloned into pUC18 to generate plasmid pT3/WSN-HAm. By using this plasmid, we were able to construct two chimeric HA plasmids, pW(H1)-H2 and pW(H1)-H3. In pW(H1)-H2 and pW(H1)-H3, the nucleotides that encode the amino acids of the loop were replaced by the corresponding HA sequences of Japan virus (H2) and HK virus (H3), respectively. As shown in Fig. 1B, of the six amino acids in the loop structure of site B, H2 and H3 HAs each differ from HA H1 at three positions. These plasmids were designed to yield chimeric viruses that express HAs with epitopes derived from different subtypes.

First, we transfected RNA derived from pT3/WSN-HAm into MDBK cells which were infected with HK-WSN(H3N1) helper virus (Fig. 2). Fresh MDBK cells were infected with 0.1 ml of different dilutions of the supernatant obtained from the transfection experiment in the presence of polyclonal anti-HK serum. A cytopathic effect was observed within 48 h postinfection. In the control experiment, in which the RNP was omitted, no cytopathic effect was detected. The yield of transfectant virus was higher than 10^3 PFU/ml of supernatant from the transfection experiment. Direct RNA sequencing of this virus indicated that the introduced silent mutation was present in the HA gene (data not shown). This confirmed that the virus was a novel transfectant virus and not contaminating wild-type WSN virus. In the same way, the chimeric HA RNAs derived from plasmids pW(H1)-H2 and pW(H1)-H3 were transfected into MDBK cells. When the supernatant from cells transfected with RNA derived from pW(H1)-H2 was used to infect fresh MDBK cells, a cytopathic effect was observed at a 10^{-2} dilution. In contrast, the yield of transfectant virus derived from pW(H1)-H3 was about 10 times lower than that derived from pT3WSN-HAm

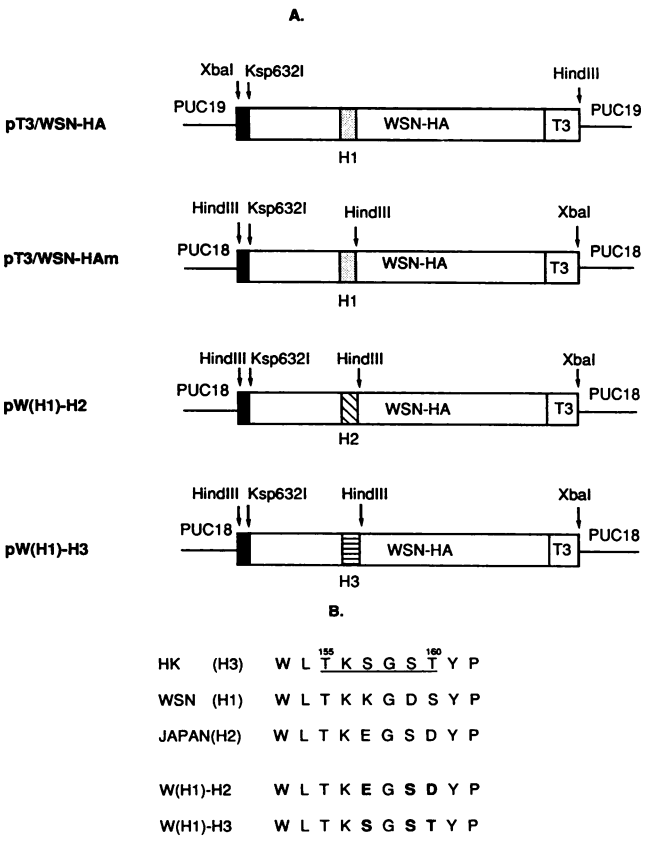

FIG. 1. Plasmid constructs of influenza virus HAs and comparison of amino acid sequences in the loop of antigenic site B. (A) pUC19-derived plasmid pT3/WSN-HA was previously described (5); it contains the complete nucleotide sequence of the HA gene of WSN influenza virus, the truncated bacteriophage T3 RNA polymerase promoter; and a *Ksp*632I restriction enzyme site. The stippled box indicates the location of the gene region that encodes the loop structure of antigenic site B (not to scale). pUC18-derived plasmid pT3/WSN-HAm contains a silent mutation resulting in a new *Hin*dIII restriction enzyme site (Materials and Methods). In plasmids pW(H1)-H2 and pW(H1)-H3, the loop structure of WSN virus HA was replaced by the corresponding regions of the Japan and HK HAs, respectively. The *Ksp*632I-digested plasmids can be used as templates for in vitro synthesis of full-length WSN virus or chimeric HA RNA. (B) The amino acids of the loop of antigenic site B in HK (H3) virus HA are underlined (16, 17). The sequences of the WSN and Japan virus HAs are also indicated (8, 9). The amino acids in the chimeric HAs which differ from those in the WSN virus HA molecule are indicated in boldface letters.

and pW(H1)-H2. This lower yield of the W(H1)-H3 chimeric virus can be explained by the fact that selection of the transfectant viruses involved the use of antiserum raised against an H3 subtype virus.

The transfectant viruses were plaque purified twice, and then their biological properties were characterized. The plaque morphology of the transfectant viruses in MDBK and MDCK cells was comparable to that of wild-type WSN virus, and the growth kinetics in single or multicycle replication was also indistinguishable from that of WSN virus (data not shown). The nucleotide sequences that encode the antigenic loop structure of the HAs of the transfectant viruses were determined by direct sequencing of the RNAs of the purified transfectant viruses. As shown in Fig. 3, the

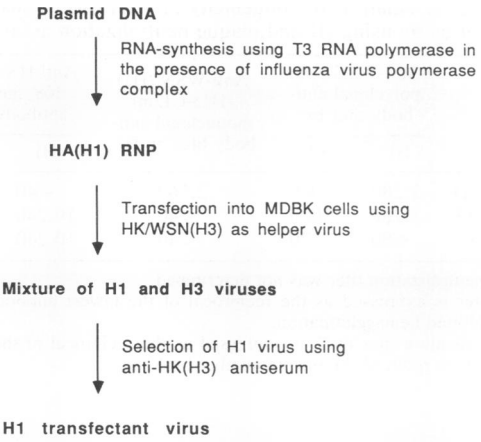

FIG. 2. Strategy for isolation of transfectant viruses containing chimeric HAs.

HA genes of viruses W(H1)-H2 and W(H1)-H3 contain the expected sequences.

W(H1)-H3 transfectant virus contains epitopes from both subtypes H1 and H3. The antigenic properties of W(H1)-H3

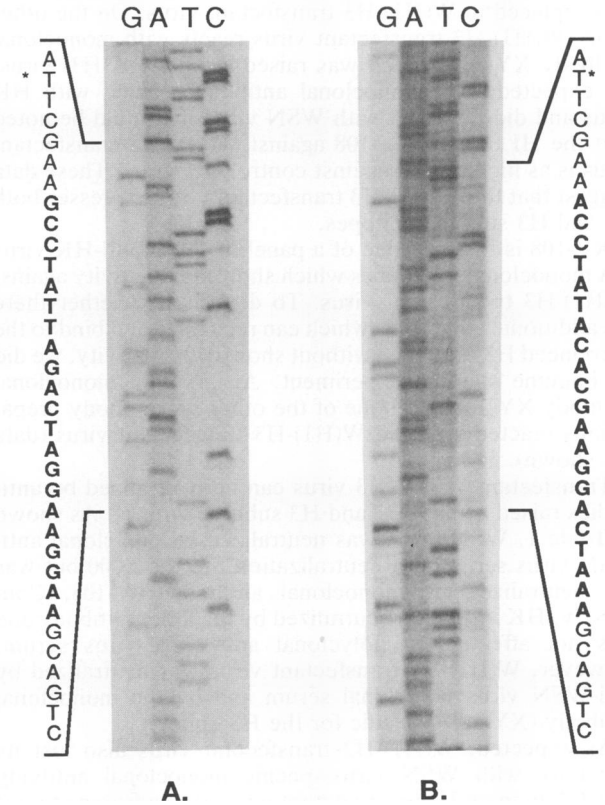

FIG. 3. RNA sequences of the HAs of W(H1)-H2 and W(H1)-H3 transfectant viruses. Purified viral RNAs of W(H1)-H2 and W(H1)-H3 transfectant viruses were used for direct RNA sequencing using a primer corresponding to positions 445 to 464 of WSN virus HA. The asterisks indicate the silent mutation (G-563 to T-563) which was introduced to generate a *Hin*dIII site in the plasmid DNA. Boldface letters indicate the introduced H2 or H3 subtype HA sequence in the chimeric HAs. Panels: A, sequence of W(H1)-H2 RNA; B, sequence of W(H1)-H3 RNA.

TABLE 1. Analysis of antigenicity of W(H1)-H3 transfectant virus by using HI and plaque neutralization assays

Virus	Anti-WSN (H1) polyclonal antibody titer by:		Anti-WSN (H1) (H15-C12m) monoclonal antibody titer by HI[a]	Anti-HK (H3) (XY-108) monoclonal antibody titer by:	
	HI[b]	NT[c]		HI	NT
WSN (H1)	1,280	2,000	2,560	<40	<20
W(H1)-H3	1,280	2,000	<40	10,240	200,000
HK (H3)	<80	<20	<40	10,240	200,000

[a] The neutralization titer was not determined.
[b] HI titer is expressed as the reciprocal of the lowest antibody dilution which inhibited hemagglutination.
[c] Neutralization titer (NT) is expressed as the reciprocal of the antibody dilution which reduced the plaque number by 50%.

transfectant virus were investigated by HI analysis and in an immune staining assay. As shown in Table 1, polyclonal rabbit anti-WSN virus serum reacted with both W(H1)-H3 transfectant virus and WSN virus at an HI titer of 1,280 but did not react with HK virus. H15-C12m is a monoclonal antibody raised against WSN virus. It reacted with WSN virus at an HI titer of 2,560 but did not react with W(H1)-H3 transfectant virus or HK virus. Although the epitope specificity of this monoclonal antibody was not known, it seems likely that this antibody recognizes the antigenic loop which was replaced in W(H1)-H3 transfectant virus. On the other hand, W(H1)-H3 transfectant virus reacts with monoclonal antibody XY-108, which was raised against HK(H3) virus. As expected, this monoclonal antibody reacted with HK virus and did not react with WSN virus. It should be noted that the HI titer of XY-108 against W(H1)-H3 transfectant virus is as high as that against control HK virus. These data suggest that the W(H1)-H3 transfectant virus expresses both H1 and H3 subtype epitopes.

XY-108 is the only one of a panel of seven anti-HK virus HA monoclonal antibodies which showed HI activity against W(H1)-H3 transfectant virus. To determine whether there are additional antibodies which can recognize and bind to the introduced H3 sequence without showing HI activity, we did an immune staining experiment. Again, only monoclonal antibody XY-108 and none of the other six antibody preparations reacted with the W(H1)-H3 transfectant virus (data not shown).

Transfectant W(H1)-H3 virus can be neutralized by antibodies raised against H1 and H3 subtype viruses. As shown in Table 1, WSN virus was neutralized by polyclonal anti-WSN virus serum at a neutralization titer of 2,000 but was not neutralized by monoclonal antibody XY-108. Conversely, HK virus was neutralized by the latter antibody and was not affected by polyclonal anti-WSN virus serum. However, W(H1)-H3 transfectant virus was neutralized by anti-WSN virus polyclonal serum and by the monoclonal antibody (XY-108) specific for the H3 epitope.

As expected, W(H1)-H2 transfectant virus also lost its reactivity with WSN virus-specific monoclonal antibody H15-C12m in an HI test (data not shown), confirming that it too lacks the loop present in the WSN virus HA molecule. Analysis using Japan virus-specific monoclonal antibody Fc125 and available polyclonal antisera failed to demonstrate the detectable expression of an H2 subtype epitope in this chimeric virus by HI analysis and immune staining assay.

Immunogenicity of W(H1)-H3 transfectant virus. The immunogenic potential of W(H1)-H3 transfectant virus was examined for the ability to elicit antibodies in mice. Sera from mice bled before immunization (prebled) and immunized with WSN or W(H1)-H3 virus were tested for HI activity against WSN, HK, and W(H1)-H3 transfectant viruses. Sera from prebled mice showed no HI activity against WSN, HK, or W(H1)-H3 transfectant virus. As expected, WSN virus was capable of inducing an immune response, with HI titers ranging from 1,280 to 5,120 against the WSN and W(H1)-H3 viruses, but no H3-specific activity was detected with these sera (Table 2). In contrast, the sera from all five mice immunized with W(H1)-H3 transfectant virus showed HI activity against all three of the viruses tested. The HI titers against WSN(H1) and W(H1)-H3 transfectant viruses were in the range of 1,280 to 5,120, and the titers against H3 subtype virus varied from 80 to 640 (third bleeding). Although the HI titers against the H3 subtype virus were lower than those against the H1 virus, the data indicate that the W(H1)-H3 transfectant virus is capable of inducing a bispecific immune response to both H1 and H3 subtype epitopes. It should be noted that sera from all bleedings demonstrated higher and more rapid responses to H1 HA than to H3 HA. The kinetic response data of the sera from mice immunized with W(H1)-H3 virus are shown in Fig. 4. Sera from W(H1)-H3 transfectant virus-immunized mice with the highest HI titer against the H3 subtype virus (mice 3 and 4 in Table 2) were then tested in a neutralization assay and found to inhibit plaque formation of WSN(H1) and HK(H3) viruses specifically (Table 2). The titer against the H3 subtype virus was also lower than that against the H1 subtype virus.

TABLE 2. Antibody titers in sera of mice immunized with W(H1)-H3 transfectant virus

Virus used for immunization and mouse no.	Titer of antibody to virus:				
	WSN (H1)		W(H1)-H3[a] (HI)	HK (H3)	
	HI[b]	NT[c]		HI	NT
W(H1)-H3					
1	1,280	ND	1,280	80	ND
2	>5,120	ND	5,120	320	ND
3	2,560	2,000	2,560	640	200
4	2,560	2,000	2,560	640	200
5	1,280	ND	1,280	320	ND
WSN (H1)					
6	2,560	ND	2,560	<40	<20
7	5,120	ND	5,120	<40	<20
8	1,280	ND	1,280	<40	ND

[a] The neutralization titer was not determined.
[b] HI titer is expressed as the reciprocal of the lowest serum dilution which inhibited hemagglutination. Sera from immunized mice were from the third bleeding (see Fig. 4). All preimmune sera lacked HI and neutralizing activities against any of the test viruses.
[c] Neutralization titer (NT) is expressed as the reciprocal of the serum dilution that reduced the plaque number by 50%. ND, not determined.

DISCUSSION

In this report, we describe the construction of influenza transfectant viruses which express chimeric HA molecules. We obtained plasmids which allowed in vitro runoff transcription of HA RNAs, and these RNAs were rescued by infectious virus particles with an RNP transfection protocol. WSN (H1) virus HA genes in which a short region was replaced with the corresponding one from Japan (H2) or HK (H3) virus were constructed. The region which was replaced

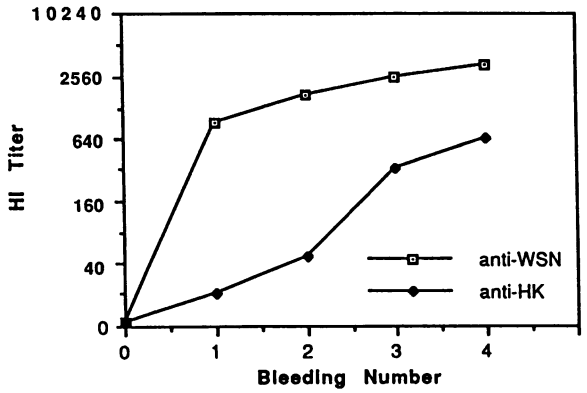

FIG. 4. Kinetics of antibody response in mice immunized with W(H1)-H3 transfectant virus. Ten micrograms of purified W(H1)-H3 virus was injected intraperitoneally into five BALB/c mice. Equal booster doses were administered intraperitoneally three times at 7-, 14-, and 14-day intervals. At 1 week after the first, second, and fourth boosts and 2 weeks after the third boost, sera were collected and treated with receptor-destroying enzyme before being tested for HI activity. HI titers represent the average titer of the sera from five individual mice measured against WSN (H1) or HK (H3) virus. The data for the third bleeding are taken from Table 2.

in the H1 HA molecule has previously been identified as antigenic site Sa (3, 8) and, on the basis of comparative amino acid sequence analysis, corresponds to site B in H3 HA (7). Site B contains an exposed loop (amino acids 155 to 160 in the H3 numbering system), as demonstrated by structure analysis (18, 19). We assumed that the secondary structures of the H1, H2, and H3 HA molecules are similar and could easily be interchanged. Although the loop contains six amino acids, there are only three amino acid changes between the H1 and H2 and H1 and H3 sequences. It should also be noted that substitutions in each of these positions have been detected in natural variants of H3 subtype viruses (7). Infectious transfectant viruses were easily obtained by using a polyclonal anti-H3 serum to select against the H3 helper virus. This rescue of W(H1)-H2 and W(H1)-H3 viruses proves that substitution in this particular site is compatible with obtainment of fully functional viruses. It should be noted, however, that the yield of the W(H1)-H3 virus was lower by a factor of 10, possibly because of suppression by the H3 antiserum used for selection. It is likely that the antiserum contained a low concentration of neutralizing antibodies that reacted with the H3 epitope on the chimeric virus.

Analysis of the chimeric viruses revealed that they lost their reactivity to an H1 HA-specific antibody in HI and neutralization tests. It is thus assumed that this monoclonal antibody recognizes site Sa (site B) in H1 HA. Thus, we suggest that chimeric transfectant viruses of the type of W(H1)-H2 and W(H1)-H3 could become useful reagents for identification of the specificity of monoclonal antibodies. Antibody escape mutants have been successfully used to map antibodies. In general, those escape mutants have single amino acid substitutions and antibodies directed to that general area may still react with some of the escape mutants. By replacing the entire epitope in an antigen, such viruses may become more effective tools in rapidly identifying antibody specificities.

Interestingly, W(H1)-H3 virus interacted in the HI test with a monoclonal antibody specific for site B of H3 HA. The HI titer against HK virus was identical to that against the chimeric virus, which suggests that monoclonal antibody XY-108 recognizes a similar structure in the H3 and chimeric HAs. However, it should be noted that attempts to identify an H2-specific antibody that recognizes W(H1)-H2 transfectant virus were not successful. This latter result is possibly due to the fact that the available H2-specific monoclonal and polyclonal antibody preparations have different specificities. Alternatively, the structure of antigenic site B in H2 HA may be sufficiently different so that exchange of only the loop amino acids and not the surrounding amino acids does not lead to regeneration of a genuine H2 epitope. Attempts are in progress to obtain monoclonal antibodies capable of reacting against both Japan virus and W(H1)-H2 transfectant virus. In any case, the fact that W(H1)-H2 no longer reacts with WSN virus-specific monoclonal antibody H15-C12m further shows that this method can be employed to isolate viruses with altered antigenic structures.

The W(H1)-H3 transfectant was further examined by immunizing mice. Following three booster immunizations, the sera of all mice showed cross-reactivity in HI tests with viruses containing H1 or H3 HA. In addition, two of the sera were tested in a neutralization assay and found to inhibit both H1 and H3 subtype viruses. This result demonstrates that a single epitope in a prominent location on the virus can direct the formation of an efficient immune response. Further studies are planned to generate chimeric viruses which carry two or more H3 epitopes, and it will be important to study their ability to stimulate a cross-protective immune response in vivo. We suggest that through the introduction of new epitopes the immunogenic potential of influenza viruses can be enhanced and that this method could be used to develop a new generation of effective live attenuated influenza viruses capable of eliciting immune responses to more than one subtype virus. Several DNA and plus-stranded RNA viruses have been used to express foreign antigens or chimeric proteins (1, 6). The present results indicate that influenza virus may also be used as an expression vector for foreign epitopes. This system, together with other expression systems, makes available new approaches to the development of effective vaccines. In particular, transfectant influenza viruses which express antigens of other respiratory viruses may prove useful for generation of a broad protective immune response against several respiratory viruses.

ACKNOWLEDGMENTS

We thank Terri Latham and Ling-Yu Chen Chiu for excellent technical assistance. We also thank T. Braciale and W. Gerhard for kindly providing us with monoclonal antibodies.

Support for this work was from NIH program project grant AI 24460 (P.P. and C.B.).

REFERENCES

1. **Bloom, B. R.** 1989. Vaccines for the third world. Nature (London) **342**:115–120.
2. **Burke, K. L., G. Dunn, M. Ferguson, P. D. Minor, and J. W. Almond.** 1988. Antigenic chimaeras of poliovirus as potential new vaccines. Nature (London) **332**:81–82.
3. **Caton, A. J., G. G. Brownlee, J. W. Yewdell, and W. Gerhard.** 1982. The antigenic structure of influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell **31**:417–427.
4. **Enami, M., W. Luytjes, M. Krystal, and P. Palese.** 1990. Introduction of site specific mutations into the genome of influenza virus. Proc. Natl. Acad. Sci. USA **87**:3802–3805.
5. **Enami, M., and P. Palese.** 1991. High efficiency formation of influenza virus transfectants. J. Virol. **65**:2711–2713.

6. **Evans, D. J., J. McKeating, J. M. Meredith, K. L. Burke, K. Katrak, A. John, M. Ferguson, P. D. Minor, R. A. Weiss, and J. W. Almond.** 1989. An engineered poliovirus chimaera elicits broadly reactive HIV-1 neutralizing antibodies. Nature (London) **339**:385–388.
7. **Fitch, W. M., J. M. Leiter, X. Li, and P. Palese.** 1991. Positive Darwinian evolution in human influenza A viruses. Proc. Natl. Acad. Sci. USA **88**:4270–4274.
8. **Gerhard, W., J. W. Yewdell, M. Frankel, and R. G. Webster.** 1981. Antigenic structure of influenza virus hemagglutinin defined by hybridoma antibodies. Nature (London) **289**:713–717.
9. **Gething, M. J., J. Bye, J. Skehel, and M. Waterfied.** 1980. Cloning and DNA sequence of double-stranded copies of hemagglutinin genes from H2 and H3 strains elucidates antigenic shift and drift in human influenza virus. Nature (London) **287**:301–306.
10. **Hiti, A. L., A. R. Davis, and D. P. Nayak.** 1981. Complete sequence analysis shows that the hemagglutinins of the H0 and H2 subtypes of human influenza virus are closely related. Virology **111**:113–124.
11. **Kawaoka, Y., S. Yamnikova, T. M. Chambers, D. K. Lvov, and R. G. Webster.** 1990. Molecular characterization of a new hemagglutinin, subtype 14, of influenza A virus. Virology **179**: 759–767.
12. **Luytjes, W., M. Krystal, M. Enami, J. D. Parvin, and P. Palese.** 1989. Amplification, expression, and packaging of a foreign gene by influenza virus. Cell **59**:1107–1113.
13. **Muster, T., E. K. Subbarao, M. Enami, B. R. Murphy, and P. Palese.** 1991. An influenza A virus containing influenza B virus 5′ and 3′ noncoding regions on the neuraminidase gene is attenuated in mice. Proc. Natl. Acad. Sci. USA **88**:5177–5181.
14. **Palese, P.** Unpublished data.
15. **Palmer, D. F., M. T. Coleman, W. R. Dowdle, and G. C. Schild.** 1975. Advanced laboratory techniques of influenza diagnosis. Immunol. Ser. **6**:51–52.
16. **Webster, R. G., W. G. Laver, and G. M. Air.** 1983. Antigenic variation among type A influenza viruses, p. 127–168. *In*: P. Palese and D. W. Kingsbury (ed.), Genetics of influenza viruses. Springer-Verlag, New York.
17. **Wharton, S. A., W. Weis, J. J. Skehel, and D. C. Wiley.** 1989. Structure, function, and antigenicity of the hemagglutinin of influenza virus, p. 153–173. *In*: R. M. Krug (ed.), The influenza viruses. Plenum Press, New York.
18. **Wiley, D. C., I. A. Wilson, and J. J. Skehel.** 1981. Structural identification of the antibody-binding sites of Hong Kong influenza hemagglutinin and their involvement in the antigenic variation. Nature (London) **289**:373–378.
19. **Wilson, I. A., J. J. Skehel, and D. C. Wiley.** 1981. Structure of the hemagglutinin membrane glycoprotein of influenza virus at 3A resolution. Nature (London) **289**:366–373.