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Biological Characteristics of a Cold-adapted Influenza A Virus Mutation Residing on a Polymerase Gene

By

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With 3 Figures

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Summary

The biological function of a cold-adapted (ca) mutation residing on the PB2 gene of an influenza A/Ann Arbor/6/60 (A/AA/6/60) ca variant virus in the viral replication cycle at 25° C was studied. The viral polypeptide synthesis of A/AA/6/60 ca variant at 25° C was evident approximately 6 hours earlier than the wild type (wt) virus and yielded twice as many products. The quantitative analysis of viral complementary RNA (cRNA), synthesized in the presence of cycloheximide, revealed that A/AA/6/60 ca variant and a single gene reassortant that contains only the PB2 gene of the ca variant with remaining genes of the wt virus produced equal amount of cRNA at 25° and 33° C, which was an amount approximately four fold greater than the wt virus' cRNA synthesized at 25° C. These results strongly suggest that the ca mutation residing on the PB2 gene of A/AA/6/60 ca variant affects the messenger RNA synthesis at 25° C in the primary transcription.

Introduction

A cold-adapted variant of A/Ann Arbor/6/60 (A/AA/6/60) influenza virus was developed by serial passages in primary chick kidney (PCK) cells by gradually lowering the incubation temperature to 25° C which restricted the growth of the wt strain (17, 18). The A/AA/6/60 variant strain has been found to possess two inheritable plaquing phenotypes. One is the cold-adapted (ca) property which is the ability to grow to high titer at 25° C. The other is the temperature-sensitive (ts) property with a shutoff tem-

perature of 37° or 38° C (16, 17, 18, 27). Reassortants that possessed the six nonglycoprotein genes of A/AA/6/60 variant and the two glycoprotein genes of a wt virus, were attenuated to both animals and humans (18, 21, 27), possessed good immunogenic qualities (5, 29, 32), and they were genetically stable (8). Thus, the A/AA/6/60 ca variant virus is currently the most useful master strain for developing live influenza vaccine lines.

Identification of the ca A/AA/6/60 variant genes responsible for the ca and ts properties have been hampered due to two commonly encountered difficulties; 1. the lack of single-gene reassortants bearing the ca or ts properties and 2. the lack of a diversity in the gene constellations of reassortant viruses. Assignment of gene constellations using a large number of ca reassortant clones, formed by mating the A/AA/6/60 ca variant and wt viruses, suggests that the ts property of the ca reassortant is not determined by a single gene of the A/AA/6/60 ca variant (6, 7, 23).

The function of the ca mutation in the viral replication cycle and the gene(s) involved in ca property of A/AA/6/60 ca variant have yet to be unequivocally identified. Previously, we developed ca and non ts phenotypic reassortant clones using canine kidney (MDCK) cells at a non-selective temperature of 33° C (23). Some of the single-gene reassortants that possessed only RNA 2 coding for the PB2 gene or RNA 5 coding for the neuraminidase (NA) gene of A/AA/6/60 ca variant, were involved in the ca and non ts phenotypes. There reassortants allowed us to determine the function of the ca mutation in viral attenuation in mice (24) as well as the biological mechanism that operates to lower the viral replication range to 25° C.

The present study describes the effects of a ca mutation residing on the PB2 gene of A/AA/6/60 variant strain. Using the PB2 single-gene ca reassortant virus, we found that the PB2-ca mutation affects both polymerase activity and messenger RNA synthesis in primary transcription at 25° C.

Materials and Methods

Viruses and Tissue Cultures

Influenza A viruses used in the present study were a 8 PI-A/Ann Arbor/6/60 (H2N2) cold-adapted (ca) variant (17), an A/Ann Arbor/6/60 (H2N2) wild type strain, an A/Alaska/6/77 (H3N2) and the ca reassortant clones of the A/Ann Arbor/6/60 ca variant and A/Alaska/6/77 virus (23).

Madin-Darby canine kidney (MDCK) cells (Flow Laboratory, Rockville, Md.) were used between the 60th and 75th passage levels. The cell cultures were maintained in an Eagle's MEM containing 10 per cent heat-inactivated fetal calf serum and were used for subsequent viral growth curve and biochemical analysis.

Viral Growth Curve Analysis

Confluent MDCK monolayers in 25 cm^2 plastic bottles were infected with the viruses at a multiplicity of infection (MOI) of 1.0 plaque-forming unit (PFU) per cell in double-strength Eagle's basal medium (2X Eagle's) containing 5 μg/ml of TPCK

trypsin. After 1 hour adsorption at room temperature, cultures were washed twice with 5 ml of Hank's balanced salt solution (HBSS). The washed cultures were then incubated with 1 ml of diluted chicken A/Ann Arbor/6/60 ca variant antiserum for A/Ann Arbor/6/60 ca variant and for the wild type virus; or with 1 ml of diluted A/Bangkok/1/79(H3)-Equine/Prague/1/56(N7) antiserum for the ca reassortant viruses (hemagglutination-inhibition titer of 1:50) for 30 minutes at room temperature to neutralize any unpenetrated viruses. The antisera were then removed by two washings and the cultures were incubated at 25° or 33° C with 2 ml of 2X Eagle's containing 5 μg/ml of TPCK trypsin. The virus released into the supernatant fluid was harvested at various time intervals. The cultures were washed once with 5 ml of prewarmed HBSS, followed by a supplement with 2 ml of prewarmed 2X Eagle's for further incubation. The virus titer in the supernatant fluid was evaluated by plaque assay on MDCK monolayer cells at 33°, as previously described (23).

Virion Associated RNA-dependent RNA Polymerase Assay

In vitro virion RNA polymerase activity was analyzed by the method described by PLOTCH and KRUG (25). Approximately 20 μg of purified virion protein was suspended in a small volume of STE (100 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) and disrupted with 10 mM of NaCl and 0.2 per cent of Triton N 101. The disrupted virion was added to the reaction mixture of a final volume of 50 μl, containing 50 mM Tris-HCl (pH 7.8), 100 mM KCl, 1 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM three ribonucleoside triphosphates, 2 μCi ^{3}H-UTP (specific activity 38.5 Ci/mmol) and 0.4 mM ApG as a primer. The duplicate samples were then incubated at either 25° or 33° C. The reactions were terminated at various time intervals by the addition of 1 ml of cold 10 per cent trochloroacetic acid (TCA) containing 0.1 M sodium pyrophosphate. After 30 minutes at 0° C, the precipitated RNA was collected on a Whatman GF/C filter, and then washed; first with 20 ml of cold 5 per cent TCA containing 0.1 M sodium pyrophosphate and then with 20 ml of cold ethanol. Radioactivity was determined in a scintillation spectrometer after adding 5 ml of aqueous counting scintillant (ACS II, Amersham Corp.).

Preparation of Infected Cell RNA

MDCK monolayer cultures grown in 75 cm^2 plastic bottles (2.2×10^7 cells/culture) were infected at an MOI of 50 PFU/cell in the presence of 100 μg/ml cycloheximide. After adsorption for 30 minutes at room temperature, the inocula were removed and the cultures were washed 3 times with 10 ml of cold HBSS. The virus infected cells were incubated at 25° C for 5 hours or at 33° C for 3 hours in the growth medium as described by SUGIURA *et al.* (28) in the presence of 100 μg/ml cycloheximide. Zero time corresponds to the time at which the infected cells were brought to the aforementioned temperatures.

At the indicated times, the infected cells were collected into reticulocytes standard buffer (0.01 M KCl, 0.0015 M $MgCl_2$, 0.01 M Tris-HCl pH 7.4), and were extracted 5 to 7 times with phenol-chloroform, as described previously (19). The ethanol precipitated RNA was desalted followed by chromatography through PD-10 column (Sephadex G-25 M) in water. The RNAs in the excluded volume were then pooled and lyophilized to dryness.

Quantitative Analysis of Virus-specific RNA

Measurements of virus specific complementary RNA (cRNA) were made by annealing with ^{3}H-labeled viral RNA (^{3}H-vRNA) extracted from purified virion. The procedures for RNA-RNA annealing experiments were based on the methods of GLASS *et al.* (9) and KRUG *et al.* (12). Three micrograms of ^{3}H-vRNA were mixed with 20 μg

of virus-infected cellular RNA in a final volume of 100 μl of 2×SSC in an Eppendorf tube. After boiling at 98° C for 5 minutes, the mixture was immersed in a 68° C water bath for 4 hours and then slowly cooled for 18 hours at 25° C. One-half of the sample was treated with 10 μg/ml of pancreatic RNase and 50 units/ml of T1 RNase for 30 minutes at 37° C .The other half was left untreated. The RNase resistant RNA was determined as described previously (12).

Synthesis of Viral Polypeptides

Confluent MDCK monolayers were inoculated at an MOI of 50 PFU/cell for 30 minutes at room temperature and then incubated in 2× Eagle's medium at 25° C. At various times after infection, the medium was replaced with 1 ml of prewarmed 2× Eagle's medium deficient in amino acids. After incubation for 45 minutes at 25° C, the infected cells were treated with 150 mM NaCl for 15 minutes before the addition of the radioactive label as described previously (15). The medium was removed and replaced with 0.2 ml of 2× Eagle's medium deficient in amino acids and containing 50 μCi/ml of ^{14}C-L-amino acid mixtures for exactly 20 minutes at 25° C. The labeling medium was then rapidly removed and cells were lysed in 100 μl of lysis buffer (14). Lysates were scraped from the culture tubes, boiled at 100° C for 1 minutes, sonicated, and frozen at —20° C.

Polyacrylamide Gel Electrophoresis (PAGE)

Virus specific polypeptides in infected cells were analyzed by high-resolution discontinuous PAGE using Tris-glycine buffer with SDS (13). This procedure was described previously in detail (23).

Results

Characteristics of Viral Growth at Various Temperatures

In this study, we attempted to determine the stage(s) in the viral replication cycle which are affected by mutation of A/AA/6/60 ca variant strain, resulting in equivalent virus yield at 25° and 33° C as previously reported by Maassab (17, 18). To investigate this phenomenon, infected cells were initially incubated at 33° C for 4 hours, followed by a temperature shift down to 25° C (Table 1). The infectious particles of the ca variant and A/AA/6/60 wt virus were detected as early as 6 hours after the temperature was shifted down. The titers of the wt virus at each time interval

Table 1. *Virus production at 25° C after temperature shift-down from 33° C*

	Virus titer (log 10 PFU/ml) at indicated times (hours)				
Viruses	6	12	18	24	30
A/AA/6/60 ca	3.0	4.0	5.0	5.2	5.9
A/AA/6/60 wt	2.5	3.9	4.3	5.1	5.2

MDCK monolayers were infected with the viruses at an MOI 10 PFU/cell and the aliquot samples were harvested at the indicated time intervals

Table 2. *Virus production at 33° C after temperature shift-up from 25° C*[a]

Viruses	Virus titer (log 10 PFU/ml) at indicated times (hours)			
	3	6	9	12
A/AA/6/60 ca	3.0	5.0	5.8	6.3
A/AA/6/60 wt	N.D.[b]	3.9	4.5	6.0
$T_2$36-3-1	4.1	5.3	5.7	6.3

[a] Immediatly after infection, the infected cells were incubated at 25° C for 6 hours. The temperature was then elevated to 33° C

[b] N.D.: not detected

were almost equal to those of the ca variant in spite of being incubated at 25° C.

Table 2 presents the results of temperature shift-up experiments in which the virus-infected cells were incubated at 25° C for 6 hours prior to

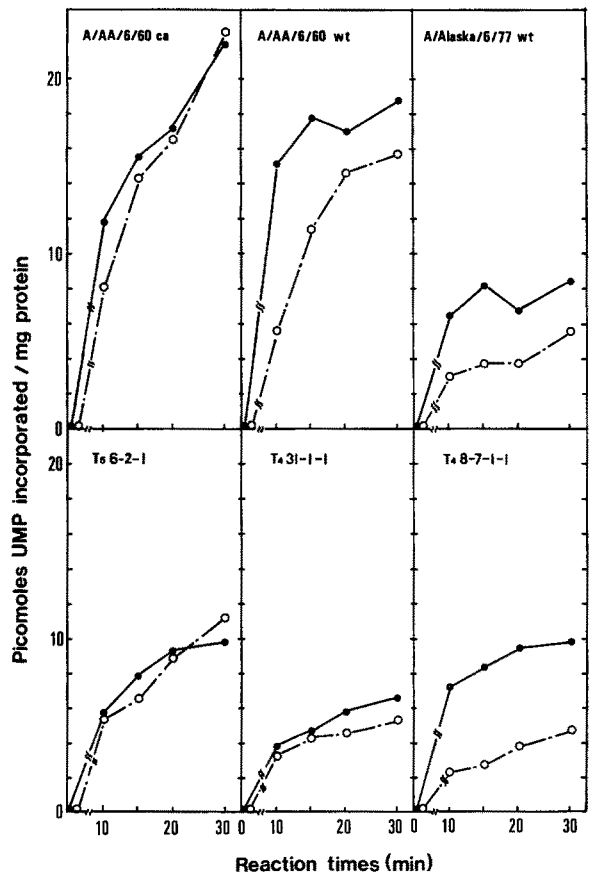

Fig. 1. Comparison of *in vitro* virion RNA polymerase activity at 25° C (o— — —o) and 33° C (•————•)

incubation at 33° C. Viral production by the ca variant occurred 3 hours earlier than that of A/AA/6/60 wt strain. The yield of the wt strain lagged behind until 12 hours after infection. Clone T_2 36-3-1, which expresses ca phenotype and bears only the PB2 gene from A/AA/6/60 ca variant with all remaining genes from wt A/Alaska/6/77 (Table 3), showed a viral growth pattern similar to that of the ca variant virus.

These experiments at latered temperatures seem to suggest that the ca mutation of A/AA/6/60 ca variant affects the early rather than late stage of viral replication, resulting in a high virus yield at 25° C.

In vitro *Virion RNA Polymerase Activity*

To determine whether the ca mutation of A/AA/6/60 ca variant can be expressed as a phenotype with higher virion RNA polymerase activity at 25° C, the polymerase activity was analyzed by the incorporation of ^{3}H-uridine triphosphates (^{3}H-UTP). The kinetics of the enzyme activity of the ca reassortants and their parental viruses in 10 repeated determinations are shown in Fig. 1. Both A/AA/6/60 ca variant and A/AA/6/60 wt strains exhibited higher polymerase activity at 33° C, however, the enzyme activity of A/Alaska/6/77 virus was considerably lower (upper panels). The maximum activities (picomoles UMP incorporated/mg protein) of these viruses were 22.00, 18.86 and 8.40, respectively. Low enzyme activity at 33° C was also observed in the ca reassortants (lower panels). These ca reassortants possess a majority of gene segments originating from A/Alaska/6/77 strain (Table 3).

Table 3. *Gene constellations and biological properties of ca reassortant viruses*[a]

		RNA segments							
Viruses	Phenotypes	1 (PA)	2 (PB2)	3 (PB1)	4 (HA)	5 (NA)	6 (NP)	7 (M)	8 (NP)
$T_2$36-3-1	ca and non ts	wt[b]	AA[c]	wt	wt	wt	wt	wt	wt
$T_4$31-1-1	ca and non ts	wt	AA	wt	wt	wt	wt	wt	wt
$T_4$8-7-1-1	ca and non ts	wt	wt	wt	wt	AA	wt	wt	wt
$T_6$6-2-1	ca and ts	wt	AA	AA	wt	AA	wt	wt	wt

[a] Taken from Odagiri *et al.* (23)
[b] wt RNA derived from A/Alaska/6/77 (H3N2) wild type virus
[c] AA RNA derived from A/Ann Arbor/6/60 (H2N2) ca variant virus

Presumably the basis for depressed enzyme activity resides in these shared genes. This low enzymic activity at 33° C might be attributed to the influence of matrix (M) protein of A/Alaska/6/77 strain as was previously shown for vesicular stomatitis virus (4) and also for influenza virus (33). Investigations are now in progress to explore this possibility.

Comparing the polymerase activity between 25° and 33° C in each strain, A/AA/6/60 ca variant and the ca reassortants which possessed the PB2 gene of the ca variant (T_6 6-2-1 and T_4 31-1-1) exhibited similar patterns of enzyme activity at 25° and 33° C. This was in contrast to the polymerase activities of two wt virus and a ca reassortant, containing three polymerase genes from A/Alaska/6/77 (T_4 8-7-1-1), which were markedly low at 25° C. These results clearly indicate that the ca mutation residing on the PB2 gene of the ca variant can be expressed as a phenotype of high polymerase activity at 25° as well as at 33° C.

Synthesis of Viral cRNA in the Primary Transcription

For measurement of viral cRNA products synthesized in the primary transcription, cRNA extracted from virus-infected cells, which were treated with cycloheximide (CM) from the beginning of infection, was annealed with ^{3}H-labeled virion RNA (^{3}H-vRNA). In preliminary experiments (data not shown), the rate of appearance of cRNA of A/AA/6/60 ca variant after infection reached a maximum between 4 and 6 hours at 25° C and between 2 and 4 hours at 33° C. The transcripts gradually decreased with further incubation. These findings correlated with the results of maximum ^{3}H-uridine incorporation into the infected cells at 5 hours (25° C) and 3 hours (33° C) in the presence of CM (data not shown).

Table 4. *Hybridization analysis of viral cRNA synthesized in the presence of cycloheximide*

Viruses	cRNA synthesized(μg)[a]		Ratio of cRNA synthesized to A/AA/6/60 ca variant	
	at 25° C	at 33° C	at 25° C (%)	at 33° C (%)
A/AA/6/60 ca	0.225	0.236	100	100
A/AA/6/60 wt	0.052	0.174	23.1	73.7
$T_2$36-3-1 ca	0.195	0.167	86.7	70.8

[a] Cytoplasmic RNA was extracted and annealed to 3 μg of ^{3}H-uridine labeled viral RNA (18,000 cpm/μg RNA to 48,500 cpm/μg RNA). The amount of viral RNA rendered RNase resistant after annealing represents the amount of viral cRNA synthesized in the infected cells

The maximum amount of cRNA synthesized by the ca and the wt viruses at 25° and 33° C is shown in Table 4. The production of cRNA by A/AA/6/60 ca variant was nearly equal at 25° and 33° C. The PB2-single gene ca reassortant T_2 36-3-1 also exhibited similar properties, although its maximum products were approximately 15 to 30 per cent less at each temperature than the ca variant strain. In contrast, the cRNA products of A/AA/6/60 wt was significantly lower at 25° C than its products at 33° C. As a result, the amount of cRNA synthesized at 25° C was a quarter of the amount synthe-

sized by the ca variant at the same temperature. These results indicate that the PB2-ca mutation enhances viral primary transcription at low temperature.

Previously, Hay *et al.* (10) reported that no detectable synthesis of unpolyadenylated cRNA, which functions as a template for vRNA synthesis,

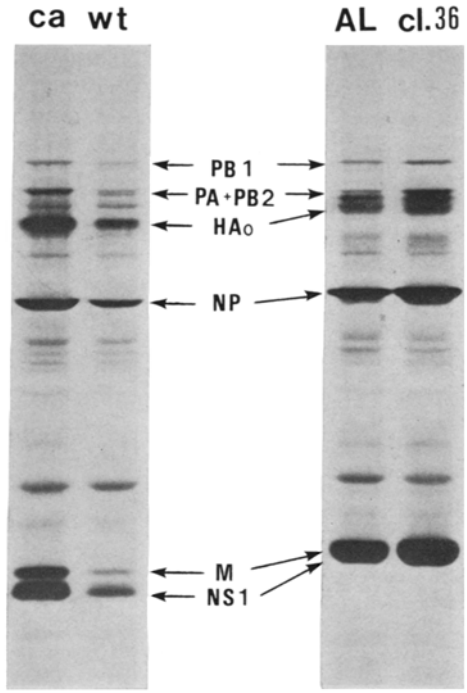

Fig. 2. Viral polypeptides translated from viral cRNA synthesized at 25° C in the presence of cycloheximide (CM). MDCK cells were infected at an MOI of 50 PFU/cell in the presence of 100 μg/ml CM and incubated for 3 hours at 25° C in the presence of CM. The cells were then treated with 2 μg/ml actinomycin D for 1 hour at 25° C. After removal of the drugs, the cells were incubated for 4 hours at 33° C and labeled with 30 μCi/ml ^{14}C-L-amino acid mixtures for 20 minutes, as described in Materials and Methods. The samples were subjected to electrophoresis on a 10 per cent gel and fluorographed for 7 days at −80° C. *ca* A/AA/6/60 ca variant; *wt* A/AA/6/60 wt strain; *AL* A/Alaska/6/77 wt strain; *cl. 36* $T_2$36-3-1 ca reassortant

occurred in infected cells treated with CM from the beginning of infection. Thus, the cRNA measured in this primary transcription experiments represented messenger RNA (mRNA). To demonstrate whether the transcripts synthesized under our experimental conditions function as mRNA in infected cells, we undertook primary translation experiments. Infected cells were incubated at 25° C for 3 hours in the presence of CM and the cells were subsequently treated with actinomycin D for 1 hour at 25° C to block further cRNA synthesis (26). The transcripts synthesized under limited

conditions by actinomycin D were translated in the cells after removal of the drugs at 33° C for 4 hours as shown in Fig. 2. The temperature of 33° C was chosen to avoid any differences in the translation rate between the ca mutants and the wt viruses. The relative amount of the ca variant and the PB2 single-gene reassortant translates were greater than those of the wt viruses.

The finding suggests that the primary transcripts affected by the PB2-ca mutation were mainly, if not all, mRNA.

Synthesis of Viral Polypeptides at 25° C

Viral polypeptides synthesis in the virus-infected cells at 25° C was characterized at various times post-infection (p.i.). Polypeptide synthesis

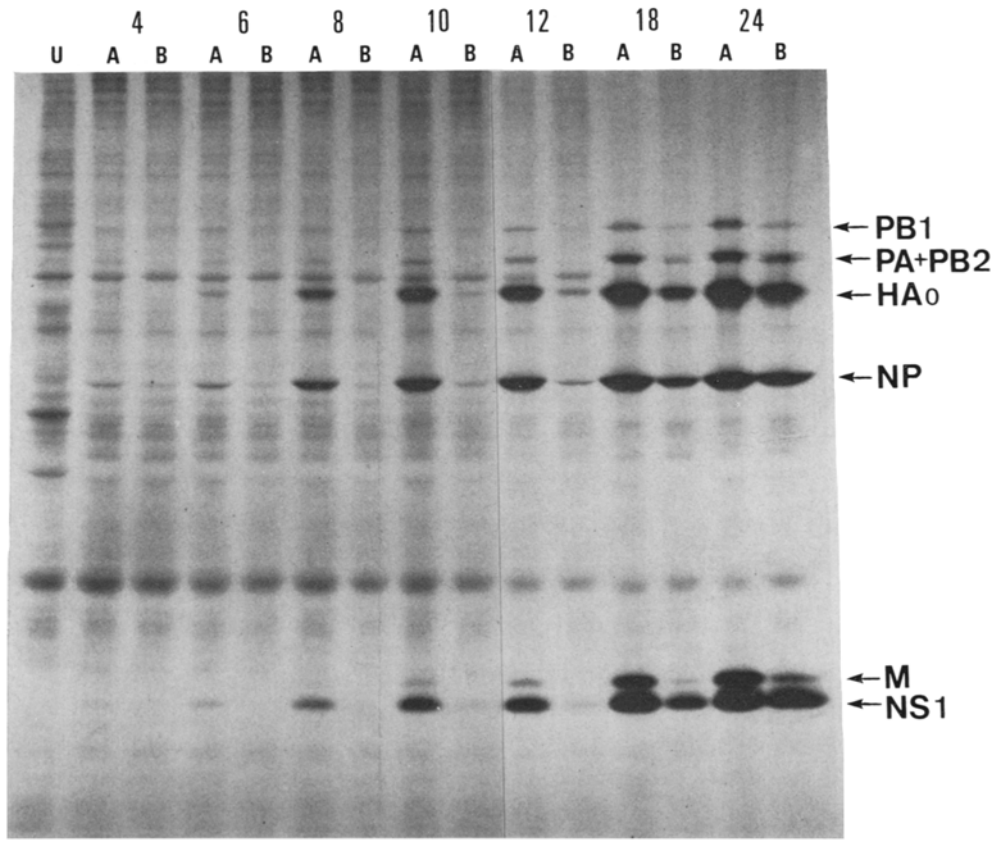

Fig. 3. Synthesis of viral polypeptides at 25° C in virus-infected MDCK cells. The infected cells were incubated at 25° C for the hours indicated at the top of each column. The samples labeled, as described in Materials and Methods, were subjected to electrophoresis on a 10 per cent gel and fluorographed for 14 days at −80° C. *U* uninfected cell; *A* A/AA/6/60 ca variant; *B* A/AA/6/60 wt strain

at 33° C which corresponds to normal infection was also examined. At this temperature the polypeptides of both ca variant and the wt viruses were detected at 2 hours p.i., with no significant differences in the amount of products observed (data not shown). However, at 25° C the time required for the appearance of each viral polypeptide was markedly different as can be seen in Fig. 3. The nucleoprotein (NP) and nonstructural proteins (NS) of the ca variant were detected at 6 hours p.i. (lane 6A). These polypeptides were gradually accumulated under subsequent incubation at 25° C. The M protein of the ca variant was detected at 10 hours p.i. (lane 10A) and all of the polypeptides attained their maximum levels by 18 hours p.i. (lane 18A). On the contrary, no visible viral polypeptide synthesis of the wt strain was observed until 10 hours p.i. (lanes 4B to 10B), with synthesis becoming significant from 12 hours p.i. (lane 12B).

Rough quantitative comparison of the polypeptide products of these two viruses was also performed by densitometer tracing. The total amount of wt viral polypeptide synthesized at 24 hours p.i. was calculated to be approximately 56 per cent of the polypeptide synthesized by the ca variant. In addition, the polypeptide synthesis of the wt virus at 24 hours p.i. corresponded to that of the ca variant at 18 hours p.i. (compare lanes 18A and 24B).

Discussion

A ca mutation contributing to the ca property of A/AA/6/60 variant strain was identified as residing on the PB2 gene of the variant strain by the following findings; i) some ca mutants which expressed only ca phenotype were "PB2 single-gene" reassortant viruses (23) and ii) electrophoretic analysis of viral polypeptides in infected cells at 33° C showed that only the PB2 protein of A/AA/6/60 variant migrated more slowly than that of the wt strain (unpublished data). The present study is the first attempt to delineate the function of the ca mutation(s) residing on the PB2 gene (PB2-ca mutation) of the ca variant strain using the PB2 single-gene reassortant virus.

The comparative analysis of viral growth curves from temperature shift experiments indicated that A/AA/6/60 ca variant grew equally well at 25° and 33° C regardless of any shifts in the incubation temperature. This was not true with the wt virus whose growth was markedly delayed and whose yield was depressed when the wt virus-infected cells were placed at the low temperature during the early stage of the viral replication. This phenomenon correlated with the finding that at 25° C synthesis of the wt polypeptides occurred 6 hours later than synthesis of the ca variant polypeptides and that the maximum polypeptide production was only half of the ca variant product. The fact that the ca virus' growth range is expanded to 25° C

strongly suggests that at least one of the ca mutations of the ca variant strains plays an important role in the early stage of viral replication.

The optimal temperature for *in vitro* enzyme activity of virion RNA polymerase is reported to be between 31° and 33° C (2). At 25° C the activities of any influenza wt strains do not surpass their activities at the optimal temperature (11, 22). The ca A/AA/6/60 variant and any ca reassortants containing the A/AA/6/60 PB2 gene, however, exhibited identical activities at both 25° and 33° C in our assay. The low polymerase activity of A/Alaska/6/77 wt strain at 25° C increases until it reaches a level similar to that at 33° C if the PB2 gene of A/Alaska/6/77 is exchanged for the PB2 gene of the ca variant by genetic reassortment, as can be seen for T_4 31-1-1. From the *in vitro* polymerase assays, it was apparent that the PB2 gene of A/AA/6/60 ca variant strain can affect the phenotype of enzyme activity at 25° C. In addition, A/AA/6/60 ca variant's polymerase in the temperature-sensitive property was considerably more heat labile than the wt polymerase when treated for 10 minutes at 39° C and this thermal lability is attributed to the PB2 protein of the ca variant strain (in preparation).

Because the ca variant and the reassortants containing only the PB2 gene from A/AA/6/60 synthesized polypeptides in twice the abundance of the wt virus' in infected cells at 25° C ,we were led to predict that at 25° C the virus strains containing the PB2-ca mutation would synthesize a greater amount of mRNA than the wt strain. Both unpolyadenylated cRNA and vRNA are not detected in the presence of cycloheximide (CM) (1, 20). Therefore, quantitative analysis of viral cRNA synthesized in the presence of CM in our experiments demonstrated that the ca variant (A/AA/6/60) produced equal amount of cRNA at 25° and 33° C and that this amount was approximately four-fold greater than the wt cRNA synthesized at 25° C. The PB2 single-gene reassortant virus acted in a similar manner. Thus the evidence strongly suggests that the PB2-ca mutation was expressed at the first step in viral mRNA synthesis at 25° C. When viral mRNA is synthesized, the PB2 protein functions in recognition and binding of the cap 1 structure as a primer during the endonuclease reaction (3, 30, 31).

Using the methods of T1 oligonucleotide mapping and RNA-RNA hybridization, Cox *et al.* (7) showed that A/AA/6/60 ca variant strain possesses mutations in all eight gene segments. It is not clear whether the ca mutation on the PB1 gene segment of the ca variant affects the initiation and elongation step in viral mRNA synthesis caused by PB1 protein (3). Furthermore the ca mutation on the A/AA/6/60 variant neuraminidase (NA) gene that presumably functions in the late stage of the viral replication remains to be determined. However, the assessment of the ca mutations using the single-gene reassortants of A/AA/6/60 variant strain as shown in the present study can be a useful approach in clarifying the mechanism of viral replication at low temperature.

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