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Characterization of an Influenza A Host Range Mutant

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A mixed infection of primary chick kidney cells at 38° with A/Ann Arbor/6/60 cold adapted virus and A/Alaska/6/77 wt virus yielded a cold-reassortant virus, CR43-clone 3, which had a host range different from that of either parent. It does not produce detectable virus when grown in Madin-Darby canine kidney cells, while growing normally in primary chick kidney cells at 33°. Both parents, however, grow well in either cell type at 33°. Genotypic analysis of viral RNA electrophoresed in polyacrylamide gels has shown that CR43-clone 3 virus has an aberrant NS gene different from the NS gene of either parent virus. Reassortant viruses made between CR43-clone 3 virus and A/California/10/78 (H1N1) virus in primary chick kidney cells at 33° showed the same host range restriction only if the NS gene was derived from the CR43-clone 3 virus. A mixed infection with these same parents, but in Madin-Darby canine kidney cells at 33°, produced reassortants that always contained the A/California/10/78 NS gene instead of the CR43-clone 3 NS gene. Ferrets inoculated intranasally with the CR43-clone 3 reassortant do not become sick or infected, based on the lack of symptoms: no rhinitis, coryza, or fever; and no detectable virus recovered from nasopharyngeal swabs, turbinate, or lung tissues at 48 hr after infection. Thus, CR43-clone 3 virus contains an aberrant NS gene and manifests a restricted host range phenotype in Madin-Darby canine kidney cells and ferrets.

INTRODUCTION

Host range mutants of influenza A viruses include both temperature dependent-host range (td-hr) and non-temperature-dependent (hr) mutants. These latter hr mutants have been primarily of two types: (1) those involving cleavage of the HA gene due to the presence of a particular NA gene (Schulman and Palese, 1977; Ghendon *et al.*, 1979; Nakajima and Suigiura, 1980) or due just to the presence of a particular HA gene (Bosch *et al.*, 1979); and (2) hr mutants with an extended host range due to a mutation in a polymerase gene (Almond, 1977). Polymerase genes were also involved in the td-hr mutants (Scholtissek and Murphy, 1978; Israel, 1980). Shimizu and collaborators have recently shown that td-hr mutants could be found in each of eight complementation groups described for A/Udorn/72 on Madin-Darby canine kidney (MDCK) cells implying that an altered host range was possible due to mutations in any of the eight RNA segments of influenza A viruses (Shimizu *et al.*, 1983).

AA-CR43 was a reassortment experiment performed in primary chick kidney (PCK) cells at 38° using the cold-adapted (ca) and temperature-sensitive (ts) A/Ann Arbor/6/60 (A/AA/60) master strain and A/Alaska/6/77 (A/Ala/77) wild-type (wt) virus (Maassab, *et al.*, 1981b). This experiment was an attempt to generate reassortants that had a wider range of gene constellations than those previously recovered from reassortment experiments performed at 25°, a temperature which might favor the selection of genes from the ca and ts A/AA/60 virus (Cox, *et al.*, 1979). One of the reassortant viruses, CR43-clone 3 (CR43-3) was found to have an NS gene which migrated differently from the NS gene of either parent on electrophoretic gels. CR43-3 virus also differed from its

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parents in that it formed no plaques in MDCK cells at 33 or 39°. Thus, the CR43-3 virus acted like a non-temperature-dependent hr mutant which could not grow in MDCK cells, possibly due to its aberrant NS gene. Experiments were performed to examine more fully its restricted host range phenotype in MDCK cells; to test for growth in the ferret animal model system; and finally, to link the restricted hr phenotype to the CR43-3 NS gene.

MATERIALS AND METHODS

Viruses and tissue culture. The viruses used were the ca and ts A/Ann Arbor/6/60 (H2N2) master strain, developed in this laboratory (Maassab, 1969), and A/Alaska/6/77 (H3N2), furnished by Flow Laboratories, Rockville, Md., for use in live virus vaccine reassortment experiments. A/California/10/78 (A/Cal/78) (H1N1) virus was plaque purified in this laboratory prior to use in the reassortment experiments, below. The CR43 reassortant viruses, CR43-3 and CR43-7, were isolated from a reassortment experiment described earlier (Maassab *et al.*, 1981). MDCK cells, also obtained from Flow Laboratories, and PCK cells were grown as described earlier (Odagiri *et al.*, 1982; Maassab, 1969).

Plaque assays. Plaque assays were performed in PCK cells by infecting confluent monolayers in 25-cm^2 plastic flasks with 1.0 ml of virus diluted in double-strength Eagle's basal medium (2× Eagle's). Virus was adsorbed for 1 hr at room temperature with continuous, gentle rocking, at which time the virus inoculum was aspirated off, and 5 ml of overlay media consisting of 1.0% purified agar (BBL Microbiological Systems, Cockeysville, Md.) in modified 199 medium (Sugiura and Kilbourne, 1965) was added. After incubation for 48 hr at 33 and 39°, or for 5 days at 25°, the monolayers were stained by adding 4 ml of 1.0% agar in the modified medium 199 containing 0.01% neutral red. Plaques were read on Days 5, 6, and 7 for the cultures incubated at 33 and 39°, and on Days 7–12 for the cultures incubated at 25°. Plaque assays in MDCK cells were similar except unmodified 199 medium plus 5 μg/ml of TPCK trypsin (Worthington Biochemical Corp., Freehold, N. J.) was present at every stage, and 1.0% Seakem ME agarose (FMC Corp., Rockland, Md.) was used in place of the purified agar.

Reassortment experiments: MD1 and CR55. MD1 reassortants were produced by infecting 10 individual culture tubes of MDCK cells with 0.3 ml of a mixture of CR43-3 and A/Cal/78 viruses, each at a multiplicity of infection (m.o.i.) ranging from 5 to 10 plaque-forming units (PFU) per cell. The virus was adsorbed for 90 min at room temperature; the inoculum from each was removed; and 1 ml of 2× Eagle's medium, without serum, containing 5 μg/ml of TPCK trypsin was added to each tube. The infected cells were incubated for 3 days postinfection (pi) at 33°, until cell lysis was evident. The progeny virus were then passed two times at 33° in MDCK cells in the presence of diluted ferret antiserum (final hemagglutination-inhibition titer, HI = 1:8) against A/Cal/78 virus. Progeny virus of these cultures were then inoculated allantoically into 11-day-old embryonated eggs, incubated at 33°, and harvested after 72 hr. The MD1 reassortants were plaque-purified three times in MDCK cells by plaque to plaque passages at 33° and working pools made in eggs, as above. The reassortant viruses were stored at −70°.

CR55 reassortants were processed in a similar manner using PCK cells in the tubes and plaque bottles, but without trypsin. The antiserum used was ferret antiserum (final HI titer = 1:4) made against CR43-3 virus.

^{32}P-labeled vRNA. ^{32}P-labeled vRNA was prepared from viruses grown in PCK cells. Confluent PCK cells in a 25-cm^2 flask were infected with virus at an m.o.i. of 10–20 PFU/cell. The virus was allowed to adsorb to the cells for 1 hr at room temperature. The inoculum was replaced with 1.0 ml of phosphate-free Eagle's MEM containing 200 μCi/ml of carrier-free ^{32}P (Amersham Corp., Arlington Heights, Ill.). After a 4-hr incubation at 33° for A/AA/60 and all reassortants, or at 35° for all wt viruses, an additional 3 ml of 2× Eagle's maintenance medium (without serum) was added to each flask and the infected cultures in-

cubated until 48 hr pi. The labeled virus was harvested from the culture media and clarified by centrifugation at 2000 rpm for 20 min at 4°. The virus was pelleted through a 30% sucrose cushion in STE buffer (0.1 *M* NaCl, 0.01 *M* Tris-HCl, 0.001 *M* EDTA, pH 7.4) by centrifugation at 22,500 rpm for 150 min in a Beckman type 30 rotor, at 4°. The virus pellet was resuspended in 0.3 ml of 0.3 *M* sodium acetate buffer, pH 6.0, and incubated at 37° for 10 min in the presence of 500 μg/ml proteinase K (Beckman Instruments, Inc., Palo Alto, Calif.). The samples were incubated an additional 10 min at 30° in the presence of 0.5% sodium dodecyl sulfate (SDS), and then shaken with one volume of (0.3 *M* Na acetate buffer, pH 6.0)-saturated phenol for 5 min at room temperature. One volume of chloroform was added, mixed, and the phases separated by centrifugation for 2 min in an eppendorf centrifuge. The aqueous phase was removed, 0.1 ml of Na acetate buffer added to the organic phase, mixed, and re-extracted as before. The two aqueous phases were pooled and ether extracted twice. Residual ether was removed with nitrogen gas, and the samples precipitated overnight with 2.5 vol of 95% ethanol at −20°.

Gel electrophoresis. Gene constellations of the reassortants were determined using two types of gels. The polymerase genes were separated on a 1.5% agarose slab gel in TBE buffer (Peacock and Dingman, 1969) containing 0.1% SDS. The remaining six genes were separated on a mixed polyacrylamide-agarose slab gel (3.0% acrylamide, 0.15% bis-acrylamide, 0.6% agarose, and 0.1% SDS) in TBE buffer. Both gels were cast between glass plates and electrophoresed in a vertical system, totally submerged in the lower chamber buffer. The lower chamber consisted of a tall, hollow-sided plexiglass container through which heated or cooled water is circulated to control the temperature of the lower buffer and hence the gel. Dimensions of the glass plates and casting details are given in Odagiri *et al.* (1982). Viral RNA was loaded onto the gels in TBE buffer plus 10% sucrose. The agarose gel was electrophoresed 16 hr at 80 V (constant voltage) at 37°, and the mixed polyacrylamide-agarose gel was electrophoresed 16 hr at 230 V (constant voltage) at 30°. The gels were dried onto Whatman 3MM filter paper in a gel drying apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) and autoradiographed for 1 to 3 days.

Ferrets. The ferret animal model system for influenza has been recently reviewed (Maassab *et al.*, 1982). Ferrets were inoculated intranasally and examined for coryza, rhinitis, and fever twice a day for the duration of the experiment. Nasopharyngeal swabs were taken daily, and on Day 3 and Day 8, ferrets were sacrificed, and 20% suspensions of turbinate and lung tissues were made in nutrient broth. Viruses in these samples were titrated in embryonated eggs.

RESULTS

Genotype Characterization

All eight genes of A/AA/60, A/Ala/77, CR43-3, and CR43-7 virus were differentiated by analyzing the corresponding viral RNA segments in two different gel systems, agarose and mixed polyacrylamide-agarose, as described under Materials and Methods. Both types of gels are used at controlled, elevated temperatures to partially denature the viral RNA (Kendal *et al.*, 1979). The different migration of partially denatured RNA segments of corresponding genes from different viruses does not necessarily reflect different molecular weights, but rather conformational differences that may exist between the RNA segments due to sequence differences. As a consistent phenomenon it provides a simple method of genotyping reassortants. The polymerase genes were best genotyped in a 1.5% agarose gel in TBE running buffer, at 37°. Figure 1 is an autoradiograph of an agarose gel used to genotype the polymerase genes of the two reassortants, CR43-3 and CR43-7. All three polymerase genes in both reassortants are derived from A/AA/60 virus. The derivation of the remaining RNA segments was determined from the autoradiograph of a

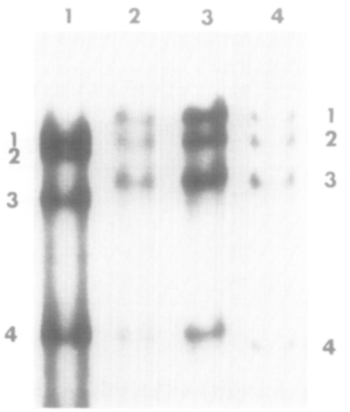

FIG. 1. Genotype determination of RNA1, 2, and 3, for CR43 reassortants. Electrophoresis conditions were described under Materials and Methods. Samples on lanes are 1 = A/A1a/77; 2 = CR43-3; 3 = CR43-7; and 4 = A/AA/60.

mixed 3.0% polyacrylamide–0.6% agarose gel in TBE running buffer, at 30°, shown in Fig. 2. The HA and NA genes for both reassortants are from A/Ala/77 virus. The assignment of RNA5 in the reassortants as the A/Ala/77 NA gene in these partially denaturing gels was verified by neuraminidase inhibition assay in this laboratory and at CDC (Dr. Alan P. Kendal, personal communication). Thus, the NP and M genes, RNA6 and RNA7, respectively, are derived from A/AA/60 virus. The CR43-7 NS gene, RNA8, is the same as the A/AA/60 NS gene. The NS gene of CR43-3 virus, indicated by "a," in Fig. 2, does not match the NS gene of either parent. Table 1 presents a summary of the genotype constellations for both reassortants compared to their two parent viruses. The CR43-7 reassortant has the genotype desired for a live virus vaccine candidate, i.e., the two surface genes of the wt parent and the six internal genes of the ca and ts master strain, A/AA/60 virus (Cox *et al.*, 1979; Maassab *et al.*, 1981a, 1982). Except for the aberrant NS gene, RNA8, the CR43-3 virus is identical to the CR43-7 virus. However, the CR43-7 virus is not host range, providing strong circumstantial evidence that the aberrant NS gene in CR43-3 virus is the determinant for the host range phenotype.

Host Range Phenotype in Cell Culture

The restricted host range phenotype of the CR43-3 virus was observed during a standard marker screening procedure in which CR43 reassortant viruses were assayed for ca and ts markers. Both PCK and MDCK cell monolayers were used, and the results shown in Table 2. As expected for reassortants with five or six internal genes from A/AA/60 virus, both CR43-3 and CR43-7 were ca and ts in PCK cells. However, when grown in MDCK cells, the CR43-3 virus produced no detectable plaques, at either 33 or 39°, while the CR43-7 virus, whose gene constellation varied from that of the CR43-3 reassortant by only the NS gene, produced plaques normally at 33° and was ts at 39°. The restricted host range phenotype and aberrant NS gene of

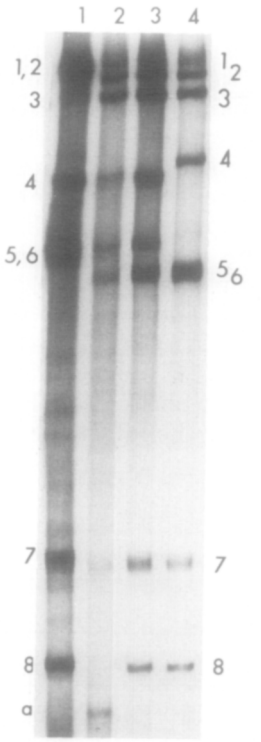

FIG. 2. Genotype determination of RNA4-8, for CR43 reassortants. Electrophoresis conditions were described under Materials and Methods. Samples on lanes are 1 = A/Ala/77; 2 = CR43-3; 3 = CR43-7; and 4 = A/AA/60.

TABLE 1

GENE CONSTELLATION OF CR43 REASSORTANTS

Virus line	Protein: RNA segment: 1	2	3	HA 4	NA 5	NP 6	M 7	NS 8
A/AA/60	A[a]	A	A	A	A	A	A	A
A/Ala/77	W[b]	W	W	W	W	W	W	W
CR43-3	A	A	A	W	W	A	A	X[c]
CR43-7	A	A	A	W	W	A	A	A

[a] (A) represents a gene derived from A/AA/60 virus.

[b] (W) represents a gene derived from A/Ala/77 virus.

[c] (X) represents the aberrant NS gene found in CR43-3 virus, which migrates differently in the genotyping gels (see Fig. 2) than either parent's NS gene.

the CR43-3 virus has remained stable throughout four consecutive passages at 33° in embryonated eggs.

The growth inhibition in MDCK cells was further examined by infecting MDCK cells in liquid overlay at an m.o.i. of 3 PFU/cell. Both parent viruses and the CR43-7 reassortant were used as positive controls. All viruses were adsorbed to the cells in 2× Eagle's maintenance media with 5.0 μg/ml of TPCK trypsin. After 1 hr adsorption of virus at room temperature, the virus inocula were removed and the cells washed once with maintenance media. Five milliliters of this media containing 1.0 μg/ml TPCK trypsin was then added to each infected culture, and the cells incubated at 33° for the A/AA/60, CR43-3, and CR43-7 viruses; or at 35° for the A/Ala/77 virus. At 48 hr pi, extensive cell lysis was observed in all of the infected cultures, even the one infected with the CR43-3 reassortant. The 48 hr pi media for each virus was plaque-titrated in both PCK and MDCK cells. The results are shown in Table 3. These results show that the CR43-3 virus-infected MDCK cells did not produce infectious virus, since the 48 hr pi media titrated at only 1000 PFU/ml in PCK cells, and <10 PFU/ml in MDCK cells. The 1000 PFU/ml of CR43-3 virus seen in the PCK cell monolayers probably represents residual infecting virus that was not completely removed by washing the monolayers after virus adsorption. Whether they represent residual infecting virus or a very low output of newly made virus they retained the hr phenotype, as evidenced by the lack of plaques in the MDCK cells. The CPE in these cells is probably similar to the CPE observed in some abortive cycles of viral replication and may indicate that the pro-

TABLE 2

BIOLOGICAL CHARACTERISTICS

Virus lines	Virus PFU/ml: MDCK 33°	MDCK 39°	PCK 25°	PCK 33°	PCK 39°	Markers: MDCK	Markers: PCK
A/AA/60	3×10^8	$<10^3$	1×10^8	3×10^8	$<10^3$	ts	ca, ts
A/Ala/77	5×10^7	8×10^6	$<10^3$	1×10^8	2×10^7	wt	wt
CR43-3	$<10^3$	$<10^3$	3×10^6	2×10^6	$<10^3$	hr	ca, ts
CR43-7	4×10^8	$<10^3$	2×10^6	1×10^8	$<10^3$	ts	ca, ts

TABLE 3

INFECTIOUS YIELD FROM MDCK CELLS INFECTED WITH CR43 REASSORTANTS AND PARENT VIRUSES

Virus line	PFU/ml	
	MDCK Cells	PCK cells
A/AA/60	4×10^7	3×10^7
A/Ala/77	6×10^6	3×10^6
CR43-3	<10	1×10^3
CR43-7	4×10^6	2×10^6

duction of virus in these cells is blocked at a late stage in viral replication.

Linkage of NS Gene to Host Range Phenotype

MD1 reassortants were made using CR43-3 and A/Cal/78 virus in MDCK cells at 33°. Because antiserum to A/Cal/78 was used to suppress the growth of the wt parent as described under Materials and Methods, and because CR43-3 virus does not grow in MDCK cells, only reassortants which lack the CR43-3 gene(s) responsible for the restricted host range should grow. Eight MD1 reassortants were isolated of which six had unique gene constellations (Table 4). Of all the genes of the CR43-3 virus only the NS gene never appeared in any of the MD1 reassortants. Although based on a limited number of reassortants, this result implies that the aberrant NS gene is the primary determinant of the restricted host range of the CR43-3 virus.

A second reassortment experiment, CR55, used the same parent viruses as MD1, but was performed in PCK cells at 33°, as described under Materials and Methods. Six independent reassortants exhibited three different gene constellations. Two of these, CR55-3 and CR55-4, shown in Table 4, had the restricted host range phenotype and one, CR55-6, lacked the restricted host range of the CR43-3 virus, and had replaced the CR43-3 NP and NS genes, found in CR55-3 and CR55-4 viruses, with those of the A/Cal/78 virus. In addition to the CR43-3 NS gene, the CR55 reassortants which retained the restricted host range phenotype, also derived their NP and RNA3 genes from the CR43-3 parent. From the MD1 reassortment experi-

TABLE 4

GENE CONSTELLATIONS OF REASSORTANTS INDEPENDENTLY DERIVED AT 33° IN PERMISSIVE AND NONPERMISSIVE HOSTS

Clones	Number of isolates[a]	Protein: RNA segment: 1	2	3	HA 4	NA 5	NP 6	M 7	NS 8	hr[b]
CR55: CR43-3 × A/Cal/78 in PCK cells										
CR55-3	2	C[c]	C	R[d]	C	C	R	R	R	yes
CR55-4	2	C	C	R	C	C	R	C	R	yes
CR55-6	2	C	C	R	C	C	C	R	C	no
MD1: CR43-3 × A/Cal/77 in MDCK cells										
MD1-1	1	C	C	R	R	C	R	C	C	no
MD1-3	2	C	C	C	R	C	R	C	C	no
MD1-4	2	C	C	C	R	R	C	R	C	no
MD1-5	1	R	R	R	R	C	R	C	C	no
MD1-9	1	C	C	R	R	R	C	C	C	no
MD1-10	1	C	C	R	R	R	C	R	C	no

[a] The number of reassortants isolated from independent culture tubes with this gene constellation.

[b] hr = host range phenotype in MDCK cells: yes, if restricted in growth; no, if not restricted in growth.

[c] (C) represents a gene derived from A/Cal/78 virus.

[d] (R) represents a gene derived from CR43-3 reassortant virus.

ment it is clear that these latter two genes are not sufficient in and of themselves or in combination with one another to manifest the host range phenotype without the CR43-3 NS gene. Thus the CR43-3 gene is the primary determinant of the restricted host range.

Host Range Phenotype in Ferrets

CR43-3 virus was inoculated intranasally into ferrets to compare its virulence with that of its parent viruses and with CR43-7 virus. All four viruses were used at equivalent titers. The results are shown in Table 5. Unlike either parent or CR43-7 virus, no evidence of infection could be demonstrated for CR43-3 virus. Our usual procedures assay virus titers in nasopharyngeal swabs and 20% suspensions of turbinate and lung tissues in nutrient broth on Days 3 and 8. Even the ca and ts A/AA/60 virus (a highly attenuated virus) grows well in the turbinate and can be isolated from nasopharyngeal swabs. CR43-3 virus, however, was never detected with these procedures, even as early as 24 hr pi. Of these four viruses only ferrets infected with A/Ala/77 virus presented any symptoms, i.e., rhinitis, coryza, and fever.

A/Cal/78, CR55-3, and CR55-6 viruses were also grown in ferrets. CR55-3, a reassortant with the CR43-3 NS gene and the restricted host range, produced no symptoms and no detectable virus was recovered from nasopharyngeal swabs or from turbinate and lung tissues. CR55-6, a non-host range reassortant, also produced no symptoms, but virus was recovered from nasopharyngeal swabs on Days 1 and 2, and from turbinate tissue on Day 3. No detectable virus was recovered from the lung tissue. CR55-6 virus resembled both A/AA/60 and CR43-7 viruses when grown in ferrets, while CR55-3 virus acted similarly to CR43-3 virus.

DISCUSSION

CR43-3 virus is a reassortant of A/AA/60 and A/Ala/77 viruses that has a mutated NS gene, will not grow in MDCK cells, and could not be recovered from ferrets after intranasal inoculation. Neither parent virus exhibits this growth restriction, growing well in both MDCK cells and ferrets. CR43-7 virus was used as a positive control in these experiments because its gene constellation was identical to that of CR43-3 except for the NS gene. The CR43-3 NS gene migrates differently in the genotyping gels from either the A/AA/60 or the A/Ala/77 NS gene. At least 15 reas-

TABLE 5

BEHAVIOR OF HOST RANGE INFLUENZA REASSORTANTS IN FERRETS

Virus line	Virus dose $\log_{10}$ EID_{50}/ml	Presence of			Virus shedding[c] (days pi)	Virus content[d]			
						Turbinates		Lungs	
		Coryza[a]	Rhinitis[a]	Fever[b]		3 (days)	8 (days)	3 (days)	8 (days)
A/AA/60	8.0	–	–	–	6	4.3	<1	<1	<1
A/Ala/77	7.0	+	+	++	5	6.0	5.0	2.5	1.5
CR43-3	7.5	–	–	–	N.D.	<1	<1	<1	<1
CR43-7	7.5	–	–	–	2	5.5	<1	<1	<1
A/Cal/78	7.0	+	+	+	6	6.5	5.5	1.5	<1
CR55-3	7.5	–	–	–	N.D.	<1	N.A.	<1	N.A.
CR55-6	7.5	–	–	–	3	4.5	N.A.	<1	N.A.

[a] (+) = presence; (–) = absence.

[b] Fever ranged between 104 and 106°F; (+) = 1 day duration, (++) = 2 days duration; (–) = normal ferret temperature, 102–103.5°F.

[c] Virus shedding was based on virus recovery from NP swabs, assayed every day after infection. N.D. = no virus detected.

[d] Virus content is expressed as $\log_{10}$ EID_{50}/ml, and is an average titer from two ferrets. N.A. = experiment not performed.

sortants made with these two parents have been examined (data not shown) and only CR43-3 virus did not grow in MDCK cells and only it has the aberrant NS gene. No other genes in any of these reassortants varied from those in the A/AA/60 or A/Ala/77 parents. Thus, the aberrant NS gene of the CR43-3 virus probably resulted from a mutation in the NS gene of either A/AA/60 or A/Ala/77 virus.

Both the CR43-3 and CR43-7 reassortants are ca and ts in PCK cells, however, in MDCK cells the CR43-3 virus will not grow at any temperature. Therefore the CR43-3 hr mutant is not a td-hr mutant, but simply a hr mutant. Host range mutants that are not td-hr mutants have been associated with polymerase genes (Almond, 1977) or with the HA and NA genes (Schulman and Palese, 1977; Ghendon, *et al.*, 1979; Bosch, *et al.*, 1979). The CR43-3 hr mutant is independent of the HA and NA genes, but as noted earlier in the CR55 reassortants, the NP and RNA3 genes from the CR43-3 parent co-reassorted with the aberrant NS gene. From the MD1 reassortants it is clear that these two genes cannot confer the hr phenotype in the absence of the CR43-3 NS gene. Thus this unique mutant represents a new class of hr mutants of influenza A viruses whose phenotype is dependent upon the NS gene.

Very few NS gene mutants have been isolated and characterized, and these have all been ts mutants (Almond *et al.*, 1977, 1979; Wolstenholme *et al.*, 1980; Koennecke *et al.*, 1981). The location of the mutations with respect to NS1 and NS2 coding regions were not known, and while these mutants varied from one another in some of their biological defects all three showed little M protein production at nonpermissive temperature. Scholtissek and Spring (1982) have reported the extragenic suppression of ts mutants in the NS gene by various polymerase genes. Thus the NS gene may function interactively with the polymerase genes. As an NS hr mutant, CR43-3 should prove to be a useful adjunct to the ts NS gene mutants in exploring the *in vivo* function(s) of the NS gene, whether the mutation affects NS1, NS2, or both proteins.

CR43-7 virus has the optimal gene constellation for a stable, attenuated live virus vaccine reassortant which derived its attenuating genes from A/AA/60 master strain. CR43-3 virus has the same gene constellation except for the aberrant NS gene. However the studies in ferrets show that CR43-3 virus is even more attenuated than the CR43-7 virus, and that it can transfer this attenuation to reassortants when they contain the aberrant NS gene. Thus, CR43-3 virus is an exciting new donor of attenuating genes, and could prove to be a useful master strain for the production of live influenza vaccines.

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