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Phil. Trans. R. Soc. Lond. B 1980 **288**, 401-415
doi: 10.1098/rstb.1980.0017

Phil. Trans. R. Soc. Lond. B **288**, 401–415 (1980)
Printed in Great Britain

Genetic approaches to attenuation of influenza A viruses for man

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The explosion of new information concerning the influenza A viral genome provides a basis for deliberate manipulation of its genes with the intent of introducing specific mutations that render influenza virus attenuated and useful for prevention of disease. Currently there is 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. In this manner new antigenic variants of influenza A virus may be satisfactorily attenuated after transfer of the mutant genes. The mutant genes must be readily identifiable by simple in-vitro techniques, thus enabling the genetic basis of attenuation to be monitored directly during all phases of vaccine development, manufacture and utilization in man. We describe our experience with two sets of *ts* mutant genes which affect viral RNA transcription or synthesis and which effect a reproducible level of attenuation in wild-type influenza A virus.

The explosion of new information concerning the structure and function of the influenza virus genome provides a basis for deliberate manipulation of its genes with the intent of introducing specific mutations into the virus that render it attenuated and potentially useful for prevention of disease in man. Viruses bearing specific, identifiable, attenuating mutations represent the live vaccine strains of the future, since the genetic basis of attenuation can be monitored directly during all phases of vaccine development, manufacture and utilization in man. Antigenic variability, which is the hallmark of influenza A virus, does not contraindicate the use of defined, identifiable, attenuating mutations for generation of live vaccine strains. On the contrary, this approach offers the most reasonable hope for controlling influenza epidemics or pandemics by live virus vaccine.

The interval between recognition of a new, potentially epidemic or pandemic virus and its spread throughout the world is rather short, perhaps 5–6 months. It is essential, therefore, that at the time of an epidemic or pandemic threat, live vaccine strains be developed and evaluated rapidly. Attenuation can be accomplished quickly by transferring mutant genes from a donor

attenuated strain to the new epidemic or pandemic strain by genetic reassortment (Maassab *et al.* 1972; McMahon & Schild 1972; Murphy *et al.* 1976). It is implicit in this approach that the mutations responsible for attenuation not affect the genes that code for the haemagglutinin and neuraminidase surface glycoproteins, otherwise these mutations could not be transferred to recombinants bearing the surface antigens of the new epidemic or pandemic virus. It is also implicit that the attenuating genetic lesions present in a recombinant influenza virus must be identifiable by simple in-vitro techniques. The time constraints imposed by the rapid spread of new pandemic viruses preclude extensive testing of the final vaccine virus. Instead, the master attenuated donor strain and its attenuating genes would be certified based upon the prior demonstration that transfer of these genes into a series of different virulent viruses had regularly brought about a satisfactory level of attenuation. Subsequently, each new recombinant bearing the surface antigens of the incipient epidemic or pandemic virus would be subjected to a limited amount of human testing before rapidly escalating its use in the general population.

The major challenge inherent in this approach to the production of live virus vaccine is the identification of a stable mutant gene or set of genes that will regularly confer a satisfactory level of attenuation. Before discussing the specific approaches that have been used to produce attenuated donor viruses, it is necessary to consider several aspects of the genetic control of virulence that are relevant to the development of live influenza virus vaccines.

It appears that a mutation in essentially any of the RNA genes of the influenza A virus that impairs viral replication can bring about a reduction of virulence. We have shown that a temperature sensitive (*ts*) mutation in the P1, P3, or NP gene results in a reduction of replication of the virus in hamster lungs (Richman *et al.* 1975; Palese & Ritchey 1977; Murphy *et al.*, unpublished observations). Scholtissek *et al.* (1977) have demonstrated that substitution of a single gene from a mammalian influenza A virus (attenuated for birds) for its corresponding gene in a highly virulent avian influenza A virus can effect attenuation of the recombinant for the natural avian host. This was shown for seven of the eight RNA segments of the influenza A virus genome. Such attenuated recombinant avian viruses with a single gene substitution are, in essence, host range (*hr*) mutants bearing an *hr* gene, derived from a mammalian virus, that causes a reduction in the efficiency of viral replication in avian cells. These observations suggest that the influenza A virus genome is subject to alteration on a broad genetic front in that mutation in at least seven of the eight influenza A virus genes can result in attenuation. Furthermore, a mutant gene or set of genes that confers attenuation can be sought in any of the six genes that do not code for the surface glycoproteins.

At this time a defined set of stable mutant genes capable of regularly effecting a satisfactory level of attenuation remains a goal rather than a reality. Several recent experiences illustrate the difficulties that must be overcome in this area. First, during passage and/or genetic reassortment, the attenuating effect of certain mutations can be diminished by subsequent mutation or possibly by the action of other proteins in the virus. The most striking example of this phenomenon is seen with temperature sensitive (*ts*) mutations, which appear to be directly responsible for restriction of viral growth *in vitro* and *in vivo* and which are easily identified and characterized by the plaque technique (Richman *et al.* 1977*b*; Spring *et al.* 1975*b*). Subtle changes in the *ts* phenotype can be detected by a small increase in the efficiency of plaque formation at restrictive temperature. In a recent study, the A/Udorn/72-*ts*-1A2 (H3N2) attenuated donor virus, which has a *ts* mutation on the P1 and P3 genes, was mated with wild-type A/Alaska/77 (H3N2) virus and the two *ts* genes from the donor were segregated into recombinant viruses.

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Two recombinants bearing the *ts* P1 gene were compared for temperature sensitivity of viral replication *in vitro* and restriction of growth in hamster upper and lower respiratory tract (figure 1). Although the *ts* P1 genes present in these two plaque purified Alaska/77-*ts*-1A2 recombinants were derived from the same *ts* donor parent, the recombinants differed in their shutoff temperature for plaque formation and restriction of growth *in vivo*. The level of replication of the two recombinants in hamster lungs correlated with the shutoff temperature for plaque formation *in vitro*. The virus bearing the unmodified *ts* P1 gene (the 38 °C recombinant, clone 6) showed a greater temperature-sensitivity of replication *in vitro* and was more restricted in replication in hamster lungs. However, both P1 recombinants replicated like wild-type virus in the cooler nasal turbinates.

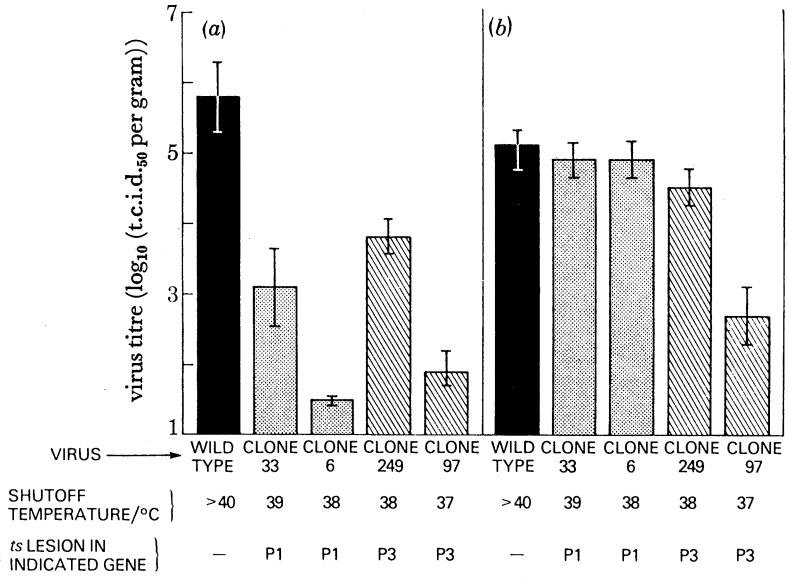

FIGURE 1. Viral replication in the lungs and nasal turbinates of hamsters infected intranasally with 10^5 t.c.i.d.$_{50}$ of Alaska/77 wild-type (w.t.) and *ts* viruses (clone numbers given). Six hamsters per virus were killed daily for 3 days, and the lungs (*a*) and nasal turbinates (*b*) were harvested. Each organ homogenate was titred individually, and the mean $\log_{10}$ titres were determined for each day. The maximum level of replication achieved for each virus over the 3 day period is indicated together with the standard error of the mean. w.t., wild-type.

Two segregants bearing the *ts* P3 gene were also analysed in a similar manner (figure 1). The two *ts* P3 gene segregants also differed in restriction of growth *in vitro* and *in vivo*. The segregant bearing the unmodified *ts* P3 gene (clone 97) was more restricted *in vitro* and was also more restricted in its growth in the lungs. This recombinant was the only recombinant that was restricted in growth in the nasal turbinates. The modifications of the P1 and P3 *ts* mutations probably occurred as a result of an additional mutation in the *ts* gene or a mutation in another gene resulting in a 'suppressor' effect. It is also possible that one or more wild-type proteins coded for by genes from the wild-type parent acted to partly correct the physiological defect of the *ts* protein through protein–protein interaction.

Similar observations have been made with recombinants derived from a cold-adapted (*ca*) donor virus (A/AA/6/60) that was also *ts*. The maximum number of genes from this donor were transferred into recombinants bearing the two surface glycoproteins of wild type A/Queensland/72 (H3N2), A/Alaska/77 (H3N2), or A/Hong Kong/77 (H1N1). Two of the

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recombinants exhibited the same temperature sensitivity as the *ca* donor virus, i.e. a 37 °C shutoff temperature for plaque formation. In contrast, the third recombinant was significantly less *ts* with a 39 °C shutoff temperature (table 3).

Another difficulty stems from the fact that the level of genetic stability of a mutation at a given locus can also be influenced by subsequent mutation and this variation may be independent of the restrictive effect of the original mutation on viral replication. We have evaluated the level of genetic stability of two A/Vic/75-*ts* recombinants, each of which has a *ts* P3 gene

TABLE 1. COMPARISON OF THE GENETIC STABILITY OF TWO *ts* VIRUSES AFTER REPLICATION IN HAMSTERS

Vic/75 *ts* virus	shutoff† temperature °C	*ts* lesion in indicated gene(s)	reduction of replication of *ts* virus compared with wild-type virus in hamster lung ($\log_{10}$(t.c.i.d.$_{50}$/ml))	percentage of lung isolates that contained ts^+ virus
Clone 81	38	P3, NP	2.9	50 (7/14)‡
Clone 65	38	P3	1.8	0 (0/44)‡

Note that the *ts* P3 genes of clones 81 and 65 originated from the same *ts* parent (A/Great Lakes/65 *ts*-1 mutant).

† Shutoff temperature is defined as the lowest temperature at which a 100-fold or greater reduction of virus titre (p.f.u. per millilitre) is observed.

‡ Number of lung suspensions yielding ts^+ virus/number tested.

that originated from the same *ts* virus (A/Great Lakes/65 *ts*-1) (Murphy *et al.* 1978*d*; Spring, *et al.* 1975*b*). Genetic stability was assessed by determining the frequency with which virus that had lost the *ts* phenotype, i.e. ts^+ virus, was isolated from hamster lungs (table 1). The clone 65 A/Vic/75 recombinant virus, which had a *ts* mutation on the P3 gene, was significantly more stable genetically *in vivo* than the clone 81 virus possessing *ts* mutations on the P3 and NP genes. No ts^+ virus was isolated from 44 hamsters infected with clone 65 virus, whereas 50% of clone 81 virus-infected hamsters yielded ts^+ virus (Murphy *et al.* 1978*d*). Although the *ts* P3 gene of the clone 65 and clone 81 recombinants ultimately originated from the same *ts* virus, the *ts* P3 gene in the clone 65 virus remained stable during passage and/or reassortment, whereas the *ts* P3 gene in the clone 81 virus underwent subsequent modification. This occurred even though the clone 65 virus replicated to a higher titre in the lungs.

The experiences just cited indicate that mutant genes can be further modified during passage and/or reassortment in a manner that can alter their attenuating effect and their genetic stability. The ideal master donor strain should possess attenuating mutant genes that resist such genetic change.

HOST RANGE MUTANTS

Three techniques are currently being used to produce attenuated donor strains of influenza A virus through mutation. The first method involves the use of a donor virus that has been passaged many times in embryonated eggs (Smorodincev 1969). This procedure leads to the emergence of host range (*hr*) mutants that grow poorly in man and that do not cause symptoms of respiratory disease.

Transfer of *hr* genes by genetic reassortment has been used by investigators in Belgium and the U.K. in an attempt to attenuate new epidemic influenza A viruses (McMahon & Schild 1972; Huygelen *et al.* 1973; Lobmann *et al.* 1977; Morris *et al.* 1975; Nicholson & Tyrrell

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1976). Since in-vitro markers of attenuation are not available, it has been necessary to assay virulence and identify suitably attenuated recombinants by tests in volunteers. Subsequently, many of the recombinants were genotyped by gel electrophoresis in an attempt to determine which constellation of genes derived from the *hr* mutant parent correlated with a satisfactory level of attenuation. Much of this work has been performed with the influenza A/PR-8/34 (H0N1) virus as the *hr* mutant, attenuated donor (Beare & Hall 1971; Beare *et al.* 1975). Since the influenza virus genome contains eight genes and since the two genes for the surface antigens, i.e. the haemagglutinin and neuraminidase, must come from the virulent parent, the maximum number of genes that can be transferred from the *hr* parent to a new recombinant is six. These include the RNA segments that code for the P1, P2, P3, NP, M and NS proteins.

TABLE 2. PARENTAL ORIGIN OF GENES IN A/PR-8/34 × A/AICHI/68 RECOMBINANT VIRUS: RELATION OF GENOTYPE TO VIRULENCE IN MAN

influenza A virus gene	parental origin of genes in X31 recombinant derived from A/PR-8/34 and A/Aichi/68 parents†		
	PR-8	X31	Aichi/68
P3	□	□	■
P1	□	□	■
P2	□	□	■
HA	□	■	■
NA	□	■	■
NP	□	□	■
M	□	□	■
NS	□	□	■
virulence for adult volunteers‡	none	moderate	not tested

□, Gene derived from PR-8/34 parent; ■, gene derived from Aichi/68 parent.
† P. Palese (personal communication).
‡ Beare *et al.* (1975).

A series of recombinant viruses that were produced by mating the A/PR-8/34 (H0N1) non-virulent virus and H3N2 wild-type virus have been evaluated in volunteers for their level of attenuation (Beare *et al.* 1975). One virus, the X-31 recombinant, received all six transferable PR-8 genes but retained moderate virulence for man (table 2). Since the PR-8 virus was non-virulent for man, it is likely that the haemagglutinin and neuraminidase genes of the PR-8 parent bear significant *hr* mutations. The use of this particular donor virus does not appear to be promising because the degree of restriction conferred by the maximum number of transferable genes was not sufficient to yield a satisfactory level of attenuation. Similar observations were made with another PR-8 × H3N2 wild-type recombinant that received five (possibly six) genes from the PR-8 parent (Oxford *et al.* 1978).

In an effort to further attenuate A/PR-8/34 recombinants, investigators at R.I.T. in Belgium have introduced a mutation into the haemagglutinin gene by selecting for mutants that are resistant to a serum inhibitor of haemagglutination (Huygelen *et al.* 1973; Lobmann *et al.* 1976). Previously it had been shown that mutation to inhibitor resistance was accompanied by a decrease in virulence of influenza A virus for man (Beare & Bynoe 1969). Of the *hr* recombinants that were subsequently made inhibitor-resistant by investigators at R.I.T.

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in Belgium and that have been evaluated experimentally in volunteers, some were found to be suitably attenuated for adults and young children (Hall *et al.* 1975; Kunz & Hofmann, 1977; Mackenzie *et al.* 1975; Rubin *et al.* 1976). Vaccine containing inhibitor-resistant A/PR-8/34 (H0N1) recombinants has been licensed in several European countries; however, it remains to be shown that transfer of the full complement of non-haemagglutinin and non-neuraminidase *hr* genes from PR-8, coupled with a mutation in the haemagglutinin gene derived from the virulent parent, will regularly yield recombinants that are satisfactorily attenuated for completely susceptible individuals.

So far no set of *hr* genes that will regularly confer a satisfactory level of attenuation on recombinant viruses has been identified. The experience with the A/PR-8/34 and A/Okuda/57 donor viruses suggests that it may not be possible to achieve this desired level of host range restriction by multiple passage of human influenza A virus in eggs. The use of *hr* genes from avian influenza A viruses to produce attenuated recombinant viruses for man has not been investigated, but this approach is worth considering. Thus, it may be possible to produce donor influenza A viruses bearing the desired *hr* mutations by performing experiments that are the reciprocal of those described by Scholtissek *et al.* (1977). One or more avian influenza A virus genes would be substituted for the corresponding genes in a human influenza A virus. Such an approach is not free from theoretical hazards (Rott *et al.* 1978; Scholtissek *et al.* 1978); it should nevertheless be investigated since recombinant viruses bearing *hr* genes that have evolved over a long period should be quite stable genetically. This is a property desirable for a live virus vaccine strain and one that has been difficult to attain through chemical mutagenesis or by cold-adaptation. The use of *hr* genes for attenuation would be hastened if it were possible to develop in-vitro techniques for identification of *hr* mutations and for detection of genetic alteration in these genes, which bring about an increase in virulence. Without such techniques the suitability of each new recombinant must be evaluated by time-consuming trials in human volunteers.

Cold-adapted (*ca*) mutants

The second technique now being actively pursued for rapid attenuation of new influenza A viruses involves the use of a donor virus that grows well at a suboptimal temperature that does not support efficient replication of wild-type virus (Maassab 1967). The A/AA/60 (H2N2) donor strain was adapted to grow well at 25 °C (Maassab 1967). This virus is also *ts* with a shutoff temperature for plaque formation of 37 °C (Maassab 1969; Spring *et al.* 1977). Genes from this cold-adapted (*ca*) master strain have been transferred to a succession of new antigenic variants of influenza A virus, and recombinant viruses bearing the *ca* and *ts* properties of the attenuated donor virus were isolated. When the RNA segments of the *ca* donor strain and a series of 35 *ca* recombinants were analysed by polyacrylamide gel electrophoresis, it was possible to identify the parental origin of each of the eight genes of the 35 recombinant viruses (Cox *et al.* 1979). Three genes from the *ca* donor virus were present in all 35 *ca* recombinants, i.e. the genes represented by RNA segments 1, 3, and 6 (NP). It is probable that one or more of these genes represent the site or sites of the *ca* and *ts* mutations.

Several *ca* recombinants derived from the A/AA/60 *ca* master strain were evaluated in volunteers and found to be attenuated (table 3) (Davenport *et al.* 1977; Hrabar *et al.* 1977; Murphy *et al.* 1979*b*). The genotype of these recombinant viruses is presented (table 3) in relation to their in-vitro shutoff temperature of plaque formation, their level of attenuation for

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man and the immune status of vaccinees. Four of the *ca* recombinants appeared to be satisfactorily attenuated and antigenic; however, the A/Scot/74 (H3N2) recombinant caused fever and systemic symptoms (Murphy *et al.* 1979*b*). Each of the *ca* recombinants possessed the *ca* phenotype, i.e. high efficiency of plaque formation at the suboptimal temperature of 25 °C.

TABLE 3. PARENTAL ORIGIN OF GENES IN COLD-ADAPTED (*ca*) RECOMBINANT VIRUSES DERIVED FROM A MATING OF A/AA/6/60 *ca* PARENT AND WILD-TYPE VIRUS OF THE H3N2 OR H1N1 SUBTYPE: RELATION TO VIRULENCE

RNA segment number	gene product	parental origin of genes in *ca* recombinant viruses derived by mating the A/AA/6/60 *ca* virus and the indicated wild-type virus				
		A/Queensland/72 H3N2	A/Scotland/74 H3N2	A/Victoria/75 H3N2	A/Alaska/77 H3N2	A/Hong Kong/77 H1N1
1	not determined	□	□	□	□	□
2	not determined	□	□	■	□	□
3	not determined	□	□	□	□	□
4	HA	■	■	■	■	■
5	NA	■	■	■	■	■
6	NP	□	□	□	□	□
7	M	□	□	□	□	□
8	NS	□	■	□	□	□
shutoff temperature/°C		37	39	39	39	37
virulence in volunteers with serum HI† antibody ⩽ 1:8 and						
(*a*) serum NI‡ ⩽ 1:2		—	—	low	—	none
(*b*) serum NI > 1:2		none	moderate	—	none	—

■, Gene derived from wild-type virus; □, gene derived from A/AA/6/60 cold-adapted parent.
† Haemagglutination-inhibiting.
‡ Neuraminidase-inhibiting.

This marker was therefore not useful in predicting satisfactory attenuation for man. The *ca* recombinants were all also temperature sensitive, but they differed in their level of temperature sensitivity. The relation between level of attenuation and temperature sensitivity was analysed because the level of temperature sensitivity of a virus can function as a determinant of its level of replication in animals and of attenuation in man (Richman *et al.* 1977*b*). The A/Scot/74 (H3N2), A/Vic/75 (H3N2), and A/Alaska/77 (H3N2) *ca* recombinants each had a 39 °C shutoff temperature, but only the A/Scot/74 virus induced febrile influenzal illness. This suggests that attenuation of the *ca* recombinants is not due solely to *ts* mutation(s) and that other mutations in these recombinants also act as determinants of reduced virulence.

Possibly a gene, or set of genes, other than those responsible for the *ca* and *ts* phenotypes contribute to attenuation of the A/AA/60 *ca* parent virus and its recombinants. If such an attenuating gene or genes were not on the RNA segments that determine the *ca* and *ts* properties, the level of attenuation and the *ca* and *ts* phenotypes could segregate independently during genetic reassortment. In this case, the level of attenuation of *ca* recombinant viruses would be determined in part by the presence or absence of these hypothetical attenuating genes. Such a non-*ts* attenuating gene has been detected in a 5-fluorouracil-induced *ts* mutant of an influenza A virus, and this non-*ts* gene segregated independently of *ts* genes (Richman *et al.* 1977*b*). The

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A/AA/60 *ca* donor virus probably contains one or more *hr* mutations since the virus was passaged 29 times in primary chick kidney tissue culture and twice in eggs (Murphy *et al.* 1979*b*). This type of passage in a heterologous host generally selects for *hr* mutations. The gene that codes for the non-structural (NS) protein is a candidate for such an attenuating *hr* gene. As shown in table 3, the A/Scot/74 *ca* recombinant did not receive its NS gene from the A/AA/60 attenuated parent, whereas each of the other recombinants received this A/AA/60 gene. If the NS gene of the A/AA/60 parent contributes significantly to attenuation of the donor virus and its recombinants, its absence from the A/Scot/74 *ca* recombinant could account for the apparent reactogenicity of this *ca* recombinant.

Analyses of virus isolates from volunteers infected with the A/Scot/74 (H3N2), A/Vic/75 (H3N2) or A/Hong Kong/77 (H1N1) *ca* recombinant indicated that the *ts* property was stable (Murphy *et al.* 1979*b*, and unpublished observations). Each of 79 isolates from 42 adult volunteers retained the *ts* property; this included 46 isolates from H1N1 vaccinees who lacked immunity to both the haemagglutinin and neuraminidase surface antigens and who shed virus for up to 9 days. The *ca* property of isolates was not as stable since virus which lost some of its efficiency of growth at 25 °C was recovered from individuals infected with the A/Scot/74 or A/Vic/75 *ca* recombinant (Murphy *et al.* 1979*b*).

The A/AA/60 *ca* mutant appears to be a promising donor virus; however, several properties of this virus require additional study before its ultimate value as a donor of attenuating genes can be resolved. First, although four *ca* recombinant viruses tested in man were satisfactorily attenuated, one recombinant retained some virulence. The genetic basis underlying this variation in attenuation remains to be determined. Incomplete attenuation could result from genetic modification of attenuating genes that occurred during development and production of vaccine or possibly from failure of a recombinant to receive one or more attenuating genes from the A/AA/60 *ca* donor. Secondly, the genetic determinants of the *ca* phenotype can undergo mutation as indicated by the recovery of ca^+ virus from infected volunteers. Thirdly, the level of temperature sensitivity of *ca* recombinant viruses can differ significantly among viruses that receive the maximum number of transferable genes from the *ca* donor virus. For example, the A/Queensland/72 (H3N2) recombinant had a 37 °C shutoff temperature for plaque formation, whereas the A/Alaska/77 (H3N2) recombinant exhibited a 39 °C shutoff (table 3). This suggests that the genetic determinants of this phenotype are not completely stable. The recovery of ts^+ virus from experimentally infected hamsters also supports this interpretation (Maassab *et al.* 1978).

To sort out these phenomena and to make more rapid progress in this area, it may be necessary to define the relative contribution to attenuation of each of the six transferable genes of the *ca* donor virus. Similarly, an assessment of the relative importance of the *ca*, *ts* and *hr* mutations in attenuation would prove most helpful. Finally, there is a need to develop in-vitro assay systems to detect change in the attenuating genes which might bring about an increase in virulence.

Temperature-sensitive mutants

The third approach to production of attenuated recombinants of influenza A virus involves the use of donor strains that possess temperature-sensitive (*ts*) mutations (Murphy *et al.* 1976). This approach takes advantage of the temperature differential that exists in the respiratory tract: the upper passages of the respiratory tract are several degrees cooler than the lungs.

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Therefore, *ts* recombinants are generated with the expectation that their *ts* mutations will suppress growth of virus in the lower respiratory tract; *ts* recombinants that are restricted in their replication *in vitro* at 37–38 °C should also grow poorly in the lower respiratory tract which has a temperature of 37 °C. However, such *ts* recombinants should grow reasonably well in the cooler passages of the upper airways, which have a temperature of 32–34 °C.

TABLE 4. PARENTAL ORIGIN OF GENES IN *ts*-1[E] RECOMBINANT VIRUSES: RELATION OF VIRULENCE TO THE GENOTYPE OF THE VACCINE STRAIN AND NEURAMINIDASE IMMUNITY OF SERUM HI ANTIBODY NEGATIVE VACCINEES

		Parental origin of genes in *ts*-1[E] recombinant viruses derived by mating indicated pairs of *ts* and wild-type (w.t.) viruses							
		GL/65-*ts*-1 × HK/68 w.t.	HK/68-*ts*-1[E] × Ud/72 w.t.			HK/68-*ts*-1[E] × GA/74 w.t.	HK/68-*ts*-1[E] × Vic/75 w.t.		
RNA segment number	gene product	clone *ts*-1[E]	clone 13	clone 16	clone 24	clone 2	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	w.t.	E	E	w.t.	E
3	P2	HK	w.t.	E	w.t.	E	E	w.t.	E
4	HA	HK	w.t.	w.t.	w.t.	w.t.	w.t.	w.t.	w.t.
5	NA	GL	w.t.	E	E	w.t.	E	w.t.	w.t.
6	NP	GL (*ts*)	w.t.	E (*ts*)	E (*ts*)	E (*ts*)	E (*ts*)	E (*ts*)	E (*ts*)
7	M	HK	w.t.	E	w.t.	E	E	w.t.	w.t.
8	NS	HK	w.t.	E	E	w.t.	E	w.t.	w.t.
pre-inoculation serum NI antibody		yes	yes	yes	yes	yes	yes	no	no
virulence† in adults		low	moderate	low	low	low	low	moderate	moderate
pre-inoculation serum NI antibody	trial A: yes / trial B: no		n.t.	n.t.	no	n.t.	n.t.	n.t.	n.t.
virulence in children	trial A: low / trial B: moderate		n.t.	n.t.	moderate	n.t.	n.t.	n.t.	n.t.

Palese *et al.* (1978) determined the order of migration of genes for HK/68 wild-type virus. The genotype of HK/68-*ts*-1[E] virus is shown to the left of the line. 'GL' designates genes derived from the GL/65-*ts*-1 parent. 'HK' designates genes derived from the HK/68 wild-type parent. The genotypes of *ts*-1[E] recombinant viruses are shown to the right of the line as determined in these studies. 'E' designates a gene derived from the HK/68-*ts*-1[E] parent; 'w.t.' designates a gene derived from the respective wild-type parent; '*ts*' designates a gene that bears a lesion that confers the temperature-sensitive phenotype. Data from Markoff *et al.* (1979).

† Low, ⩽5% of vaccinees developed fever and/or systemic symptoms; moderate, between 10 and 20% of vaccinees developed fever and/or systemic symptoms.

n.t., not tested.

It is implicit in this approach that the *ts* mutations are responsible for attenuation and that they can be monitored by a simple in-vitro technique during vaccine development and later during usage in man. The *ts* phenotype can be assayed easily by studying the effect of temperature upon growth of virus, and the number and identity of the *ts* genes can be ascertained by the complementation–recombination technique with the use of prototype single mutants with lesions on defined genes as genetic probes (Spring *et al.* 1975*b*). Evidence from a number of sources indicates that *ts* mutants exhibit a temperature dependence of replication *in vivo* suggesting that *ts* mutation(s) is (are) responsible for suppression of growth in the respiratory

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tract and hence for attenuation. This further implies that any recombinant containing a given set of *ts* genes should exhibit a predictable level of attenuation.

Two sets of *ts* mutant genes have been evaluated for their usefulness in attenuating new influenza A virus antigenic variants; these are designated *ts*-1[E] and *ts*-1A2 (Spring *et al.* 1975*a*, Murphy *et al.* 1978*c*). Polyacrylamide gel electrophoresis of viral RNA from *ts*-1[E] recombinants and their *ts* and wild type parents indicated that the two *ts*-1[E] mutations were located on the RNA genes that code for the P3 and NP proteins (Palese & Ritchey 1977). The P3 protein is involved in complementary RNA (cRNA) synthesis and the NP protein plays a role in virion RNA (vRNA) synthesis (Krug *et al.* 1975; Palese *et al.* 1977). Thus, the *ts*-1[E] recombinants are defective in both aspects of RNA synthesis. The data summarized in table 4 present the genotype of the *ts*-1[E] recombinant viruses in relation to their level of attenuation in adults and children and also to the immune status of the vaccinees (Murphy *et al.* 1972, 1973, 1978*b*; Richman *et al.* 1976, 1977*a*; Douglas *et al.* 1979; Kim *et al.* 1976; Wright *et al.* 1975). Although a limited number of *ts*-1[E] recombinants were evaluated, the following three relations could be deduced from the data. 1. *ts*-1[E] recombinant viruses that possessed both the *ts* P3 and *ts* NP genes were satisfactorily attenuated for adults and children that possessed serum neuraminidase-inhibiting (NI) antibody but who lacked serum haemagglutination-inhibiting (HI) antibody at the time of vaccination. However, the *ts*-1[E] recombinants were not satisfactorily attenuated for adults or children who lacked detectable HI and NI antibodies. 2. Absence of the NP *ts* gene in the Udorn/72 clone 13 recombinant was associated with an increase in virulence of the virus. This indicated that the *ts* NP gene was an attenuating gene. 3. Substitution of wild type genes for those of the *ts*-1[E] attenuated donor parent at any locus other than those coding for P3 and NP did not affect the level of attenuation of the resulting recombinant. These observations suggested that the attenuating genes of the *ts*-1[E] parent were indeed the P3 and NP genes that bore *ts* mutations and these two attenuating genes conferred a predictable level of attenuation. This level of attenuation was satisfactory for vaccinees with pre-existing neuraminidase immunity, while attenuation was not complete for individuals who lacked both haemagglutinin and neuraminidase immunity.

Importantly, *ts*-1[E] virus infection provided significant protection against experimental homologous wild-type virus challenge in adults (Murphy *et al.* 1972, 1978*b*; Richman *et al.* 1976). In addition, Wright *et al.* (1977) observed that infection of infants and young children with the A/Hong Kong/68-*ts*-1[E] (H3N2) recombinant induced significant protection against natural influenzal disease caused by the heterologous A/Port Chalmers/73 (H3N2) virus. This broad protective effect was of some interest since it was expressed against a virus that was two antigenic 'drifts' beyond the vaccine virus (Wright *et al.* 1977). Generally, inactivated vaccine does not provide this type of broad protection.

The *ts*-1[E] virus isolated from adult volunteers retained the *ts* phenotype (Murphy *et al.* 1976), whereas virus that had lost the *ts* phenotype was isolated from 25% of young vaccinees who had no prior experience with influenza A virus (Wright *et al.* 1975; Kim *et al.* 1976). This indicated that the HK/68-*ts*-1[E] genes lacked sufficient genetic stability for use in such completely susceptible individuals. When immunity to both surface antigens is absent only the degree of defectiveness of the vaccine virus determines attenuation and in this situation defectiveness must be greater than that produced by the *ts*-1[E] mutations. For this reason a recombinant virus, designated *ts*-1A2, was constructed by using a set of *ts* mutations that specified a greater degree of defectiveness than that seen with the *ts*-1[E] recombinants. This was

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accomplished by combining two highly defective and genetically stable *ts* genes into a single virus by genetic reassortment (Murphy *et al.* 1978*c*). The resulting recombinant virus (designated A/Udorn/72-*ts*-1A2) had a 37 °C shutoff for plaque formation and was hence more temperature sensitive than the *ts*-1[E] (38 °C shutoff) recombinants (Murphy *et al.* 1978*d*). In addition, the 1A2 recombinant was more restricted in its replication in the upper and lower respiratory tract of experimental animals than the *ts*-1[E] recombinants. Genetic studies indicated that the *ts*-1A2 lesions were located on the genes coding for the P1 and P3 proteins (Murphy *et al.* 1978*c*). Both of these large proteins are thought to be involved in the synthesis of complementary, i.e. messenger, RNA (Krug *et al.* 1975; Palese *et al.* 1977). Thus, the 1A2 recombinant had defects in both proteins required for the initial step in viral replication.

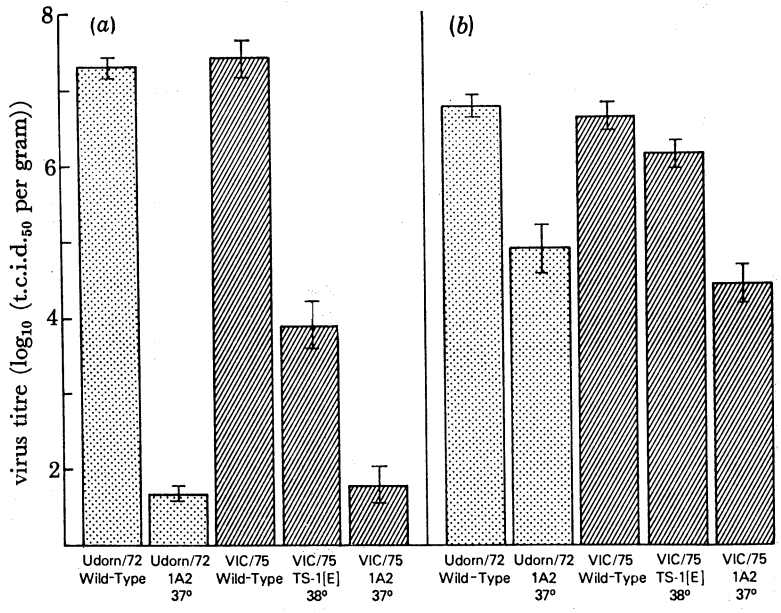

FIGURE 2. Viral replication in the lungs (*a*) and nasal turbinates (*b*) of hamsters infected intranasally with 10^4 t.c.i.d.$_{50}$ of Udorn/72 wild-type, Udorn/72-*ts*-1A2, Vic/75 wild-type, Vic/75-*ts*-1[E] or Vic/75-*ts*-1A2 virus. Eight hamsters per virus were killed daily for 4 days, and the lungs and nasal turbinates were harvested. Each organ homogenate was titred individually, and the mean $\log_{10}$ titres were determined for each day. The maximum level of replication achieved for each virus over the 4 day period is indicated together with the standard error of the mean. Data from Murphy *et al.* (1978*d*).

The two mutant genes of the A/Udorn/72-*ts*-1A2 recombinant were then transferred into the A/Vic/3/75 (H3N2) wild-type virus and the resulting A/Victoria/75-*ts*-1A2 recombinants were evaluated in hamsters (Murphy *et al.* 1978*a*,*d*). The Vic/75-*ts*-1A2 recombinant had a 37 °C shutoff temperature, failed to grow in the lungs and was restricted approximately 100-fold in its growth in the nasal turbinates compared with wild-type virus (figure 2). This degree of growth restriction was greater than that seen with the A/Vic/75-*ts*-1[E] recombinant or any of its *ts*-1[E] predecessors. Furthermore, there was no evidence of reversion of the A/Vic/75 1A2 recombinant to wild-type *in vivo*. Thus, the 1A2 recombinant appeared to be quite stable genetically. Four additional A/Vic/75-*ts*-1A2 recombinants were studied, and each behaved identically to the clone depicted in figure 2. In all respects, the five A/Vic/75-*ts*-1A2 recombinants behaved like their A/Udorn/72-*ts*-1A2 parent, indicating that transfer of the two *ts*-1A2 mutant genes conferred upon each recombinant a predictable level of attenuation

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and genetic stability. Furthermore, infection of hamsters with the *ts*-1A2 recombinants induced resistance to subsequent challenge with the homologous wild-type virus (Murphy *et al.* 1978*d*). The Udorn/72-*ts*-1A2 *ts* genes have also been transferred into the A/Alaska/77 (H3N2) virus, and the A/Hong Kong/77 (H1N1) virus, and five resulting recombinants were found to have the same genetic and biological properties as their *ts* parent (Murphy, unpublished observations). Significantly, these recombinants exhibited the same restriction of growth in hamster lungs and the same genetic stability as the parent Udorn/72-*ts*-1A2 (figure 3). These data provided the basis for evaluation of these promising recombinants in volunteers.

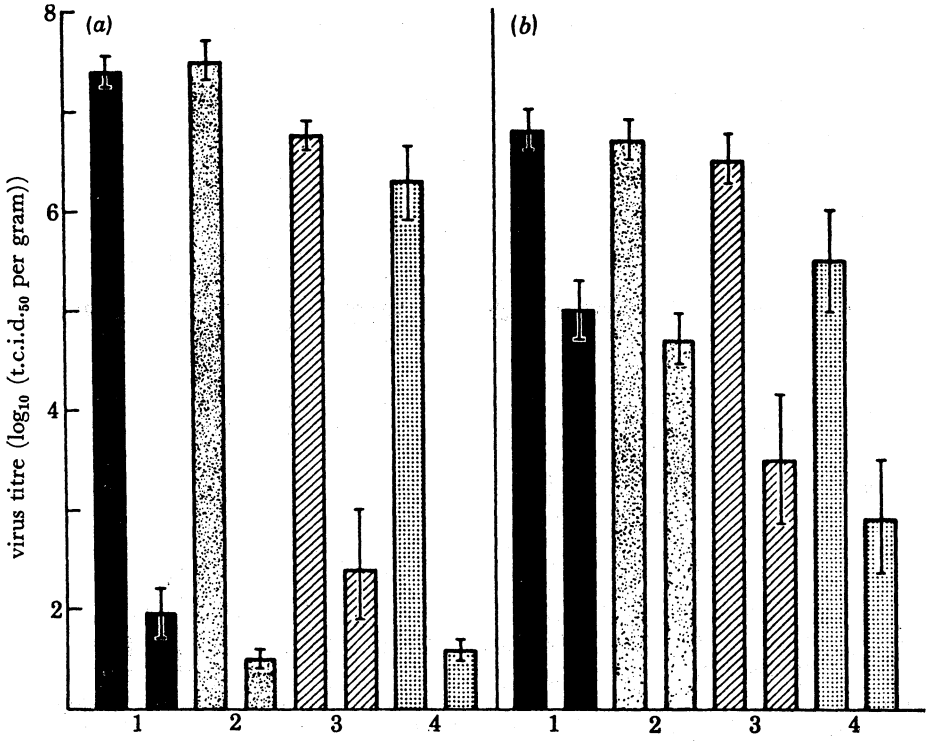

FIGURE 3. Viral replication in the lungs (*a*) and nasal turbinates (*b*) of hamsters infected intranasally with 10^4 t.c.i.d.$_{50}$ of *ts*-1A2 recombinant (right column of each pair) or wild-type (left column of each pair) viruses of the following strains: 1, A/Udorn/72 (H3N2); 2, A/Vic/75 (H3N2); 3, A/Alaska/77 (H3N2): 4, A/Hong Kong/77 (H1N1). See figure 2 for method of calculating titres.

A/Vic/75 (H3N2), A/Alaska/77 (H3N2) and A/Hong Kong/77 (H1N1) *ts*-1A2 viruses and their wild-type viruses have been administered to volunteers (table 5). The A/Vic/75-*ts*-1A2 recombinant infected 90% of 42 doubly seronegative adult volunteers without causing any systemic symptoms or fever (table 5). Vaccinees shed significantly less virus than individuals infected with wild-type virus of A/Vic/75-*ts*-1[E] virus (Murphy *et al.* 1979*a*). Each of 18 isolates from the A/Vic/75-*ts*-1A2 vaccinees retained the *ts* phenotype, indicating that the recombinant was stable genetically in doubly seronegative adults, i.e. individuals that lacked serum antibody for both surface glycoproteins. Wright also found the A/Vic/75-*ts*-1A2 virus to be safe in doubly seronegative children, and 77 isolates from 13 children who shed virus retained the *ts* phenotype (Wright *et al.*, unpublished studies). Finally, infection of vaccinees with the *ts*-1A2 recombinant induced significant resistance to subsequent challenge with A/Vic/75 wild-type virus (table 6) (Murphy *et al.* 1979*a*).

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Table 5. Response of seronegative adult volunteers to *ts*-1A2 recombinants of the H1N1 and H3N2 subtype

virus administered	dose (t.c.i.d.$_{50}$)	number of volunteers	percentage infected	percentage that shed virus	percentage with immunological response	percentage with fever and/or systemic symptoms	percentage with upper respiratory tract symptoms	percentage with any illness
(*a*) H3N2 subtype								
Vic/75-*ts*-1A2	$10^{7.5}$	42	90	24	86	0	5	5
Vic/75 wild-type	$10^{5.2}$	19	100	95	67	53	68	74
Alaska/77-*ts*-1A2	$10^{6.5}$	28	75	28	71	3	0	3
Alaska/77 wild-type	$10^{4.0}$	8	100	100	87	38	50	50
(*b*) H1N1 subtype								
HK/77-*ts*-1A2	$10^{6.5}$	23	70	26	70	4	9	13
HK/77 wild-type	$10^{4.0}$	6	100	100	100	84	66	84

Volunteers had serum HI antibody titre of not more than 1:8. All Vic/75 and HK/77 volunteers lacked serum neuraminidase-inhibiting antibody (less than 1:4), while 64% of Alaska/77 volunteers also lacked this antibody.

The A/Alaska/77-*ts*-1A2 (H3N2) recombinant was also attenuated. It should be noted that 18 of the 28 volunteers were doubly seronegative, i.e. they lacked detectable serum HI and NI antibodies. Only 1 of 28 vaccinees had a significant clinical response consisting of mild fever lasting less than 24 h. Each of the seven isolates from these infected volunteers retained the *ts* phenotype. However, virus with an altered *ts* phenotype was recovered from a doubly seronegative child given the A/Alaska/77-*ts*-1A2 recombinant by Kim and Parrott at the Children's Hospital National Medical Center of the District of Columbia. The isolates were more temperature sensitive than wild-type virus but less temperature sensitive than the *ts*-1A2 recombinant. Nevertheless, the vaccinee did not develop significant symptoms.

TABLE 6. RESISTANCE TO HOMOLOGOUS WILD-TYPE VIRUS CHALLENGE† INDUCED BY VIC/75-*ts*-1A2 RECOMBINANT

		number with indicated response		
virus previously administered	number of volunteers	fever and/or systemic symptoms	upper respiratory symptoms	any symptoms
Vic/75-*ts*-1A2	8	0	2	2
none	19	10	13	14
		$P < 0.025$		$P < 0.05$

† Vaccinees and controls received $10^{5.2}$ t.c.i.d.$_{50}$ of Vic/75 wild-type virus. Vaccinees were challenged intranasally 60 days after administration of Vic/75-*ts*-1A2.

‡ The controls had a serum HI antibody titre of not more than 1:8.

The A/HK/77-*ts*-1A2 (H1N1) recombinant was given to 23 doubly seronegative adults. Only one individual developed a definite systemic reaction, but this was mild and transient (less than 12 h in duration). Hence this recombinant was significantly attenuated compared with wild-type virus. Each of 14 isolates from the adult vaccinees retained the *ts* phenotype.

These observations indicate that acquisition of the two *ts*-1A2 genes by H3N2 and H1N1 subtype viruses was regularly associated with a level of attenuation satisfactory for individuals lacking antibody to both surface antigens of the virus. However, the experience with the A/Alaska/77-*ts*-1A2 recombinant indicated that genetic instability remains a formidable problem that must be solved. If in-vitro assays predictive of genetic instability could be developed, it might be possible to select regularly for *ts* recombinants that remain stable during replication in completely susceptible individuals.

The quest for a live influenza A virus vaccine has indeed been difficult and the goal elusive. Many problems associated with the current candidate donor viruses remain to be solved. Perhaps new opportunities created by DNA recombinant technology will allow us to manipulate DNA copies of the influenza A virus genome in a manner previously not possible with viral RNA. We may thus be able to create more stable mutations, such as deletions, that will render the virus satisfactorily attenuated.

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