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Characterization of a Cold-Sensitive (cs) Recombinant between Two Influenza A Strains

ASTRID BREUNING AND CHRISTOPH SCHOLTISSEK

Institut für Virologie, Justus-Liebig-Universität Giessen, D-6300 Giessen, Federal Republic of Germany

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Recombinants between fowl plague virus (FPV, H7N1) and the Hong Kong (H3N2) or Singapore (H2N2) influenza virus strains carrying the hemagglutinin of FPV and the neuraminidase of the human strains form only very tiny plaques at 33°C, but normal plaques at 37°C. One recombinant (113/Ho) has been studied in more detail. It multiplies only very slowly at 33°C, the nonpermissive temperature. Adsorption and penetration are normal at 33°C, but synthesis of protein is impeded. Temperature-shift experiments suggest that the synthesis of viral mRNA is slowed at 33°C. 113/Ho does not agglutinate chicken erythrocytes at 40°C, as the parent viruses do. 113/Ho can be adapted to grow normally at 33°C. The frequency of adaptation is comparable to reversion of a single point mutation (ca. 10⁻⁵). Recombinants which grow well at 37°C but not at 33°C are called cold-sensitive (cs) recombinants.

INTRODUCTION

Temperature-sensitive (ts) mutants and recombinants of influenza A viruses have been isolated and characterized in several laboratories. These mutants and recombinants multiply at low temperatures as well as the wild-type strains, but are restricted in multiplication at temperatures around 40°C. With these isolates the functional significance of various genes has been studied (for a review see Mahy, 1983).

In this communication we will describe and characterize recombinants which are restricted in multiplication at 33°C, while they multiply to normal titers at 37 or 40°C. By analogy to ts recombinants, we call these isolates cold-sensitive (cs) recombinants. Cs mutants of poliovirus (Wright and Cooper, 1973), of a murine sarcoma virus (Somers and Kit, 1973), and of a murine coronavirus (Stohlman et al., 1979) have been isolated and their properties have been described.

MATERIALS AND METHODS

Virus strains, recombinants, and cells. The following virus strains were investigated: A/FPV/Rostock/34, H7N1, plaque isolate 145 (F); A/PR/8/34, H1N1 (P); A/Singapore/1/57, H2N2 (S); A/Hong Kong/1/68, H3N2 (Ho); A/chick/Germany/"N"/49, H10N7 (N); A/equine/Miami/68, H3N8, Eq2 (E); A/swine/1976/31, H1N1 (Sw); A/turkey/England/63, H7N3 (T). Recombinants between fowl plague virus (FPV) and these strains were obtained by double infection of chick embryo cells either with specific ts mutants of FPV or FPV wild-type and the other prototype strains, and extended plaque purifications as described by Scholtissek et al. (1976) and Rott et al. (1979). They are listed in Table 1.

Primary chick embryo cells were used for multiplication of viruses and for most of the plaque tests. In a few experiments MDCK cells were also investigated.

Biological tests. Hemagglutinating activity was determined with 1% chicken erythrocytes either at room temperature or at 40°C. Neuraminidase activity was determined according to Seto and Rott (1966) at 33, 37, or 40°C. Plaque tests were carried out in chick embryo cells at different temperatures (Klenk et al., 1972). The hemadsorption test was performed according to Nakamura and Homma (1974).

To whom reprint requests should be addressed.
### TABLE 1

| Strains | Derivation of vRNA segments | Plaque morphology (maximum size in mm) |
|---------|-----------------------------|--------------------------------------|
|         | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 33°C | 37°C | 40°C |
| FPV     | F  | F  | F  | F  | F  | F  | F  | F  | 2.5, clear | 6, clear | 6, clear |
| 3/S 1   | S  | F  | F  | F  | F  | F  | F  | F  | 2, clear | 4.5, clear | 5, clear |
| 90/S 1  | S  | S  | F  | F  | F  | F  | F  | F  | 2, clear | 5, clear | 5, clear |
| 90/S 2  | F  | S  | F  | F  | F  | F  | F  | F  | 2, clear | 5, clear | 5, clear |
| 263/S 1 | S  | S  | S  | F  | F  | F  | F  | F  | 1.5, clear | 5, clear | 3, clear |
| 263/S 2 | F  | F  | S  | F  | F  | F  | F  | F  | 2, clear | 5, clear | 3, clear |
| 263/S 3 | F  | F  | S  | F  | F  | S  | F  | S  | 1, turbid | 4, clear | 3, clear |
| 263/S 4 | S  | F  | S  | F  | F  | S  | F  | S  | 0.5, clear | 3, clear | 2, clear |
| 113/S 1 | S  | F  | F  | F  | F  | S  | F  | F  | Micro, clear | 6, clear | 5, clear |
| 113/P 1 | P  | F  | F  | F  | F  | F  | F  | F  | 0.5, clear | 5, clear | 4, clear |
| 113/P 2 | F  | F  | F  | F  | F  | F  | F  | F  | 0.5, clear | 3, clear | 2, clear |
| 113/P 3 | F  | F  | F  | F  | F  | F  | F  | F  | 1, clear | 3, clear | 1, clear |
| T/3     | F  | T  | T  | F  | F  | T  | F  | T  | 2, clear | 5, clear | 2, clear |
| 113/N   | F  | F  | F  | F  | F  | N  | F  | F  | 2, turbid | 5, clear | 3, clear |
| 113/Sw 2| F  | F  | F  | F  | Sw | F  | F  | F  | 2, clear | 5, clear | 5, clear |
| 113/E 1 | F  | F  | F  | F  | F  | E  | F  | F  | 1, clear | 6, clear | 4.5, clear |
| 113/E 2 | E  | F  | F  | F  | F  | E  | F  | F  | 0.5, clear | 6, clear | 5, clear |
| KE t    | F  | F  | F  | F  | E  | F  | E  | E  | 0.3, clear | 2.5, clear | 0.5, turbid |
| 3/Ho 5  | Ho | F  | F  | F  | F  | F  | F  | F  | 2, clear | 3.5, clear | 3.5, clear |
| 90/Ho 1 | F  | Ho | F  | F  | F  | F  | F  | F  | 2.5, clear | 3, clear | 2, clear |
| 263/Ho 3| F  | F  | Ho | F  | F  | F  | F  | F  | 1, clear | 0.5, clear | 2, clear |
| 113/Ho 1| Ho | F  | F  | F  | F  | Ho | F  | F  | Micro, clear | 5, clear | 4, clear |

*The morphology of the plaques was determined after 3 days incubation at the corresponding temperature. Between 50 and 100 plaques of each recombinant were examined. Because of chance delay the plaque population is not completely homogeneous. Therefore, the maximum sizes, which are the sizes of about 50% of the plaques examined, are listed. The error width of the measurement is between 20 and 40%, depending on the plaque size.

Most of the recombinants were obtained by rescue of specific ts mutants of FPV (F) (represented by the first number) by corresponding prototype strains (Scholtissek et al., 1976). S = A/Singapore/1/57 H2N2; P = A/PR/8/34 (H1N1); N = A/chick/Germany "N"/49 (H1N7); Sw = A/swine/1976/31 (H3N1); E = A/equine/Miami/68 (H3N8); Ho = A/Hong Kong/1/68 (H3N2). T3 and KEt were recombinants obtained after double infection of chick embryo cells with A/turkey/England/63 (H7N3) or A/equine/Miami/68, respectively, and FPV, and picking plaques at random (Rott et al., 1979).

The vRNA segments 1, 2, and 3 code for the three polymerase proteins PB2, PB1, and PA, respectively. Segment 4 = hemagglutinin gene; segment 5 = nucleoprotein gene, segment 6 = neuraminidase gene; segment 7 = membrane protein gene; segment 8 = nonstructural protein gene.

#### Labeling of viral proteins by [³⁵S]methionine and polyacrylamide gel electrophoresis.

Chick embryo cells were infected with 10 to 50 PFU/cell under different temperature conditions. At 4 hr or later after infection 40 µCi [³⁵S]methionine (800 Ci/mmol; Amersham, England) per culture was added. Three hours thereafter cells were processed and the proteins separated by polyacrylamide gel electrophoresis (Bosch et al., 1979).

#### RESULTS

**Plaque Morphology of FPV and Recombinants**

When plaque tests were performed on chick embryo cells at 37 or 40°C with recombinants between FPV and other prototype influenza virus strains it was found that the plaque morphology of most of these recombinants was similar to that of FPV. However, at 33°C, 2 out of 21 recom-
binants selected formed only microplaques. It is shown in Fig. 1 and Table 1 that these recombinants carry the neuraminidase (NA) of the N2 serotype (S and Ho) and the hemagglutinin (HA) of FPV. These recombinants also have, in addition to the NA genes, RNA segment 1 derived from the Hong Kong or Singapore strains, respectively. Unfortunately, there is no recombinant available in which only RNA segment 6 of FPV is replaced by the corresponding gene of the Hong Kong or Singapore strains. However, recombinants in which only RNA segment 1 is replaced form plaques at 33° of 2 mm in diameter (Table 1). Thus, replacement of segment 1 by itself is not responsible for microplaque formation, although the overall gene constellation has a certain influence on the plaque morphology (see Table 1). Furthermore, several independently isolated recombinants with gene constellations identical to 113/Ho 1 and 113/S 1 all were able to produce only microplaques at 33° and normal plaques at 37° (not shown here).

In the following, the isolate 113/Ho has been studied in more detail. If an allantoic fluid obtained after infection with 113/Ho at 37° is titrated at 33° and at 37°, the

![Plaque morphology of FPV and recombinants thereof at 33°. Plaque tests were performed on chick embryo cells in dishes of 5 cm in diameter. The cells were stained with neutral red after 3 days incubation at 33°.](image)
plaque titer at 37°C is up to a factor of 100 higher than at 33°C. Furthermore, at 33°C the plaque number does not correlate with the dilution. For example, when at a given dilution the number of countable plaques was about 200, in the next dilution by a factor of 10 only between 2 and 5 plaques were visible, although 20 plaques had been expected. These observations suggest that most of the plaques at 33°C are so tiny that they cannot be seen by the naked eye. Only if several plaques are located close enough to fuse to a larger one do they become visible.

Indeed, if the plaque test at 33°C was read not after 3 days but after 6 days, the number of plaques increased by a factor of at least 10. However, under these conditions the plaques became somewhat heterogeneous, which possibly is due to the appearance of "adapted" virus particles within a plaque. If after incubation at 37°C plaques were picked, these viruses again formed microplaques at 33°C.

The same observation concerning the plaque morphology of 113/Ho and 113/S at the various temperatures was made when MDCK cells were investigated. Thus, the choice of the host cell seems not to play an important role in this phenomenon.

Selection of Virus from the 113/Ho Stock, which Forms Normal Plaques

If chick embryo cells were infected with 113/Ho at a multiplicity of <1 PFU/cell,

| TABLE 2 |
|-------------------|-------------------|-------------------|
| **YIELD AND PLAQUE MORPHOLOGY OF DILUTED PASSAGES OF 113/Ho AT 33°C FOR 36 HR** |
| Plaque test no. | Passage | Maximal plaque diameter (mm) |
| --- | --- | --- |
| 33°C | 1 | 1 x 10² | 2 mm, clear |
| | 2 | 3 x 10⁴ | 2 mm, clear |
| | 3 | 2.5 x 10⁶ | 2 mm, clear |
| 40°C | 4 | 4.5 x 10² | 4 mm, clear |
| | 5 | 5 x 10⁴ | 4 mm, clear |
| | 6 | 3.5 x 10⁶ | 4 mm, clear |

*For the first passage, the multiplicity of infection was <1 PFU/cell. After 36 hr at 33°C, the supernatant fluid was diluted 1:20 for the next passage, etc.*
diameter of 2 mm. In the other seven fluids too many microplaques were seen at $10^5$ infectious particles per dish to recognize plaques of normal size at $33^\circ$. Thus, the frequency of adaptation is roughly the same (ca. $10^{-5}$) as the rate of reversion of a ts point mutation (Scholtissek and Spring, 1981).

**Multiplication of 113/Ho**

In a single-cycle multiplication experiment (infection at a multiplicity of 10-50 PFU per cell) at $33^\circ$, the yield of infectious 113/Ho was very low when compared with the multiplication at $37^\circ$ or with the adapted strain or FPV (Fig. 2). Also, by the hemadsorption test after infection with 113/Ho at $33^\circ$ no significant hemagglutinin could be detected in the plasma membrane at 6 hr after infection. At 37 or 40$^\circ$ the hemagglutinin expressed at the cell surface was the same as after infection with the parent viruses or 113/Ho ad. at any of the three temperatures (not shown).

In a multiple-cycle experiment (infection at a multiplicity of <1 PFU per cell), the results are very similar (Fig. 3). There is not only a retardation in the multiplication of 113/Ho at $33^\circ$, but also the final yield is very low.

**Temperature Shift Experiments**

First it was examined whether the rate-limiting step at $33^\circ$ during the multiplication of 113/Ho is adsorption and/or penetration. Therefore, chick embryo cells were infected with 113/Ho either at $33^\circ$ or at $37^\circ$. After half an hour of incubation, they were washed with ice-cold 0.9% NaCl at pH 2.5 to inactivate most of the virus.

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**Figure 3.** Multiplication of 113/Ho, 113/Ho ad., and FPV at $33^\circ$ and $37^\circ$ in a multiple-cycle experiment. Cultures were infected with <1 PFU/cell. At different times after infection (abscissa) the cultures were processed and the virus titrated at $37^\circ$. For further details see the legend to Fig. 2.
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at the surface which had not yet penetrated (N. Dimmock, personal communication; Koennecke et al., 1981). After two further washings with PBS, the cells were incubated at either 33 or 37° or were shifted after 1 hr incubation at 37° down to 33° according to the schedule outlined in Table 3. Ten hours later, the HA titers and PFU were determined. As can be seen in Table 3, if cells were infected at 33° and after washing at pH 2.5 were further incubated at 37° the yield was the same as if the cells were incubated all the time at 37°. If the cells were incubated for 1 hr at 37° immediately after infection at 33° and further incubated at 33°, the yield was as low as if the cells were incubated all the time at 33°. These results indicate that adsorption and penetration proceed normally at 33°, but a step thereafter is impeded at 33°.

One observation deserves attention: If 113/Ho is grown in chick embryo cells in culture at 37°, HA can be detected by the hemagglutination test despite the fact that the infectivity titer is relatively low (Table 3, upper part, lines a and c). This implies either that more HA is synthesized compared with infectious particles, or that the quality of HA is different from that of the parent strain under identical conditions.

Next, protein synthesis has been studied by incorporation of [35S]methionine into viral proteins. As can be seen in Fig. 4, in 113/Ho-infected cells at 33° very little protein is labeled when compared with cells infected with the adapted strain or the parent viruses. About 10 times more isotope has to be applied in order to get a comparable incorporation (not shown here). Under these conditions, it was found that at 33° the HA of 113/Ho is cleaved into HA1 and HA2. Labeling of 113/Ho protein did not increase if the [35S] pulse was applied later in the infectious cycle (not shown here). These results indicate that a step before virus maturation is impeded at 33° in 113/Ho-infected cells, since labeling of viral proteins is already slowed down at this temperature.

To further define the step which is slowed down at 33°, corresponding temperature shift experiments have been performed. After infection, the cells were kept for 4 hr at either 33 or 37° prior to the pulse at 37 or 33° for 3 hr, respectively. As can be seen in Fig. 5, if cells infected by 113/Ho were kept at 33° and the pulse was given at 37°, the viral proteins were much less labeled when compared with conditions the other way around. For comparison, data on the adapted strain are included. The results suggest that at 33° the synthesis of viral mRNA is slowed down rather than that of viral protein itself. Otherwise, after preincubation at 33° and pulse at 37° labeling of viral proteins should not have been impeded.

**Neuraminidase and Hemagglutination Activities of 113/Ho**

Since all recombinants tested with the surface antigen composition H7N2 formed only micro, tiny, or turbid plaques at 33°, the neuraminidase and hemagglutination activities of 113/Ho have been compared with those of FPV and the adapted strain. When the neuraminidase activity tested at different temperatures was correlated to the HA titers or PFU of egg-grown virus, no significant differences between these

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**Table 3**

**Multiplication of 113/Ho and FPV under Temperature-Shift Conditions**

| Condition     | PFU at 37° | HA units |
|---------------|------------|----------|
| **113/Ho**    |            |          |
| a             | $1.2 \times 10^6$ | 4        |
| b             | $5 \times 10^6$  | 0        |
| c             | $7.5 \times 10^4$ | 4        |
| d             | $1 \times 10^4$  | 0        |
| **FPV**       |            |          |
| a             | $8 \times 10^6$  | 32       |
| b             | $5 \times 10^6$  | 0        |
| c             | $5.5 \times 10^4$ | 32       |
| d             | $2.5 \times 10^4$ | 4        |

*Notes.* Cells were infected with 10 to 50 PFU/cell. (a) Infection at 37°, incubation at 37°; (b) Infection at 33°, incubation at 33°; (c) Infection at 33°, incubation at 37°; (d) Infection at 33°, incubation for 1 hr at 37°; thereafter shift to 33°. All cells were treated at pH 2.5 after infection and processed at 10 hr after infection.
strains were found. However, if the hemagglutination test was performed at 40° with 113/Ho no agglutination of chicken erythrocytes was visible, in contrast to the adapted strain, or FPV, or Ho. Furthermore, erythrocytes once agglutinated at room temperature by 113/Ho form a central pellet in the cups of the test plate after about 1 hr, depending on the virus dilution, while the agglutination patterns of the other strains stay stable for at least 6 hr or longer. Since there is no difference in neuraminidase activity, it is suggested that, when compared with the other strains, 113/Ho crosslinks red blood cells less firmly after release of some neuraminic
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acid from the cell receptors. Thus, the observed difference between 113/Ho and the parent strains seems to be related, rather, to the hemagglutinin moiety.

DISCUSSION

Recombinants and mutants of influenza viruses which multiply well at low but not at the elevated temperatures have been isolated in many laboratories. It might be assumed that certain gene products of these isolates either by themselves or in cooperation with other gene products are impeded in their function by some kind of a melting process or conformational changes at the elevated temperature. However, there are no reports on influenza virus isolates with the opposite property; this means normal multiplication at body temperature but restriction at lower temperatures when compared with the parent strains. Such isolates, if they exist, are expected to have defects in the function of glycoproteins or the membrane (M) protein, which interact with lipid bilayers during their multiplication or assembly, since membranes become more rigid at low temperatures.

In this communication, we describe recombinants between FPV and human influenza A strains carrying a N2 neuraminidase which multiply normally at 37°C but only very slowly at 33°C when compared with the parent strains. This property seems to be related to a specific combination of the surface glycoproteins, H7N2. Recombinants with this gene constellation produce only very tiny or turbid plaques at 33°C (see Table 1). Recombinants between FPV and the Hong Kong strain (113/Ho) have been studied in more detail. At 33°C, they adsorb and penetrate normally, although the hemagglutinin of these recombinants binds less firmly to red blood cells. Since this latter property is more strongly expressed at elevated temperature, it is not clear whether it is rather circumstantial and not related to the slower multiplication of 113/Ho at low temperatures.

At 33°C labeling of viral proteins of 113/Ho is significantly slowed down. However, since after preincubation at 33°C and pulse at 37°C viral proteins are much less labeled than under conditions the other way around (Fig. 5), it is suggested that viral mRNA synthesis is impeded at low temperatures rather than that of viral proteins. The possible impairment of viral mRNA synthesis at 33°C is difficult to explain. Interaction of viral glycoproteins during their synthesis on internal membranes might play a role in the regulation of the synthesis of viral mRNA in a way which is not yet understood.

It is possible that during reassortment a new "gene constellation" is generated, as in the case of 113/Ho, in which an intrinsically cs protein that has been suppressed on the parental strain is now allowed to express a cs phenotype. The recombinant 113/Ho can easily be adapted to grow normally at 33°C. The frequency of adaptation corresponds roughly to that of reversion of a single point mutation to wild type (ca. 10⁻⁵). Thus, a single mutation might bring either one of the glycoproteins or another gene product into such a conformation that it cooperates normally again at 33°C.

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REFERENCES

BOSCH, F. X., ORLICH, M., KLENK, H.-D., and ROTT, R. (1979). The structure of the hemagglutinin, a determinant for the pathogenicity of influenza virus. Virology 95, 197-207.

KLENK, H.-D., ROTT, R., and BECHT, H. (1972). On the structure of influenza virus envelope. Virology 47, 579-591.

KOENNECKE, I., BOSCHEK, C. B., and SCHOLTISSEK, C. (1981). Isolation and properties of a temperature-sensitive mutant (ts 412) of an influenza virus recombinant with a ts lesion in the gene coding for the nonstructural protein. Virology 110, 16-25.

MAHY, B. W. J. (1983). Mutants of influenza virus. In "Genetics of Influenza Viruses" (P. Palese, and D. W. Kingsbury, eds.). Springer-Verlag, Wien, N. Y., in press.

NARAMURA, K., and HOMMA, M. (1974). Cell fusion by HeLa cells persistently infected with hemadsorption type 2 virus. J. Gen. Virol. 25, 117-124.
ROTT, R., ORLICH, M., and SCHOLTISSEK, C. (1979). Correlation of pathogenicity and gene constellation of influenza A viruses. III. Non-pathogenic recombinants derived from highly pathogenic parent strains. *J. Gen. Virol.* **44**, 471-477.

SCHOLTISSEK, C., HARMES, E., ROHDE, W., ORLICH, M., and ROTT, R. (1976). Correlation between RNA fragments of fowl plaque virus and their corresponding gene functions. *Virology* **74**, 332-344.

SCHOLTISSEK, C., and SPRING, S. B. (1981). Suppressor recombinants and suppressor mutants. In "Genetic Variation among Influenza Viruses" (D. P. Nayak, ed.), pp. 399-413. ICN-UCLA Symposia on Molecular and Cellular Biology XXI. Academic Press, New York/London/Toronto/Sydney/San Francisco.

SETO, J. T., and ROTT, R. (1966). Functional significance of sialidase during influenza virus multiplication. *Virology* **30**, 731-737.

SOMERS, K., and KIT, S. (1973). Temperature-dependent expression of transformation by a cold-sensitive mutant of murine sarcoma virus. *Proc. Nat. Acad. Sci. USA* **70**, 2206-2210.

STOHLMAN, S. A., SAKAGUCHI, A. Y., and WEINER, L. P. (1979). Characterization of the cold-sensitive murine hepatitis virus mutants rescued from latently infected cells by cell fusion. *Virology* **98**, 448-455.

WRIGHT, P. J., and COOPER, P. D. (1973). Isolation of cold-sensitive mutants of poliovirus. *Intervirology* **2**, 20-24.