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Generation of Seal Influenza Virus Variants Pathogenic for Chickens, because of Hemagglutinin Cleavage Site Changes

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Influenza virus A/seal/Mass/1/80 (H7N7) was adapted to grow in MDCK cells and chicken embryo cells (CEC) in the absence of exogenous protease. The biological properties of the virus variants obtained coincided with intracellular activation of the hemagglutinin (HA) by posttranslational proteolytic cleavage and depended on the cell type used for adaptation. MDCK cell-adapted variants contained point mutations in regions of the HA more distant from the cleavage site. It is proposed that these mutations are probably responsible, through an unknown mechanism, for enhanced cleavability of HA in MDCK cells. Such virus variants were apathogenic in chickens. CEC-adapted variants, on the other hand, contained an insertion of basic amino acids at the HA cleavage site, in addition to scattered point mutations. The insertions converted the cleavage sites in the variant virus HAs so that they came to resemble the cleavage site found in highly pathogenic avian influenza viruses. CEC variants with such cleavage site modifications were highly pathogenic for chickens. The lethal outcome of the infection in chickens demonstrated for the first time that an influenza virus derived from a mammalian species can be modified during adaptation to a new cell type to such an extent that the resulting virus variant becomes pathogenic for an avian species.

Since the pandemic of influenza in 1918–1919, the specter has always existed of another highly pathogenic human influenza virus emerging. The presence of pantropic avian influenza viruses has provided some support for such an occurrence. The event in 1979–1980, when seals experienced an apparent epidemic of influenza related to the H7 avian viruses, further supported the potential for interspecies transmission of pantropic viruses. Influenza virus A/seal/Mass/1/80 (seal virus, H7N7) was first isolated when approximately 20% of the harbor seal (*Phoca vitulina*) population on the New England coast of the United States died in 1979–1980 because of acute hemorrhagic pneumonia (8). The virus was antigenically characterized as H7N7 serotype, similar to some naturally occurring avian influenza viruses. Hybridization analyses of the genomic RNA of the seal virus and strains of avian or mammalian origin revealed that each of the eight seal virus RNA segments was closely related to those found in different avian influenza viruses rather than in mammalian viruses. Nevertheless, the virus from seals has a host range similar to that of mammalian influenza viruses. Thus, in addition to harbor seals, in which the virus causes respiratory illness, the virus replicates in pigs, ferrets, cats, and the conjunctiva of humans, whereas it grows poorly and produces no signs of disease after experimental infection of avian species (1, 25, 26).

The hemagglutinin (HA) glycoprotein, present as spikelike protrusions on the surface of influenza viruses, plays an important role in initial steps of infection and can affect host range. Thus, not only does the HA attach to cell receptors, it is also involved in a membrane fusion step necessary for genome penetration. This latter activity is demonstrated only if the HA has been proteolytically cleaved into the disulfide-linked polypeptides HA_1 and HA_2 by cellular proteases. Virus particles which have been produced in a host cell deficient in appropriate proteases and which have an uncleaved HA are noninfectious. They can be converted ("activated") into the infectious form by in vitro treatment with arginine-specific proteases, such as trypsin (14, 15). The structure at the HA cleavage site of avian viruses has been found to be a major determinant of whether cleavage occurs in infected host cells and in turn affects the severity of disease resulting from virus infection of chickens (3, 22). Thus, unlike the HA of human epidemic influenza viruses (subtypes H1 to H3) and the majority of nonpathogenic influenza viruses from birds and other animals (subtypes extend to H13), the HAs of the two subtypes H5 and H7 can, in some avian influenza viruses, possess a series of basic amino acids at the cleavage site. This structure permits HA cleavage to occur in a wide range of cell types. Such avian viruses are highly pathogenic for chickens, causing a pantropic infection (for review, see references 13 and 27).

Nucleotide sequence analysis indicates that the cleavage site between HA_1 and HA_2 of the seal virus H7 HA contains a single arginine (17). The lack of multiple basic amino acids in the connecting peptide between HA_1 and HA_2 therefore is in keeping with the finding that the seal virus is apathogenic when infected into chickens.

In this article we report changes in the structure and properties of the seal virus HA which occurred during adaptation of egg-grown virus to Madin Darby canine kidney (MDCK) cells or chicken embryo cells (CEC), originally not permissive for the production of infectious virus without exogenous trypsin. These changes were found to be determined by the cell type used for adaptation. When the virus was adapted to CEC, mutations occurred at the HA cleavage site which resulted in cleavability by ubiquitous proteases and enhanced pathogenicity for chickens. On the other hand, adaptation to MDCK cells led to mutations more distant from the cleavage site. Increased cleavability of the HA of these variants occurred only in MDCK cells, and the virus was still apathogenic for chickens.

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MATERIALS AND METHODS

Viruses and cells. The influenza virus A/seal/Mass/1/80 (H7N7) (seal virus) (8), grown in 11-day-old hen's eggs, was used. Adaptation experiments were performed in MDCK cells and in primary cultures of CEC. In some experiments BHK, GMK, and Vero cells were used. Infected cells were overlaid with reinforced Eagle medium without serum. Virus growth and plaque assays were done in the absence or presence of trypsin (5 μg/ml) in the medium described before (14).

Hemagglutination tests and experimental infection of adult white Leghorn chickens were done as described before (18).

SDS-PAGE. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), monolayers of cells on 5-cm plastic petri dishes were labeled by adding [^{35}S]methionine (10 μCi per culture) for the times indicated in the figures. At the end of the incubation period, the cells were washed three times with phosphate-buffered saline and lysed. The cell lysates were immunoprecipitated with a monoclonal antibody directed against the seal virus HA. Slab gels were analyzed by fluorography (3).

Two-dimensional gel analysis. The procedure for two-dimensional gel analysis and isoelectric point determination of immunoprecipitated HA followed the protocol of Bosch et al. (2).

Nucleotide sequence analysis. The virus used for RNA sequencing was grown in 11-day-old hen's eggs. Virus was purified by adsorption to and elution from chicken erythrocytes and subsequent centrifugation (18). The viral RNA was extracted by the hot phenol method described by Maniatis et al. (16). RNA sequence determination was done by the dideoxynucleotide chain termination method (23) with purified viral RNA as the template and reverse transcriptase (4). The oligonucleotide primers used were complementary in sequence to nucleotides 5 to 15, 307 to 318, 573 to 583, 739 to 750, 912 to 924, 1108 to 1118, 1306 to 1315, and 1591 to 1602 of the seal virus RNA gene for HA (17).

RESULTS

Generation of adaptation variants. Wild-type seal virus replicates poorly in MDCK cells and in CEC in the absence of trypsin (Fig. 1). Virus variants were selected by a series of passages in each cell type, starting with untreated egg-grown wild-type virus. Adaptation to MDCK cells was successful after 7 to 10 passages in the absence of trypsin in the culture medium, and three variants, SM1, SM2, and SM3, were isolated from three different plaques produced without exogenous trypsin. On the other hand, adaptation to CEC was more difficult. The adaptation procedure followed the protocol described in detail in Table 1. For the first passage, CEC were infected with a multiplicity of infection of 10^{-1} PFU of infectious allantoic fluid per cell, and for the following passages with the progeny (culture medium or plaques) of the preceding passages. Trypsin was added to the culture medium in the concentrations mentioned in Table 1. The virus progeny of the 20th passage were further passaged in two separate series. The series which led to the generation of SC102 after 81 additional passages in CEC (not mentioned in Table 1) were performed similarly to those which led to SC32 and SC35. SC32 and SC35 were obtained from a single plaque of passages 31 and 34, respectively, and their progeny were used for mass production in chicken eggs. It must be noted, however, that mutation is always a random process, so that the outcome of host-cell-selected virus variants will necessarily be accidental. This is exemplified by the different properties of SC35 and SC102 (see below).

Biological properties of host range variants. Depending on the cell type used for adaptation, the variants selected exhibited different biological properties. MDCK-adapted variants only produced infectious virus in MDCK cells. This was shown by their ability to form plaques in these cells and their lack of ability to multiply under multiple cycle conditions (Fig. 1) and to produce plaques in other cell types, including CEC, BHK, GMK, and Vero cells. However, the MDCK variants retained the ability to grow efficiently in embryonated eggs, like the wild-type virus. They did not acquire pathogenicity for chickens; after intramuscular infection of chickens with about 10^6 PFU of the MDCK-adapted variants, no clinical signs were seen during a 14-day observation period.

Adaptation to CEC occurred stepwise in the series SC20, -32, and -35. In contrast to the wild-type virus, the variant virus SC20, isolated after 20 passages in CEC, was able to produce turbid plaques, 1 to 2 mm in diameter, in CEC in the absence of trypsin in the agar overlay. Addition of trypsin (5 μg/ml), however, increased the plaque size threefold and the plaque number about 1,000-fold. With SC32, addition of trypsin to the agar overlay increased the plaque size significantly without affecting plaque number. Variant SC35 produced plaques in CEC without trypsin that resembled highly pathogenic H7 avian viruses; trypsin treatment influenced neither plaque morphology nor plaque number (Fig. 2). Variant SC20 replicated to some degree only in CEC in the absence of added trypsin. Under these conditions, about 4 hemagglutinating units were obtained 72 h after infection in the culture medium of CEC, whereas no hemagglutinating virus was found after infection of other cell types (not shown). With the CEC-adapted variants SC32 and SC35, on the other hand, production of infectious virus and plaque formation were not restricted to CEC. Both of the variants were also produced in infectious form in the absence of trypsin in MDCK, BHK, GMK, and Vero cells (Table 2, SC35).

The CEC variants SC32 and SC35 also differed from the wild-type virus in their pathogenic properties, whereas the variant SC20, like the parental virus, was apathogenic for chickens. Intramuscular inoculation of chickens with 10^6 PFU of SC32 and SC35 led to systemic infection and death. In both cases the pathological alterations corresponded to those found in birds infected with the highly pathogenic reference fowl plaque virus A/FPV/Rostock/34 (H7N1). The incubation period consistently differed by 1 to 2 days between SC32 and SC35, in that the chickens died 4 to 5 days after infection with the former virus but after only 2 days with the latter variant.

The CEC-derived variant SC102 was similar to SC20 in its limited ability to grow and produce plaques in CEC. However, in contrast to SC20, it produced plaques in MDCK cells. The appearance and number of the plaques were not influenced by trypsin treatment. Like the MDCK-adapted variants, SC102 was restricted to growth in MDCK cells and was not pathogenic for chickens. In this respect it did behave like the MDCK cell-derived variants.

Processing of the HA after adaptation. Alterations in the biological properties of the adaptation variants coincided with the susceptibility of their HAs to proteolytic activation in those cells which produced infectious virus. The HA of the original seal virus grown in CEC and MDCK cells was in an uncleaved form, but was cleaved after in vitro trypsin treatment (Fig. 3 and 4). With the variants, however, cells

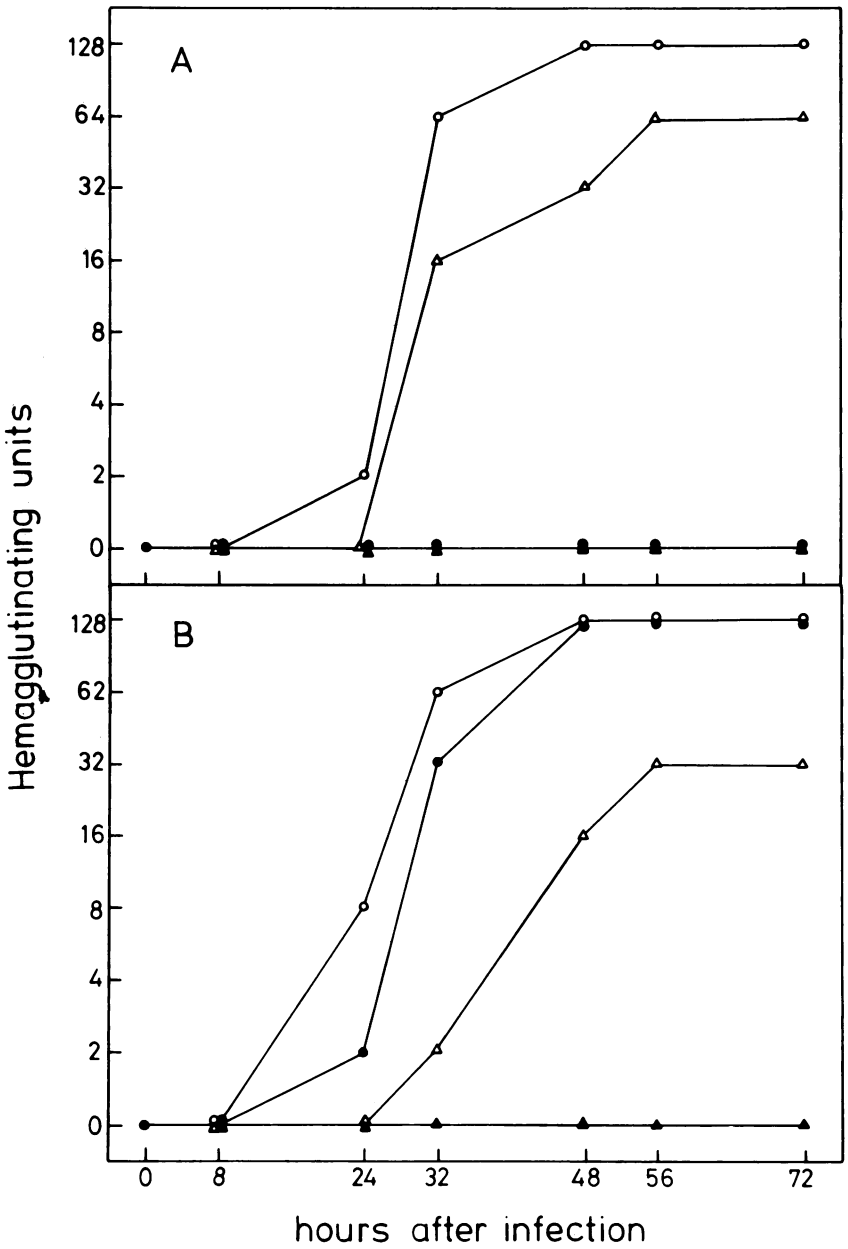

FIG. 1. Growth of wild-type seal influenza virus and its variant SM1 in CEC and MDCK cells. The monolayer of cells was infected at 10^{-3} PFU/cell with wild-type virus (A) or the MDCK variant SM1 (B). Symbols: ○, ●, MDCK cells; △, ▲, CEC; ○, △, with trypsin (5 μg/ml); ●, ▲, without trypsin.

producing infectious virus were shown to contain cleaved HA. Thus, the HA of the MDCK variants was cleaved only in MDCK cells, whereas the HAs of the CEC variants SC32 and SC35 were susceptible to cellular proteases from a wide range of cells. In CEC or MDCK cells infected with SC32, some uncleaved HA was still distinguishable even after a labeling period of 16 h. The HA of SC35 appeared to be completely cleaved, like that of the highly pathogenic avian influenza viruses (3, 14). However, by PAGE analysis, no cleavage was seen in the intracellular HA of SC20 (Fig. 4). It must be assumed, therefore, that with SC20 only small amounts of HA are cleaved that are sufficient in released virions to permit limited plaque formation by the variant, even though the cleaved HA is not detectable by PAGE.

The HA of SC102 was found to be uncleaved when produced in CEC but became cleavable when the virus variant was grown in MDCK cells (data not shown). Presumably, as with SC20, small, undetectable amounts of cleaved HA were produced in CEC, but cleavage was much more efficient in MDCK cells.

Structural requirements for increased HA cleavability of the seal virus variants. To elucidate the basis for the differences in HA cleavability, the nucleotide sequences of the HA genes of the seal virus and its variants were analyzed and their amino acid sequences were deduced. When the HA gene of the wild-type seal virus was resequenced, one deviation from the originally published nucleotide sequence (17) was found (A at 1531 to G). The variations in the

TABLE 1. Passage history of CEC-derived variants[a]

Passage no.	Virus dilution (log_{10})	Incubation time (h)	Trypsin concn (μg/ml)	Hemagglutinating units
1	1 (M)	48	5	32
2	1 (M)	72	5	64
3	1 (M)	48	5	32
4	1 (M)	96	0	4
5	2 (M)	48	5	24
6	2 (M)	72	0	4
7	3 (M)	72	5	64
8	3 (M)	96	0	8
9	1 (M)	24	5	32
10	1 (M)	24	5	32
11	1 (M)	48	0	128
12	3 (M)	48	5	64
13	1 (M)	24	0	64
14	2 (M)	96	0	16
15	2 (M)	96	0	2
16	1 (M)	24	5	64
17	1 (M)	24	0	64
18	P from medium 17	48	5	32
19	P from medium 18	72	5	64
20 (SC20)	3 (M)	24	5	96
21	3 (M)	24	1	64
22	4 (M)	48	1	32
23	4 (M)	72	0.3	16
24	4 (M)	24	1	64
25	3 (M)	24	0.5	64
26	3 (M)	72	0	16
27	3 (M)	72	0	4
28	3 (M)	48	0	4
29	1 (M)	72	1	64
30	1 (M)	24	0	16
31 (SC32)[b]	1 (M)	72	0	16
32	3 (M)	48	0	32
33	5 (M)	48	0	32
34	3 (M)	24	0	32
35 (SC35)[b]	P from medium 34	24	0	64

[a] For infection of subsequent passages, either culture medium (M) or the progeny of plaques (P) of the preceding passage were used.
[b] SC32 and SC35 were obtained from a single plaque developed from virus in the culture medium of passages 31 and 34, respectively.

primary structure of the host range variants are shown in Table 3. Based on the three-dimensional structure of an H3 influenza virus HA (28), the relatively few mutations found on the HA of the MDCK variants did not appear to be localized at biologically important domains, such as the receptor-binding site or the cleavage site. There was also no accumulation of base substitutions in a distinct region of the HA. The prominent feature among the three MDCK variants

TABLE 2. Growth of CEC-derived variant SC35 of seal influenza virus in different cell types[a]

Time post-infection (h)	Hemagglutinating units									
	BHK		CEC		GMK		MDCK		Vero	
	−T	+T	−T	+T	−T	+T	−T	+T	−T	+T
8	0	0	0	0	0	0	0	0	0	0
24	16	16	16	32	2	4	8	8	0	0
32	32	32	32	32	16	16	32	32	0	0
48	32	32	32	32	16	16	32	32	4	4
56	32	32	32	32	16	16	32	32	8	8
72	32	32	32	32	16	16	32	32	8	8

[a] Cells were infected with 10^{-3} PFU/cell and incubated for the time indicated with (+T) or without (−T) trypsin.

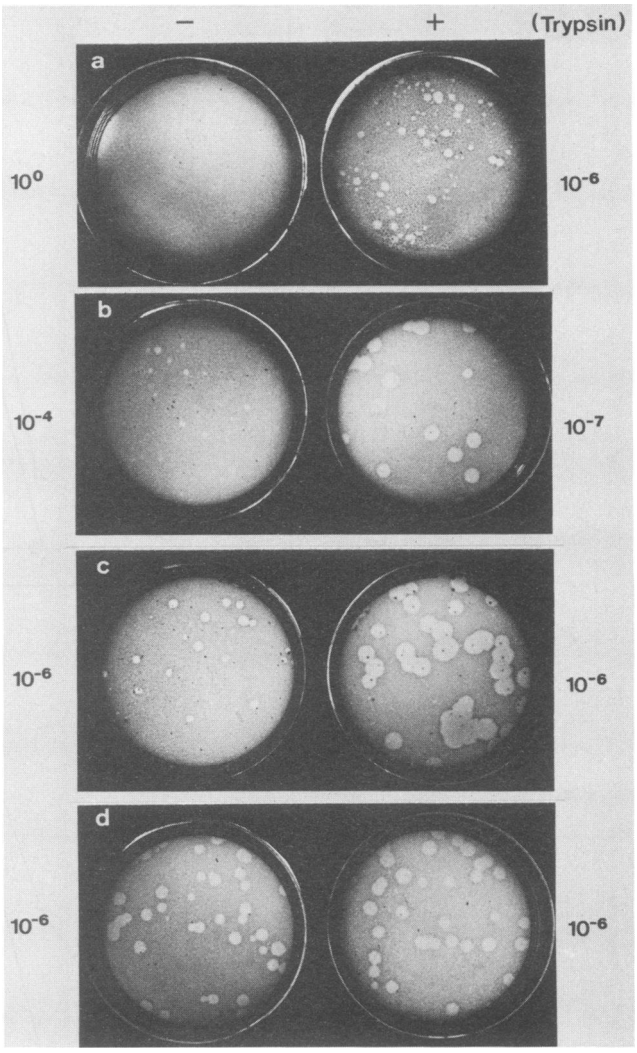

FIG. 2. Alterations in plaque formation during adaptation of seal influenza virus to CEC. The plaque test was done in the presence (+) or absence (−) of trypsin (10 μg/ml) in the agar overlay. Cells were stained 3 days after infection. (a) Wild-type seal virus; (b,c,d) CEC variants SC20, SC32, and SC35, respectively.

was the replacement of Thr by Ser (SM1 and SM2) or by Ala (SM3) at HA_1 position 125. Interestingly, substitution in the neighboring Arg-131 by Ser was found in SC102, the HA of which was also cleaved in MDCK cells. These exchanges therefore suggest that the mutations in this region may influence the increased HA cleavability.

In all of the CEC variants, including SC20 and SC102, two point mutations were in identical positions, which should exclude their significance in extended HA cleavability. It is, however, not possible to decide whether and how the point mutations contributed to enhanced growth of the CEC variants. In addition to the point mutations with the CEC variants SC32 and SC35, in which the enhanced HA cleavability led to striking changes in their biological properties, an insertion of nine nucleotides, CGGAGGGGG, was found in the region of the cleavage site (Fig. 5). With SC32 the insertion corresponds to the amino acid sequence Arg-Arg-Gly, which is directly adjacent to the NH_2-terminal Gly of HA_2. With SC35 there was an additional alteration at the cleavage site caused by the substitution of the original

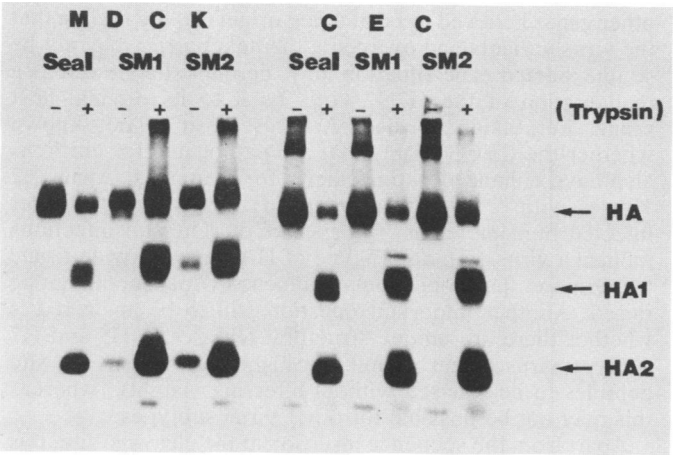

FIG. 3. HA polypeptides of wild-type seal influenza virus and its MDCK variants SM1 and SM2. Infected CEC or MDCK cells were labeled with [^{35}S]methionine and incubated with (+) or without (−) trypsin in the culture medium. Cell lysates were immunoprecipitated with HA-specific monoclonal antibody and analyzed by SDS-PAGE.

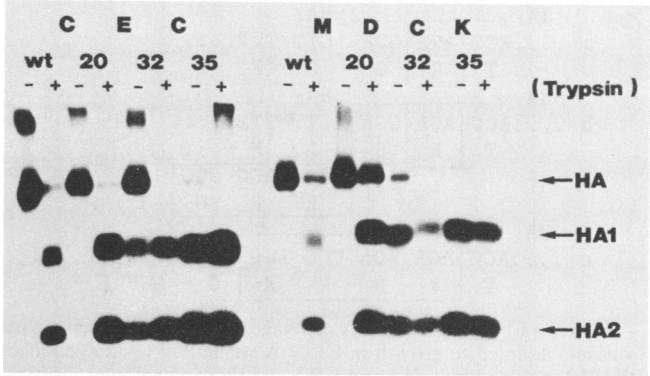

FIG. 4. SDS-polyacrylamide gel of HAs from seal influenza virus and its CEC-derived variants SC20, SC32, and SC35 produced in CEC or MDCK cells. For experimental conditions, see Fig. 3 legend. wt, Wild-type seal virus.

NH_2-terminal Gly by another Arg. By means of enhancement of the basic charge, the cleavage site of the SC32 and SC35 HAs became comparable to those of the highly pathogenic avian influenza viruses, the HAs of which are also cleaved in a broad range of host cells (13, 27).

When the inserted nucleotide sequence at the HA gene of SC32 and SC35 is translated and the encoded extra basic amino acids between HA_1 and HA_2 are eliminated by the cleavage reaction (7), the HAs should possess different isoelectric points before and after cleavage. To test this, a mixture of cleaved and uncleaved HA was analyzed by two-dimensional electrophoresis. Such differences existed, and there was a progressive diversity from wild-type virus to SC32 and on to SC35 (Fig. 6). This means that the insert of nine nucleotides found by sequencing was in fact translated and that the resulting new connecting peptide between HA_1 and HA_2 of the variants SC32 and SC35 was eliminated by proteolytic cleavage.

DISCUSSION

Host range variants of the H7 seal influenza virus were generated with different alterations in biological properties depending on the cell type used for adaptation. The variants' biological properties correlated with the susceptibility of their HAs to posttranslational proteolytic cleavage in different cell types. Together with several previous findings on host range variants of other influenza viruses (12, 18, 21; M. Orlich, D. Khatchikian, A. Teigler, and R. Rott, Virology, in press), this study furnishes evidence that MDCK cells can select for cleavable-HA variants in which the susceptible cell range is increased only to this particular cell type. On the other hand, CEC screen for variants which grow in infectious form with cleaved HA in a wide variety of cell types tested. With the H5 and H7 subtype viruses, cleavage activation of the HA in a broad range of host cells is a prerequisite for rapid spread of the virus in the infected animal and thus for pathogenicity (3, 11, 18, 22). This concept has been confirmed in this study. It is, however, novel that a virus isolated from a mammalian species could become pathogenic for chickens. The molecular basis for the dramatic change in the host range of the CEC-adapted

TABLE 3. Nucleotide and amino acid changes in the HAs of seal influenza virus variants

HA affected	Nucleotide changes		Amino acid changes		MDCK variants			CEC variants[a]			
	Position	Change	Position	Change	SM1	SM2	SM3	SC20	SC32	SC35	SC102
HA_1	353	C → T	93	T → I				+	+	+	+
	448	A → T	125	T → S	+	+					
	448	A → G	125	T → A			+				
	468	A → T	131	R → S							+
	511	A → G	146	N → D			+				
	524	C → A	150	A → E							+
	613	A → G	180	T → A							+
	626	G → A	184	R → K	+						
	662	G → A	196	G → E			+				
	739	G → A	222	D → N				+	+	+	+
	1026	T → A	317	N → K							+
HA_2	1039	G → A	1	G → R						+	
	1047	T → G	3	F → A				+			
	1081	G → A	15	E → K			+				
	1091	T → C	18	I → T		+					
	1211	A → G	58	K → R							+
	1292	G → A	85	R → E							+

[a] CEC variants SC32 and SC35 have a nucleotide insert in the region of the HA gene encoding the cleavage site (see Fig. 5).

```
     HA1 <------------------> HA2
a ....ACC | AGA | GGA   CTT TTT....
        T |  R  |  G    L   F
     HA1 <------------------> HA2
b ....ACC | AGA | GGA | CTT TTT....
        T |  R  |  G  | L   F
                      | CGG AGG GGG
                      |  R   R   G
     HA1 <------------------------------> HA2
c ....ACC | AGA AGA CGG AGG | GGG CTT TTT....
        T |  R - R - R - R  |  G - L - F -
```

FIG. 5. Changes of the HA cleavage site of seal influenza virus variants adapted to growth in CEC. A single Arg residue connects the HA polypeptides HA_1 and HA_2 of the original seal virus (a). With the variant SC32 (b), there is an insertion of nine nucleotides adjacent to the 5′-terminal codon of HA_2. With variant SC35 (c), the cleavage site became further changed by substitution of the original NH_2-terminal Gly of HA_2 by another Arg. The connecting peptides are boxed.

variants can almost certainly be attributed to the insertion of basic amino acids at the cleavage site. This change converts the peptide connecting the HA_1 and HA_2 polypeptides to become similar to that of the highly pathogenic avian influenza viruses (for a review, see references 13 and 27). Although it remains to be determined what mechanism underlies this insertion, it can be assumed that a similar event could support the development of highly pathogenic avian influenza viruses in nature. This would imply that the pathogenic avian strains belonging to the H5 and H7 subtypes have evolved from nonpathogenic avian influenza viruses whose HA_1 and HA_2 are connected by a single arginine.

In view of the fact that seal virus contains H7 HA and other genes believed to be of avian origin (1), the finding that the virus variants had overcome the host barrier may not be so unexpected. The situation may, perhaps, be viewed as a readaptation of the H7N7 virus back to its original host range, i.e., avian species. At present, it is not known whether the CEC variants that are pathogenic for chickens also have enhanced pathogenicity for mammals. Avian H7 viruses with or without cleavable HA have rarely if ever infected humans. Thus, the specter of a human infectious influenza virus with an H7 type of HA having a wide tissue tropism (i.e., pantropic human influenza virus) should not be raised. Another important question still to be answered is whether there are unique structural features in H5 and H7 subtype viruses that permit the highly basic cleavage site peptides to be inserted without affecting viability, whereas this may not be possible for other virus subtypes.

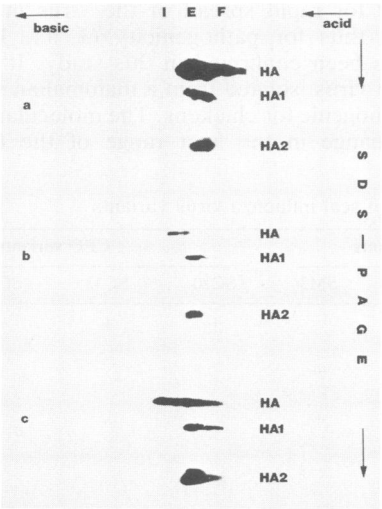

FIG. 6. Two-dimensional analysis of the HAs of seal virus (a) and its CEC variants SC32 (b) and SC35 (c) before and after cleavage. CEC were pulse labeled with [^{35}S]methionine 5 h after infection for 10 min, followed by a 2-h chase period. With the parental seal virus, the cells were treated with 10 μg of trypsin per ml of medium for 10 min after the chase period. Cell lysates were immunoprecipitated with an anti-HA monoclonal antibody. The immunoprecipitates were analyzed by PAGE by isoelectric focusing (IEF) under nonreducing conditions in the horizontal direction, followed by SDS-PAGE under reducing conditions in the vertical direction. The pH gradient was linear in the range of pH 5.0 to 7.0.

Apart from the sequence insertion at the cleavage site, it is probable that mutations more distant from the cleavage site may also influence HA cleavability, as shown from results with the MDCK cell variants here and in previous studies (21; Orlich et al., in press). This kind of mutation was found to vary between individual variants. Furthermore, known functionally important domains of the HA molecule were not involved. It is difficult, therefore, to elucidate the mechanism by which such structural changes could so markedly influence the host-dependent differences in HA cleavage activation. Clearly, a suggestive implication of this observation is that this type of single point mutation, perhaps in concert with other amino acid substitutions far from the cleavage site, could determine the pathogenic phenotype of mammalian influenza viruses. Comparative studies will be required to determine whether the degree of pathogenicity in mammals is in fact related to point mutations far from the cleavage site that result in differences in HA activation in the respective host cell. The role of proteases secreted by potentially coinfecting bacteria must also be considered (24).

It is remarkable that influenza viruses are more readily adaptable to MDCK cells than to CEC. This may be due to the lack in CEC of a proteolytic enzyme which has as broad a substrate specificity as the activating enzyme of MDCK cells. It should be noted, however, that some cell types, such as endodermal cells of the chorioallantoic membrane of chicken eggs, exhibit enzymes which are able to activate the HA of all influenza viruses (22). Based on the data available, we propose that, because the mammalian cells that are the primary target sites of infection may contain proteases with a broader substrate specificity than those found in avian cells, such as in CEC, there is never a need for mutants of mammalian viruses to arise with a multibasic peptide at the HA cleavage site. This hypothesis could explain why pantropic mammalian influenza viruses have not been found.

It has become clear that several structural requirements influence cleavage activation of influenza virus HA: a series of basic residues in the connecting peptide between HA_1 and HA_2 (2, 10, 11, 18), masking of the cleavage site by an adjacent carbohydrate site chain (9), an insertion of foreign amino acids at the cleavage site, which renders it susceptible to the action of ubiquitous cellular proteases by bulging the cleavage site (12; Orlich et al., in press), and point mutation more distant from the cleavage site, perhaps to generate an appropriate conformation for enzyme recognition, as shown here and in previous publications (21; Orlich et al., in press). There is evidence that with the F protein of paramyxoviruses also, besides the number of basic amino acid residues in the connecting peptide, other structural features may be important for cleavage activation (19, 24a).

It is known that in addition to enhanced HA cleavability, any mutation in the HA which affects the receptor-binding site (5, 6, 20) may provide a selective growth advantage in the respective host system. However, the precise coincidence reported here and previously between viral growth and HA cleavability suggests that this process is the major limiting step in adaptation of influenza viruses to cell types and therefore tissue tropism and pathogenicity in a susceptible host animal.

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