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TS MUTATIONS IN THE GENOME OF COLD-ADAPTED VARIANTS OF HUMAN INFLUENZA VIRUS A/KRASNODAR/101/59 (H2N2) AT DIFFERENT PASSAGE LEVELS AND THEIR REPRODUCTION IN LUNGS OF HAMSTERS

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Summary. — Human influenza virus A/Krasnodar/101/59 (H2N2) was passaged in chick fibroblast cultures in the presence of trypsin at suboptimal temperature. The virus which underwent 16 passages at 28 °C possessed cold-adapted (ca) and temperature sensitive (ts) phenotypes and formed larger plaques at the optimal temperature (33 °C). Its reproduction in the lungs of hamsters was decreased as evidenced by approximately 2.5 $\log_{10}$ lower titres; only one of 9 virus isolates from the lungs of hamsters acquired the ts $\pm$ phenotype, although it had retained a ca phenotype. Recombination of this variant with ts mutants of fowl plague virus (FPV) revealed a ts mutation only in gene 4 of this variant coding for haemagglutinin (HA). The virus which had had 25 passages at 28 °C possessed the same properties as the previous variant, but all eight virus isolates from the lungs of hamsters retained the ts phenotype; the genome of this variant contained ts mutations in genes 1, 3, 4, 5 and 6. The mutation found in gene 8 was not a ts mutation. The virus, which underwent 25 passages at 28 °C and additional 15 passages at 27 °C, formed large plaques and alike to the previous variants it possessed the ca and ts phenotypes; however, its reproduction in the lungs of hamsters was decreased by 4.0 $\log_{10}$ and occurred in the lungs only of 4 out 16 infected animals. This variant contained ts mutations in genes 1, 3, 4, 5, 6 and 7 and a non-ts mutation in gene 8.

Key words: influenza virus; cold-adapted variants; ts mutations; hamsters

Introduction

Much attention has been focussed till now on the ca ts variants of influenza virus; these have been shown to possess a decreased pathogenicity for susceptible hosts and may be suitable attenuated donors in producing recombinant strains for live influenza vaccines (Kendal *et al.*, 1981). However, to achieve a sufficient degree of attenuation and to retain genetic stability, such strains should have mutation lesions in a number of genes.

The aim of the present study was to obtain cold-adapted variants of influenza virus A/Krasnodar/101/59 (H2N2) which would have a different number of passages at suboptimal temperature, and to study the ca and ts phenotypes of these variants, degrees of reproduction in the lungs of hamsters, reversions of the ca and ts phenotypes during reproduction in the lungs of hamsters and to identify genes containing ts mutations.

Materials and Methods

Viruses and cells. Human influenza virus A/Krasnodar/101/59 (H2N2) was used for passages at suboptimal temperature. Recombination tests were performed with the following ts mutants of FPV strain A/Weybridge (H7N7): ts 29, ts 131, ts 166, ts 5 and ts 303/1 containing ts mutations in genes 1, 2, 3, 6 and 7, respectively (Ghendon *et al.*, 1982, 1983*a*, *b*) and with ts mutants of FPV strain A/Rostock/34 (H7N1): ts 46, ts US1 and ts mN3 containing ts mutations in genes 4, 5 and 8 (Almond *et al.*, 1979). The ts mutants of FPV strain A/Rostock/34 were kindly provided by Dr. B. W. J. Mahy (Cambridge, England). The viruses were grown in 11-day-old chick embryos (CE). Passages of influenza virus and recombination tests were carried out in primary CE fibroblast cultures (CEF).

Obtaining of ca variants. Human influenza virus A/Krasnodar /101/59 (H2N2) strain was cloned by 3 plaque-passages in CEF cultures in the presence of trypsin and then passaged in CEF cultures (1—2 PFU/cell 72—96 hr) under liquid medium (Eagle MEM) in the presence of trypsin (2.5 μg/ml, crystalline, SPOFA, Prague). The initial 25 passages were carried out at 28 °C and the following passages at 27 °C. After 16, 25 and 40 passages, the materials were cloned twice by plaque-passages in CEF culture and used for further analysis (variants K/16, K/25 and K/40, respectively).

Determination of ca, ts and S phenotypes. The ability of the variants obtained to reproduce at suboptimal temperature (ca phenotype) was determined by titration using a plaque technique in CEF cultures in the presence of trypsin (5 μg/ml) at the optimal (33 °C) and suboptimal (27 °C) temperatures. The ability of the variants to reproduce at high temperature (ts phenotype) was determined by titration in CEs at 33 °C and 39 °C. To determine the size of plaques (S phenotype) formed in CEF cultures in the presence of trypsin, the diameters of approximately 200 plaques were measured 4 days post-infection (p.i.).

Studies of virus reproduction in the lungs of hamsters. According to the method described by Spring *et al.* (1975), 6-week-old Syrian hamsters were inoculated intranasally with 10^5 EID_{50} of the virus under hexobarbital narcosis. Animals of the same sex were used in each experiment. Animals were sacrificed 48 hr p.i., the lungs were removed and used to prepare 10% homogenates (w/v) in Eagle's MEM and clarified by centrifugation for 10 min at 3 000 rev/min. The amount of virus per gram of tissue homogenate was determined by titration in 11-day-old CE using 10-fold dilutions of the lung suspensions. Ts phenotype of viruses grown in the lungs of hamsters was studied by infection of CE with the 10% organ suspensions. After incubation at 39,3 °C for 48 hr, titrations were performed in CE at 33 °C. The ca phenotype of virus grown in the lungs of hamsters was determined by comparison of the plating efficiency at suboptimal and optimal temperatures. The ratio of 0.5 — 1 accounted for the acquisition of ca marker.

Recombination test. The recombination in CEF cultures was performed according to the method of Ghenkina and Ghendon (1979) described in detail elsewhere (Lisovskaya *et al.*, 1981). In brief: CEF cultures were infected with virus variants under study (1 — 2 EID_{50}/cell) and with various 10-fold dilutions of the FPV ts mutants. The monolayer of infected cells was overlaid with trypsin-free agar and incubated at permissive (36 °C) and non-permissive (40 °C or 42 °C) temperatures for 72 hr; thereafter the plaques were counted. For recombination in CE, coinfection was done with the viruses under study at a dose of 10^3 EID_{50}. In control experiments , CE were individually infected with each virus. After incubation at 33 °C for 48 hr, the ts phenotype of the virus population formed was determined by titration of the allantoic fluid of CE at 33 °C and 40 °C, and the recombinants possessing the ts^+ phenotype were further analysed in a haemagglutination-inhibition (HI) test.

Genome analysis of the ca recombinants. This was done using the method of Hay *et al.* (1977) described in detail earlier (Ghendon *et al.*, 1979). In brief: CEF cultures were infected with

viruses under study (100 EID_{50}/cell) and incubated in the presence of cycloheximide (100 μg/ml) for 1 hr at 36 °C. ^3H-uridine (3.7 MBq/ml) was then added, and the incubation continued for a further 4 hr. Complementary RNA (cRNA) extracted from the cells was hybridized with an excess of unlabelled virion RNA (vRNA) isolated from purified virions. Double-stranded complexes thus obtained were treated with nuclease S1 and analysed by electrophoresis in 7.5% polyacrylamide gel containing 6 mol/l urea (Hay *et al.*, 1979).

Results

Ca and ts phenotypes of the variants of A/Krasnodar/101/59 virus passaged at suboptimal temperature

The variants of influenza virus A/Krasnodar/101/59 which had had 16, 25 and 40 passages at suboptimal temperature reproduced in CEF cultures at 27 °C practically to the same degree as at 33 °C, although the parent A/Krasnodar/101/59 strain failed to reproduce at 27 °C (Table 1). Having acquired the ca phenotype, all variants lost their ability to reproduce in CE at 39.3 °C, although the parent virus A/Krasnodar/101/59 reproduced in this system to the titre of 10^7 EID_{50}/ml. All variants formed significantly larger plaques in CEF cultures at 33 °C than did the parent virus. Plaques formed at 27 °C 4 days p.i. were smaller (2—3 mm in diameter) as compared to the plaques formed at 33 °C (5.5 mm in diameter), but increased with time.

Reproduction of the ca variants of A/Krasnodar/101/59 virus in the lungs of hamsters

The results shown in Table 2 demonstrate that the variants which had 16 and 25 passages at 28 °C reached in the lungs of hamsters titres by more than 2 $\log_{10}$ lower than did the parent strain. In addition, when the hamsters were infected with the parent strain, the virus was recovered from lungs

Table 1. Ca, ts and S markers of the variants of A/Krasnodar/101/59 virus passaged at suboptimal temperature

Variant	Ca phenotype* at		Ts phenotype				S phenotype***
			in chick embryos at		in CEF cultures* at		
	27 °C	33 °C	33 °C	39.3 °C	33 °C	39.3 °C	
A/Krasnodar/101/59 (parent strain)	< 1.0	7.48	8.15**	7.0**	7.48	6.0	2.5 ± 0.43
The variant after 16 passages at 28 °C (K/16)	7.11	7.36	8.3	< 3.0	7.36	< 1.0	5.5 ± 0.68
The variant after 25 passages at 28 °C (K/25)	7.18	7.70	8.4	< 3.0	7.7	< 1.0	5.5 ± 0.43
The variant after 25 passages at 28 °C + 15 passages at 27 °C (K/40)	7.00	7.11	8.5	< 3.0	7.11	< 1.0	5.5 ± 0.51

* Titres in CEF cultures ($\log_{10}$ PFU/ml) in the presence of trypsin. ** $\log_{10}$ EID_{50}/ml. *** Plaque size in CEF cultures at 33 °C (diameter in mm).

Table 2. Reproduction of cold-adapted variants of A/Krasnodar/101/59 virus in the lungs of hamsters

Variant	Titre	Number of animals
A/Krasnodar/101/59 (parent virus)	5.98 ± 0.4*	14/14**
K/16	3.69 ± 0.39	9/11
K/25	3.28 ± 0.74	8/10
K/40	2.0***	4/16

* log EID_{50}/g of lung tissue. ** Numerator = number of hamsters from which virus was recovered; denominator = number of infected animals. *** The lowest virus titre detectable was 2.0 $\log_{10}$/g.

of each animal, but when the hamsters were infected with the variants K/16 and K/25, the virus was recovered from 80% of animals only. The variant which had had 40 passages at suboptimal temperature reproduced in the lungs of hamsters even to a lesser degree the virus having been recovered from 25% of infected animals only.

Analysis of ts phenotype of the isolates from the lungs of infected hamsters showed (Table 3) that one from 9 isolates of animals infected with the variant K/16 possessed a ts ± phenotype (the titre of this isolate after reproduction in CE at 39.3 °C was $10^{4.1}$ EID_{50}/ml and at 33 °C $10^{7.3}$ EID_{50}/ml). Nevertheless, it should be noted that the isolate which possessed a ts± phenotype, has retained the ability to reproduce at suboptimal temperature. All 8 isolates from animals infected with the variant K/25 retained the ts phenotype. As can be seen from Table 2, the variant K/40 was recovered only from 4 of 16 animals infected with this virus. In order to create similar conditions for all variants to detect the revertant virus, further experiments were performed with variant K/40, and 6 additional isolates from the lungs of hamsters were obtained. It must be noted that in these experiments likewise in our previous studies, the virus was recovered only from 25% of the infected animals. The data in Table 3 show that all 10 isolates retained the ts phenotype.

Identification of genes ca variants containing ts mutations

The data presented in Table 4 show that when the ca variant K/16, which had had 16 passages at 28 °C, was crossed with FPV ts mutants containing ts mutations in genes 1, 2, 3, 5, 6, 7, 8, ts^+ recombinants capable of inducing plaques at the nonpermissive temperature were formed. Since clones capable of forming plaques at the non-permissive temperature may result from intracistronic complementation during interaction of influenza virus ts mutants (Heller, Scholtissek, 1980; Thierry *et al.*, 1980), we decided to find out whether intracistronic complementation might occur in our experiments. Clones were isolated from plaques formed at the non-permissive temperature (10 clones in the experiments with each of ts mutants), and their ts phenotype was studied. As all the clones proved to possess a ts phenotype (not shown), we could exclude that an intracistronic complementation did occur. The results

Table 3. Ts and ca phenotypes of the virus isolated from the lungs of infected hamsters

Variant used for infection	No. of isolates	Virus titre (log EID_{50}/ml) in CE at		Ts and ca phenotypes after one egg passage at 39 °C*	
		33 °C	39.3 °C	log EID_{50}/ml at 39.3 °C	Ratio of PFU/ml at 27 °C and 33 °C
K/16	1	2.6	< 1.0	< 1.0	0.5
	2	3.1	< 1.0	< 1.0	0.5
	3	2.97	< 1.0	< 1.0	0.8
	4	3.1	< 1.0	4.1	0.7
	5	3.4	< 1.0	< 1.0	0.5
	6	3.97	< 1.0	< 1.0	0.9
	7	3.97	< 1.0	< 1.0	1.0
	8	3.4	< 1.0	< 1.0	1.0
	9	3.97	< 1.0	< 1.0	1.0
K/25	1	3.4	< 1.0	< 1.0	1.0
	2	2.97	< 1.0	< 1.0	0.7
	3	4.1	< 1.0	< 1.0	0.6
	4	4.1	< 1.0	< 1.0	0.9
	5	3.1	< 1.0	< 1.0	0.8
	6	3.4	< 1.0	< 1.0	0.9
	7	2.9	< 1.0	< 1.0	1.0
	8	2.43	< 1.0	< 1.0	0.5
K/40	1	1.6	< 1.0	< 1.0	0.9
	2	1.6	< 1.0	< 1.0	0.8
	3	1.6	< 1.0	< 1.0	1.0
	4	1.6	< 1.0	< 1.0	0.7
	5	≦ 2.0	< 1.0	< 1.0	0.8
	6	≦ 2.0	< 1.0	< 1.0	1.0
	7	≦ 2.0	< 1.0	< 1.0	0.6
	8	≦ 2.0	< 1.0	< 1.0	0.6
	9	≦ 2.0	< 1.0	< 1.0	0.5
	10	≦ 2.0	< 1.0	< 1.0	0.6

* Test for revertant virus.

obtained allowed us to conclude that the variant K/16 contained no ts mutations in genes 1, 2, 3, 5, 6, 7 and 8.

Table 1 shows that the variant K/16 had a distinct ts phenotype. For this reason we suggest that this variant might contain a ts mutation in gene 4 coding for HA. To study this possibility, experiments were carried out in CE which were co-infected with the ca variant K/16 and FPV mutant ts 46 containing ts mutation in gene 4 coding for HA (Almond *et al.*, 1979). In control experiments, CE were co-infected with the ca variant K/16 and FPV ts mutant US1 containing a ts mutation in gene 5 coding for nucleoprotein; this mutant also recombined with the variant K/16 (Table 4). As seen in Table 5, no infectious virus reproducing at 40 °C and, consequently, no ts^+

Table 4. Recombination of FPV ts mutants with ca variants of A/Krasnodar/101/59 influenza virus

Ts mutants	Mutant genes	Mutant proteins	Plaque formation in CEF cultures infected with FPV ts mutants and variants of human influenza virus									
			K/16		K/25		K/40		A/Krasnodar/ 101/59		FPV ts mutants only	
			36 °C	40° or 42 °C	36 °C	40° or 42 °C	36 °C	40° or 42 °C	36° C	40° or 42 °C	36 °C	40° or 42 °C
29	1	PB2	1×10^8	2×10^7	1×10^8	$<10^2$	2×10^8	$<10^2$	1×10^8	2×10^7	1×10^8	$<10^2$
131	2	PB1	1×10^8	8×10^5	9×10^7	7×10^5	1×10^8	8×10^5	1×10^8	1×10^6	1×10^8	$<10^2$
166	3	PA	2×10^7	3×10^6	2×10^7	$<10^2$	1×10^7	$<10^2$	3×10^7	3×10^6	2×10^7	$<10^2$
US1	5	NP	6×10^6	1×10^5	5×10^6	$<10^2$	6×10^6	$<10^2$	8×10^6	1×10^6	7×10^6	$<10^2$
5	6	NA	9×10^6	4×10^5	1×10^7	$<10^2$	1×10^7	$<10^2$	1×10^7	8×10^5	1×10^7	$<10^2$
303/1	7	M	3×10^6	3×10^5	4×10^6	5×10^5	3×10^6	$<10^2$	1×10^7	3×10^6	1×10^7	$<10^2$
mN3	8	NS	1×10^7	2×10^5	1×10^7	2×10^5	1×10^7	2×10^5	2×10^7	5×10^5	1×10^7	$<10^2$

Recombinations performed in CEF cultures as described in Materials and Methods.

Table 5. Studies of recombination in CE of the variants K/16 and FPV of ts mutants having a ts mutation either in HA (ts 46) or in NP (ts US1)

Strains crossed	Titres in CE		Titres in CEF cultures a t 33 °C
	33 °C	40 °C	
ts 46 × ts 46	6.5*	< 1.0	1.5×10^{7}**
K/16 × K/16	6.5	< 1.0	< 1.0
K/16 × ts 46	6.5	< 1.0	1.0×10^{7}
ts US1 × ts US1	6.5	< 1.0	1.5×10^{7}
K/16 × ts US1	6.5	3.0	1.0×10^{7}

CE were infected with viruses under study (10^3 EID_{50} of each strain on recombination, and 2×10^3 EID_{50} on self crossing), incubated at 33 °C for 48 hr. Virus titres in the allantoic fluid were determined using either CE (at 33 °C and 40 °C) or CEF cultures under agar overlay in the absence of trypsin at 33 °C.
* expressed in $\log_{10}$ EID_{50}/0.1 ml; ** expressed in PFU/ml.

recombinants, were detected on recombination of the variant K/16 with FPV mutant ts 46. At the same time, recombination of the variant K/16 with FPV mutant ts US1 yielded ts^+ recombinants. This was confirmed by the presence of infectious virus capable of reproduction at 40 °C and by the absence of virus capable of reproduction at this temperature in the allantoic fluid of CE infected only with ts US1 mutant or with the variant K/16. When the virus-containing allantoic fluid from CE co-infected with ts US1 mutant and/or the variant K/16 had been passaged at 40 °C, the ts^+ phenotype was retained. In addition, analysis of this material in a HI test with sera against A/Krasnodar/101/59 (H2N2) virus and FPV Rostock (H7N1) strain showed that the ts^+ recombinant possessed antigenic specificity of the HA of FPV Rostock strain (not shown), which indicated formation of ts^+ recombinants between the variant K/16 and ts US1 mutant in CE. The fact that the variant K/16 and ts 46 mutant failed to recombine with each other was not due to the interference of the viruses during their co-reproduction in CE, since the titres of FPV ts mutants, including ts 46, were essentially the same both in self-crossing and in recombination with the variant K/16 (Table 5). These data indicate that the ca variant K/16 contains a ts mutation in gene 4 coding for HA.

Table 4 shows that recombination of the ca variant K/25 which had had 25 passages at 28 °C with FPV mutants ts 131, ts 303/1 and ts mN3 having ts mutations in genes 2, 7 and 8, respectively, yielded ts^+ recombinants capable of forming plaques at non-permissive temperature. The analysis of ts phenotype of clones isolated from these plaques showed that they all retained the ts^+ phenotype which exclude the possibility of intracistronic complementation (not shown). Recombination of the variant K/25 with FPV mutants ts 29, ts 166, ts US1 and ts 5 containing ts mutations in genes 1, 3, 5 and 6, respectively, did not yield recombinants capable of forming plaques under nonpermissive conditions. The results of recombination of the variant K/25 with the mutant ts 46 having a ts mutation in gene 4 showed that this mutant

did not recombine with the variant K/25 on infection of CE by the above described technique (not shown). The results obtained allow us to make the conclusion that further passages of A/Krasnodar/101/59 virus at 28 °C led to the appearance of a variant (K/25) which contained ts mutations in genes 1, 3, 5 and 6 coding for proteins PB2, PA, NP and NA, respectively, and retained the ts mutation in gene 4 coding for HA.

The data in Table 4 also show that when the ca variant K/40 which had had 25 passages at 28 °C and 15 additional passages at 27 °C was crossed with FPV mutants ts 29, ts 166, ts US1, ts 5 and ts 303/1 having mutations in genes 1, 3, 5, 6 and 7, no ts$^+$ recombinants were formed. When it was crossed with mutants ts 131, ts mN3 having mutations in genes 2 and 8, ts$^+$ recombinants forming plaques at non-permissive temperature (Table 4) were obtained. The clones isolated from the plaques retained the ts$^+$ phenotype, which allows us to exclude intracistronic complementation (not shown). Recombination in CE of the variant K/40 with the mutant ts 46 containing a mutation in gene 4, using the same technique as used in the studies of the variant K/16, did not yield ts$^+$ recombinants (not shown). Therefore, the data obtained made it possible to conclude that the variant which had had 15 additional passages at 27 °C as compared to the variant K/25, acquired a ts mutation in gene 7 coding for the M protein and retained ts mutations in genes 1, 3, 4, 5 and 6 coding for PB2, PA, HA, NP and NA proteins, respectively.

As can be seen from Table 4, none of ts mutants formed plaques at a higher temperature in control experiments. But when they were crossed with a wild type strain of A/Krasnodar/101/59 virus, recombinants forming plaques at high temperature were obtained with all ts mutants. This is in good agreement with earlier data (Lisovskaya *et al.*, 1981) indicating that the parent strain A/Krasnodar/101/59 possessing the ts$^+$ phenotype contained no ts mutations in any of its genes.

Studies of the genome of ca variants

The data in literature indicate that analysis of nuclease S1-treated double-stranded cRNA/vRNA hybrids of influenza virus by electrophoresis in a 7.5% polyacrylamide gel containing 6 mol/l urea detects differences even of a single nucleotide (Hay *et al.*, 1979). Since passages of viruses at suboptimal temperature might involve the acquisition of other than ts mutations not detected in the recombination test (e.g. hd mutations), we decided to apply the analysis of double-stranded cRNA/vRNA hybrids for detection of mutations in individual genes of the ca variants of influenza virus. The results shown in Fig.1 (Plate XLIX) indicate that by the electrophoresis technique it was difficult to separate genes 1, 2 and 3, while genes 4, 5, 6, 7 and 8 could be easily analysed. The hybridization of vRNA of the parent virus A/Krasnodar/101/59 with cRNA of the variant K/16 showed certain changes in the electrophoretic mobility of segment 4 coding for HA; no differences concerning the electrophoretic mobilities of other segments (5, 6, 7, 8) could be observed. When vRNA of the A/Krasnodar/101/59 virus was hybridized with cRNA of the variant K/25, considerable differences were observed in the electrophoretic

mobilities of segments 4, 5, 6 and slight differences of segment 8. Analysis of the variant K/40 revealed considerable differences for segments 4, 5 and 6 and slight differences for segments 7 and 8.

Studies of the genome by hybridization of cRNA/vRNA allowed us to analyse the genes 4, 5, 6, 7 and 8. We managed to confirm that the variant K/16 has a ts mutation only in gene 4 coding for HA. The HA of the variants K/25 and K/40 had apparently undergone additional mutation changes, since the changes in the electrophoretic mobility of segment 4 of these variants were expressed more distinctly than in the variant K/16. In addition, we detected a mutation in segment 8 of the variants K/25 and K/40 which apparently was not a ts mutation since it could not be revealed in the recombination test with FPV ts mutants.

Discussion

The results obtained showed that all variants of influenza virus A/Krasnodar/101/59 which had had 16, 25 and 40 passages at suboptimal temperature both acquired the ability to reproduce at 27 °C (ca phenotype) and possessed a distinct ts phenotype. In recombination test, the virus which had had 16 passages at 28 °C, was shown to have a ts mutation only in the gene 4 coding for HA. These data were confirmed by hybridization of cRNA/vRNA. At the same time the variant which had had 25 passages at 28 °C contained ts mutations in five genes (1, 3, 4, 5 and 6) and a non-ts mutation in gene 8. Fifteen additional passages at 27 °C resulted in the appearance of one more ts mutation in gene 7. Thus, the data obtained indicate that the main events in the process of passages of influenza virus at suboptimal temperature resulting in the appearance of ts mutations in many genes, or in the selection of variants containing a maximal number of ts mutations occurred between passages 16 to 25 at 28 °C. It sould be noted, however, that in our studies in each case only one cloned variant was analysed at passages 16, 25 or 40 and the possibility can not be ruled out that there were in the population of the 16th passage virions containing ts mutations in more than one gene.

The variants obtained differed from the parent strain and from one another in the degree of reproduction in the lungs of hamsters. The reproduction in the lungs of hamsters of the variants which had had 16 and 25 passages at 28 °C was decreased; the titres were approximately by 2.0 $\log_{10}$ lower, but viral replication was detected in the lungs of the majority of hamsters. The variant which had had 40 passages at suboptimal temperature showed a considerably reduced ability to reproduce in the lungs of hamsters as evidenced by decrease of both, virus titres and the number of infected animals. Apparently, hyperattenuation occurred in this variant having ts mutations in 6 genes which caused a drastic reduction in the ability of the virus to reproduce in the organism of susceptible animals. Such hyperattenuation could be undesirable from the standpoint of using ca variants as donors of attenuation in obtaining a recombinant influenza virus vaccine, since such a drastic reduction of the growth of virus in a susceptible host may have a negative effect on its immunogenic activity.

The analysis of the ca and ts phenotypes of virus isolates from the lungs of infected hamsters showed that one from nine isolates of the variant K/16 acquired the ts± phenotype, while all eight isolates of the variant K/25 and 10 isolates of the variant K/40 retained the ts phenotype. These data indicate that if a ts mutation is contained in one gene only, as in the variant K/16, it does not ensure stability of the ts phenotype during reproduction of virus in a susceptible animal; at the same time the presence of ts mutations in several genes, as in the variant K/25 and K/40 ensures stability of the ts phenotype.

Of interest is the fact that the isolate which acquired the ts± phenotype retained its ability to reproduce at suboptimal temperature (ca phenotype). Dissociation of ca and ts phenotypes was also described by Spring *et al.* (1977) and Odagiri *et al.* (1982) in their studies of the ca variant of A/Ann Arbor/6/60. These data indicate that mutations resulting in the appearance of the ca and ts phenotypes of virus involve different regions of the genome.

Thus, our results showed that passages of influenza virus at suboptimal temperature led to the emergence of cold-adapted mutants, in which ts mutations appeared in the process of passages. The number of these ts mutations increased in the course of passages. Taking into consideration the data of Odagiri *et al.* (1982) that a reduction in pathogenicity of ca influenza virus strains possessing the ts phenotype, first of all is due to ts mutations and the ca property being of secondary importance, we can make the conclusion that the presence of ts mutations in several genes is an important prerequisite for obtaining a highly attenuated and stable ca variant of influenza virus.

A variant having that number of passages at low temperature leading to ts mutations in 4—5 genes coding for non-glycosylated proteins seems to be an optimal attenuated donor which is stable but not hyperattenuated.

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Explanation of Figure (Plate XLIX)

Fig. **1**. Electrophoretic analysis of the mobility of ds RNA obtained after hybridization of ^{3}H-labelled cRNA from ca variants with vRNA of influenza virus strain A/Krasnodar/101/59. CEF cultures were infected with viruses and incubated in the presence of cycloheximide and (^{3}H)-uridine for 3 hr at 36 °C, cRNA was then extracted and hybridized with an excess of unlabelled vRNA. The samples were treated with S1 nuclease and analyzed by electrophoresis in a 7.5% polyacrylamide gel containing 6 M urea.
1, 3, 3, 4, 5, 6, 7, 8 = location of corresponding double-stranded complexes of cRNA/vRNA (Kr = A/Krasnodar/101/59^{e} C/16, C/25 and C/40 = ca variants).