INFECTION AND IMMUNITY, Oct. 1979, p. 280-286
0019-9567/79/10-0280/07$02.00/0

Vol. 26, No. 1

Relationship of Genotype of Recombinants of Influenza A/Hong Kong/68-*ts*-1[E] Virus Used as Live Virus Vaccines to Virulence in Humans

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Received for publication 1 June 1979

Influenza A/Hong Kong/68-*ts*-1[E] virus is a temperature-sensitive mutant developed for use as a live virus vaccine (B. R. Murphy, E. G. Chalhub, S. R. Nusinoff, J. Kasel, and R. M. Chanock, J. Infect. Dis. **128**:479-487, 1973). This virus and temperature-sensitive recombinants derived by mating it with A/Udorn/72, A/Georgia/74, or A/Victoria/75 wild-type virus have been administered to volunteers in clinical trials on the assumption that the *ts*-1[E] temperature-sensitive genetic lesions on a polymerase gene (P3) and on the nucleoprotein gene (NP) would determine a satisfactory and reproducible level of attenuation regardless of the genetic constitution of *ts*-1[E] recombinants at other loci (B. R. Murphy, D. D. Richman, S. B. Spring, and R. M. Chanock, Postgrad. Med. **52**: 381-388, 1976). In this paper, the parental origin of genes in the *ts*-1[E] recombinants was determined by using the technique of polyacrylamide gel electrophoresis of virion ribonucleic acid segments in the presence of a denaturing agent (urea). When tested in individuals who lacked immunity to hemagglutinin antigen, attenuation of the *ts*-1[E] recombinants appeared to correlate with inheritance of the *ts*-1[E] temperature-sensitive genes at the P3 and NP loci and with the level of preinfection neuraminidase immunity. There was no evidence that other genes from the *ts*-1[E] donor virus played a role in attenuation.

The genome of the influenza A virion consists of eight segments of single-stranded ribonucleic acid (RNA), each of which codes for a specific polypeptide (11). The segmented nature of the influenza A virus genome makes it possible to reassort genes with high frequency during mixed infection (20). Recently there has been considerable effort to develop a defined set of mutant genes that confer a specific and desired level of attenuation upon any viral recombinant into which they are transferred. Temperature-sensitive (*ts*) donor viruses have been developed with the hope that the transfer of a gene or genes bearing *ts* mutations would reproducibly attenuate each new variant of influenza A virus (10).

One such donor virus is the Hong Kong/68-*ts*-1[E] virus (HK/68), which has a 38°C shutoff temperature for plaque formation and *ts* lesions on the genes coding for a polymerase (P3) and the nucleoprotein (NP) (6, 12, 20). To determine whether the two *ts*-1[E] *ts* genes could confer a specific level of attenuation, the HK/68-*ts*-1[E] virus was mated with A/Udorn/72 (Ud/72), A/Georgia/74 (Ga/74), or A/Victoria/75 (Vic/75) wild-type H3N2 virus. *ts*-1[E] recombinant clones derived from these H3N2 variants were then evaluated in adults and children for level of attenuation and antigenicity (2, 4, 9, 15, 17). Although each of the *ts*-1[E] recombinants was attenuated with respect to wild-type virus, the levels of attenuation achieved by these viruses were not identical (2, 4, 17). One factor which appeared to contribute to this variable response was pre-existing neuraminidase (NA) immunity. *ts*-1[E] vaccinees who lacked pre-existing NA-inhibiting antibody developed more systemic symptoms than did individuals with such antibody (4, 9, 17). However, the possibility that NA immunity was responsible for the observed differences in virulence of the *ts*-1[E] recombinants was offered as a tentative explanation, because the *ts*-1[E] recombinant viruses were not completely characterized with respect to genotype. The serological and genetic tests used to characterize each *ts*-1[E] recombinant virus permitted identification of the parental origin of only four of the eight genes in the *ts*-1[E] recombinants, namely the hemagglutinin (HA), NA, P3, and NP genes (7). The parental origins of the other four genes, i.e., the genes coding for the P1 and P2 polymerases, the membrane (M) pro-

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tein, and the nonstructural (NS) protein, were not established. Therefore, the contribution of these genes to the level of attenuation of the *ts*-1[E] recombinant viruses could not be determined. In the present study, the *ts*-1[E] recombinants were genotyped by polyacrylamide gel electrophoresis (PAGE) of virion RNA, permitting an assessment of the contribution of the *ts* and non-*ts* genes of the HK/68-*ts*-1[E] donor virus to attenuation of the *ts*-1[E] recombinants previously evaluated in volunteers.

MATERIALS AND METHODS

Viruses. Influenza A strains evaluated in these experiments included the following cloned *ts*-1[E] recombinants previously administered to volunteers: HK/68-*ts*-1[E] ($H3_{68}N2_{65}$); Ud/72-*ts*-1[E] clones 13 ($H3_{72}N2_{72}$), 16 ($H3_{72}N2_{65}$), and 24 ($H3_{72}N2_{65}$); Ga/74-*ts*-1[E] clone 2 ($H3_{74}N2_{74}$); and Vic/75-*ts*-1[E] clones 67 ($H3_{75}N2_{65}$), 81 ($H3_{75}N2_{75}$), and 113 ($H3_{75}N2_{75}$). All *ts*-1[E] recombinants except Ud/72 clone 13 had a 38°C shutoff temperature for plaque formation and possessed the two *ts*-1[E] *ts* genes. Clone 13 had a 39°C shutoff temperature for plaque formation and possessed only one of the two *ts*-1[E] *ts* genes, that which belonged to complementation group 1 (18). Also included were the corresponding wild-type H3N2 parent strains, Ud/72 ($H3_{72}N2_{72}$), Ga/74 ($H3_{74}N2_{74}$), and Vic/75 ($H3_{75}N2_{75}$).

Preparation and extraction of virion RNA. A total of 50 9- to 11-day-old eggs (Truslow Farms, Rockville, Md.) were inoculated with 0.1 ml of an allantoic fluid suspension of virus diluted to contain approximately 10^5 50% tissue culture infective doses per ml. Eggs were incubated at 34°C for 72 h, chilled, and harvested to yield approximately 400 ml of allantoic fluid. This material was clarified by low-speed centrifugation, and virus was then pelleted by centrifugation at 20,000 rpm for 3 h in a Beckman Spinco L2-65B ultracentrifuge. Pelleted virus was resuspended in 2 to 3 ml of phosphate-buffered saline, pH 7.4. Concentrated virus in phosphate-buffered saline was then layered on a discontinous gradient of 30 and 60% (wt/vol) sucrose buffered with 0.1 M sodium chloride–0.02 M tris(hydroxymethyl)aminomethane (pH 7.5)–0.001 M ethylenediaminetetraacetate or reticulocyte standard buffer and centrifuged at 36,000 rpm for 90 min (SW40 rotor; Beckman Spinco L2-65B).

Approximately 1.5 ml of concentrated purified virus banded at the interface of the discontinuous gradient was then collected by using a Pasteur pipette. The whole virus suspension was diluted to 3 ml with 0.1 M sodium chloride–0.02 M tris(hydroxymethyl)aminomethane (pH 7.5)–0.001 M ethylenediaminetetraacetate or reticulocyte standard buffer, and virion RNA was extracted by the method described by Palese and his collaborators after proteinase K (Boehringer Mannheim) and sodium dodecyl sulfate treatment (13, 14). A 50- to 100-μg amount of RNA was usually recovered from 250 ml of allantoic fluid with an HA titer (determined on microtiter plates) of 1:64.

Separation of virion RNA by PAGE. A 1- to 3-μg amount of virion RNA dissolved in Loening buffer [0.036 M tris(hydroxymethyl)aminomethane, pH 7.8, 0.03 M phosphate, 0.001 M ethylenediaminetetraacetate] was applied to the slot (3 by 0.75 mm) of an 11-cm (Bio-Rad Corp.) or a 15-cm (National Institutes of Health) 2.6% polyacrylamide slab gel. Specific conditions of PAGE are described below. The basic technique was similar to that employed by Floyd et al. (3), as modified by Palese and his collaborators (13, 14), except that the concentration of urea in the gel was reduced from 6.0 to 4.5 M. Reducing the concentration of the denaturing agent permitted detection of increased conformational differences between RNA molecules very similar or identical in molecular weight (1, 3). Differences in migration rate observed between corresponding genes of these closely related viruses are due to such conformational differences, not apparent in 6.0 M urea, rather than to differences in molecular weight (1; unpublished data). Using 4.5 instead of 6.0 M urea did not result in an alteration of the order of migration of RNA segments of a given strain of virus.

Visualization of RNA bands on polyacrylamide gels. After PAGE for 8 to 12 h, 2.6% polyacrylamide gels were bathed for 20 to 30 min at room temperature in Loening buffer to which ethidium bromide (Aldrich Chemical Co.) had been added to a final concentration of approximately 1.0 μg/ml. Gels were then transferred to an ultraviolet light table (Ultra-Violet Products model 0-61), and fluorescent RNA bands were photographed.

RESULTS

Genotyping of the Ud/72-, Ga/74-, and Vic/75-*ts*-1[E] recombinant viruses. Recombinants bearing one or both of the *ts*-1[E] *ts* genes were prepared by mating the HK/68-*ts*-1[E] virus with the Ud/72, Ga/74, or Vic/75 wild-type virus. Virion RNA from recombinant and parental viruses was then studied by PAGE. Electrophoretic mobility of each of the eight RNA segments of the HK/68-*ts*-1[E] virus differed under the chosen conditions from the mobilities of the corresponding RNA segments of the three wild-type viruses, thus enabling us to determine the parental origin of genes in each of the recombinants studied (Fig. 1 through 4 and Table 1). The virion RNA segments of HK/68 wild-type and HK/68-*ts*-1[E] viruses had previously been assigned to the gene products of the virus by Ritchey and others (19). Therefore, the genotype of each *ts*-1[E] recombinant could be deduced from the presence or absence of a given gene from the HK/68-*ts*-1[E] parent at each of the eight loci.

Three Ud/72-*ts*-1[E] recombinants produced by mating the HK/68-*ts*-1[E] virus and the Ud/72 wild-type virus were analyzed by PAGE. A PAGE separation analysis of Ud/72-*ts*-1[E] clone 16 recombinant virus and its *ts* and wild-type parents is shown in Fig. 1. RNA segments are numbered 1 through 8 in order of migration from the origin. Ud/72-*ts*-1[E] clone 16 virus

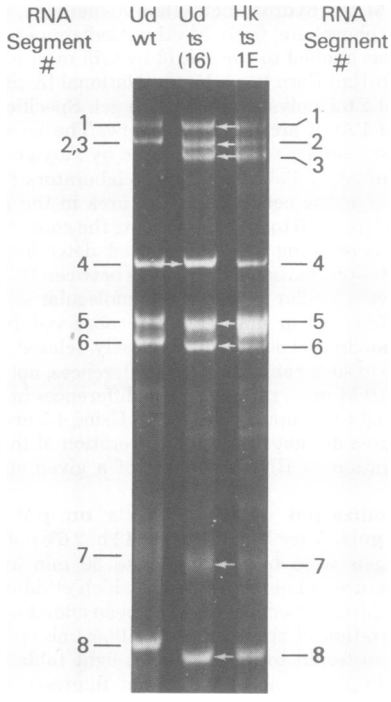

FIG. 1. *PAGE of virion RNA from influenza A/Ud/72-ts-1[E] recombinant clone 16 (center lane) and its parental strains, Ud/72 wild type (wt; left lane) and HK/68-ts-1[E] (right lane). A 2-μg amount of each RNA preparation was separated on an 11-cm 2.6% slab gel in the presence of 4.5 M urea. PAGE was carried out for 10 h at 30°C and 70 V (6.4 V/cm). Positions of virion RNA segments 1 through 8 for each parental strain are indicated by left- and right-hand numbering, respectively. Direction of arrows indicates the parental origin of each gene in the recombinant. RNA 7 of Ud/72 wild-type forms a broad diffuse band under these conditions, but its major position, slightly closer to the origin than RNA 7 of HK/68-ts-1[E] and the recombinant, is a consistent finding. Both RNAs 7 are well resolved in the presence of 6.0 M urea, but migration rate differences are difficult to detect.*

received all RNA segments from the HK/68-*ts*-1[E] parent except RNA 4, which codes for the Ud/72 HA, in keeping with the results of serological analysis of this recombinant. The parental origin of the genes of Ud/72-*ts*-1[E] recombinant clones 13 and 24 was determined in a similar manner (data not shown). The genotypes of these two clones are summarized in Table 1. PAGE analysis of clone 13 supported the previous genetic evidence, which demonstrated that this recombinant received only one of the two *ts* genes from the HK/68-*ts*-1[E] parent, RNA segment 1 coding for the P3 polymerase (12, 18).

The PAGE analysis of the Ga/74-*ts*-1[E] recombinant clone is shown in Fig. 2 and summarized in Table 1. Ga/74-*ts*-1[E] received its genes from the *ts* parent, except for those genes represented on RNAs 4, 5, and 8, which must code for HA, NA, and NS proteins. Although the parental origin of RNA 4 is not apparent in Fig. 2, additional experiments which are not shown and which were performed under slightly different conditions readily demonstrated that the Ga/74-*ts*-1[E] recombinant received RNA 4 from the Ga/74 wild-type parent.

The parental origin of the genes present in Vic/75-*ts*-1[E] recombinant clones 67, 81, and 113 was similarly determined (Fig. 3 and 4). Clone 67 received each of its genes from the

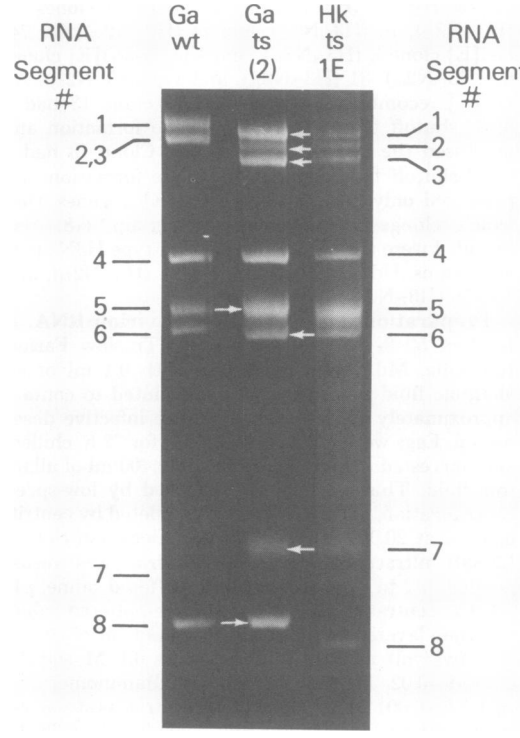

FIG. 2. *PAGE of virion RNA from influenza A/Ga/74-ts-1[E] recombinant clone 2 (center lane) and its parental strains, Ga/74 wild type (wt; left lane) and HK/68-ts-1[E] (right lane). A 2-μg amount of each RNA preparation was run on an 11-cm slab gel of 2.6% polyacrylamide in the presence of 4.5 M urea. PAGE was carried out for 9.5 h at 31°C and 70 V (6.4 V/cm). Numbering of virion RNA segments and the significance of the arrows are the same as in Fig. 1. RNA 4 (HA) cannot be assigned in this experiment, but migration rate differences between these parental genes were evident in other experiments (Table 1). RNA 7 (M) of Ga/74 wild-type virus forms two faint bands, one diffuse, under these conditions, probably due to the secondary structure of the molecule. These bands are resolved in the presence of 6.0 M urea.*

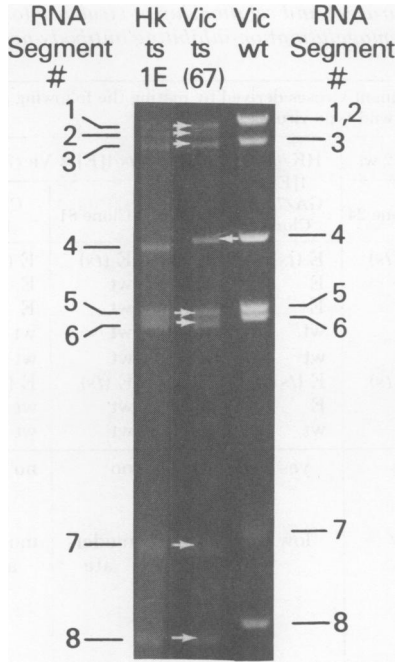

FIG. 3. *PAGE of virion RNA from influenza A/Vic/75-ts-1[E] recombinant clone 67 (center lane) and its parental strains, HK/68-ts-1[E] (left lane) and Vic/75 wild type (wt; right lane). A 15-cm 2.6% polyacrylamide slab gel with 4.5 M urea was employed. PAGE was carried out for 12 h at 27°C and 90 V (6 V/cm). Numbering of virion RNA segments and the significance of the arrows are the same as in Fig. 1.*

HK/68-*ts*-1[E] virus except for RNA 4, which codes for the Vic/75 HA. The genotypes of clones 81 and 113 were similar to each other except at the P1 and P2 loci (RNAs 2 and 3) (Table 1).

Although the order of migration of the genes of the wild-type parental viruses was not investigated directly, the results of PAGE analysis of the *ts*-1[E] recombinants permit inferences to be made. The genes of Ud/72 wild-type virus appeared to migrate in the same order as those of HK/68-*ts*-1[E] virus in the presence of 6.0 M urea (12, 19) or 4.5 M urea (Fig. 1 through 4; unpublished data). We suspect that the same is true for Ga/74 and Vic/75 wild-type viruses. However, the data do not allow certain distinctions to be made. For Ga/74 wild type, RNA 2 or 3 codes for either P1 or P2, and RNA 4 or 5 codes for either HA or NA. For Vic/75 wild type, RNA 2 or 3 codes for either P1 or P2, and RNA 7 or 8 codes for either M or NS. The order of migration of the genes at these loci in Ga/74- and Vic/75-*ts*-1[E] recombinants may differ from the order of migration in HK/68-*ts*-1[E]. However, the parental origin of these genes in these recombinants is not in doubt, because we were able to demonstrate differences in the mobility of each of the eight genes of HK/68-*ts*-1[E] virus and of the corresponding genes from the Ga/74 and Vic/75 wild-type viruses (Fig. 1 through 4; unpublished data), as previously stated.

Relationship of the virulence of *ts*-1[E] recombinant viruses to the genotype of the vaccine virus and to pre-inoculation NA immunity. The *ts*-1[E] viruses indicated in Table 1 were administered to serum hemagglutination-inhibiting antibody-negative (hemagglutination-inhibiting antibody titer, ≤1:8) adults

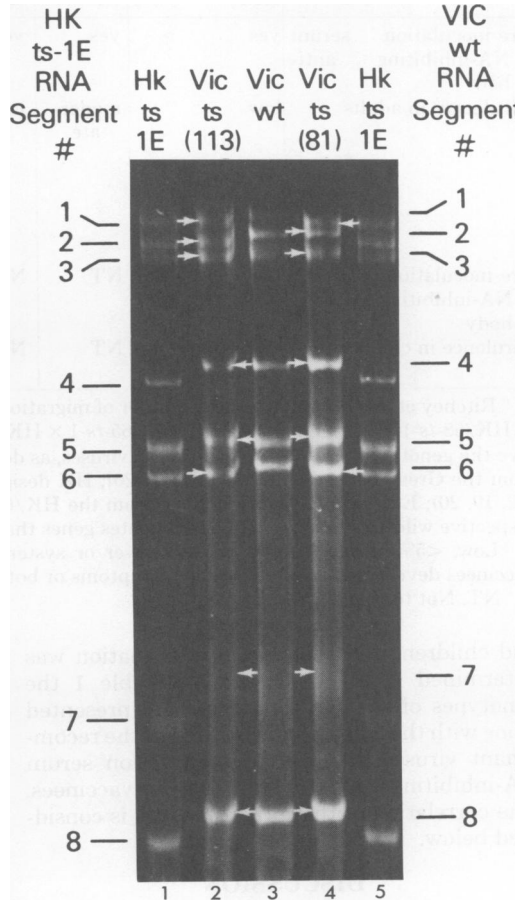

FIG. 4. *PAGE of virion RNA from influenza A/Vic/75-ts-1[E] recombinant clones 113 and 81 (lanes 2 and 4, respectively) and parental strains, Vic/75 wild type (wt; lane 3) and HK/68-ts-1[E] (lanes 1 and 5). An 11-cm 2.6% polyacrylamide slab gel with 4.5 M urea was employed. PAGE was carried out for 10.5 h at 30°C and 70 V (6.4 V/cm). Parental strain RNA segments are numbered. Direction of arrows indicates parental origin of genes.*

TABLE 1. *Parental origin of genes in ts-1[E] recombinant viruses[a] and relationship of virulence to the genotype of the vaccine strain and to NA immunity of serum hemagglutination-inhibiting antibody-negative vaccinees*

RNA segment no.	Gene product	Parental origin of genes in *ts*-1[E] recombinant viruses derived by mating the following pairs of *ts* and wild-type viruses:							
		GL/65-*ts*-1 × HK/68 wt, clone *ts*-1[E]	HK/68-*ts*-1[E] × Ud/72 wt			HK/68-*ts*-1[E] × GA/74 wt, Clone 2	HK/68-*ts*-1[E] × Vic/75 wt		
			Clone 13	Clone 16	Clone 24		Clone 67	Clone 81	Clone 113
1	P3	GL (*ts*)	E (*ts*)	E (*ts*)	E (*ts*)	E (*ts*)	E (*ts*)	E (*ts*)	E (*ts*)
2	P1	HK	E	E	wt	E	E	wt	E
3	P2	HK	wt	E	wt	E	E	wt	E
4	HA	HK	wt	wt	wt	wt	wt	wt	wt
5	NA	GL	wt	E	E	wt	E	wt	wt
6	NP	GL (*ts*)	wt	E (*ts*)	E (*ts*)	E (*ts*)	E (*ts*)	E (*ts*)	E (*ts*)
7	M	HK	wt	E	wt	E	E	wt	wt
8	NS	HK	wt	E	E	wt	E	wt	wt
Pre-inoculation serum NA-inhibiting antibody		yes	yes	yes	yes	yes	yes	no	no
Virulence[b] in adults		low	moderate	low	low	low	low	moderate	moderate
		Trial A	Trial B						
Pre-inoculation serum NA-inhibiting antibody		yes	no	NT[c]	NT	no	NT	NT	NT	NT
Virulence in children		low	moderate	NT	NT	moderate	NT	NT	NT	NT

[a] Ritchey et al. (19) determined the order of migration of genes for HK/68 wild-type (wt) virus. The genotype of HK/68-*ts*-1[E] virus is given in the GL/65-*ts*-1 × HK/68 wt, clone *ts*-1[E] column (12, 19); the other columns give the genotypes of *ts*-1[E] recombinant viruses, as determined in these studies. GL designates genes derived from the Great Lakes/65-*ts*-1[E] parent (20); HK designates genes derived from the HK/68 wild-type parent (12, 19, 20); E designates genes derived from the HK/68-*ts*-1[E] parent; wt designates genes derived from the respective wild-type parent; and *ts* designates genes that bear a lesion which confers the *ts* phenotype (12, 20).

[b] Low, <5% of vaccinees developed fever or systemic symptoms or both; moderate, >10% but <20% of vaccinees developed fever or systemic symptoms or both.

[c] NT, Not tested.

and children, and the level of attenuation was determined (2, 4, 9, 15, 17). In Table 1 the genotypes of the *ts*-1[E] viruses are presented along with the levels of attenuation of the recombinant viruses and the pre-inoculation serum NA-inhibiting antibody status of the vaccinees. The correlation of these three factors is considered below.

DISCUSSION

The genetic constitution of seven influenza A *ts*-1[E] recombinant vaccine strains was determined by PAGE of recombinant and parental virion RNAs under partially denaturing conditions (Table 1). The results of these studies are consistent with previous data concerning the genetic constitution of these recombinants with respect to HA, NA, P3, and NP genes. The HA and NA surface antigenic subtype had been determined previously by serological technique, and the P3 and NP genes of the HK/68-*ts*-1[E] *ts* parent vaccine strain had been shown to be determinants of the *ts*-1[E] *ts* phenotype by genetic testing for complementation of plaque formation, using *ts* virus probes with a single *ts* lesion in the P3 or NP gene (6, 7, 15, 18). In the present study the parental origin of P1, P2, M, and NS genes, for which serological or genetic markers are not available, was determined by PAGE.

The HK/68-*ts*-1[E] donor virus has two *ts* genes (P3 and NP) and one non-*ts* gene (NA) derived from its *ts* parent, Great Lakes/65-*ts*-1 virus (6, 12). The remaining five genes were derived from wild-type HK/68 virus (Table 1). The *ts*-1[E] virus is attenuated with respect to

its HK/68 wild-type parent by all in vitro and in vivo assessments (6). Because of the evidence for the association of the *ts* phenotype and attenuation (5, 16), we inferred that the level of attenuation of the *ts*-1[E] donor virus was a function of its two *ts* genetic lesions. We also inferred that a *ts*-1[E] recombinant would be attenuated to the level of the donor virus, if it received the *ts*-1[E] *ts* genes, without respect to its remaining genotype. Thus, wild-type genes other than those coding for P3 and NP (the sites of the *ts*-1[E] mutations) would not be expected to influence attenuation or abrogate the attenuating effect of the *ts*-1[E] *ts* genes. The inheritance of HK/68-*ts*-1[E] genes among recombinants suggests that these inferences are correct.

Verification of the identity of the attenuating genes in the HK/68-*ts*-1[E] donor virus is made difficult by two factors. First, the gene constellations of the recombinants examined represent less than 10% of all possible combinations of *ts*-1[E] and wild-type genes (if six loci, other than P3 and NP, can be substituted in a *ts*-1[E] virus). It is possible that certain constellations of genes in *ts*-1[E] recombinant viruses that were not represented could have rendered a *ts*-1[E] recombinant virus either more virulent or more attenuated than those *ts*-1[E] recombinants that were evaluated. Second, some vaccinees possessed NA immunity, whereas others did not. Comparison of two clones of *ts*-1[E] recombinant viruses for differences in their level of attenuation requires that the viruses be given to volunteers with a similar background of NA immunity.

Despite these difficulties, it appears possible to identify two attenuating *ts*-1[E] genes by reference to the genotypes of recombinants alone. Correlation of the genotypes of *ts*-1[E] recombinant viruses with level of attenuation for adults who possessed NA immunity, but lacked serum hemagglutination-inhibiting antibody for HA, i.e., clones 2, 13, 16, 24, and 67, revealed that the presence of both *ts*-1[E] *ts* genes (P3 and NP) was associated with satisfactory attenuation. In contrast, substitution of a wild-type NP gene for its *ts* counterpart (Ud/72-*ts*-1[E] clone 13) was associated with an increase in virulence (Table 1), although this virus was still attenuated with respect to its wild-type parent. This observation suggests that the HK/68-*ts*-1[E] NP gene contributes to attenuation of *ts*-1[E] recombinants that possess it. Among the seven *ts*-1[E] recombinants, there was an example of inheritance of a wild-type parental gene at each locus except P3. The absence of an HK/68-*ts*-1[E] gene at the P1, P2, HA, NA, M, or NS locus did not alter the level of attenuation of the respective *ts*-1[E] recombinants. Each of the seven recombinants was attenuated with respect to wild type, and the only *ts*-1[E] gene they had in common was the P3 gene. Therefore, inheritance of the HK/68-*ts*-1[E] P3 gene by a recombinant resulted in attenuation. The fact that the *ts*-1[E] recombinants (clones 2, 16, 24, and 67), representing three H3N2 antigenic variants, were attenuated to the level of HK/68-*ts*-1[E] parent virus in adults with NA immunity is consistent with the hypothesis that the presence of both the P3 and NP *ts*-1[E] genes in a recombinant resulted in a specified level of attenuation irrespective of inheritance at the other loci. That is, the other genes of the *ts*-1[E] viruses neither contributed detectably to attenuation nor modified the attenuating effect of the *ts* genes. This conclusion is also supported by data on restriction of replication of the *ts*-1[E] recombinant viruses in hamsters compared with wild-type virus (7, 18).

Since NA immunity in the host reduces virulence of wild-type viruses (8), we expected that this would also be true for attenuated influenza A viruses. The HK/68-*ts*-1[E] virus and the Ud/72-*ts*-1[E] clone 24 virus exhibited low virulence in vaccinees with NA immunity, whereas moderate virulence was evident in children lacking such immunity (4, 17). Based on those results, the moderate virulence of Vic/75-*ts*-1[E] recombinants (clones 81 and 113) was attributed to the fact that adult volunteers lacked antibody against the Vic/75 NA ($N2_{75}$) (2, 9). The low virulence of Vic/75-*ts*-1[E] recombinant clone 67 was attributed to the presence of pre-existing antibody against the NA of this virus ($N2_{65}$) (2). This interpretation presupposed that the virulence of *ts*-1[E] recombinants would not be affected by the inheritance of genes from the wild-type H3N2 parent virus. The present studies support that hypothesis with one reservation. We cannot rule out the possibility that the moderate virulence of clones 81 and 113 was a specific function of the Vic/75 M gene or NS gene or both (Table 1). We can argue by extrapolation that this is unlikely, since the virulence of Ud/72 clone 24 and Ga/74 clone 2 *ts*-1[E] recombinants was not affected by their respective wild-type M and NS genes.

Our observations support the view that it is possible to transfer *ts* genes that confer a specific and reproducible level of attenuation from a *ts* donor virus to newly emerging variants of influenza A virus. In the case of the HK/68-*ts*-1[E] donor virus, the P3 and NP genes are the attenuating genes, whereas the genes at the other loci do not appear to significantly modify virulence. Although the level of attenuation conferred by

the two *ts*-1[E] *ts* genes is not sufficient for vaccinees who lack both HA and NA immunity, these *ts* genes effected a satisfactory level of attenuation for vaccinees with NA immunity.

LITERATURE CITED

1. **Desselberger, U., and P. Palese.** 1978. Molecular weights of RNA segments of influenza A and B viruses. Virology **88**:394-399.
2. **Douglas, R. G., L. J. Markoff, B. R., Murphy, R. M. Chanock, R. F. Betts, F. G. Hayden, M. M. Levine, G. A. Van Blerk, S. B. Sotman, and D. R. Nalin.** 1979. Live Victoria/75-*ts*-1[E] vaccines in adult volunteers: role of hemagglutinin immunity in protection against illness and infection caused by influenza A virus. Infect. Immun. **26**:274-279.
3. **Floyd, R. W., M. P. Stone, and W. K. Joklik.** 1974. Separation of single stranded RNA by acrylamide agarose-urea gel electrophoresis. Anal. Biochem. **59**:599-609.
4. **Kim, H. W., J. O. Arrobio, C. D. Brandt, R. H. Parrott, B. R. Murphy, D. D. Richman, and R. M. Chanock.** 1976. Temperature-sensitive mutants of influenza A virus. Response of children to the influenza A/Hong Kong/68-*ts*-1[E] (H3N2) and influenza A/Udorn/72-*ts*-1[E] (H3N2) candidate vaccine viruses and significance of immunity to neuraminidase antigen. Pediatr. Res. **10**:238-242.
5. **Murphy, B. R., E. G. Chalhub, S. R. Nusinoff, and R. M. Chanock.** 1972. Temperature-sensitive mutants of influenza virus. II. Attenuation of *ts* recombinants for man. J. Infect. Dis. **126**:170-178.
6. **Murphy, B. R., E. G. Chalhub, S. R. Nusinoff, J. Kasel, and R. M. Chanock.** 1973. Temperature-sensitive mutants of influenza virus. III. Further characterization of the *ts*-1[E] influenza A recombinant (H3N2) virus in man. J. Infect. Dis. **128**:479-487.
7. **Murphy, B. R., N. T. Hosier, S. B. Spring, S. R. Mostow, and R. M. Chanock.** 1978. Temperature-sensitive mutants of influenza A virus: production and characterization of A/Victoria/3/75-*ts*-1[E] recombinants. Infect. Immun. **20**:665-670.
8. **Murphy, B. R., J. A. Kasel, and R. M. Chanock.** 1972. Association of serum anti-neuraminidase antibody with resistance to influenza in man. N. Engl. J. Med. **286**: 1329-1332.
9. **Murphy, B. R., L. J. Markoff, N. T. Hosier, H. M. Rusten, R. M. Chanock, A. P. Kendal, R. G. Douglas, R. F. Betts, T. R. Cate, Jr., R. B. Couch, M. M. Levine, D. H. Waterman, and H. P. Holley, Jr.** 1978. Temperature-sensitive mutants of influenza A virus: evaluation of A/Victoria/3/75-*ts*-1[E] recombinant viruses in volunteers. Infect. Immun. **20**:671-677.
10. **Murphy, B. R., D. D. Richman, S. B. Spring, and R. M. Chanock.** 1976. Recent progress in the development and assessment of live attenuated vaccines. Postgrad. Med. **52**:381-388.
11. **Palese, P.** 1977. The genes of influenza virus. Cell **10**:1-10.
12. **Palese, P., and M. B. Ritchey.** 1977. Live attenuated influenza virus vaccines: strains with temperature-sensitive defects in the P3 protein and nucleoprotein. Virology **78**:183-191.
13. **Palese, P., and J. L. Schulman.** 1976. Differences in RNA patterns of influenza A viruses. J. Virol. **17**:876-884.
14. **Palese, P., and J. L. Schulman.** 1976. Mapping of the influenza virus genome: identification of the HA and NA genes. Proc. Natl. Acad. Sci. U.S.A. **73**:2142-2146.
15. **Richman, D. D., B. R. Murphy, R. B. Belshe, H. M. Rusten, R. M. Chanock, N. R. Blacklow, T. A. Parrino, F. B. Rose, M. M. Levine, and E. Caplan.** 1977. Temperature-sensitive mutants of influenza A virus. XIV. Production and evaluation of influenza A/Georgia/74-*ts*-1[E] recombinant viruses in human adults. J. Infect. Dis. **136**:256-262.
16. **Richman, D. D., B. R. Murphy, and R. M. Chanock.** 1977. Demonstration of a non-temperature-sensitive growth-restricting mutation in a *ts* mutant of influenza A virus: implications for live virus vaccine development. Virology **83**:356-364.
17. **Richman, D. D., B. R. Murphy, R. M. Chanock, J. M. Gwaltney, Jr., R. G. Douglas, R. F. Betts, N. R. Blacklow, F. B. Rose, T. A. Parrino, M. M. Levine, and E. S. Caplan.** 1976. Temperature-sensitive mutants of influenza A virus. XII. Safety, antigenicity, transmissibility, and efficacy of influenza A/Udorn/72-*ts*-1[E] recombinant viruses in human adults. J. Infect. Dis. **134**:585-594.
18. **Richman, D. D., B. R. Murphy, S. B. Spring, M. T. Coleman, and R. M. Chanock.** 1975. Temperature-sensitive mutants of influenza virus. IX. Genetic and biological characterization of *ts*-1[E] lesions when transferred to a 1972(H3N2) influenza A virus. Virology **66**: 551-562.
19. **Ritchey, M. B., P. Palese, and J. L. Schulman.** 1976. Mapping of the influenza genome. III. Identification of genes coding for nucleoprotein, membrane protein, and nonstructural protein. J. Virol. **20**:307-313.
20. **Spring, S. B., S. R. Nusinoff, J. V. Mills, D. D. Richman, E. L. Tierney, B. R. Murphy, and R. M. Chanock.** 1972. Temperature-sensitive mutants of influenza virus. VI. Transfer of *ts* lesions from the Asian subtype of influenza A virus (H2N2) to the Hong Kong subtype (H3N2). Virology **66**:522-532.