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Influenza B Viruses with Site-Specific Mutations Introduced into the HA Gene

WENDY S. BARCLAY AND PETER PALESE*

Department of Microbiology, Mount Sinai School of Medicine, New York, New York 10029

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We have succeeded in engineering changes into the genome of influenza B virus. First, model RNAs containing the chloramphenicol acetyltransferase gene flanked by the noncoding sequences of the HA or NS genes of influenza B virus were transfected into cells which were previously infected with an influenza B helper virus. Like those of the influenza A viruses, the termini of influenza B virus genes contain *cis*-acting signals which are sufficient to direct replication, expression, and packaging of the RNA. Next, a full-length copy of the HA gene from influenza B/Maryland/59 virus was cloned. Following transfection of this RNA, we rescued transfectant influenza B viruses which contain a point mutation introduced into the original cDNA. A series of mutants which bear deletions or changes in the 5′ noncoding region of the influenza B/Maryland/59 virus HA gene were constructed. We were able to rescue viruses which contained deletions of 10 or 33 nucleotides at the 5′ noncoding region of the HA gene. The viability of these viruses implies that this region of the genome is flexible in sequence and length.

Although influenza B viruses are important human pathogens and in some years are responsible for the majority of influenza cases, they have been studied less than the influenza A viruses. Both influenza A and B viruses contain eight segments of single-stranded RNA of negative polarity, but there are differences in the *cis*-acting signals (2), coding strategies (6, 22), proteins (18), host ranges, and biological properties (8) of these virus types. In order to be able to address the basis for these differences, and also as an effort toward developing live attenuated vaccines which would effectively prevent influenza morbidity, we have established a protocol for the genetic manipulation of influenza B viruses. We describe here the rescue of transfectant influenza B viruses and demonstrate that this methodology will be useful in delineating the functions of various regions of the influenza B virus genome.

Transfection of model influenza B virus RNPs results in amplification and expression of the RNA. It has been shown that ribonucleoprotein complexes (RNPs) of influenza A virus can be assembled in vitro by transcribing synthetic RNAs in the presence of purified polymerase proteins (PB1, PB2, and PA) and nucleoprotein (NP) (17). Provided that such RNAs contain the noncoding sequences from influenza virus genes at their termini, the RNPs are replicated and expressed when introduced by transfection into cells previously infected with a helper virus (14). We attempted to adapt this protocol for the transfection of synthetic RNPs with influenza B virus sequences. The plasmids we used direct transcription of two model RNAs which contain the coding region of the chloramphenicol acetyltransferase (CAT) gene in the negative sense flanked by noncoding sequences from either the NS (NSBCAT, previously described as IVBCAT [16]), or the HA (HABCAT) gene of influenza B/Lee/40 virus RNAs (Fig. 1a). We next purified influenza B/Yamagata/83 virus polymerase proteins and NP from allantoic fluids of infected eggs using glycerol gradient centrifugation followed by cesium chloride gradient centrifugation (17). Cells infected with influenza B/Lee/40 helper virus were then transfected with RNPs formed by transcription of NSBCAT or HABCAT RNAs in the presence of the polymerase protein preparation. Twenty-four hours after transfection, cells were harvested and the extracts were analyzed for CAT enzyme activity (Fig. 1b). CAT activity was present in cells transfected with RNAs from either plasmid. Activity was dependent upon previous infection with helper virus, indicating that the amplification of RNA was carried out by the helper virus replicative machinery (data not shown). CAT activity was also dependent upon the presence of purified polymerase proteins and NP. That is, no CAT activity was found after transfection of naked RNA (data not shown). The CAT conversion by extracts from HABCAT-transfected cells was always less than that from NSBCAT-transfected cells (Fig. 1b), an observation which is opposite to the situation found for the analogous influenza A virus model RNAs (11, 12). We also found that the level of CAT expression was higher when the cells were incubated at 34°C than at 37°C after infection and transfection. It should be noted that NP and polymerase proteins isolated from B/Lee/40 virions have also been used to reconstitute functional RNPs (data not shown).

Model influenza B virus RNPs are packaged into novel infectious particles. We confirmed and extended earlier studies (4, 15) indicating that transfection of MDBK cells by RNPs using either the DEAE-dextran or DOTAP lipofection method was more efficient than transfection of MDCK cells. This observation presented a dilemma, since although influenza B viruses can replicate in MDBK cells, high titers of infectious virus are not released after infection, but we ultimately wanted to use the RNP transfection technique to recover novel transfectant influenza B viruses. The small amount of infectious influenza virus particle formation from MDBK cells is thought to be due to incomplete cleavage of the HA (hemagglutinin) protein and in some instances is overcome by digestion with trypsin (19). We therefore incubated the transfected cells in media containing a high concentration (4 μg/ml) of trypsin. Although the MDBK cells showed extensive damage as a result of this treatment, the level of CAT activity expressed after transfection of HABCAT RNPs was as high as that obtained in

* Corresponding author. Mailing address: Department of Microbiology, Mount Sinai School of Medicine, One Gustave L. Levy Pl., New York, NY 10029.

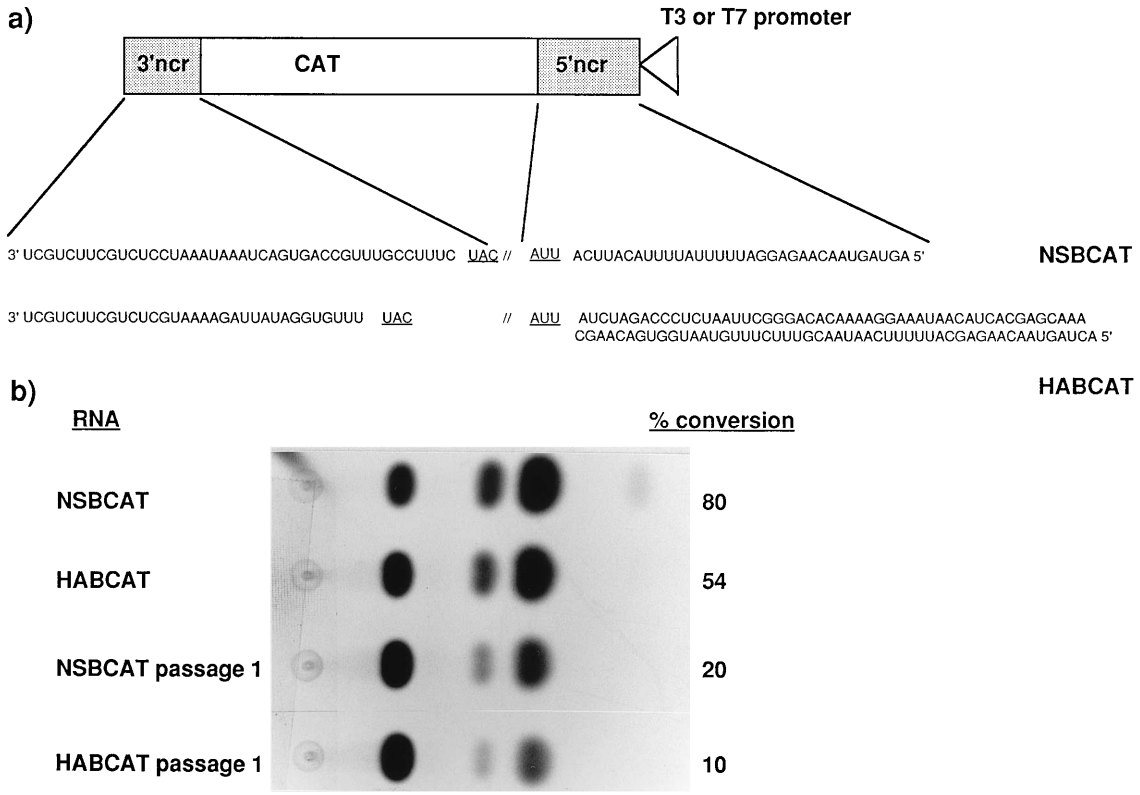

FIG. 1. (a) Plasmid pT7NSBCAT has been previously described as plasmid IVBCAT (16). The construction of plasmid pT3HABCAT was essentially the same as that for pT7NSBCAT (16) and utilized PCR cloning techniques with appropriate primers. The RNAs transcribed from these plasmids contain the coding region of the CAT gene in the negative sense flanked by the noncoding regions of influenza B virus NS or HA genes. The nucleotides of the noncoding sequences (3′ncr and 5′ncr) of NSBCAT and HABCAT RNAs are indicated. (b) RNP complexes formed in vitro by transcribing NSBCAT or HABCAT RNAs from 1 μg of *Hga*I-linearized DNA template in the presence of 3 to 5 μg of purified polymerase subunits and NP protein. MDBK cells were infected at a multiplicity of infection of 1 with influenza B/Lee/40 helper virus 1 h prior to transfection. Following overnight incubation at 34°C, transfected cells were harvested into 100 μl of 250 mM Tris-HCl (pH 7.5). The supernatants were treated with RNase A (1 μg/ml) at 37°C for 30 min and then used to infect MDCK monolayers. Following overnight incubation at 34°C, the cells were harvested into 100 μl of Tris-HCl (pH 7.5). Aliquots (50 μl) of cell extracts were processed for detection of CAT enzyme activity as described previously (5).

the absence of trypsin (data not shown). Furthermore, when the supernatants from these cells were treated with RNase A and passaged onto fresh MDCK monolayers, substantial CAT activity was present in extracts from the second monolayer (Fig. 1b), suggesting that the input RNA had been authentically replicated and that novel RNAs derived from the transfected gene were packaged into virus particles.

Transfection of a full-length HA gene and rescue of viruses containing novel RNAs. In order to rescue transfectant influenza B viruses, we transfected RNPs assembled from synthetic RNAs which represented a whole influenza B virus segment. We attempted to rescue viruses containing a novel HA gene, since we possessed monoclonal antibodies (MAbs) against the B/Lee/40 HA protein which specifically neutralize B/Lee/40 virus without affecting replication of viruses with different HAs, for example, those containing the HA of B/Maryland/59 (B/Md/59) virus. Therefore, we could select viruses which contained transfected B/Md/59 HAs from a background of B/Lee/40 helper virus by passage in the presence of such antibodies (4, 10).

We cloned the HA gene from influenza B/Md/59 virus downstream of a T3 promoter and upstream of an *Hga*I restriction enzyme site such that in vitro transcription using T3 RNA polymerase (4) would give rise to an RNA 1,882 nucleotides long containing authentic 3′ and 5′ termini. RNPs were formed in the presence of polymerase proteins and NP and transfected into MDBK cells previously infected with influenza B/Lee/40 virus as the helper virus. Supernatants were passaged twice either undiluted or diluted 10-fold in MDCK cells in the presence of two MAbs which neutralize B/Lee/40 virus (MAbs 123 and 142, used at concentrations of 12 and 28 μg/ml, respectively). Finally, progeny were isolated by growth of plaques on MDCK cells by using a solid agar overlay which also contained MAbs 123 and 142. At 3 days postinfection, large distinct plaques were visible. These viruses were shown by hemagglutination inhibition to have an influenza B/Md/59 virus HA. We next introduced a point mutation into the cDNA of influenza B/Md/59 virus HA by which we could tag the transfectant viruses we recovered. A single C-to-T substitution at position 1755 (of the sequence and numbering for positive-sense RNA) resulted in the creation of an *Xba*I restriction enzyme site, and the cDNA was named pT3MdHAX (Fig. 2a). Following transfection of RNPs transcribed from the MdHAX clone, viruses containing influenza B/Md/59 virus HA were recovered. RNA was purified from eggs infected with these progeny and subjected to reverse transcription followed by PCR amplification of the sequences at the 5′ end of the viral RNA (vRNA) (Fig. 2b). The 209-bp product was digested with *Xba*I into two fragments 53 and 156 bp long (lane 4). In contrast, the fragment obtained from the MdHA transfectant virus was not cut (lane 3). Furthermore, direct sequence analysis of the PCR products revealed the presence of the point mutation in the virus recovered after MdHAX transfection (Fig. 2c).

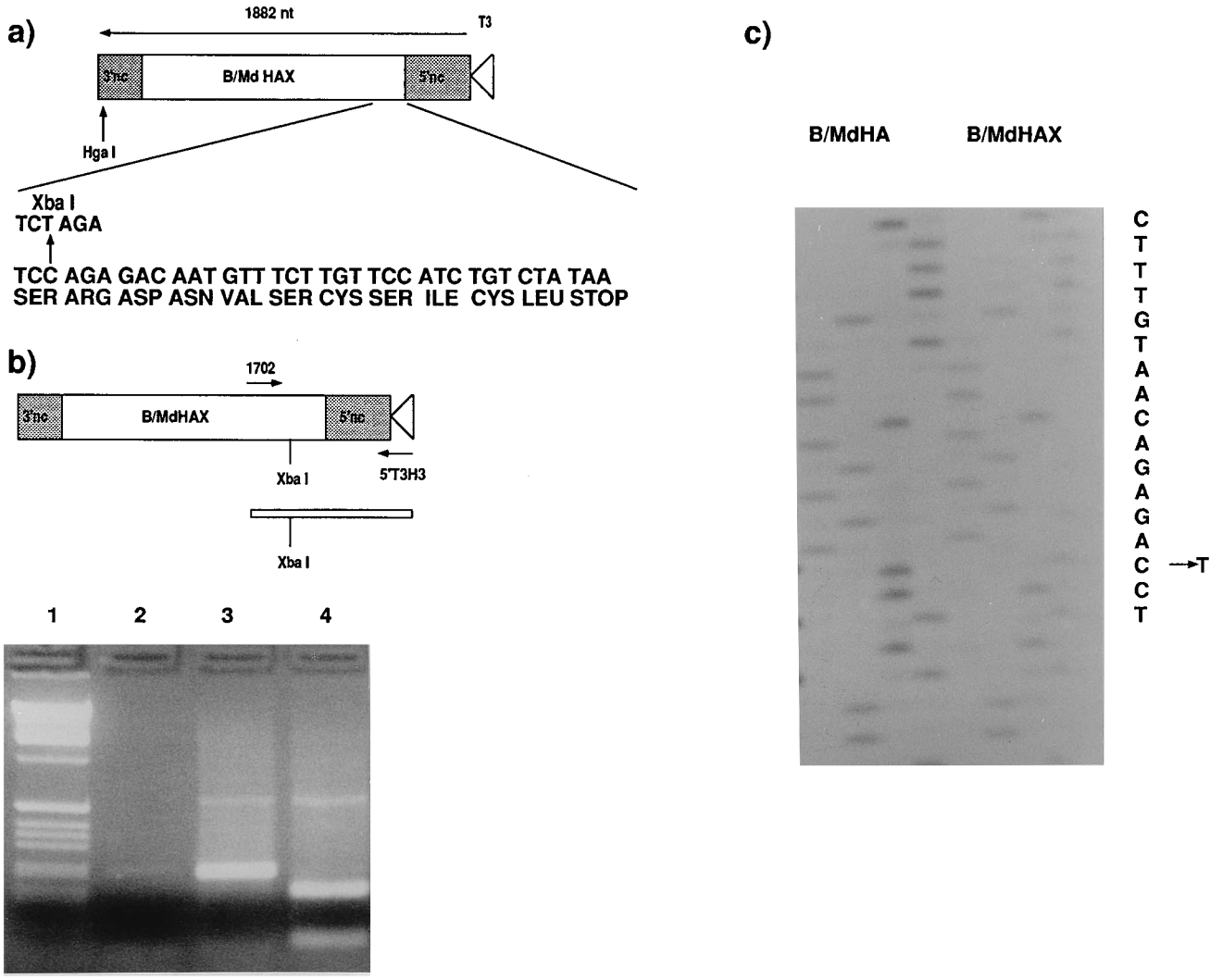

FIG. 2. (a) Plasmid pT3B/MdHAX was constructed by reverse transcription of purified influenza B/Md/59 vRNA using avian myeloblastosis virus (AMV) super reverse transcriptase (Molecular Genetics Resources) and primer XbaB3′, which anneals to the first nine conserved 3′-terminal sequences of influenza B vRNAs. The product was amplified by PCR with Vent polymerase by using the same 3′ primer (XbaB3′) and primer 5′T3H3, which anneals to the first 22 nucleotides (nt) of the HA cDNA. These primers also introduce the T3 polymerase promoter sequence at the 5′ end of the HA gene and an *Hga*I restriction enzyme site at the 3′ end, as well as appropriate restriction enzyme sites to allow the entire cDNA to be cloned into pUC19. The sequences of oligonucleotides used as primers were based on the published sequence of the HA gene of influenza B/Md/59 virus (9). The point mutation at position 1755 was introduced by overlapping PCR and standard cloning techniques, with appropriate primers. nc, noncoding sequence. (b) Reverse transcription of vRNA was performed with AMV reverse transcriptase as described above and primer 1702, which anneals to nucleotides 1702 through 1716 of B/Md/59 HA vRNA (numbering is of positive-sense RNA). PCR amplification of the cDNA products used primers 1702 and 5′T3H3. The 209-bp products obtained after amplification of vRNA from viruses recovered after transfection of MdHA or MdHAX RNAs were digested with *Xba*I, and fragments were analyzed on a 3% agarose gel stained with ethidium bromide. Lane 1, kilobase ladder (GIBCO BRL); lane 2, no template; lane 3, MdHA vRNA template; lane 4, MdHAX vRNA template. (c) Reverse transcriptase PCR products from vRNA of MdHA (four left-hand lanes) and of MdHAX (four right-hand lanes) viruses were sequenced directly by incorporating dimethyl sulfoxide into the annealing and labelling mixes (21). Sequencing reaction mixtures were loaded left to right in the order A, G, C, T.

Rescue of HA transfectant viruses with deletions in their 5′ noncoding regions. The extreme termini of the eight RNA segments of influenza B virus share highly conserved sequences. At the 5′ ends of the viral RNAs (vRNAs) are long stretches of noncoding sequences up to 100 nucleotides long which have no counterparts in the influenza A vRNAs and whose functions are unknown (23). Analysis of sequence data for the HA genes of different influenza B virus strains (1, 9) reveals that the HA 5′ noncoding regions of viruses isolated many decades apart are conserved in length and sequence (Fig. 3a). We have investigated the putative role of these sequences in the influenza B virus life cycle by deleting some or all of them from both model RNAs and from full-length influenza virus HA RNAs. We deleted 10, 33, or 67 nucleotides from the 5′ noncoding region between the HA2 protein termination codon and the stretch of U residues thought to act as a polyadenylation signal during mRNA synthesis (12, 13). We also replaced these sequences with analogous sequences from the influenza B virus NA gene. The sequences of the 5′ noncoding regions between the HA2 protein termination codon and the 5′ terminus of the mutant cDNAs we constructed are shown in Fig. 3b. When model RNAs harboring these deletions and mutations were transfected, all of the replicons produced comparable levels of CAT activity which could be passaged to a second monolayer (data not shown). Although the levels of CAT enzyme were not quantified, the data indicated that all of

a)

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                1786                                                                                                1882
B/Lee/40        TGAGGGAGATTAAGCCCTGTGTTTTCCTTTACTGTAGTGCTCATTTGCTTGTCACCATTACAAAGAAACGTTATTGAAAAATGCTCTTGTTACTACT 5'
B/Bonn/43       TGAGGGAGATTAAGCCCTGTGTTTTCCTTTACTGTAGTGCTCgTTTGCTTGTCACCATTACAAAG-AACGTTATTGAAAAATGCTCTTGTTACTACT 5'
B/Md/59         TaAGGGAaATTAAGCCCTGTGTTTTCCTTTAtTGTAGTGCTCgTTTGCTTGTCACCATTACAAAGAAACGTTATTGAAAAATGCTCTTGTTACTACT 5'
B/Sing/72       TaAGGaAGATTAAGCCCTGTaTTTTCCTTTAtTGTAGTGCTtgTTTGCTTGTtACCATTACAAA-AAACGTTATTGAAAAATGCTCTTGTTACTACT 5'
B/HK/73         TGAGGaAaATTAAGCCCTGTaTTTTCCTTTAtTGTAGTGCTtgTTTGCTTGTtACCATTACAAA-AAACGTTATTGAAAAATGCTCTTGTTACTACT 5'
B/Or/80         TaAGGaAaATTAAGCCCTGTaTTTTCCTTTAtTGTAGTGCTtgTTTGCTTGTtACCATTACAAA-AAACGTTATTGAAAAATGCTCTTGTTACTACT 5'
B/Eng/82        TagGGagGATTgAGCCCTGTaTTTTCCTTTgtTGTgGTGCTtgTTTGCTTGTtACCATTACAgA-AAACG.........................
B/USSR/83       TaAGGaAaATTAAGCCCTGTaTTTTCCTTTAtTGTAGTGCTtgTTTGCTTGTtACCATTACAAA-AAACGTTATTGAAAAATGCTCTTGTTACTACT 5'
B/ND/83         TaAGGaAaATTAAGCCCTGTaTTTTCCTTTAtTGTAGTGCTtgTTTGCTTGTtAtCATTACAAA-AAACGTTATTGAAAAATGCTCTTGTTACTACT 5'
B/Vic/85        TaAGGaAaATTAAGCCCTGTaTTTTCCTTTAtTGTAGTGCTtgTTTGCTTGTtACCATTACAAA-AAACGTTATTGAAAAATGCTCTTGTTACTACT 5'
B/Mem/86        TaAGGaAaATTAAaCCCTGTaTTTTCCTTTAtTGTAGTGCTtgTTTGCTTGTtACCATTACAAA-AAACGTTATTGAAAAATGCTCTTGTTACTACT 5'
B/Vic/87        TagGGagGATTgAGCCCTGTaTTTTCCTTTgtTGTgGTGCTtgTTTGCTTGTtACCATTACAgA-AAACG.........................
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b)

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pT3B/MdHAX      TAAGGGAAATTAAGCCCTGTGTTTTCCTTTATTGTAGTGCTCGTTTGCTTGTCACCATTACAAAGAAACGTTATTGAAAAATGCTCTTGTTACTACT 5'
pT3B/MdHAXd10   TAAGATC----------TGTGTTTTCCTTTATTGTAGTGCTCGTTTGCTTGTCACCATTACAAAGAAACGTTATTGAAAAATGCTCTTGTTACTACT 5'
pT3B/MdHAXd33   TAAGATC----------------------------------TCGTTTGCTTGTCACCATTACAAAGAAACGTTATTGAAAAATGCTCTTGTTACTACT 5'
pT3B/MdHAXd67   TAAGATC------------------------------------------------------------------TGAAAAATGCTCTTGTTACTACT 5'
pT3B/MdHAX5'NA  TAAGATCTAGAGG-
                ATGGTTGGATCTGTTCTAAACCCTTTGTTCCTATTTTATTTGAACAGTTGTTCTTACTAGATTTAATTGTTTCTGAAAAAATGCTCTTGTTACTACT 5'
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FIG. 3. (a) Sequences of the 5′-terminal nucleotides of HA genes of influenza B virus strains (1, 9) aligned with that of influenza B/Lee/40 virus. Consensus is shown by capital letters, and deviations are indicated by lowercase letters or by dashes for deletions. B/Eng/82 and B/Vic/87 full-length sequences were not available. The numbering shown is for positive-sense B/Md/59 HA RNA. (b) Plasmids with sequences of influenza B virus HA mutant cDNAs with deletions or changes in the 5′ noncoding region constructed by PCR cloning methods, with appropriate primers. The 5′ noncoding sequences from the HA2 protein termination codon (underlined) to the extreme 5′ terminus are shown for each mutant. A GGA triplet near the termination codon was exchanged for nucleotides ATC in order to create a *Bgl*II restriction enzyme site which facilitated further cloning. The changed nucleotides are shown in boldface type. Plasmid pT3B/MdHAX5′NA was found to contain two additional changes upon sequencing. These are indicated in boldface type and include an additional A nucleotide in the polyadenylation signal and an A residue deleted from position 1799. Sequencing of all other plasmids (with Sequenase [U.S. Biochemicals]) confirmed that the engineered mutations were present.

the mutated noncoding sequences were able to direct replication and packaging of an influenza virus-like RNA within an infected cell. However, when we transfected derivatives of the MdHAX RNA which bore the same deletions or mutations at the 5′ noncoding region of an otherwise full-length HA gene, only viruses with 10 and 33 nucleotides deleted were recovered and no virus with 67 nucleotides deleted or with the HA sequences replaced by those from the NA gene was obtained (Fig. 3b). These transfections were repeated on five separate occasions, and at all times we were able to rescue wild-type (MdHAX) viruses but we were not successful in the recovery of the MdHAXd67 or the MdHAX5′NA mutant. This implies that loss of nucleotides 1826 to 1860 either is lethal to virus infectivity or attenuates the virus to an extent that we are unable to recover such transfectants at present. An attenuated mutant would be rescued much less efficiently than a wild-type transfectant by our negative selection system. This difference is due to the increased frequency of formation of pseudotypes in which the transfected gene is packaged within virions whose envelopes contain a high proportion of helper HA and are therefore susceptible to neutralization by the selecting antibodies.

Although we did not rescue viruses with deletions or changes beyond nucleotide 1826, derivatives of the HABCAT model RNA bearing the 67-nucleotide deletion or the 5′ NA exchange were amplified and expressed in influenza B virus-infected cells. It is possible that the gene expression from these mutants was altered by only a slight degree such that the differences were not readily observed by the CAT system but were sufficient to prevent recovery of transfectants. Alternatively, the sequences after nucleotide 1826 may harbor a signal which is absolutely required to mediate a function not measured by our model RNA system.

We verified that the MdHAXd10 and MdHAXd33 mutants bore the expected deletions in their vRNAs by a primer extension assay using end-labelled oligonucleotide 1702 to prime reverse transcription of the purified vRNAs (Fig. 4). The full-length HA template directs transcription of a 180-nucleotide product (lane 1), whereas the products obtained from MdHAXd10 and MdHAXd33 vRNAs were 10 and 33 nucleotides shorter, respectively (lanes 2 and 3). As an internal control, a primer specific for the NS gene (BNS5′SEQ) was also used in a primer extension assay with each vRNA as a template. A product 90 nucleotides long was transcribed from

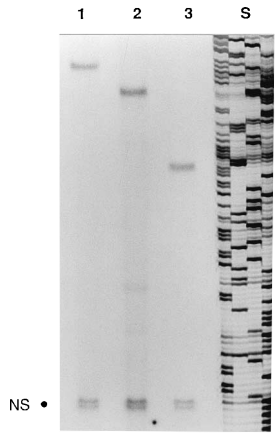

FIG. 4. Primer 1702 was end labelled with [^{32}P]phosphate by use of polynucleotide kinase. This oligonucleotide (300,000 cpm) was used as the primer for reverse transcription of 200 ng of purified vRNA, and the products were analyzed on a 7 M urea–6% polyacrylamide gel. A plasmid sequence was loaded onto the same gel as a size ladder (S). Primer NS5′SEQ (which anneals to nucleotides 950 through 967 of the NS gene [numbered as for positive-sense RNA]) was also end labelled for use in a primer extension assay. This primer anneals at a site 90 nucleotides from the 5′ terminus of the NS RNA. The doublet seen after primer extension of NS5′SEQ may indicate that the oligonucleotide was not synthesized as a pure product. The products of the two reactions for each vRNA were mixed and loaded together onto the gel. Lane 1, MdHAX; lane 2, MdHAXd10; lane 3, MdHAXd33.

all three templates, and the ratio of HA to NS product was the same for each virus. This indicates that only the HA gene has been altered in the transfectant viruses and that the novel segment is packaged at the same frequency as the wild-type HA vRNA.

We also used the vRNA from each virus as a template for reverse transcription followed by PCR amplification as described above. Direct sequencing of these PCR products confirmed that the 5′-terminal sequences of the MdHAXd10 and MdHAXd33 viruses were identical to the cDNAs used in their transfections (data not shown).

MdHAXd10 and MdHAXd33 viruses were propagated in MDCK cells and in the allantoic cavities of chicken eggs, and each attained titers which were comparable with titers of wild-type influenza B/Md/59 virus. Viruses with deletions in the 5′ noncoding region of the HA gene have not been isolated from nature even though they appear to grow well both in tissue culture and in eggs. Some attenuation of these viruses may be evident in humans. Such a finding might explain why the influenza B viruses appear to have retained this part of their genome during evolution over several decades. Indeed, recent genetic manipulations of the genomes of other small RNA viruses have shown that both negative- and positive-strand RNA viruses also retain sequences which are not essential for their replication in tissue culture (3, 7, 20).

In conclusion, the genetic manipulation of influenza B viruses is now possible. This allows us to attempt to engineer stably attenuated viruses for use as live vaccines and to investigate the potential of influenza B viruses as viral vectors. Already, we have identified a region of the genome of influenza B viruses whose length and sequence are flexible and which may be a target for the insertion of foreign sequences.

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REFERENCES

1. **Air, G. M., A. J. Gibbs, W. G. Laver, and R. G. Webster.** 1990. Evolutionary changes in influenza B are not primarily governed by antibody selection. Proc. Natl. Acad. Sci. USA **87:**3884–3888.
2. **Desselberger, U., V. R. Racaniello, J. J. Zazra, and P. Palese.** 1980. The 3′ and 5′ terminal sequences of influenza A, B, and C virus RNA segments are highly conserved and show partial inverted complementarity. Gene **8:**315–328.
3. **Dildine, S. L., and B. L. Semler.** 1989. The deletion of 41 proximal nucleotides reverts a poliovirus mutant containing a temperature-sensitive lesion in the 5′ noncoding region of genomic RNA. J. Virol. **63:**847–862.
4. **Enami, M., and P. Palese.** 1991. High-efficiency formation of influenza virus transfectants. J. Virol. **65:**2711–2713.
5. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
6. **Horvath, C. M., M. A. Williams, and R. A. Lamb.** 1990. Eukaryotic coupled translation of tandem cistrons: identification of the influenza B virus BM2 polypeptide. EMBO J. **9:**2639–2647.
7. **Iizuka, N., M. Kohara, K. Hagino-Yamagishi, S. Abe, T. Komatsu, K. Tago, M. Arita, and A. Nomoto.** 1989. Construction of less neurovirulent polioviruses by introducing deletions into the 5′ noncoding region sequence of the genome. J. Virol. **63:**5354–5363.
8. **Kingsbury, D. W.** 1990. Orthomyxoviridae and their replication, p. 1075–1091. *In* B. N. Fields and D. Knipe (ed.), Virology, 2nd ed. Raven Press, New York.
9. **Krystal, M., J. F. Young, P. Palese, I. A. Wilson, J. J. Skehel, and D. C. Wiley.** 1983. Sequential mutations in hemagglutinins of influenza B virus isolates: definition of antigenic domains. Proc. Natl. Acad. Sci. USA **80:**4527–4531.
10. **Li, S., J. L. Schulman, T. Moran, C. Bona, and P. Palese.** 1992. Influenza A virus transfectants with chimeric hemagglutinins containing epitopes from different subtypes. J. Virol. **66:**399–404.
11. **Li, X., G. Luo, M. Bergmann, and A. Garcia-Sastre.** Unpublished data.
12. **Li, X., and P. Palese.** 1994. Characterization of the polyadenylation signal of influenza virus RNA. J. Virol. **68:**1245–1249.
13. **Luo, G., W. Luytjes, M. Enami, and P. Palese.** 1991. The polyadenylation signal of influenza virus RNA involves a stretch of uridines followed by the RNA duplex of the panhandle structure. J. Virol. **65:**2861–2867.
14. **Luytjes, W., M. Krystal, M. Enami, J. D. Parvin, and P. Palese.** 1991. Amplification, expression, and packaging of a foreign gene by influenza virus. Cell **59:**1107–1113.
15. **McKimm-Breschkin, J. L.** 1994. Analysis of DOTAP- and DEAE-dextran-mediated transfection of influenza virus-derived ribonucleoprotein complexes (RNPs) by determination of CAT expression and immunofluorescence. Biochemica **11:**22–24.
16. **Muster, T., E. K. Subbarao, M. Enami, B. P. Murphy, and P. Palese.** 1991. An influenza A virus containing influenza B virus 5′ and 3′ noncoding regions of the neuraminidase gene is attenuated in mice. Proc. Natl. Acad. Sci. USA **88:**5177–5181.
17. **Parvin, J. D., P. Palese, A. Honda, A. Ishihama, and M. Krystal.** 1989. Promoter analysis of influenza virus RNA polymerase. J. Virol. **63:**5142–5152.
18. **Racaniello, V. R., and P. Palese.** 1979. Influenza B virus genome: assignment of viral polypeptides to RNA segments. J. Virol. **29:**361–373.
19. **Schulman, J. L., and P. Palese.** 1977. Virulence factors of influenza A viruses: WSN virus neuraminidase required for plaque production in MDBK cells. J. Virol. **24:**170–176.
20. **Schnell, M. J., T. Mebatsion, and K.-K. Conzelmann.** 1994. Infectious rabies viruses from cloned cDNA. EMBO J. **13:**4195–4203.
21. **Seto, D.** 1990. An improved method for sequencing double stranded plasmid DNA from minipreps using DMSO and modified template preparation. Nucleic Acids Res. **18:**5905–5906.
22. **Shaw, M. W., P. W. Choppin, and R. A. Lamb.** 1983. A previously unrecognized influenza B virus glycoprotein from a bicistronic mRNA that also encodes the viral neuraminidase. Proc. Natl. Acad. Sci. USA **80:**4879–4883.
23. **Stoeckle, M. Y., M. W. Shaw, and P. W. Choppin.** 1987. Segment-specific and common nucleotide sequences in the noncoding regions of influenza B virus genome RNAs. Proc. Natl. Acad. Sci. USA **84:**2703–2707.