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# A Single Amino Acid in the PB2 Gene of Influenza A Virus Is a Determinant of Host Range

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**The single gene reassortant virus that derives its PB2 gene from the avian influenza A/Mallard/NY/78 virus and remaining genes from the human influenza A/Los Angeles/2/87 virus exhibits a host range restriction (*hr*) phenotype characterized by efficient replication in avian tissue and failure to produce plaques in mammalian Madin-Darby canine kidney cells. The *hr* phenotype is associated with restriction of viral replication in the respiratory tract of squirrel monkeys and humans. To identify the genetic basis of the *hr* phenotype, we isolated four phenotypic *hr* mutant viruses that acquired the ability to replicate efficiently in mammalian tissue. Segregational analysis indicated that the loss of the *hr* phenotype was due to a mutation in the PB2 gene itself. The nucleotide sequences of the PB2 gene of each of the four *hr* mutants revealed that a single amino acid substitution at position 627 (Glu→Lys) was responsible for the restoration of the ability of the PB2 single gene reassortant to replicate in Madin-Darby canine kidney cells. Interestingly, the amino acid at position 627 in every avian influenza A virus PB2 protein analyzed to date is glutamic acid, and in every human influenza A virus PB2 protein, it is lysine. Thus, the amino acid at residue 627 of PB2 is an important determinant of host range of influenza A viruses.**

Most viruses exhibit a relatively restricted host range, with efficient viral replication occurring in the natural host and complete or partial restriction of viral replication occurring in other host species. Many avian influenza A viruses exhibit a host range phenotype characterized by restricted replication in the respiratory tract of nonhuman primates (10). We previously isolated a single gene reassortant (SGR) virus that derived its PB2 gene from such a virus, the avian influenza A/Mallard/NY/78 virus, and the remaining genes from the human influenza A/Los Angeles/2/87 (A/LA/2/87) virus. This reassortant virus had an unusual in vitro host range phenotype which consisted of efficient replication in avian cells (primary chick kidney [PCK] cells) and restricted replication in mammalian cells (Madin-Darby canine kidney [MDCK] cells) (4). The influenza A/Mallard/NY/78 and A/LA/2/87 parent viruses each replicated efficiently in both PCK and MDCK cells. The host range restriction (*hr*) phenotype of this PB2 SGR virus was also associated with restricted replication in vivo in the respiratory tract of squirrel monkeys and humans (4). Further study of this virus was undertaken in an attempt to identify the genetic basis for this *hr* phenotype. Four phenotypic *hr* mutants of the PB2 SGR virus that had acquired the ability to replicate efficiently in mammalian cells were generated independently, and a single nucleotide substitution in the PB2 gene that predicted a single amino acid substitution was identified as being responsible for loss of the *hr* phenotype.

## MATERIALS AND METHODS

**Viruses.** The isolation and biological cloning of the PB2 SGR virus that derives its PB2 gene from the avian influenza virus A/Mallard/NY/6750/78 and remaining genes from the human influenza virus A/LA/2/87 has been described previously (4). This virus as well as the two parental strains were grown in 9-day-old specific-pathogen-free eggs. The parental origin of the PB2 gene of the PB2 SGR virus was confirmed by sequence analysis as described (4).

**Isolation of the *hr* mutants of the PB2 SGR virus.** Four *hr* mutants of the PB2 SGR virus were generated independently by passage of the PB2 SGR virus in MDCK cells. The first *hr* mutant was recovered by direct inoculation of a monolayer of MDCK cells with the PB2 SGR virus at a multiplicity of infection of 0.1. The virus present in this culture supernatant, which was harvested 48 h after inoculation and subsequently passaged twice, contained virus that produced plaques efficiently on MDCK cells. This virus was biologically cloned by plaque purification three times on MDCK cells. Three additional *hr* mutants were generated by first deriving three independent suspensions of the PB2 SGR virus by terminally diluting the virus in the allantoic cavity of 9-day-old embryonated eggs. Each of these three biologically cloned virus suspensions manifested the host range phenotype of greatly restricted replication in MDCK cells. The allantoic fluid from these terminal dilutions were then inoculated onto monolayers of MDCK cells at a multiplicity of infection of 0.007 to 0.02, and virus present in the culture supernatants was plaque purified three times on MDCK cells as for the first *hr* mutant. The *hr* mutants were designated 1 through 4 and were amplified in the allantoic cavity of 9-day-old embryonated eggs.

**Growth characteristics of the *hr* mutant viruses.** The in vitro growth characteristics of the PB2 SGR virus, the avian virus parent A/Mallard/NY/78, the human virus parent A/LA/2/87, and the four *hr* mutant viruses were compared in monolayers of PCK and MDCK cells prepared as previously described (10).

**Segregational analysis.** Segregational analysis was carried out by separately coinfecting PCK cells with each of the four *hr* mutant viruses and the A/LA/2/87 wild-type virus each at a multiplicity of infection of 5. Culture supernatants were harvested at 24 h, and the virus suspension was plaqued on

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monolayers of PCK cells. Between 80 and 100 plaques were picked for each of the reassortments; for comparison, a similar number of plaques was also picked for the A/LA/2/87 and the PB2 SGR parent viruses. Each plaque was amplified in the allantoic cavity of two 9-day-old embryonated eggs, and the infectivity of each of the plaque progeny in MDCK cells was measured. Allantoic fluid suspensions that had a hemagglutination titer of ≥1:2 and that exhibited restricted replication ($<10^{3.0}$ 50% tissue culture infectious doses [$TCID_{50}$]/ml) in MDCK cells were considered to have the *hr* phenotype.

**Cloning and nucleotide sequence analysis of the PB2 gene of the PB2 SGR and *hr* mutant viruses.** cDNA copies of the PB2 virion RNA were produced in three overlapping segments from a purified viral RNA template by the polymerase chain reaction (PCR), using a programmable thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.) as previously described (5). Oligonucleotide primers were designed with an *Eco*RI site for use in cloning. Synthetic oligonucleotide primers used for PCR and sequencing were prepared in an Applied Biosystems model 380A DNA synthesizer. Each of the PCR products was purified from low-melting-point agarose gels, digested with *Eco*RI, and cloned separately into the *Eco*RI site in the polylinker region of plasmid pUC19 (Life Technologies, Inc., Gaithersburg, Md.). The plasmid was then transformed into maximum efficiency *Escherichia coli* DH5α (Life Technologies). Plasmid DNA was purified from 125-ml cultures of the transformed *E. coli*, using Qiagen maxiprep kits (Qiagen, Chatsworth, Calif.). Dideoxy-chain termination sequencing of the plasmid DNA was carried out by using 12 oligonucleotide primers, [$^{35}$S]dATP αS (Amersham Corp., Arlington Heights, Ill.), and the Sequenase kit (U.S. Biochemical, Cleveland, Ohio) as previously described (11). The terminal 50 nucleotides were sequenced directly from PCR-derived cDNA. Nucleotide substitutions were confirmed by directly sequencing an independently derived PCR product from each of the viruses.

**Comparison of the level of replication of parental, reassortant, and *hr* mutant viruses in the respiratory tract of squirrel monkeys.** The level of replication of the PB2 SGR virus and the A/LA/2/87 parent virus in the respiratory tract of squirrel monkeys has been previously described (4). The level of replication of each of the four *hr* mutant viruses was compared with the levels of replication of these two viruses. An inoculum of $10^{7.0}$ $TCID_{50}$ of each virus was administered intratracheally on day 0; the upper respiratory tract was sampled daily by obtaining nasal washes for 10 days, and the lower respiratory tract was sampled by tracheal lavage on days 2, 4, and 6. Virus titrations of the respiratory tract secretions were carried out in MDCK cells or embryonated eggs as described elsewhere (3, 15).

**Nucleotide sequence accession number.** The nucleotide sequence for the PB2 gene of the PB2 SGR virus has been submitted to the GenBank nucleotide sequence data base and assigned accession number L02352.

## RESULTS

**Isolation and characterization of the *hr* mutant viruses.** The PB2 SGR virus has the property of efficient replication in avian tissue such as eggs and PCK cells but is restricted in replication in mammalian cells such as MDCK cells. This phenotype is referred to as the *hr* phenotype. Four *hr* mutant viruses were generated independently from the PB2 SGR virus by passage in MDCK cells. Each of the four *hr* mutant viruses acquired the ability to replicate efficiently in MDCK cells (Table 1) and was analyzed further.

TABLE 1. Levels of replication in avian and mammalian cells of influenza A parental, host range-restricted reassortant viruses, and their *hr* mutants

| Virus | Titer in PCK cells ($log_{10}$ $TCID_{50}$/ml) | Titer in MDCK cells ($log_{10}$ $TCID_{50}$/ml) |
|---|---|---|
| Parental | | |
| A/Mallard/NY/78 | 7.2 | 7.9 |
| A/LA/2/87 | 7.5 | 8.0 |
| Reassortant PB2 SGR (A/Mallard/NY/78 × A/LA/2/87) | 7.3 | <3.0 |
| *hr* mutant | | |
| 1 | 6.8 | 6.8 |
| 2 | 7.5 | 7.8 |
| 3 | 7.3 | 6.5 |
| 4 | 7.0 | 7.5 |

**Segregational analysis of the *hr* mutant viruses.** Segregational analysis was carried out to determine whether the new phenotype occurred as a result of an extragenic mutation or an intragenic mutation in the PB2 SGR virus. If the new phenotype was due to an extragenic mutation, approximately 25% of the progeny viruses resulting from reassortment of the virus with the human A/LA/2/87 parent virus would be expected to exhibit the *hr* phenotype. On the other hand, if the new phenotype resulted from a mutation in the PB2 gene itself, the mutation could not be segregated from the gene conferring the *hr* phenotype, and therefore, each of the progeny viruses of the reassortment described above would retain the *hr* mutant (i.e., non-host range restricted) phenotype. None of the 80 to 98 plaque progeny obtained from separate reassortment of each of the four *hr* mutant viruses with the human A/LA/2/87 parent virus were restricted in replication in MDCK cells, indicating that the new phenotype in each instance resulted from a mutation in the PB2 gene.

**Comparison of the low level of replication of the PB2 SGR virus and the four *hr* mutant viruses in the respiratory tract of squirrel monkeys.** In addition to the phenotype of restricted replication in mammalian cells in vitro, the PB2 SGR virus was markedly restricted in replication in the respiratory tract of nonhuman primates (squirrel monkeys) and adult human volunteers (4). We evaluated each of the *hr* mutant viruses for the level of replication in squirrel monkeys in order to determine whether the *hr* phenotype in vitro was linked to restriction of replication in the respiratory tract of monkeys. The level and duration of virus replication of each of the four *hr* mutant viruses was intermediate between that of the PB2 SGR virus from which they were derived and their human A/LA/2/87 virus parent (Table 2).

**Identification of the site of the intragenic mutation.** PB2 cDNA was generated from the PB2 SGR virus and its four *hr* mutant viruses by PCR. These cDNAs were cloned and sequenced in their entirety (2,318 nucleotides). The PB2 genes of the four *hr* mutant viruses were identical, and they differed from the sequence of the PB2 SGR virus by a single nucleotide substitution of an A for a G at position 1886. This change resulted in a substitution of lysine for glutamic acid at amino acid position 627 in the predicted amino acid sequence (Table 3). Each nucleotide substitution was confirmed by sequencing an independently derived PCR product from each of the *hr* mutant viruses and the PB2 SGR virus.

TABLE 2. Comparison of the level of viral replication of the A/LA/2/87 wild-type, PB2 SGR, and *hr* mutant viruses in the respiratory tract of squirrel monkeys

| Virus | No. of monkeys | Upper respiratory tract | | Lower respiratory tract | |
|---|---|---|---|---|---|
| | | Mean peak virus titer ± SE ($log_{10}$ $TCID_{50}$/ml) | Mean duration of virus shedding (days) | Mean peak virus titer ± SE ($log_{10}$ $TCID_{50}$/ml) | Mean duration of virus shedding (days) |
| A/LA/2/87 (wild type)[a] | 6 | 3.5 ± 1.1 | 8.2 ± 1.6 | 3.3 ± 1.4 | 5.7 ± 2.3 |
| *hr* mutant | | | | | |
| 1 | 4 | 2.2 ± 0.9 | 5.0 ± 3.2 | 3.2 ± 0.8 | 2.5 ± 1.0 |
| 2 | 4 | 2.6 ± 0.5 | 4.8 ± 2.2 | 2.3 ± 0.3 | 4.0 ± 1.6 |
| 3 | 4 | 2.3 ± 0.5 | 6.8 ± 1.9 | 1.8 ± 0.9 | 3.5 ± 2.5 |
| 4 | 4 | 2.8 ± 0.5 | 7.0 ± 1.2 | 2.0 ± 1.1 | 4.0 ± 2.8 |
| PB2 SGR[a] | 6 | 0.9 ± 0.9 | 1.3 ± 3.3 | 0.8 ± 0.7 | 0.7 ± 1.6 |

[a] Four of the six monkeys reported here were studied and reported previously (4).

The PB2 gene of the SGR virus is derived from the avian influenza A/Mallard/NY/78 virus. Twenty-six differences from the published sequence of the PB2 gene of the A/Mallard/NY/78 virus (16) were identified in the PB2 SGR parent virus, and these differences were also present in the four *hr* mutant viruses. There are two possible explanations for the differences from the published sequence of the PB2 gene of A/Mallard/NY/78 virus. First, the PB2 SGR virus was derived after multiple crosses of the A/Mallard/NY/78 virus and the A/LA/2/87 wild-type virus (4), and some of the changes may have been introduced during successive passages involved in preparing suspensions of SGR viruses. Second, the original nucleotide sequence of the genes of the A/Mallard/NY/78 virus was determined by direct sequencing of viral RNA, while the sequence reported here was obtained and confirmed by analysis of cDNA, which often provides less ambiguous results.

TABLE 3. Comparison of amino acid sequence at position 627 in the PB2 genes of a variety of influenza viruses

| Virus | Efficient replication in MDCK cells | Amino acid at position 627[a] |
|---|---|---|
| Avian influenza A | | |
| A/FPV/Rostock/34 (H7N7) | + | Glutamic acid |
| A/Turkey/MN/833/80 (H4N2) | + | Glutamic acid |
| A/Seal/MA/133/82 (H4N5) | + | Glutamic acid |
| A/Mallard/NY/6750/78 (H2N2) | + | Glutamic acid |
| A/Ruddy Turnstone/NJ/47/85 (H4N6) | + | Glutamic acid |
| A/Budgerigar/Hokkaido/1/77 (H4N6) | + | Glutamic acid |
| A/Gull/MD/704/77 (H13N6) | + | Glutamic acid |
| A/Gull/Astrakhan/227/84 (H13N6) | + | Glutamic acid |
| Avian-human reassortant influenza | | |
| PB2 SGR (H3N2) | − | Glutamic acid |
| hr mutant 1 (H3N2) | + | Lysine |
| hr mutant 2 (H3N2) | + | Lysine |
| hr mutant 3 (H3N2) | + | Lysine |
| hr mutant 4 (H3N2) | + | Lysine |
| Human influenza A | | |
| A/WSN/33 (H1N1) | + | Lysine |
| A/PR/8/34 (H1N1) | + | Lysine |
| A/Chile/1/83 (H1N1) | + | Lysine |
| A/Singapore/1/57 (H2N2) | + | Lysine |
| A/Ann Arbor/6/60 (H2N2) | + | Lysine |
| A/Korea/426/68 (H2N2) | + | Lysine |
| A/NT/60/68 (H3N2) | + | Lysine |
| A/Udorn/72 (H3N2) | + | Lysine |
| A/Vic/75 (H3N2) | + | Lysine |
| A/Memphis/8/88 (H3N2) | + | Lysine |
| Human influenza B/Ann Arbor/1/66 | + | Lysine |
| Human influenza C/JJ/50 | + | Lysine |

[a] Data from reference 6 and this report.

## DISCUSSION

Previous studies identified the PB2 gene as a determinant of the host range of certain avian influenza A viruses that are restricted in replication in mammalian cells (1, 2, 9, 12). The PB2 gene of the influenza A virus was first identified as a single gene that controlled the host range of an avian influenza virus, fowl plaque virus (1, 2, 9). We have extended these studies and identified a site on the PB2 gene that is associated with host range phenotype.

The PB2 SGR virus that derives its PB2 gene from the avian influenza virus A/Mallard/NY/78 and remaining genes from the human influenza A/LA/2/87 virus was previously reported to possess the unusual phenotype of efficient replication in avian tissue and restricted replication in mammalian cells (4). A similar phenotype was also observed for a reassortant virus that derived its PB2, NP, and M genes from the avian virus A/Pintail/Alberta/119/79 (H4N6) and remaining genes from the human influenza virus A/Washington/897/80 (H3N2), suggesting that this *hr* phenotype could result from an interaction of any avian PB2 gene with genes encoding other polymerase proteins of human influenza A virus origin (3, 4, 14). An intragenic mutation that was responsible for the loss of the host range restriction of A/Mallard/NY/78 × A/LA/2/87 PB2 SGR was a single nucleotide substitution at position 1886 that results in a predicted amino acid change from glutamic acid to lysine at amino acid 627. The importance of this amino acid substitution was apparent when the predicted amino acid sequences of the PB2 gene products of several avian and mammalian influenza viruses were compared (Table 3). Each of the avian influenza viruses, including the A/Mallard/NY/78 and A/Pintail/Alberta/119/79 viruses, have a glutamic acid at position 627, while all of the mammalian influenza A viruses for which sequence information was available with the exception of equine influenza A viruses had a lysine at this position (6). In fact, lysine at this position was conserved in H1N1, H2N2, and H3N2 influenza A viruses from 1933 to 1975 as well as among influenza B and C viruses (Table 3). The four *hr* mutant viruses that had lost the *hr* phenotype each had sustained a lysine substitution at amino acid position 627, suggesting that the host range restriction resulting from the interaction of the avian PB2 gene and other human influenza A genes could be reversed by substituting a lysine for glutamic acid at position 627. Our results have implications that could explain the observations that antigenic shift

viruses of 1957 and 1968 acquired their PB1 genes from avian viruses, while their PB2 genes were derived from the human influenza A virus parent (7, 13). Reassortant viruses that derived their PB2 genes from the avian virus parent may have been selected against because of inefficient replication in mammalian cells.

In an attempt to establish the genetic basis for attenuation of avian-human reassortant influenza A viruses, we evaluated a series of SGR viruses that derived a single non-surface glycoprotein gene from the avian virus and remaining genes from the human influenza A/LA/2/87 virus (4). We found that the PB2 SGR virus was highly restricted in replication in the respiratory tract and was attenuated for nonhuman primates and adult humans (4). The observation that the four *hr* mutant viruses in the present study exhibited a level and duration of replication in the respiratory tract of squirrel monkeys that was intermediate between those of the PB2 SGR and the human influenza virus parent, A/LA/2/87, indicates that the PB2 SGR has two separate mechanisms leading to attenuation of the virus for squirrel monkeys. First, the host range phenotype was specified by the constellation of an avian PB2 gene in the context of human PB1 and PA genes, and this appeared to be the major genetic determinant of growth restriction. Second, the finding that the PB2 SGR *hr* mutants that had lost the host range phenotype were still partially restricted in replication in squirrel monkeys indicated that other nucleotide or amino acid sequence differences in the PB2 genes of the avian and human viruses tested also contributed to attenuation.

The *hr* phenotype of the PB2 SGR virus is of particular interest for two reasons. First, such reassortant viruses have several characteristics that are desirable in a live virus vaccine, namely, efficient replication in the substrate used for vaccine production (i.e., the allantoic cavity of embryonated hen eggs) but restricted replication in vivo. However, reassortant viruses that are attenuated solely by gene incompatibility of an avian influenza A virus PB2 gene in the context of other human influenza A virus genes would seem to have two serious limitations for use as a live, attenuated virus vaccine: (i) overattenuation and (ii) instability of the attenuation phenotype documented in this study. Second, the *hr* phenotype makes the PB2 SGR virus a candidate for use as a helper virus in experiments designed to rescue a synthetic human PB2 gene (8). The level of the genetic stability of this phenotype currently remains a problem in our attempts to rescue a synthetic PB2 gene. The identification of the site of the mutation, however, may allow us to generate a more stable host range-restricted virus for future use. This study clearly identifies amino acid 627 of the PB2 protein as an important determinant of host range and virulence of influenza A viruses for primates.

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