Comparative Studies of Wild-Type and "Cold-Mutant" (Temperature-Sensitive) Influenza Viruses: Polypeptide Synthesis by an Asian (H2N2) Strain and Its Cold-Adapted Variant

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The structure and replication of a cold-adapted, temperature-sensitive (TS) mutant of an Asian (H2N2) influenza virus was compared with that of its wild-type (WT) parent. Viruses were grown in a chicken kidney cell system, and at the nonpermissive temperature of 40 C, production of infectious TS virus was about 100,000-fold less than at 35 C, in contrast to WT virus. Major structural polypeptides of each virus grown at 35 C were similar, except that the hemagglutinin glycopolypeptide (HA) of the TS virions was slightly more heterogenous than that of WT virions. Synthesis of viral polypeptides was examined by sodium dodecyl sulfate acrylamide gel electrophoresis of pulse-labeled infected cells. This revealed a defect in the synthesis of TS viral hemagglutinin that was most pronounced at the nonpermissive temperature. Other TS viral polypeptides appeared to be synthesized normally at 40 C. A defect in the TS virus hemagglutinin was also indicated by serological studies that demonstrated that TS virus hemagglutinin had lost antigenic sites present on the WT virus. Thus, it is concluded that the virus mutant examined contains lesions in the hemagglutinin gene, although the possibility of additional unrecognized lesions is not excluded.

Temperature-sensitive (TS) mutants have been isolated from many viruses by passage at reduced temperatures or, more commonly, by treatment with chemical mutagens, which were reviewed by Ghendon (6). For the influenza viruses, several sets of TS mutants have been obtained by mutagenesis and grouped on the basis of gross physiological defects, recombination, and complementation (27-29, 36, 40, 41). Because TS viruses often have low virulence, their potential use as live vaccine has also been evaluated (30, 31). In this laboratory, influenza viruses have been adapted to grow at reduced temperature ("cold-adapted"), as an alternative to mutagenesis for the preparation of attenuated virus (3, 22, 24, 25). Although wild-type (WT) influenza will not replicate at 25 C, the cold-adapted mutants grow efficiently at

this temperature (22). Such "cold-mutant" viruses are also temperature-sensitive (22, 24, 25), and it is of practical and fundamental interest to determine the nature of the TS lesions and any association with adaptation to grow efficiently at reduced temperature. In this initial study of the problem, polypeptide synthesis by WT virus and a prototype cold-mutant TS virus were compared by demonstrating a temperature-sensitive defect in the synthesis of hemagglutinin by the mutant virus.

MATERIALS AND METHODS

Viruses. The virus strain studied was the Asian influenza virus A/AA/6/60 (H2N2), which was isolated in these laboratories in the chicken kidney tissue culture (CKTC) system (21). After isolation, the virus was grown once in eggs and then cloned by three consecutive plaque purifications in CKTC. Preparation of the cold-adapted variant was by slowly lowering the growth temperature of virus in stages to 25 C using the CKTC system and has been previously de-

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scribed (22). After adaptation to growth at 25 C, which included 10 passages in CKTC at this temperature, the variant virus was cloned by three consecutive plaque purifications at 25 C in CKTC by using previously described methods (23). Subsequently, the variant virus was passaged four times in CKTC at 25 C and 20 times in eggs at 25 C, so that in total the virus had been passaged 37 times at 25 C. WT virus had a similar passage level, but had been grown exclusively at 35 to 36 C. The mutant adapted to growth at 25 C will be referred to as TS virus, because the yield of this virus dropped by 10^4 to 10^5 infectious units when grown at 40 C (22, Table 1).

Cells. Preparation of primary cell cultures from chicken kidneys (CK cells) was as described previously (23). The cells were grown to confluence at 35 C by using medium 199 supplemented with 10% calf serum. Maintenance medium was Eagle basal medium, containing twice the normal amount of amino acids, glutamine, and vitamins ($2 \times$ Eagle medium). Comparative studies of viral polypeptide synthesis at different temperatures were all conducted by using cell monolayers prepared at the same time. At least 8 h prior to infection, fresh maintenance medium was added to all monolayers, and half of the cultures were then transferred from 35 to 40 C. Experiments with BHK cells were conducted under similar conditions. These cells had originally been purchased from Microbiological Associates, Inc., and subsequently were grown by using medium 199 supplemented with 10% fetal calf serum.

Virus purification. Labeled virus grown in tissue culture was harvested in the maintenance medium, clarified by low-speed centrifugation, and then purified after adding 0.5×10^6 hemagglutinin (HA) units of unlabeled virus (A/AA/6/60) as carrier. This carrier virus had been grown in eggs and purified by previously described centrifugation procedures (11). The mixtures, containing labeled virus and unlabeled carrier virus, were centrifuged at $70,000 \times g$ for 1 h, and the pellets were suspended in 0.1 M Tris-hydrochloride buffer, pH 7.5, and sonicated for 1 min with a Raytheon 10 KC oscillator. Virus purification was

then achieved by centrifugation through 6 to 30% linear sucrose gradients, buffered with 0.1 M Tris-hydrochloride, pH 7.5, for 30 min at 25,000 rpm in the Spinco SW39 rotor. The opalescent virus band two-thirds down each tube was recovered in fractions collected from the bottom of the tubes, diluted with 0.1 M Tris-hydrochloride, pH 7.5, and pelleted by centrifugation at $100,000 \times g$ for 1 h.

Serology. Pools of antisera to the WT and TS viruses were obtained from mice 3 days after infection with 10^5 mean egg infective doses (EID₅₀) of the viruses. Hemagglutinin inhibition (HI) tests were conducted by using sera treated with receptor-destroying enzyme and chicken erythrocytes (33). Neuraminidase inhibition (NI) tests were performed by using fetuin substrate (13). Untreated virus in allantoic fluid was used as antigen for each test.

Virus infectivity. Virus infectivity was titrated in 11-day-embryonated chicken eggs at 35 C, which were harvested for allantoic fluid at 48 h postinfection and tested for hemagglutinin (24).

Isotopes. All isotopes were obtained from Amersham-Searle.

Measurement of neuraminidase synthesis in influenza-infected cells. Neuraminidase in influenza-infected cells was determined by adding substrate to monolayers in situ, similarly to the method previously described (9). Substrate was sialo-lactose purified from bovine colostrum (10). To each cell monolayer (about 10^6 cells) was added 0.5 ml of prewarmed sialo-lactose (150 μ g of sialic acid per ml) in 0.1 M phosphate buffer, pH 6.0. After 15 min of reaction at 37 C, the substrate solution was recovered, and free sialic acid in 0.4-ml samples was determined by the method of Aminoff (1). Triplicate cultures were assayed in every case, and uninfected cells also were assayed as a control for possible endogenous neuraminidase.

Preparation of 3 H-amino acid-labeled VS virus. Growth and purification of 3 H-amino acid-labeled vesicular stomatitis (VS) virus was as previously described (16).

Acrylamide gel electrophoresis. This was performed in 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS), 0.5 M urea, and buffered at pH 7.4. All samples were prepared under reducing conditions prior to electrophoresis, and these procedures, together with gel preparation for scintillation counting, were as previously described (12).

RESULTS

Temperature sensitivity of growth by A/AA/6/60 influenza virus variants. Chicken kidney cell monolayers were infected with WT or TS variants of influenza virus A/AA/6/60 and maintained at either 35 or 40 C. Cells were then frozen and thawed once, clarified by low-speed centrifugation, and titrated for infectious virus and viral HA. Table 1 illustrates that although the yield of WT virus was only about $0.5 \log_{10}$ infectious units less at 40 than 35 C, the reduction in yield of TS virus was as much as $5 \log_{10}$ infectious units at the higher temperature. Re-

TABLE 1. Production of infectious virus and viral hemagglutinin by influenza virus A/AA/6/60 variants at 35 and 40 C in chicken kidney cell monolayers

Virus variant	Growth temp (C)	Virus yield at 24 h post-infection ^a	
		Infectious virus	Hemagglutinin
Wild type	35	7.0-7.5 ^b	32-64
Wild type	40	6.7-7.0	32-64
Temperature-sensitive	35	6.8-7.3	32
Temperature-sensitive	40	1.7-2.5	2-8

^a Range of values in three experiments.

^b Median infectious doses in eggs at 37.5 C, $\log_{10}$ units.

duction in HA production by the TS virus also occurred at 40 C, but traces were detected (Table 1). These results confirm previous findings with virus of a shorter passage history that adaptation of influenza virus to grow at low temperatures (25 C) is paralleled by an increase in temperature sensitivity of the virus (22, 24, 25).

Difference in growth between the WT and TS viruses at 40 C was such that the temperature of 40 C was clearly satisfactory to adopt as the standard nonpermissive temperature for subsequent experiments designed to investigate the nature of the restrictions operating on the TS virus mutant.

Identification of structural components of influenza virus A/AA/6/60 grown in chicken kidney cells. Although the general structural features of influenza viruses have now been well established, there may be size differences in the viral glycopolypeptides depending on the type of cell used and the presence of serum in the medium (2, 7, 20, 32). Additionally, most descriptions of the polypeptide compositions of human influenza A viruses have been performed with strains of antigenic type HON1 (2, 5, 7, 8, 19, 32, 34, 38, 39), whereas our investigations concerned Asian (H2N2) influenza virus not characterized at the time of this study. Therefore, to identify virus-structural polypeptides in chicken kidney cells infected with A/AA/6/60, it was first necessary to determine the polypeptide composition of this virus grown in the same cells. WT virus, grown in CKTC and labeled with ^{14}C -amino acid was purified and mixed with ^3H -amino acid-labeled VS virus as a source of internal size markers. Electrophoresis on SDS-polyacrylamide gels under reducing conditions revealed only three polypeptides from influenza virus A/AA/6/60 (Fig. 1). VS virus resolved into the four structural polypeptides L, G, N, and M (42), and the most recently determined sizes of these VS virus polypeptides (43) were used to calibrate the gels. Thus, sizes of the influenza polypeptides were estimated as about 70,000, 55,000, and 20,000 daltons. As shown later, the 70,000-dalton polypeptide is glycosylated and is identified as the largest form of the hemagglutinin glycopolypeptide designated HA (14). From their sizes and relative positions, the other influenza virion polypeptides are identified as the nucleoprotein (NP) and membrane protein (M). There was no evidence for extensive cleavage of the HA into heavy and light chain (HA_1 and HA_2), in contrast to several other systems (17-19, 20, 37). Lack of identification of a neuraminidase subunit (NA) is not surprising, because neuramini-

FIG. 1. Identification of structural components of chicken kidney cell-grown influenza virus A/AA/6/60. Monolayers of 2×10^7 chicken kidney cells were infected with WT influenza virus A/AA/6/60 at multiplicity of infection of 10 EID₅₀/cell. Unabsorbed virus was washed off after 1 h and cells were maintained at 35 C with 100 ml of serum-free Eagle minimal essential medium containing 250 μCi of ^{14}C -serine (171 mCi/mmol). The medium was recovered 24 h postinfection, and the virus was purified from the tissue culture medium by differential and density gradient centrifugation (see Materials and Methods) and finally suspended in 0.15 ml of 0.1 M Tris-hydrochloride buffer, pH 7.5. A 20- μl sample was mixed with 20 μl of purified ^3H -amino acid-labeled vesicular stomatitis virus (VSV), Indiana strain, employed as a source of protein size markers. The virus mixture was heated to 100 C for 2 min in 0.1 M phosphate buffer, pH 7.4, containing 1% SDS, 0.5 M urea, and 1% 2-mercaptoethanol, and subjected to electrophoresis in 7.5% acrylamide gels for 8.5 h at 7.4 mA. VSV proteins (solid arrows) are designated following the proposal of Wagner et al. (42), and influenza polypeptides (dashed arrows) as proposed by influenza workshop I (14). The glycosylated nature of the largest influenza polypeptide (HA) is illustrated in Fig. 2.

dase comprises only 5 to 10% of the total protein in Asian influenza virus and has a subunit size of about 50,000 daltons, similar to the viral nucleoprotein (10, 11, 14).

Comparison of WT and TS virus structural polypeptides. Chicken kidney cell monolayers were infected in parallel with either WT or TS virus, which were then grown in the presence of ^3H -valine and ^{14}C -glucosamine. Because the yield of TS virus was very low at 40 C (Table 1), the viruses were grown at the permissive temperature of 35 C. Purified viruses were then analyzed on SDS-polyacrylamide gels, again resolving three main species of polypeptides (Fig. 2). Use of the carbohydrate label clearly

Fig. 2. Polypeptide composition of WT and TS influenza A/AA/6/60 virions grown in chicken kidney cells at 35 C. Monolayers of 2×10^6 chicken kidney cells were infected with either WT or TS variants of influenza virus A/AA/6/60 at an MOI of 10 EID₅₀/cell. After removal of unadsorbed virus, infected cells were maintained at 35 C with 100 ml of serum-free Eagle minimal essential medium containing only 25% of the standard dextrose concentration. ³H-valine (31.6 Ci/mmol) was included in the medium at 2.5 μ Ci/ml and ¹⁴C-glucosamine (318 mCi/mmol) at 0.5 μ Ci/ml. After 24 h of growth at 35 C, virus was purified by differential centrifugation and density gradient centrifugation (see Materials and Methods) and finally suspended in 0.15 ml of 0.1 M Tris-hydrochloride buffer, pH 7.5. A 20- μ l sample of each virus preparation was heated to 100 C for 2 min in pH 7.4 buffer containing 1% SDS, 0.5 M urea, and 1% 2-mercaptoethanol, and subjected to electrophoresis in 7.5% acrylamide gels. A, TS virion structural polypeptides. B, WT virion structural polypeptides. Polypeptides are designated as proposed by influenza workshop I (14). To compare the degree of glycosylation of each virus HA, ¹⁴C-to-³H ratios were calculated. For fractions 13, 14, and 15, the ratio was 0.60, 0.60, and 0.40, respectively, for the TS virus, and the ratio was 0.52, 0.48, and 0.31, respectively, for the WT virus.

identified the largest polypeptide as glycosylated, confirming its identity as an uncleaved HA. Polypeptide patterns of the two viruses were comparable, except for the glycosylated HA which was distributed over a slightly broader peak for the TS virus than for the WT virus. However, the ¹⁴C-glucosamine-to-³H-valine ratio was similar in equivalent fractions across the HA peak for both viruses, indicating

that after synthesis, the HA of each virus was equally glycosylated. In addition to the large peak of HA, both viruses contained a small peak of glycopolypeptide with a size of about 45,000 daltons, smaller than neuraminidase subunit (11), but corresponding to the size expected for the HA₁. Thus, slight cleavage of HA may have occurred in these virus preparations.

Identification of structural polypeptides of influenza virus A/AA/6/60 in viral-infected chicken kidney cell monolayers. Virus-infected cell monolayers were pulse-labeled with ³H-valine from 6 to 6.45 h postinfection, and a sample of the labeled cells was subjected to coelectrophoresis with ¹⁴C-amino-acid-labeled virus. Pulse-labeled mock-infected cells were analyzed in parallel. Several size ranges of host-cell polypeptides were prominent in the mock-infected cells, and for reference purposes, these were numbered C1 through C7 (Fig. 3). In chicken-kidney cell monolayers infected with WT virus A/AA/6/60 at 35 C, there was a consistent inhibition of host-cell polypeptide synthesis, as evidenced particularly by a decrease in amounts of host proteins C1 and C7 (Fig. 3). The viral polypeptides HA and NP were present in sufficient quantities to be readily distinguished, even though their sizes were similar to some of the cell components. In addition to the HA and NP components, a large peak was present in the virus-infected cells that overlapped the viral membrane protein M. The breadth of this peak in infected cells suggested that it contained both viral M protein and the slightly smaller nonstructural polypeptide (NS) described by Lazarowitz et al. (19). An even smaller polypeptide was also present in the virus-infected cells, but not apparent in the control cells. This polypeptide might be a further nonstructural polypeptide, similar to that with a size of 11,000 daltons observed by Skehel (37). For reference purposes, the two presumed nonstructural polypeptides are referred to in this text as NS₁ and NS₂ (Fig. 3). Additional nonstructural proteins may also be present (4) that are not readily detected by SDS acrylamide gel electrophoresis.

Comparison of polypeptide synthesis by chicken kidney cells infected with WT and TS variants of influenza A/AA/6/60. Chicken kidney cells infected with WT or TS virus were double-labeled with ³H-valine and ¹⁴C-glucosamine, and polypeptide synthesis was examined by SDS-polyacrylamide gel electrophoresis of pulse-labeled cells. Figure 4 shows that at 35 C, the polypeptide pattern of WT virus-infected cells was similar to that found above (cf. Fig. 3), and the additional presence of a carbohydrate label in this case confirmed that hemagglutinin

FIG. 3. Identification of host cell and virus-specified polypeptides in chicken kidney cell monolayers infected with influenza virus A/AA/6/60. Monolayers of 10^6 chicken kidney cells were mock-infected or infected with 10^6 EID₅₀/cell of WT virus A/AA/6/60. After 1 h of adsorption at 35 C, unadsorbed virus was washed off, and 5 ml of maintenance medium added. At 5.5 h postinfection, maintenance medium was removed and replaced with medium deficient in amino acids and dextrose. After 0.5 h, the deficient medium was removed and replaced with 1 ml of deficient medium, to which was added 5 μ Ci of 3 H-valine (31.6 Ci/mmol). A 5-ml portion of complete maintenance medium was added 0.25 h later, and after a 0.5 h chase period, the medium was removed, cells were washed with chilled 0.05 M phosphate buffer, pH 7.4, and finally scraped into 0.15 ml of this buffer. A 50- μ liter sample of virus-infected cells was mixed with 20 μ liters of 14 C-serine-labeled purified influenza virus (as analyzed in Fig. 1), and prepared for electrophoresis as described for Fig. 1. This sample (A) and a 50- μ liter sample (B) of mock-infected cells were analyzed on parallel 7.5% acrylamide gels. Distinctive host cell polypeptides are designated as cell components 1 through 7 (C_1 to C_7) for descriptive purposes. In virus-infected cells, two nonstructural polypeptides were designated as NS_1 and NS_2 , and other virus-specified polypeptides were designated as proposed by influenza workshop I (14).

heavy and light chains HA_1 and HA_2 were absent in the cells. This is consistent with the belief that hemagglutinin is synthesized as a large polypeptide of about 70,000 to 80,000

daltons (17, 19, 20). At 35 C, synthesis of viral NP, M plus NS_1 , and NS_2 appeared similar for cells infected with either virus (Fig. 4). However, the TS virus HA peak was poorly resolved, probably for two reasons: (i) TS virus did not inhibit host cell protein synthesis to the degree observed with WT virus, as evidenced by the relative amounts of the characteristic host cell polypeptides C_1 and C_7 synthesized in cells infected with each variant; and (ii) judged by the distribution of carbohydrate label, TS viral HA glycopolymer was synthesized in lower amounts than WT HA. Because the 14 C-glucosamine-to- 3 H-valine ratio is similar in the HA of both virus variants, 14 C-glucosamine is a good label for comparing the HA synthesis of the viruses, particularly as control cells did not contain 14 C-labeled glycoproteins (Fig. 4).

A defect in the synthesis of the TS virus HA glycoprotein was more apparent when polypep-

FIG. 4. Comparison of polypeptides synthesized at 35 C in chicken kidney cell monolayers infected with WT and TS variants of influenza virus A/AA/6/60. Monolayers of 10^6 chicken kidney cells were mock-infected, or infected with either WT or TS variants of influenza virus A/AA/6/60. Conditions of virus growth and polypeptide labeling were similar to those described for Fig. 3, except that cells were simultaneously double-labeled with 5 μ Ci of 3 H-valine (31.6 Ci/mmol) and 5 μ Ci of 14 C-glucosamine (55 mCi/mmol). A, Mock-infected cells, 14 C-glucosamine label only is shown, as the pattern of 3 H-valine was similar to that in Fig. 2B; B, cells infected with the TS influenza variant; C, cells infected with WT influenza virus. Host cell polypeptides are designated by reference to Fig. 3B.

tides were labeled in cells grown at the nonpermissive temperature of 40 C (Fig. 5). Although peaks of NP, M plus NS₁, and NS₂ were comparable for WT and TS viruses, the amount of TS virus HA synthesized was about 50 to 75% less than for the WT virus, judged by amino acid or carbohydrate label. The distribution of the carbohydrate label also suggested that the TS virus HA was heterogenous, containing some components smaller than present for the WT virus. This heterogeneity was reproducible in several experiments.

Polypeptide synthesis in BHK cells infected with WT and TS virus variants. To verify that the patterns of polypeptide synthesis of WT and TS virus variants were not significantly influenced by the host cell system, similar experiments to those above were repeated, but by using BHK cell monolayers. Procedures throughout were identical to those employed with CK cell monolayers. At 35 C, peaks corresponding to HA, NP, and M plus NS₁ were observed for both viruses, and a small, broad peak was also present in the region where NS₂ migrated in the CK cells (Fig. 6). When polypeptide synthesis was also examined at 40 C, the only viral component affected was the TS virus HA glycoprotein, which was present in reduced amounts (Fig. 7). Judged by the distri-

FIG. 6. Comparison of polypeptides synthesized at 35 C in BHK cell monolayers infected with WT or TS variants of influenza virus A/AA/6/60. Monolayers of 10⁶ BHK cells were infected with either WT or TS influenza virus A/AA/6/60 at an MOI of 10 EID₅₀/cell. Conditions of virus growth and polypeptide labeling were similar to those described for Fig. 3, except that cells were simultaneously double-labeled with ³H-valine (5 μCi) and ¹⁴C-glucosamine (5 μCi). A, TS virus-infected cells; B, WT virus-infected cells. Virus-specific polypeptides, designated similarly to Fig. 3, were clearly superimposed over BHK host cell polypeptides in mock-infected cells (not shown).

FIG. 5. Comparison of polypeptides synthesized at 40 C in chicken kidney cell monolayers infected with WT or TS variants of influenza virus A/AA/6/60. The experiment was conducted in parallel to that described for Fig. 4, except that cells were maintained at 40 C throughout. A, Cells infected with the TS influenza variant; B, cells infected with WT influenza virus. Host cell polypeptides are designated by reference to Fig. 3B.

bution of carbohydrate label, the TS viral HA synthesized at 40 C was also heterogenous. These observations confirm those made by using the CK cell system that the virus mutant studied has a temperature-sensitive defect in its ability to synthesize HA glycopolymer.

Production of neuraminidase in chicken kidney cell monolayers infected with WT and TS influenza variants. Because the NA subunit was not readily distinguishable in polyacrylamide gel patterns of influenza-infected cells, the synthesis of neuraminidase was investigated by direct measurement of its enzymic activity. At 35 C, similar amounts of neuraminidase were present on the plasma membrane of cells infected with WT or TS influenza virus A/AA/6/60 (Table 2). Production of WT virus neuraminidase was virtually unaffected by growth of the virus at 40 C, whereas the rate of production of the TS virus neuraminidase was significantly reduced at the nonpermissive temperature. Nevertheless, as shown by measuring TS virus neuraminidase on

FIG. 7. Comparison of polypeptides synthesised at 40 C in BHK cell monolayers infected with WT or TS variants of influenza virus A/AA/6/60. The experiment was conducted in parallel to that described for Fig. 6., except that cells were maintained at 40 C throughout. A, TS virus-infected cells; B, WT virus-infected cells.

TABLE 2. Production of neuraminidase in influenza-infected chicken kidney cell monolayers

Growth temp (C)	Neuraminidase activity of cells infected with WT or TS variants of influenza virus A/AA/6/60		
	WT 7 h PI ^a	TS	
		7 h PI	12 h PI
35	26 ^{a, b}	21	22
40	21	8	18

^a PI, Postinfection.

^b Measured in micrograms of sialic acid per 15 min per 10⁶ cells. At 0 h postinfection, activity was <1 μg sialic acid per 15 min per 10⁶ cells.

cells at different times after infection, synthesis of the TS virus enzyme was sustained at 40 C, so that levels were achieved comparable to those at 35 C with the TS virus and similar to levels of WT virus neuraminidase. Hence, it is unlikely that the lack of production of infectious TS virus at the nonpermissive tempera-

ture resulted primarily from a lack of neuraminidase at the cell plasma membrane.

Antigenic specificities of WT and TS virus glycoproteins. Previous results suggested that cold-adaptation of influenza virus and successive passage at reduced temperatures resulted in a virus that lost antigenic site(s) from its hemagglutinin (25). To confirm this, viruses from the same stocks used to infect cells for polypeptide analysis were also administered to mice, and resulting serum was tested by cross-hemagglutination-inhibition reactions. As shown in Table 3, a pool of serum from mice infected with WT virus contained antibodies that inhibited equally the hemagglutination activity of the WT and TS viruses. In contrast, a pool of serum from mice infected with TS virus contained antibodies reacting almost exclusively against the homologous TS virus. The results are similar to earlier observations of asymmetric cross-reactivity between the hemagglutinins of these WT and TS viruses (25). Because this finding has been reproduced with different pools of sera and with sera from mice infected with virus of various passage levels (25), it is unlikely to be an artifact due to selection of virus mutants during replication of the viral antigens in mice.

Cross-reactions between the neuraminidases of the WT and TS virus variants were also examined with the same sera by a chemical inhibition (NI) test (Table 4), and the neuraminidases of the viruses showed only a small divergence. Although the HI and NI tests measure quite different parameters, results of the

TABLE 3. Cross hemagglutination-inhibition reactions of influenza virus A/AA/6/60 variants

Virus antigen	Mouse Serum	
	Wild-type	Temperature-sensitive
Wild type	512	8
Temperature sensitive	512	512

TABLE 4. Cross neuraminidase-inhibition reactions of influenza virus A/AA/6/60 variants

Virus antigen	Mouse Serum	
	Wild-type	Temperature-sensitive
Wild type	375 ^a	200
Temperature sensitive	320	220

^a Serum dilution inhibiting 50% of the activity of a standard dose of enzyme incubated with normal mouse serum.

two serological tests suggest that only the HA glycoprotein of the TS virus is defective.

DISCUSSION

The variant of A/AA/6/60 studied here is a prototype cold-adapted influenza virus (22). This mutant is now being employed as a parent in recombination experiments designed to rapidly obtain attenuated influenza viruses containing hemagglutinin and neuraminidase antigens of new epidemic strains (H. F. Maassab, *in* R. D. Barry and B. W. J. Mahy, ed., *Negative strand viruses*, *in press*). It is recognized that the genome of influenza viruses is probably segmented, facilitating the possibility for recombination (35) during which process there may be enhanced opportunities for virus mutations to occur (15, H. F. Maassab, *in press*). Therefore, it is particularly important to determine the nature of any lesions in the A/AA/6/60 TS variant and their significance to the attenuation of this virus, so as to identify any biochemical markers that are transferred to progeny virus during the process of "attenuation by recombination."

Results described here have demonstrated that even at the nonpermissive temperature of 40 C, cells infected with the TS mutant synthesized amounts of most viral structural polypeptides comparable to those observed with the WT virus. Apparently, therefore, the lesions in the TS virus do not affect viral adsorption, uncoating, or transcription and translation of the entire viral genome. Insofar as it was possible to identify any nonstructural viral specific polypeptides, these also were synthesized by TS virus-infected cells at the nonpermissive temperature. The only defect that could be observed in the TS virus was its reduced efficiency to synthesize HA glycopolymer. At the permissive temperature of 35 C, there appeared to be a small reduction in the quantity of HA synthesized by the TS virus, and judged by observations with isolated virions, the HA glycopolymer synthesized at 35 C was slightly more heterogeneous than WT virus HA. At the nonpermissive temperature of 40 C, quantitative and qualitative deficiencies in TS virus HA synthesis could be observed in infected cells, although it was not feasible to examine HA in virions at the nonpermissive temperature because little virus is made at 40 C.

Biochemical evidence for a qualitative defect in the mutant virus HA is supported by serological evidence of a loss of antigenic sites from the HA. It may be noteworthy that raising the growth temperature from 35 to 40 C results in about a 10^4 to 10^5 logs loss of yield of infectious

TS virus, yet only a 50 to 75% reduction in synthesis of HA glycopolymer, suggesting that the HA component made at the nonpermissive temperature is nonfunctional. Alternately, there may be additional unidentified lesions in the TS virus genome, affecting perhaps the maturation of virions.

Further insight into this problem may be gained with recombinant viruses. By using attenuated A/PR/8/34 (HON1) as one parent, it was previously found that virulent influenza virus strains could be cold-adapted by recombination independently of hemagglutinin type (26). More recently, the same result has been obtained by using the A/AA/6/60 mutant as the donor of the cold-adaptation property (H. F. Maassab, *in press*). This does indeed suggest that the A/AA/6/60 mutant has lesions additional to those in the hemagglutinin gene. It is anticipated that further studies of cold-adapted viruses and their recombinants will provide valuable information about the alterations of viral functions arising as a consequence of attenuation by cold-adaptation and will contribute to an understanding of the variability of influenza viruses.

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