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Studies on the adaptation of mumps virus to chick embryo

H. Naruse[2], T. Morishima[2], S. Isomura[2], M. Hamaguchi[1], T. Toyoda[1], T. Yoshida[1], and Y. Nagai[1]

[1] Research Institute for Disease Mechanism and Control,
[2] Department of Pediatrics, Nagoya University School of Medicine, Nagoya 466, Japan

Abstract. About 15 serial passages of wild type mumps virus (Sasazaki strain) in the amnion sac of chick embryo (CE) yielded a CE-adapted strain which was poorly replicative and did not form plaques in Vero cells where the wild strain grew well. In the course of this limited replication of the CE-adapted strain in Vero cells, we have analysed the viral protein and RNA synthesis. It was found that protein synthesis took place very efficiently at least early in infection by 12 h. The subsequent rate of synthesis remained, however, at a low level without showing the progressively increasing synthesis observed with the wild strain. Furthermore, 50S genomic RNA was synthesized early in the limited infection, but the subsequent synthesis was markedly suppressed. In addition, the other virus-specific RNA species could not be detected throughout. Thus the amplified RNA synthesis observed in the permissive CE cells and in the wild strain-infection of Vero cells seemed not to occur in the limited replication. Neither interferon nor DI (defective interfering) RNA was involved in the limited virus growth. When Vero cells were infected with the wild strain 6 to 8 h before inoculation of CE-adapted strain, growth restriction was overcome and the yield of the latter virus was greatly enhanced by a factor more than 10^3. These results suggest that through adaptation to CE, mumps virus may be altered in such a way that there is a restriction, probably at a step (s) involved in amplification of the viral RNA synthesis in Vero cells and that the restriction may be overcome by the simultaneous genome expression of the prototype wild strain.

Introduction

A major strategy in the development of virus vaccines has been adaptation of the virus to a different or unnatural host, which results in alteration of the nature of the host-virus interaction. For mumps virus, a member of the paramyxovirus group, this has involved adaptation of human isolates to the chick embryo (Enders et al. 1946; Buynak

* *Offprint requests to:* H. Naruse

and Heilleman 1966). Earlier studies with chick-adapted vaccine strains demonstrated that they could no longer be propagated in mammalian cells (Henle and Deinhardt 1955; Deinhardt and Henle 1957; Brandt 1961; Gresser and Enders 1961). Mumps virus is frequently cytopathic in culture, causing syncytia formation characteristic of paramyxoviruses, but the cytopathogenicity for a given in vitro cell culture may be determined by the „adaptive status" of the virus (Gresser and Enders 1961). A recent study with a prototype vaccine strain Enders and a green monkey kidney cell line (Vero cells) has demonstrated that the virus is significantly noncytopathic, allowing the balance between virus replication and cell survival; that is, a persistent infection, and that the generation of defective interfering particles has been the likely factor modulating this persistent infection (Mc Carthy et al. 1981). In another report, an abortive replication of mumps virus in murine cell lines has been analysed (Yamada et al. 1984). It was shown that interferon produced endogenously during the virus infection might be responsible for that abortive replication.

There are several of chick-adapted mumps virus strains. Their passage histories have, however, not been definitely documented; furthermore, the respective prototype wild strains are often unavailable, which would hamper precise comparative studies between wild type and vaccine strains. In the present study we first attempted to obtain a chick-adapted strain from a cloned stock of a human isolate (Sasazaki strain) and then analysed their replication patterns in Vero cells. The results obtained have suggested that the adaptation of the virus to chick embryo may bring about a defect (s) in the step of amplification of the viral genome in Vero cells, thus resulting in poor virus growth in these cells and that coinfection with the wild strain under certain conditions may be able to complement this defect. Thus our data suggest another novel mechanism which may be responsible for the alteration of mumps virus-host interactions.

Materials and methods

Viruses and cells. A human isolate of Sasazaki strain was provided by Dr. M. Hayami, National Institute of Health, Japan. The virus was isolated and grown in Vero cells, and used in this study as the prototype wild strain after plaque purification in the same cells. The chick embryo (CE)-adapted strain was obtained by serial propagations of the wild strain in the amnion sac of 7-day-old chick embryos as described in the text. Vero cells were cultured in minimum essential medium (MEM) containing 10% fetal calf serum. The preparation of primary chick embryo (CE) cells was described previously (Nagai et al. 1972).

Virus titrations. The infectivity of the wild and CE-adapted strains were assayed by plaquing on the monolayers of Vero and CE cells, respectively. The infected monolayers were overlayed with MEM containing 1% fetal calf serum and 0.9% noble agar followed by the secondary overlay with the same medium containing 0.0001% neutral red, after 4 and 8 days, respectively. Hemagglutination titrations were described previously (Naruse et al. 1981).

Viral protein synthesis. Viral protein synthesis in infected cells was analysed essentially as described (Naruse et al. 1981). Vero cells infected with either wild or CE-adapted

strain at a multiplicity of 10 PFU/cell were pulse labelled for 1 h with [^{35}S]methinine (10 μCi/ml) at various times after infection. The virus-specific proteins were recovered from the cell lysates by immunoprecipitation (Kessler 1975) and electrophoresed on 10% polyacrylamide slab gels (Laemmli 1970). The gels were processed for fluorography and exposed to Kodak X-Omat films (Naruse et al. 1981).

Viral RNA synthesis. Infected cells were labelled with [^{3}H]uridine (10 μg Ci/ml) for 3 h at various times after infection with a multiplicity of 10 PFU/cell. Actinomycin D was present from 1 h before labelling at the concentrations of 10 and 1 μg/ml for infections of Vero and CE cells, respectively. The cells were processed as described previously (East and Kingsbury 1971). Briefly, the cells were homogenized in RSB (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$) with 1% Triton X-100. After centrifugation at 1000 rpm for 5 min, the supernatant was made 1% and 500 μg/ml in SDS and proteinase K, respectively, in STE (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA), and centrifuged through a linear gradient (15–30%) on a 60% cushion of sucrose in STE containing 0.5% SDS. Centrifugation was at 19.000 rpm in an SW 27 rotor for 16 h at 20°C. The virion 50S RNA isolated from CE-adapted strain grown in CE cells as well as chick ribosomal 28S and 18S RNAs were parallelly run in companion tubes.

Isolation of intracellular viral nucleocapsids. Virus-infected Vero cells were labelled with [^{3}H]uridine for 15 h in the presence of actinomycin D (10 μg/ml). The cells were scraped off into PBS by a rubber policemen, pelleted and processed for isolation of nucleocapsids by solubilization with 1% NP40 and two cycles of equilibrium density centrifugation in CsCl, according to the methods described by Roux and Holland (1979) and modified by Yoshida et al. (1982). The RNA species contained were analysed by sucrose gradient centrifugation as described above.

Chemicals and radioisotopes. Actinomycin D was obtained from P-L Biochemicals Inc., Milwaukee, Wisconsin. L-[^{35}S]Methionine (> 400 Ci/mmol) and [5-^{3}H]Uridine (> 25 Ci/mmol) were purchased from New England Nuclear Corporation, Boston, Massachusetts.

Results

Growth of chick embryo (CE)-adapted and wild strains of mumps virus in vero and primary CE cells

A human isolate of mumps virus (Sasazaki strain) was plaque-purified in Vero cells and the stock virus was grown in the same cells. The virus was then inoculated into the amnion sack of 7-day-old CE at a dilution of 10^{-2}. The virus recovered from CE (passage no. 1) was serially propagated in the same host at dilutions of 10^{-2} to 10^{-4}. At each passage, hemagglutination units (HAU), plaque forming units (PFU) and plaque size were determined. As summarized in Table 1, the virus of the initial passage in CE as well as wild strain produced large clear plaques in Vero cells, whereas HAU remained at a low level, thus giving a high infectivity to HAU (IH) ratio. In contrast, these virus-

Table 1. Serial passage of mumps virus in the amnion sac of chick embryo[a]

Passage number	HAU	Vero			CE		
		PFU/ml ($\times 10^{-6}$)	diameter (mm)	PFU/HAU ($\times 10^{-6}$)	PFU/ml ($\times 10^{-6}$)	diameter (mm)	PFU/HAU ($\times 10^{-6}$)
0	4	30	2.0 - 3.0	7.5	0	–	–
1	4	15	2.0 - 3.0	3.8	0	–	–
5	8	4.5	1.5 - 2.0	0.6	1.7	0.5	0.2
10	80	3.0	<0.5	0.04	22	1.0	0.3
15	80	1.5	<0.5	0.02	33	2.0 - 3.0	0.4

[a] A human isolate of mumps virus which was plaque-purified and propagated in Vero cells was serially passaged in the amnion sac of 7-day-old chick embryo at dilutions of 10^{-2} to 10^{-4}. At various passages, hemagglutination units (HAU) and plaque forming units (PFU) as well as plaque diameter in Vero and primary chick embryo (CE) cells were measured. The virus of passage number 15 was propagated once in chick embryos and used as CE-adapted strain

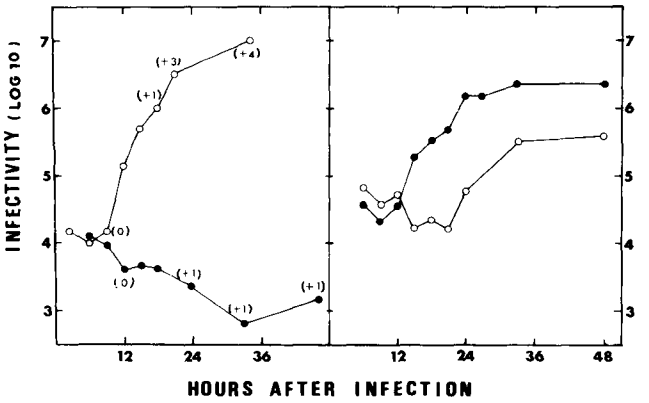

Fig. 1. Growth of CE-adapted (●) and wild (○) strains in Vero (left) and chick embryo (right) cells. MOI:1.0 PFU/cell. (0), no cytopathic effect (cpe); (+1), development of small foci of fused cells; (+4), extensive cpe manifested by cell fusion of entire monolayer

es did not form plaques in primary chick embryo (CE) cells. By the fifth passage in CE, the virus became able to produce plaques in CE cells, but their sizes were still small. After 15 passages the plaque size reached the maximum, and the virus exhibited a high IH ratio in CE cells, although the ratio was still lower than that of wild strain in Vero cells. Further passages in CE cells resulted in no increase in either plaque size or IH ratio. Thus it appeared that some 15 passages under the conditions employed might be enough to yield a strain adapted to CE. The CE-adapted strain now showed extremely low PFU and small plaque sizes in Vero cells with a IH ratio about 400 fold lower than that of the wild strain. The growth kinetics indicate that the CE-adapted virus can no longer multiply in and is not very cytopathic for Vero cells, whereas it has acquired a capacity to grow well in CE cells (Fig. 1).

Viral protein synthesis

Since there had been no difference in the number of antigen positive cells between the infections of Vero cells with CE-adapted and wild strains under single cycle growth conditions at various multiplicities of infection, both viruses appeared to adsorb to and penetrate into the cells equally well (not shown). Thus we examined then the genome expression by monitoring the viral protein synthesis. Vero cells infected with either virus were pulse-labelled for 1 h with ^{35}S-methionine at various times after infection, and the virus specific proteins recovered by immunoprecipitation were analysed by polyacrylamide gel electrophoresis. As shown in Fig. 2, the proteins of CE-adapted strain were detected even sooner than those of wild strain and exhibited the maximum synthesis rate already at 12 h when the latter became detectable. However, the subsequent protein synthesis of CE-adapted strain remained at a lower level, if compared with the time course of wild strain. With the wild strain, all the viral proteins except for P and P47 (a phosphorylated form of P) (Naruse et al. 1981) underwent progressively increasing synthesis with time up to 24 h. Essentially the same amplified synthesis was observed when CE cells were analysed after infection with CE-adapted strains used here

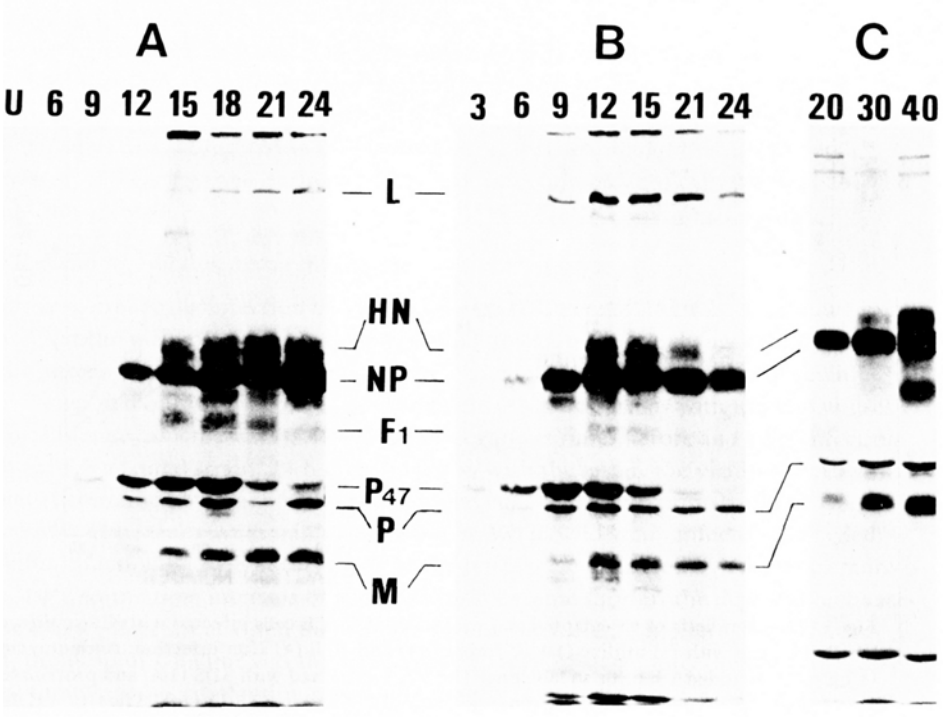

Fig. 2. Synthesis of proteins of wild (A) and CE-adapted strain (B) in Vero cells. The stock viruses were grown in Vero and primary chick embryo (CE) cells, respectively and inoculated to Vero cells at a multiplicity of 10 PFU/cells. The cells were labelled with ^{35}S-methionine (10 μCi/ml) for 1 h at various hours after infection and the virus specific proteins were recovered by immunoprecipitation and analysed by polyacrylamide gel electrophoresis. The hours of labelling were indicated on the top of the gel lanes. U, uninfected. The infection of CE cells with the CE-strain was similarly analysed (C)

(Fig. 2) and previously (Naruse et al. 1981). These data indicate that restricted genome expression may be a likely factor involved in the limited replication of CE-adapted mumps virus in Vero cells. It remains to be elucidated why the synthesis rates of P and P47 have fluctuated markedly during both wild and CE-virus infections.

Viral RNA synthesis

Figure 3A shows sedimentation patterns of CE-adapted virus specific RNAs isolated from the permissive CE cells. The infected cells were labelled with ^{3}H-uridine for 3 h at 15 h and 30 h after infection. Actinomycin D was added 1 h prior to the labelling. The labelled RNAs were extracted and centrifuged through sucrose gradients as described in "Materials and methods." To identify the peak of viral genomic RNA, isolated RNA from the virions grown in CE cells and labelled with ^{3}H-uridine were parallelly run (Fig. 3B). The data indicate that the fastest sedimenting peak coincides exactly with that of 50S virion RNA. Additional virus specific radioactivities were present in the components sedimenting at about 18S and other small peaks between 18S and 50S.

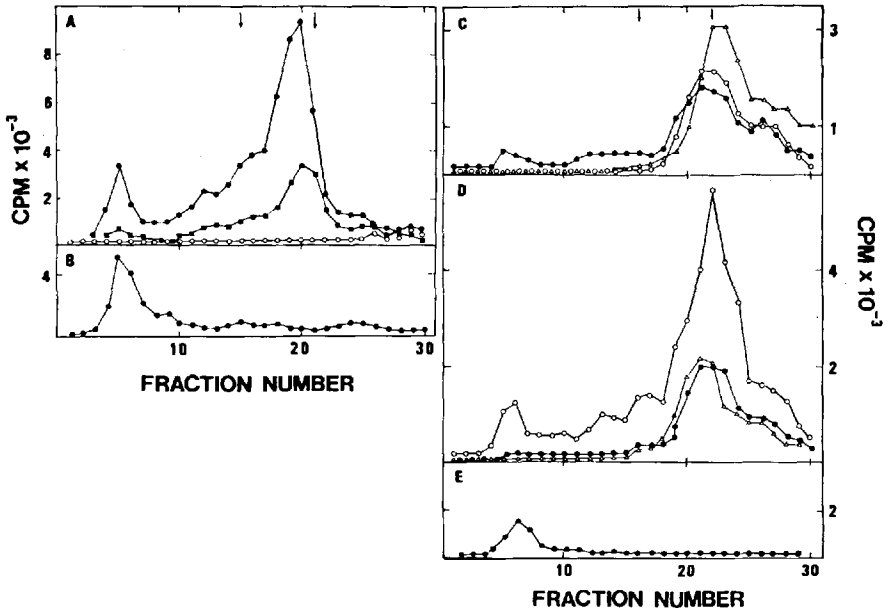

Fig. 3. RNA synthesis of CE-adapted and wild strain. (A), CE cells infected with CE-strain were labelled for 3 h with ^{3}H-uridine (10 μCi/ml) 15 (■) and 30 h (●) after infection. Actinomycin D (1 μg/ml) was added 1 h prior to labelling. The RNA extracted with SDS (1%) and proteinase K (500 μg/ml) was centrifuged through a sucrose gradient (15–30%) in STE (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA) with 0.5% SDS for 16 h at 19,000 rpm in a Spinco SW 41 rotor. Acid insoluble radioactivity was measured in fractions of the gradient. Arrows (fractions 15 and 21) represent the peaks of 28S and 18S ribosomal RNAs, respectively. (○), uninfected. (B), 50S virion RNA similarly extracted and centrifuged. (C, D), similar sedimentation analysis of RNA extracted from Vero cells infected with CE adapted (●) and wild (○) strains. The cells were labelled for 3 h at 6 h (C) or 15 h (D) after infection in the presence of actinomycin D (10 μg/ml). (△), uninfected. (E), sedimentation pattern of RNA contained in the nucleocapsids recovered from Vero cells infected with CE-strain and labelled for 24 h with ^{3}H-uridine (10 μCi/ml).

These peaks might represent 18S, 22S and 35S mRNAs (Bratt and Hightower 1977) and replicative intermediates (East and Kingsbury 1971). It must be noted that all the virus specific peaks increased greatly during 12 h and 24 h after infection, indicating an efficiently amplified viral RNA synthesis in this permissive system. When Vero cells infected with CE-adapted strain were similarly analysed, small but significant synthesis of 50S RNA was observed 6 h after infection (Fig. 3C). However, this peak of 50S RNA became almost undetectable at 15 h (Fig. 3D). Little significant synthesis of other RNA species could be detected throughout the infection with CE-adapted strain (Fig. 3C, D). This may be at least in part due to insufficient shutoff of the host synthesis by actinomycin D, which yielded a relatively high background, sedimenting broadly to fractions 15 to 30. When Vero cells infected with the wild strain were analysed, little virus-specific components were detected 6 h after infection (Fig. 3C). Later on, however, the peaks of 50S as well as the other RNA species became quite distinct (Fig. 3D). Thus, the synthesis of the viral RNAs were found to be efficiently amplified in these cells.

These results suggested that Vero cells infected with the CE-adapted strain might produce a small amount of the viral RNA but not allow such amplified synthesis as is the case when the wild strain is grown in Vero cells or when the CE-adapted strain is grown in CE cells.

The nucleocapsids isolated from Vero cells infected with the CE-adapted strain were examined for RNA by centrifugation as described above. As shown in Fig. 3E, the RNA was found to sediment to a 50S position. Thus the infection might generate no detectable defective interfering RNAs of smaller sizes. It was also found that no detectable interferon was synthesized during the course of infection of Vero cells with CE-adapted strain (not shown).

Intracellular interaction between CE-adapted and wild strains

Upon simultaneous coinfection of Vero cells with CE-adapted and wild strains, we were aware that the yield of the former was slightly but significantly enhanced, whereas that of the latter was reduced. To investigate these phenomena in more detail, the coinfection was carried out with different intervals between inoculations with the two viruses. Vero cells were infected with the wild strain at various times before and after infection with the CE-adapted strain. 24 h after infection with the latter, the yields of both viruses were determined by plaquing on the monolayers of Vero and primary CE cells, respectively. The results are summarized in Fig. 4. When wild-strain infection preceded CE-virus-infection by 6–8 h, the yields of the latter were enhanced by factors of more than 10^3. As the time intervals of superinfection became shorter, the degree of enhancement sharply decreased. When the wild strain was inoculated 2 h after CE-virus infection, the yield of CE-virus was the same as the control of single infection with the same virus. Simultaneous coinfection resulted in slightly higher yield of CE-virus than in the case of 2 h preinfection with the wild strain.

Figure 4 further indicates that yields of wild strain have been reduced under some conditions of coinfection when compared with those of single infections with the virus. Thus the CE-adapted strain was found to interfere with the replication of wild strain. This interference was, so far tested, the most extensive, when the wild strain-infection was done 2 h after that with CE-adapted strain. The interference was less significant upon simultaneous coinfection. This may be related to the above-described relatively

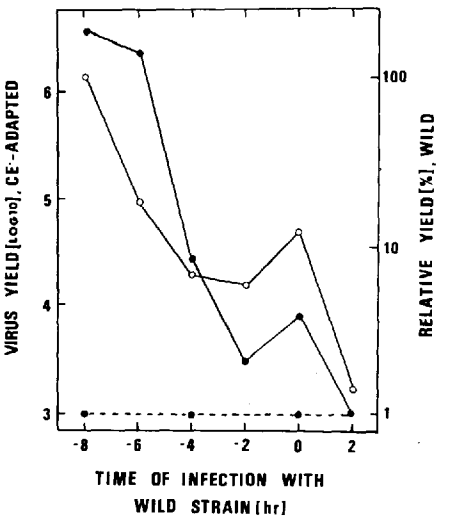

Fig. 4. Interaction between the wild and CE-adapted strains in Vero cells. Vero cells were infected with wild strain at various times from 8 h before (-) to 2 h after infection with CE-adapted strain. At 24 h after infection with the latter, the yields of CE-adapted (●——●) and wild (○——○) strains are assayed by plaquing on primary CE and Vero cells, respectively. The plaques of both strains in the latter cells were discriminated from each other by their size difference (Table 1). The titers of wild strains were expressed by relative yields to the respective controls of single infection with the same virus. (● - - - - ●), single infection with CE strain. All the infections were done at an input multiplicity of 2 PFU/cell

high degree of enhancement of CE-virus under the same condition. The interference was again distinct when infected with the wild strain 2 to 4 h prior to CE-virus-infection, but completely abolished by 8 h.

Neither the enhancement of CE-virus production by the wild strain nor the interference with the latter by the former was observed when either virus was UV irradiated, indicating that the virus replication might be essential for both phenomena.

These data taken together with those shown in the previous sections indicate that CE-adapted strain may be defective probably in a step of amplification of viral RNA synthesis in Vero cells and that this defect may be able to be complemented by the wild strain. For this complementation to take place, it appears that the wild strain should be inoculated sufficiently before infection with the CE-adapted strain so that the former undergoes a certain level of its genome expression without suffering from extensive interference by the latter.

Discussion

The aim of this study has been to elucidate the mechanism of change in mumps virus-host interactions caused by the virus adaptation to chick embryo (CE), by comparing a newly obtained CE-adapted strain with its prototype wild strain. Under the conditions employed, some 15 passages of the virus in CE yielded a strain fully adapted to CE. This CE-adapted strain showed very low capability to grow in Vero cells which were

fully permissive for the wild strain. The CE-adapted and wild strains appeared to adsorb to and penetrate into Vero cells equally well. Neither DI particles nor interferon might be involved in the limited growth of CE-adapted virus in Vero cells. The most striking difference between the CE adapted and wild strains was observed when the viral RNA synthesis was compared. Vero cells have been found to support the synthesis of CE-virus genomic RNA early in infection but not to allow any efficiently amplified viral transcription and replication late in infection. Thus no such progressively increasing RNA synthesis has been observed in these cells as found with the permissive CE cells infected with the same virus as well as Vero cells infected with the wild strain. These results may be compatible with the finding that the CE-virus protein synthesis in Vero cells has undergone a distinct restriction not early but late in infection. It is, therefore, likely that the poor growth of CE-adapted mumps virus in Vero cells is due to the lack or inefficient amplification of the viral RNA synthesis.

Recent papers have provided evidence which suggests that generation of DI particles as well as interferon production may be responsible for similar limited mumps virus replication in certain host cells (Mc Carthy et al. 1981; Yamada et al. 1984). Since either factor may not be involved in our system as mentioned above, our data indicate another novel mechanism for the limited virus growth, and suggest that the factors responsible may differ, depending on the strain and host systems analysed.

Our data have shown further that the growth restriction of the CE-adapted strain in Vero cells can be overcome by preinfecting wild strain. Assuming that the restriction may be due to the block of amplified viral RNA synthesis, this defect may be complemented by the products of wild strain. There may be also the possibility that the CE-virus might have utilized some components of the wild strain for its assembly into virions. The intracellular interactions between the two viruses seem to be complex since CE-virus both interfered with and was rescued by the wild strain. Thus for the wild strain to induce maximum enhancement of CE-virus production, the former should be inoculated sufficiently before infection with the latter so that the former was no longer interfered with but was able to multiply fully.

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