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GENETICS

Studies on mutation lesions and physiology of fowl plague virus *ts* mutants

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[Plate 1]

Temperature-sensitive (*ts*) mutants of fowl plague virus (FPV) were divided into six complementation groups. Experiments with *ts* mutants having defects of transcription showed that in FPV strain Weybridge, protein P1 coded by gene N2 takes part in primary transcription, and protein P3 coded for by gene N1 takes part in secondary transcription. *Ts* mutants of FPV with lower pathogenicity were present in all six complementation groups under study. Simultaneous inoculation of chickens with two pathogenic *ts* mutants of FPV caused death of the chickens and a pathogenic virus with ts^+ phenotype was isolated from their organs. By recombination of *ts* multimutant FPV with human influenza virus a recombinant was obtained that contained genes coding for the haemagglutinin and neuraminidase of human influenza virus; all other genes were derived from FPV. In experiments on volunteers this recombinant appeared to be non-reactogenic but capable of inducing antibody formation.

Mutations of viruses, according to their phenotypic manifestations, may be divided into three main groups: (1) mutations showing phenotypic manifestations under normal reproduction conditions, such as changes in the size of the plaques produced by the virus; (2) lethal mutations in which the synthesis or the function of the vitally important virus protein is completely impaired under any propagation conditions; and (3) conditional-lethal mutations in which the defective virus protein functions normally under optimal reproduction conditions but is unable to function under non-permissive conditions, either at a higher temperature (such mutants are called temperature-sensitive, *ts*) or when certain cells are used (these are host-dependent or host-restricted mutants, *hd* or *hr*).

Recently, much attention has been given to conditional-lethal mutants with the *ts* phenotype. The investigations have shown that *ts* phenotype is not due to defects in any one special gene but may be the result of mutations in any gene coding for the protein whose function is vitally important for virus reproduction. Thus, *ts* mutants may be produced which would have mutation defects in practically all virus genes.

Proceeding from this important circumstance, *ts* mutants of viruses were used successfully for the determination of the number of genes contained in virus genomes, for identification of the products of individual genes and for the elucidation of the exact role of individual virus proteins in virus reproduction. Besides, the very important evidence that *ts* mutations are not infrequently accompanied by a decrease or loss of virus pathogenicity for the susceptible host led to the investigations of a possible use of *ts* mutants as live virus vaccines.

In investigations with orthomyxoviruses, *ts* mutants were produced with mutagens by several authors. Three groups of research workers obtained *ts* mutants of human influenza

virus, WSN strain (Simpson & Hirst 1968; Mackenzie 1970; Sugiura *et al.* 1972). The results of studies on *ts* mutants of WSN influenza virus have been presented at length in recently published reviews (Hightower & Bratt 1977; Palese 1977).

In investigations with fowl plague virus (FPV), a typical member of type A orthomyxoviruses, *ts* mutants were prepared and studied, also by three groups of workers (Scholtissek *et al.* 1974, 1975, 1976, 1978; Almond *et al.* 1977; Ghendon *et al.* 1973*a*, *b*, 1975; Markushin & Ghendon 1973, 1979; Genkina & Ghendon 1979).

We should like to dwell on some examples of using FPV *ts* mutants for investigations associated with the genome structure and functions of proteins of orthomyxoviruses.

Table 1. Recombination of FPV *ts* mutants

	ts 43	*ts* 15	*ts* 16	*ts* 166	*ts* 206	*ts* 250	*ts* 29	*ts* 131	*ts* 5
ts 43	< 2	< 2	< 2	< 2	< 2	< 2	5.1†	5.1	5.6
ts 15		< 2	< 2	< 2	< 2	< 2	5.5	5.8	5.0
ts 16			< 2	< 2	< 2	< 2	5.3	5.4	5.4
ts 166				< 2	< 2	< 2	5.3	5.4	5.3
ts 206					< 2	< 2	5.5	5.6	5.4
ts 250						< 2	5.3	5.2	5.3
ts 29							< 2	5.1	5.6
ts 131								< 2	6.1
ts 5									< 2

† Log_{10}(p.f.u./ml). The cells were infected with two mutants, incubated at 42 °C for 10 h and the virus titre was determined at 42 °C.

Table 2. Complementation between FPV *ts* mutants

	complementation	level of complementation						extent of complementation					
mutants	groups	*ts* 43	*ts* 166	*ts* 29	*ts* 131	*ts* 5	*ts* 303	*ts* 43	*ts* 166	*ts* 29	*ts* 131	*ts* 5	*ts* 303
ts 43	A		8×10^5	5×10^5	300	400	7		76	5	55	40	4.6
ts 166	B			20	10	5000	9×10^4			10	1.5	33	30
ts 29	C				9×10^4	10	7				1.8	1.7	1.4
ts 131	D					50	40					9.8	11
ts 5	E						300						1.5
ts 303	F												

The cells were infected with two mutants, incubated at 42 °C for 10 h and the virus titre was determined at 36 °C.

One of the important problems still remaining unresolved is the true number of genes present in the genome of orthomyxoviruses. Most research workers believe that the genome of orthomyxoviruses contains eight genes corresponding to the number of RNA fragments usually observed (Palese 1977; Scholtissek 1978). At the same time, there is some evidence indicating a possible presence in the influenza virus genome of one or maybe several more small genes (Skehel 1972; Hay *et al.* 1977; Lamb *et al.* 1978). In the solution of this problem, studies with *ts* mutants could be very useful because by complementation–recombination tests the number of genes present in the genome may be determined accurately. The use of these tests in studies on the genome of orthomyxoviruses is also particularly expedient because the segmented structure of the orthomyxovirus genome and the capacity of each segment to function independently give a very high frequency of recombination with mutants defective in different genes, as can be seen from the results of recombination test with our FPV *ts* mutants (table 1).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

In the complementation test, the degree and level of complementation are also quite high (table 2); this permits one to divide the mutants understudy into complementation groups each of which corresponds to the mutation defect in one gene.

In our experiments with FPV *ts* mutants, six complementation groups were distinguished (Markushin & Ghendon 1973; Genkina & Ghendon 1979). The same number of recombination groups was found in studies on FPV, the Rostock strain, *ts* mutants produced by Scholtissek & Bowles (1975) and J. W. Almond (personal communication). In studies with influenza virus, WSN strain, *ts* mutants, the maximum number of complementation–recombination groups so far demonstrated is eight (Hirst 1973; Nakajima & Sugiura 1977). Thus, eight genes have so far been found in the genome of orthomyxoviruses by means of complementation–recombination tests.

TABLE 3. PROPERTIES OF *ts* MUTANTS OF FPV, WEYBRIDGE

complementation group	synthesis of cRNA	synthesis of vRNA	synthesis of proteins	synthesis of HA and NA	maturation	defects
A	+	−	+	−	−	replication
B	+	−	+	−	−	replication
C	±	−	+	−	−	transcription
D	∓	−	∓	−	−	transcription
E	+	+	+	+	−	maturation
F	+	+	+	−	∓	formation of normal HA

It should be mentioned that the production of *ts* mutants belonging to new complementation groups is a quite complicated procedure. It would be much simpler to conduct cooperative experiments on complementation–recombination analysis of orthomyxovirus *ts* mutants already produced and characterized in various laboratories as has been done with *ts* mutants of vesicular stomatitis virus and herpes virus produced by different research workers. Studies of this kind, in particular with FPV *ts* mutants produced in three laboratories, could reveal new complementation groups and elucidate the possible presence of more than eight genes in the genome of orthomyxoviruses.

At the present time, at least eight virus-specific proteins of orthomyxoviruses are known (see Scholtissek 1978). While there are certain notions concerning the functions of haemagglutinin and neuraminidase, the exact role of other virus proteins in virus reproduction remained obscure until recently. This problem was resolved as a result of investigations on orthomyxovirus *ts* mutants (Palese 1977; Scholtissek 1978).

As an example of such studies, we shall present the results of studies on the physiology of FPV *ts* mutants produced by our group (Ghendon *et al.* 1973*a*, 1975, and unpublished results). The results of these experiments (table 3) showed, in particular, that in mutants belonging to four complementation groups the synthesis of virus-specific RNAs was impaired: in two groups, the synthesis of cRNA was impaired, i.e. the process of transcription was blocked, and in the other two groups there was no vRNA synthesis, i.e. replication was impaired. These data permitted a conclusion that at least four viral proteins take part in the synthesis of orthomyxovirus RNA, of which two are responsible for transcription and the other two participate in viral RNA replication.

In these experiments, polyfunctional capacities of some viral proteins were demonstrated

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

(figure 1). Thus, if the cells infected with *ts* 131 and *ts* 29 mutants, in which under non-permissive conditions the process of transcription is impaired, are first incubated for 3 h at the optimal temperature (i.e. under conditions when a sufficient amount of viral proteins taking part in transcription are formed) and then at a non-permissive temperature, no synthesis of virion RNA is observed although the proteins taking part in replication had no mutation defects in these mutants. These results suggest that virus proteins taking part in transcription also take part in viral RNA replication, that is they are polyfunctional.

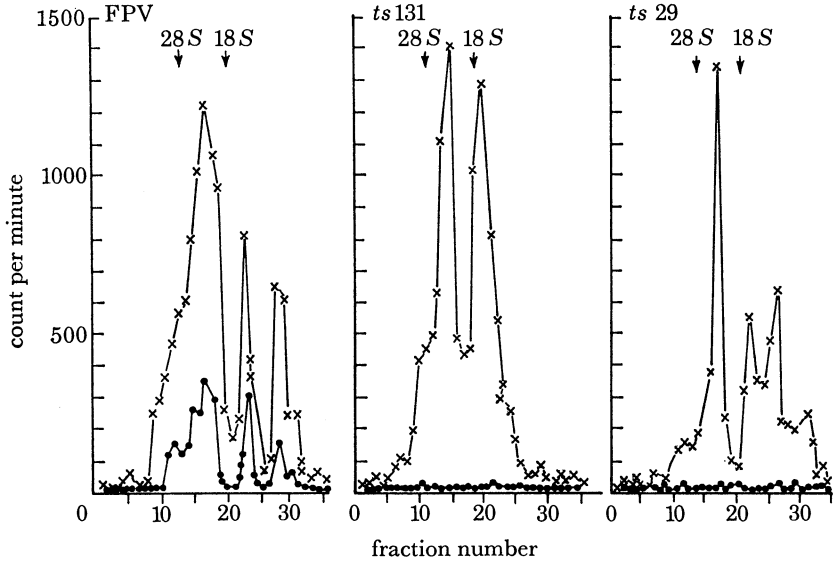

FIGURE 1. Investigation of RNA synthesis in FPV *ts* mutants. The infected cells were incubated at 36 °C for 3 h, actinomycin D was added (5 μg/ml), followed by 45 min of incubation by [^{3}H]uridine (20 μCi/ml), after which incubation continued at 36 or 42 °C for 2 h. RNA was analysed by electrophoresis in a cylindrical polyacrylamide gel. ×, Incubation with [^{3}H]uridine at 36 °C; •, incubation with [^{3}H]uridine at 42 °C.

TABLE 4. PROPERTIES OF MUTANTS *ts* 131 AND *ts* 29 FPV, WEYBRIDGE

mutant	in-vitro activity of virion transcriptase at 42 °C	transcription, synthesis of poly(A)+ cRNA	transcription, synthesis of poly(A)− cRNA	translation, synthesis of proteins	replication, synthesis of vRNA	defects
ts 131	reduced	reduced	absent	reduced	absent	primary transcription
ts 29	not reduced	synthesis of all types of poly(A)+ cRNA	absent	synthesis of all types of polypeptides	absent	secondary transcription

We studied in greater detail mutants *ts* 131 and *ts* 29 with defects in transcription. Mutant *ts* 131 was found (table 4) to have defects in practically all stages of reproduction although this virus could adsorb onto and penetrate into the cells under non-permissive conditions. It should be noted that at a higher temperature this mutant had defects in the function of virion transcriptase *in vitro*. In cooperative studies with A. Hay, *ts* 29 mutant was found to have synthesis of all kinds of poly(A)-containing cRNAs but no synthesis of cRNAs containing no poly(A). On the basis of the data by Hay *et al.* (1977) it may be concluded that in this mutant, reproduction is blocked at the stage of secondary transcription. At the same time, *ts* 131 mutant appears to have defect in the protein taking part in primary transcription.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

On the basis of *ts* 131 and *ts* 29 mutants, we tried to determine which genes and, accordingly, which proteins of FPV take part in primary and secondary transcription. For this purpose it was necessary to determine the defective gene and the protein it coded for in these mutants. For these studies we prepared recombinants of *ts* mutants and a human influenza A/Krasnodar/101/59 (H2N2) wild strain in which genome segments and proteins had somewhat different electrophoretic mobilities from those of FPV. As a result, we obtained recombinants having a ts^+ phenotype (that is, capable of multiplication at a non-permissive temperature) because in their genome the defective gene was replaced by the corresponding

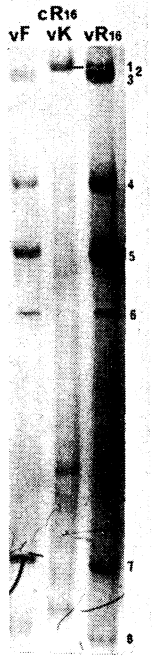

FIGURE 2. Analysis of the genome of recombinant obtained by recombination of FPV *ts* 29 mutant with human influenza A/Krasnodar/101/59 virus. The cells were inoculated with the viruses (500 e.i.d.$_{50}$ per cell) and incubated in the medium containing cycloheximide and [^{3}H]uridine for 4 h. Then RNA (cRNA) was isolated and hybridized with unlabelled vRNA isolated from purified virions. The samples were treated with S_1 nuclease and examined by electrophoresis in a slab polyacrylamide gel (40 g/l). cR16, recombinant 16 cRNA; vF, FPV vRNA; vK, A/Krasnodar vRNA; vR16, recombinant 16 vRNA. The numbers 1–8 show the locations of the corresponding double-stranded RNA fragments.

non-defective gene of the other partner. Then we analysed the composition of the genome and proteins of the recombinants to detect the defective gene that the recombinants had derived from the non-defective parent. At present, very sophisticated methods have been developed for the analysis of the genome composition of influenza virus recombinants clearly showing which genes were derived by the recombinant from which parents. These methods are based on differences in the electrophoretic mobility of virion RNA fragments of influenza virus strains (Palese 1977), on various degrees of hybridization of individual virion RNA fragments with complementary viral RNA (Scholtissek *et al.* 1976), and on the analysis of the electrophoretic mobility of nuclease-treated double-stranded vRNA and cRNA hybrids (Ito & Joklik 1972). We used the latter method as modified by Hay *et al.* (1977) for studies on orthomyxoviruses.

In these experiments, virion RNA isolated from both parent strains is hybridized with cRNA isolated from the recombinant-infected cells. The resulting double-stranded hybrids are treated

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

with nuclease. If vRNA and cRNA fragments are homologous, the double-stranded hybrid is resistant to nuclease; if they are not homologous, they are destroyed by nuclease completely or partly, and the subsequent analysis by polyacrylamide gel electrophoresis detects these non-homologous fragments and determines which genes of the recombinants are derived from one parent and which from the other. An analysis of the recombinant genomes showed that all of the recombinants of *ts* 29 mutant had derived gene no. 1 from influenza A/Krasnodar virus. The analysis of the genome of one of such recombinants is presented in figure 2.

One can see that in the process of hybridization the recombinant vRNA with the cRNA of the same recombinant eight bands are revealed, corresponding to eight virus genes. If the recombinant cRNA is hybridized with FPV parent strain vRNA, one can see bands corresponding to all genes except for no. 1. At the same time, in the process of hybridization with vRNA of another partner (A/Krasnodar influenza virus) only one band is revealed corresponding to gene no. 1. Accordingly, it was this gene that appeared to be defective. Similar experiments with *ts* 131 mutant recombinants showed gene no. 2 to be defective in this mutant (results not shown).

A comparative study of the electrophoretic mobility of proteins of the parent strains and recombinants demonstrated that in mutant *ts* 131 recombinants protein P1, and in mutant *ts* 29 recombinants protein P3, are derived from human influenza virus A/Krasnodar/101/59 (not shown).

Thus, these results permit a conclusion that in FPV protein P1, coded for by gene no. 2, takes part in primary transcription, and protein P3, coded for by gene no. 1, takes part in secondary transcription.

Similar studies were carried out by Scholtissek *et al.* (1974, 1975, 1976), Rohde *et al.* (1977) and Harms *et al.* (1978) with *ts* mutants of the Rostock strain of FPV prepared by them. The authors demonstrated that four genes (1, 2, 3 and 5) and proteins coded for by them (P1, P2, P3 and NP) take part in viral RNA synthesis.

According to Palese and coworkers (Palese *et al.* 1977; Ritchey & Palese 1977), who analysed human influenza virus, WSN strain, *ts* mutants, genes 1 and 2 and proteins P3 and P1 coded for by them take part in virus genome transcription, genes 3 and 5 and proteins P2 and NP coded for by them in RNA replication, and gene 7 and protein M coded for by it, are important for virion assemblage.

Investigation of *ts* mutant physiology occasionally reveals previously unknown information on the function of some virus proteins. Thus, our study on one FPV mutant, *ts* 303 (Markushin *et al.* 1979), showed that in the cells infected with this mutant and incubated at 36 °C, haemagglutinin cleavage and some degree of glycosylation were impaired; the virion population showed considerable polymorphism and size variation, the bulk consisting of non-infectious virus-like particles with a reduced content of haemagglutinin and protein M (figure 3).

Determinations of the defective gene showed this mutant to have a single defect in the gene coding for haemagglutinin. These results suggest that influenza virus haemagglutinin is extremely important not only for primary stages of virus infection, virus adsorption on cells, but plays an important role in the final stages of reproduction in assembling of infectious virions, determining the proper formation of the inner virion membrane consisting of protein M as well as controlling geometry and size of virions.

As mentioned above, *ts* mutations are frequently accompanied by a decrease or loss of pathogenicity for susceptible hosts. This permits one to investigate the role of individual genes

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

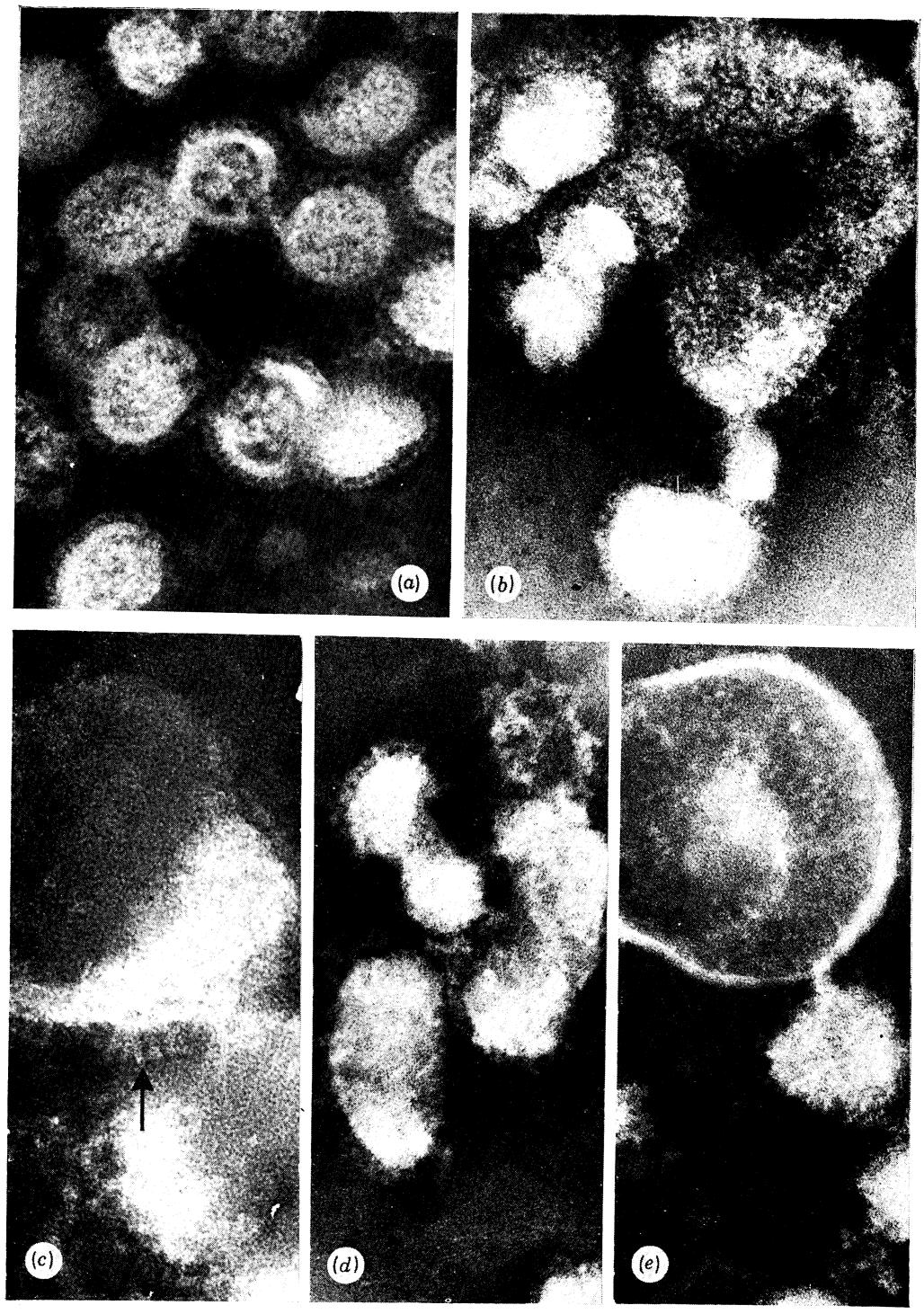

FIGURE 3. Electron micrographs of the parental FPV strain (*a*) and *ts* 303 mutant (*b*, *c*, *d*, *e*) populations. (*a*) Population of FPV virions characterized by stable size, shape, electron optic density, and permanent presence of haemagglutinin spikes on the surface. (Magn. × 200 000.) (*b*) A group of *ts* 303 mutant virions of irregular shape and varying size with vague haemagglutinin spikes. (Magn. × 200 000.) (*c*) A large virion of *ts* 303 mutant 200 nm in diameter. A part of the surface is seen containing haemagglutinin spikes (indicated by arrows) twice as long as normally observed. (Magn. × 300 000.) (*d*) A group of virions of *ts* 303 mutant of irregular shape; the surfaces are partly covered with haemagglutinin spikes of varying length. (Magn. × 300 000.) (*e*) A large virion of *ts* 303 mutant about 250 nm in diameter, without haemagglutinin spikes. (Magn. × 200 000.)

(*Facing p.* 388)

in the manifestation of a very important virus property, that of pathogenicity. In studies on FPV *ts* mutants belonging to six complementation groups (Ghendon *et al.* 1973*a*, and unpublished results) we found (table 5) that *ts* mutants with lower pathogenicity were present in all the complementation groups under study. In two *ts* mutants retaining their virulence the virulence was due to revertants emerging in chickens. Thus, these data indicate that *ts* mutation of any of at least six FPV genes leads to a decrease or loss of virus pathogenicity for the susceptible host.

Table 5. Pathogenicity of FPV *ts* mutants for chickens

complementation group	mutant	pathogenicity for chickens	
		1.5 months old†	1 day old‡
A	*ts* 43	0/2	2.0
A	*ts* 17	0/2	0.7
B	*ts* 159	0/2	1.3
B	*ts* 166	2/2	13
C	*ts* 10	0/2	1.1
C	*ts* 29	0/2	0.8
D	*ts* 130	0/2	0.5
D	*ts* 131	0/2	1.6
E	*ts* 5	2/2	15
E	*ts* 207	0/2	0.4
F	*ts* 303	0/2	1.2
	FPV, wild-type	2/2	20

† Number of dead chickens/number of infected chickens.
‡ $L.d._{50}$/p.f.u.

A very sophisticated study of this problem was made by Scholtissek *et al.* (1977). In their experiments the defective gene of FPV *ts* mutants was replaced by recombination by the corresponding non-defective gene of the other orthomyxovirus, and the pathogenicity of these recombinants for chickens was studied. The authors also concluded that a change of even one gene may result in a loss of pathogenicity. These studies confirm the concept that pathogenicity is a complex property of viruses which is controlled by many genes.

The reduced pathogenicity of *ts* mutants underlie the use of such viruses as live influenza vaccines. Investigations on the use of such strains are being carried out in the U.S.S.R. and the U.S.A., but the strains used by various authors usually have defects in not more than two genes (Palese & Ritchey 1977; Kendal *et al.* 1978) which, on the one hand, may cause an insufficient level of attenuation and, on the other, a possibility of emergence of pathogenic recombinants due to recombination of these mutant vaccine strains with naturally circulating influenza virus variants of low pathogenicity which is due to defects in other genes than in the vaccine strains. The possibility of emergence of pathogenic recombinants in the susceptible host infected with two non-pathogenic *ts* mutants with defects in different genes was demonstrated in our studies with FPV *ts* mutants (Genkina & Ghendon 1979). It will be seen in table 6 that the chickens inoculated individually with each *ts* mutant under study remained alive, whereas simultaneous inoculation with two mutants caused death of the chickens, and a pathogenic virus with a ts^+ phenotype was isolated from their organs.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

To reduce the possibility of emergence of such recombinants in the susceptible host, we constructed a strain the genome of which contained five genes with *ts* mutations by mutual recombination of FPV *ts* mutants belonging to five complementation groups. Simultaneous inoculation of chickens with this strain and any *ts* mutant was never accompanied by emergence of pathogenic recombinants in chickens (Ghendon *et al.*, unpublished results).

Table 6. Recombination of FPV *ts* mutants in chickens

	pathogenicity for chickens							*ts* phenotype of viruses isolated from dead chickens infected with mixture of mutants *ts* 131 and *ts* 29		
		days after infection							titre (p.f.u./ml)	
mutant	infection dose (p.f.u.)	1	2	3	4	5	7	material under study	at 36 °C	at 42 °C
ts 131	0.04	0/4†	0/4	0/4	0/4	0/4	0/4	chicken N1	7.9	7.6
ts 29	0.04	0/4	0/4	0/4	0/4	0/4	0/4	chicken N2	7.0	7.0
ts 131+	0.02+	0/4	0/4	0/4	0/4	1/4	4/4	chicken N3	7.3	6.7
ts 29	0.02							chicken N4	6.7	5.9
								ts 29 (original)	8.7	<2.0
								ts 131 (original)	7.7	<2.0

† Number of dead chickens/number of infected chickens.

Table 7. Trials of R/Kr/*ts* FPV recombinant in volunteers

			haemagglutinin-inhibition antibody†	
volunteer	age	untoward reactions	before vaccination	after vaccination
N.G.	37	no	<1	4.5
A.V.	39	no	<1	4.0
M.V.	25	no	<1	4.0
M.E.	24	37.2 °C, one day	2.5	4.5
N.L.	31	no	2.5	5.0
M.B.	41	no	3.0	3.5
F.I.	37	no	3.5	5.5
K.B.	28	mild catarrh, one day	3.5	5.5
O.A.	35	37.4 °C one day	3.5	7.0
A.A.	37	no	4.5	5.0
G.C.	31	no	6.0	6.5
N.G.	22	no	6.0	6.0

The volunteers were given $10^{6.5}$ e.i.d.$_{50}$ of virus intranasally and observed for 14 days.
† Results expressed as binary logarithms.

By recombination of this multimutant FPV with human influenza A/Krasnodar/101/59 (H2N2) virus we obtained a recombinant that contained genes coding for the external membrane proteins (haemagglutinin and neuraminidase) of human influenza virus; all the other genes were derived from FPV, five of these genes having a *ts* mutation. When this recombinant was used for immunization of adult human volunteers (unpublished results) none of the volunteers showed any untoward reactions (table 7) and eight of them developed a significant rise of haemagglutination-inhibiting antibody.

We believe that the use, as vaccine strains, of recombinants deriving genes coding for the external membrane proteins from human influenza virus and all the other genes from animal influenza virus may be quite expedient in the control of influenza infection.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Further study of orthomyxovirus *ts* mutants may give much important information on the genome structure, the function of virus proteins, and, which is most important, may be quite useful in the development of new approaches to the construction of influenza virus vaccine strains.

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FIGURE 2. Analysis of the genome of recombinant obtained by recombination of FPV *ts* 29 mutant with human influenza A/Krasnodar/101/59 virus. The cells were inoculated with the viruses (500 e.i.d.$_{50}$ per cell) and incubated in the medium containing cycloheximide and [^{3}H]uridine for 4 h. Then RNA (cRNA) was isolated and hybridized with unlabelled vRNA isolated from purified virions. The samples were treated with S_1 nuclease and examined by electrophoresis in a slab polyacrylamide gel (40 g/l). cR16, recombinant 16 cRNA; vF, FPV vRNA; vK, A/Krasnodar vRNA; vR16, recombinant 16 vRNA. The numbers 1–8 show the locations of the corresponding double-stranded RNA fragments.

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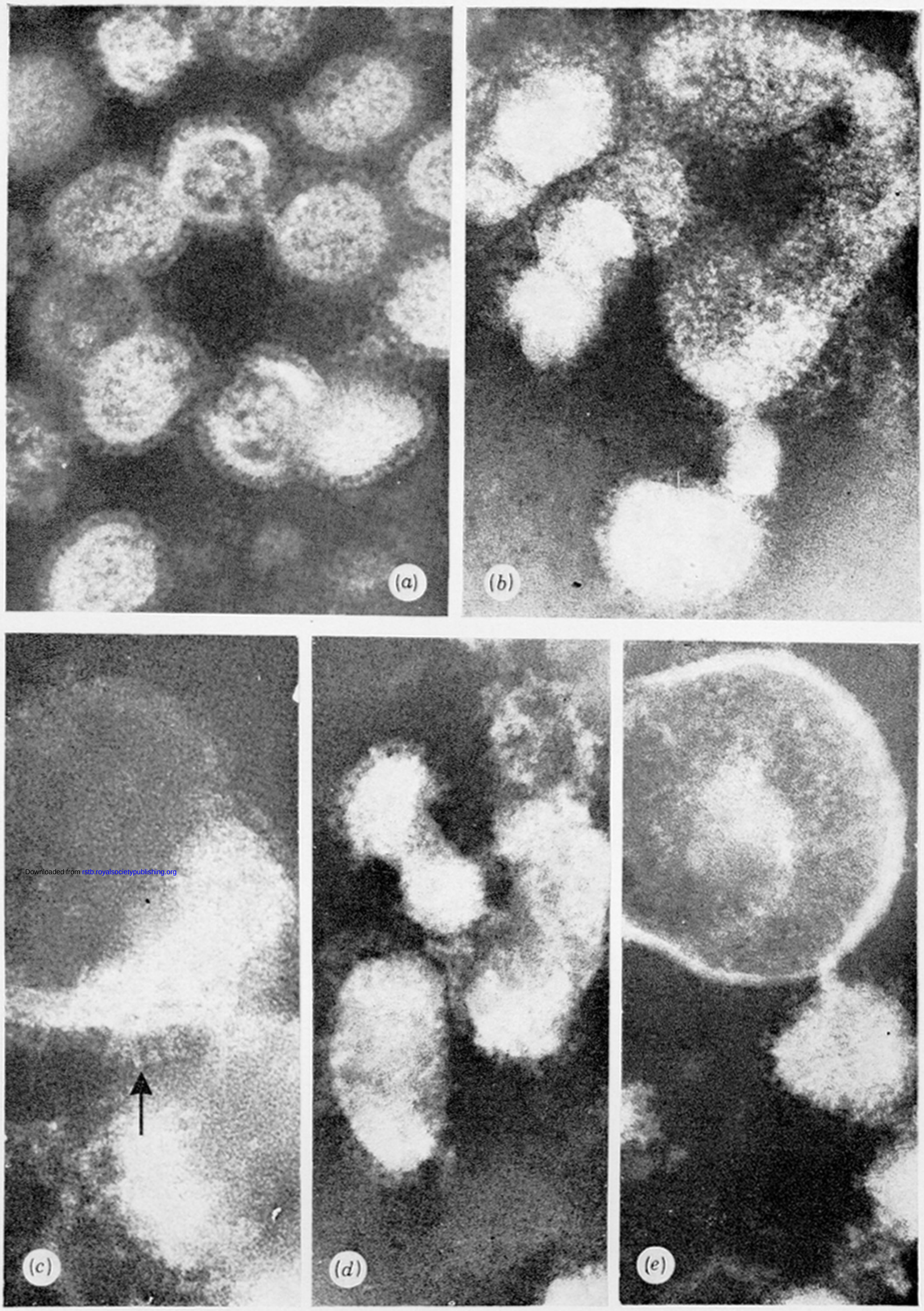

FIGURE 3. Electron micrographs of the parental FPV strain (*a*) and *ts* 303 mutant (*b*, *c*, *d*, *e*) populations. (*a*) Population of FPV virions characterized by stable size, shape, electron optic density, and permanent presence of haemagglutinin spikes on the surface. (Magn. ×200000.) (*b*) A group of *ts* 303 mutant virions of irregular shape and varying size with vague haemagglutinin spikes. (Magn. ×200000.) (*c*) A large virion of *ts* 303 mutant 200 nm in diameter. A part of the surface is seen containing haemagglutinin spikes (indicated by arrows) twice as long as normally observed. (Magn. ×300000.) (*d*) A group of virions of *ts* 303 mutant of irregular shape; the surfaces are partly covered with haemagglutinin spikes of varying length. (Magn. ×300000.) (*e*) A large virion of *ts* 303 mutant about 250 nm in diameter, without haemagglutinin spikes. (Magn. ×200000.)