

Permissiveness of Mouse, Monkey and Hybrid Cells to Encephalomyocarditis (EMC) Virus

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SUMMARY

Encephalomyocarditis (EMC) virus replicates to high titre in permissive mouse kidney (MKS) cells but poorly in monkey kidney (CV₁) cells. The permissiveness of monkey-mouse hybrid cells varies according to their chromosomal content.

In monkey cells, the synthesis of both single-stranded and double-stranded virus RNA is restricted; in semi-permissive hybrid clones, the double-stranded RNA is synthesized normally, whereas the synthesis of the single-stranded RNA is inhibited. Thus, it seems that more than one restrictive event is responsible for the low permissiveness of monkey cells to EMC virus.

INTRODUCTION

Cell permissiveness to virus replication might be defined as the capacity of the virus particle to replicate in a sensitive cell after its fixation on a membrane binding site. This definition implies an unaltered capacity of the cell to uncoat the virus. In contrast, in non-permissive cells, the virus can attach and penetrate into the cell and the blockage occurs in some steps involving the synthesis of the virus particle. This event might vary from one cell system to another and could take place during an early or late stage of the synthesis of the virion.

The factors involved in this blockage can either be induced by the virus (such as the interferon system) or be present in the cell. This latter possibility has been postulated by Cassingena & Tournier (1968) and Cramer (1969) who showed the presence of an inhibitor in cells non-permissive to SV 40 and polyoma virus, which could block the expression of the virus structural antigens. In contrast, it has also been proposed that permissive cells contain proteins which might induce SV 40 DNA synthesis and even virus particles in non-permissive SV 40-transformed cells (Suarez *et al.* 1972).

We have studied the replication of EMC virus in permissive and semi-permissive cells. EMC replicates to high titre in permissive mouse cells but poorly in semi-permissive monkey cells. The existence of a well-defined somatic hybrid monkey-mouse cell system (Kit *et al.* 1970) enabled us to study the role of the monkey genome present in such hybrid cells in the permissiveness of different hybrid clones.

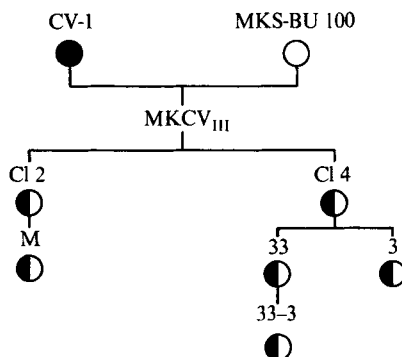


Fig. 1. Monkey-mouse hybrid cell system.

METHODS

Cells. Mouse L cells are routinely maintained in the laboratory. The parental monkey CV₁ is a continuous cell line originating from African green monkey, *Cercopithecus aethiops* (Jensen *et al.* 1964). The MKS-BU 100 stem from mouse kidney cells transformed by SV 40 and resistant to 5-bromodeoxyuridine, thus lacking thymidine kinase activity (Dubbs *et al.* 1967). The monkey-mouse hybrid cell line MKCV_{III} was selected by Kit *et al.* (1970) and cultivated in the presence of the selective HATG medium (10^{-4} M-hypoxanthine, 10^{-5} M-aminopterin, 4×10^{-5} M-thymidine, 10^{-5} M-glycine) described by Littlefield (1965). From this hybrid population, a great variety of cell clones was isolated in soft agar in the presence of HATG, according to the method of Montagnier & MacPherson (1964). The hybrid clones were used between the following passages: Cl 2: 26th to 35th; Cl 2/Cl M: 11th to 20th; Cl 4: 30th to 53rd; Cl 4/Cl 3: 14th to 20th; Cl 4/Cl 33: 13th to 59th; Cl 4/Cl 33-3: 19th to 21st (Fig. 1). All cells were cultivated in Eagle's medium (MEM) containing 10 % calf serum.

Virus. EMC virus was routinely propagated in L cells; it contained 5×10^9 p.f.u./ml.

Purification of EMC virus. After three freezing (-80°C) and thawing cycles, the supernatant fluid of infected L cells was precipitated by the addition of protamine sulphate (150 mg/ml). After 1 h at 4°C , the precipitate was centrifuged for 15 min at 4400 g; the supernatant fluid was then centrifuged in a 40 rotor of a Spinco ultracentrifuge for 90 min at 40000 rev/min. The virus pellet was treated with 100 $\mu\text{g}/\text{ml}$ of trypsin (Worthington $3 \times$ crystallized) in phosphate buffer (0.02 M), pH 7, for 1 h at 37°C and layered on a calcium phosphate column. The virus adsorbing to the column was eluted with phosphate buffer (0.02 M to 0.3 M), pH 7. Virus haemagglutinating activity (HA) was recovered in the fractions eluting in 0.3 M-phosphate.

Extraction of RNA from EMC. Bentonite (0.2 ml) at a concentration of 9 mg/ml and freshly distilled phenol (4 ml) saturated with tris buffer (0.1 M-NaCl, 0.01 M-tris, pH 7.4) were added to the purified virus (4 ml). After mixing for 15 min at 0°C , the preparation was centrifuged and the aqueous phase collected. The residual RNA present in the phenol phase and interphase was recovered after a second extraction with tris buffer (1 ml). The two aqueous phases were mixed and two more phenol extractions (7 ml) were performed. The RNA present in the final aqueous phase was precipitated with 0.1 vol. of 20 % sodium acetate and 2 vol. of absolute ethanol at -20°C for at least 24 h. The RNA precipitate was dissolved and dialysed with tris buffer. The RNA was then stored at -80°C .

Titration of virus RNA. The RNA extracted from the purified virus was titrated by the plaque forming method on L cells. The cells cultivated in Petri dishes were pre-incubated with 1 ml of DEAE-dextran (Pagano, McCutchan & Vaheri, 1967), dissolved in PBS at a concentration of 1 mg/ml for 10 min at 37°C. RNA (0.5 ml) was then added and the mixture incubated for 10 min. The supernatant fluid was removed and the agarose medium layered on the cells. The plaques were counted 48 h later. The titre of the RNA was about 10³-fold lower than that of the virus.

Infection of cells by EMC RNA. DEAE-dextran (300 µg/ml) was dissolved in 0.2 ml PBS and added to the monolayers for 10 min at 37°C. After the addition of RNA (0.1 ml) for a further 15 min, the mixture was removed and replaced by MEM. At selected time intervals after incubation at 37°C, samples of the infected cells were frozen at -80°C and the virus yield assayed.

Extraction of virus RNA from EMC-infected cells. Monolayers were infected with EMC virus at a m.o.i. = 20. One hour later, 1 ml MEM containing 4 µg/ml of actinomycin D was added to MKS cells and 15 µg/ml to CV₁ cells. These amounts of actinomycin D were sufficient to block 92 to 98 % of ribosomal RNA synthesis. Virus RNA synthesis was assayed by pulse-labelling the cells with [³H]-uridine (2 µCi/ml). The cells were then incubated at 37°C and at 1 h intervals the RNA was precipitated as follows: 1 ml SDS (0.6 %) was added and 20 min later 0.4 ml of 50 % trichloroacetic acid (TCA) was added at 4°C. Ten min later, the precipitate was filtered through a Whatman GF/C filter and rinsed twice with 5 % TCA and with ethanol. After drying, the filters were counted with toluene-PPO-POPOP liquid in a Packard scintillation counter.

Extraction of single-stranded and double-stranded RNA from EMC-infected cells. Monolayers in Roux bottles were infected with EMC at a multiplicity of 50. After 1 h adsorption, the virus was replaced by MEM (30 ml). Three hours after infection, actinomycin D was added (2 µg/ml for MKS and hybrid cells, 10 µg/ml for CV₁ cells); 1 h later, [³H]-uridine (5 µCi/ml) was added for 5 h. RNA was extracted 3 times with SDS-phenol as described by Bratt & Robinson (1967). After ethanol precipitation, RNA was dissolved in tris buffer (0.1 M-NaCl, 0.01 M-tris, pH 7.4, 0.001 M-EDTA) and layered on a 5 to 30 % buffered sucrose gradient. After 18 h centrifuging (20000 rev/min) at 4°C in a SW 25.1 rotor of a Spinco L3 ultracentrifuge, the gradient was passed through an ISCO spectrometer and then 1 ml fractions were collected. A sample of each fraction (0.5 ml) was treated with pancreatic RNase (20 µg/ml) for 1 h at room temperature. After addition of bovine albumin (500 µg), RNA was precipitated with 10 % TCA (0.5 ml) at 4°C. The precipitates were collected on filters as described above.

Karyotypic analysis. Cell suspensions (100000 cells/ml) were cultivated in Leighton tubes at 37°C. The medium was removed 24 h later and replaced by MEM (2 ml) supplemented with 10 % calf serum, containing colchicine (2 µg/ml). After 5 h at 37°C, the coverslips were dipped in hypotonic solution (1 part PBS, 3 parts distilled water) for 35 min. After fixation for 1 h (25 % acetic acid, 75 % methanol), the coverslips were dried, treated with trypsin (10 µg/ml) for 3 min and rinsed with a Clark and Lubs buffer, pH 6.75. The cells were stained with 10 % Giemsa stain for 8 min, rinsed and dried, and mounted for observation.

Chemicals. Actinomycin D was a gift of Merck, Sharp and Dohme (New Jersey, U.S.A.). [³H]-uridine (sp. act. 20 Ci/mmol) was obtained from the Biology Dept. of the Centre de l'Energie Atomique (Saclay, France). DEAE-dextran, mol. wt. = 2×10^6 , $\eta = 0.07$, and nitrogen content = 3.2 %, was purchased from Pharmacia (Uppsala, Sweden).

Table 1. *Multiplication of EMC virus in parental and hybrid cell lines**

Cell line	Virus yield	
	p.f.u./cell	%†
MKS	1.3×10^3	100
CV ₁	8.1×10^2	6.2
MKCV _{III} Cl 2	5.5×10^3	420
Cl 2/Cl M	3.4×10^3	250
Cl 4	2.5×10^2	19
Cl 4/Cl 3	5.2×10^2	40
Cl 4/Cl 33 (early)	3.9×10^2	30
Cl 4/Cl 33 (late)	2.5×10^3	190
Cl 4/Cl 33-3	2.1×10^3	160

* After 1 h adsorption at 37 °C, the dishes containing the monolayers were washed 3 times with MEM. Five ml of MEM were added, the dishes incubated at 37 °C for 15 h and frozen at -80 °C.

† EMC virus yield in CV₁ and hybrid clones is compared to the 100 % yield in MKS cells.

RESULTS

Virus multiplication in different cell lines

After one replicative cycle (m.o.i. = 10 p.f.u./cell) each mouse MKS cell released between 1 000 and 5 000 virus particles and each monkey CV₁ cell 10 to 100 particles. Thus, compared to the yield in mouse cells, production of EMC virus in monkey cells was between 2 and 7 %.

With the hybrid clones at different passage levels, the EMC virus yield per cell varied from one clone to another. In Cl 2, Cl 2/Cl M, Cl 4/Cl 33 (late passage) and Cl 4/Cl 33-3, the virus yield was similar or slightly higher than in the parental mouse cells, while it was consistently lower in Cl 4, Cl 4/Cl 3 and Cl 4/Cl 33 (early passage), where it reached 10 to 30 % (Table 1).

In further experiments, the kinetics of the first replicative cycle of EMC virus were investigated. In parental MKS cells, the eclipse period was about 5 h and the highest virus yield was obtained 9 h after infection (Fig. 2). In monkey CV₁ cells, the eclipse lasted 7 h and the highest virus yield was reached 11 h after infection. In the fully permissive Cl 4/Cl 33 hybrid cells, the replicative cycle and virus yield were comparable to those in MKS cells, while in the semi-permissive hybrid Cl 4/Cl 3, the eclipse period was significantly longer (6 h) and the virus yield reached levels between those obtained in the parental cells.

No significant differences in virus adsorption could be observed in these different cells regardless of the m.o.i. employed. Thus, the high or low capacity of MKS, CV₁ and hybrid cells to synthesize EMC virus was probably not due to an inability of the virus to bind to the cell membrane.

Infection of cells with virus RNA

To determine whether inhibition of virus particle synthesis occurred before or after the liberation of virus RNA in the cell, we studied virus replication using infectious RNA instead of the whole virus particle. Infectious RNA extracted from the virus particle was added to the cells in the presence of DEAE-dextran. The yield of virus particles produced by inoculation of infectious virus RNA was 10 to 100 times lower in CV₁ cells than in the MKS and hybrid Cl 2 and Cl 4/Cl 33 (Table 2). The kinetics of virus particle production showed that in the MKS cells the eclipse period was slightly shorter than after inoculation of the virus. In monkey CV₁ cells weakly permissive to EMC the eclipse period (7 h) was 2 h longer than in MKS. Consistently, 10 to 100 times fewer virus particles were produced

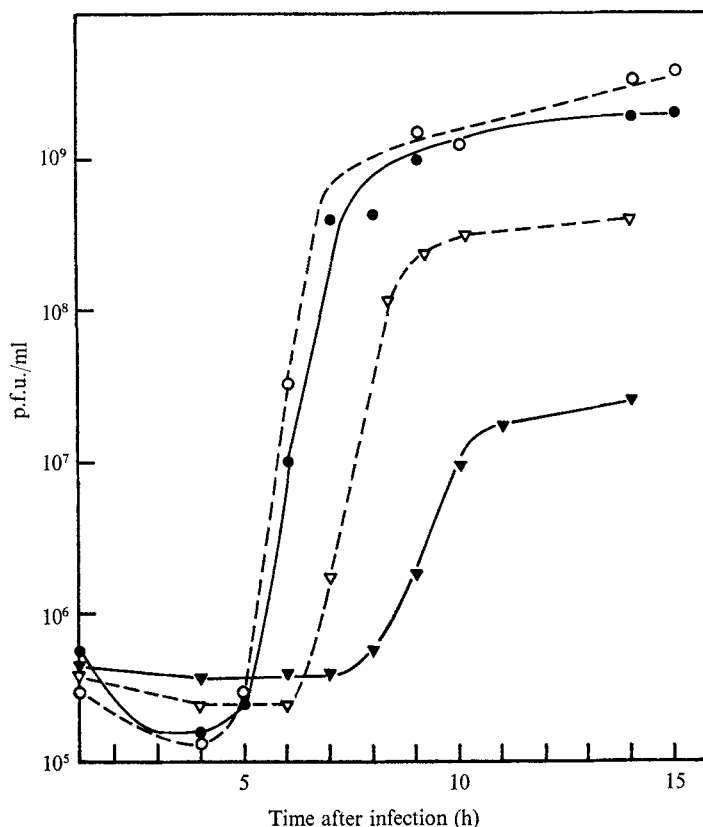


Fig. 2. EMC virus multiplication in parental and hybrid cells. Cells were infected with EMC at a m.o.i. = 20. One hour later, the monolayers were washed, 1 ml of Eagle's MEM was added, and the cells incubated at 37° C. Every hour an infected monolayer was frozen at -80° C. Total virus produced was titrated by the plaque assay method. ●—●, MKS; ▼—▼, CV₁; ▽---▽, Cl 4/Cl 3; ○---○, Cl 4/Cl 33.

Table 2. *EMC virus production by different cell lines infected with EMC RNA*

Cells	Expt. 1	Expt. 2	Expt. 3
MKS	7.5×10^5 *	1.3×10^5	5.2×10^4
CV ₁	2.5×10^4	4.5×10^3	9.5×10^2
MKCV _{III} Cl 2	ND†	ND	7.2×10^4
Cl 4/Cl 33 (late)	4.6×10^5	1.6×10^5	1×10^4

* Virus yield is expressed in p.f.u. using L cells after 1 replicative cycle.

† ND = not done.

in CV₁ cells than in MKS cells (Fig. 3). Thus, the low permissiveness of simian cells for EMC is not related to penetration or uncoating. The restrictive event could affect a step in the synthesis of virus proteins, resulting in a decrease of virus RNA synthesis or the inhibition of the formation of a structural protein. We therefore explored the synthesis of virus RNA in a further series of experiments.

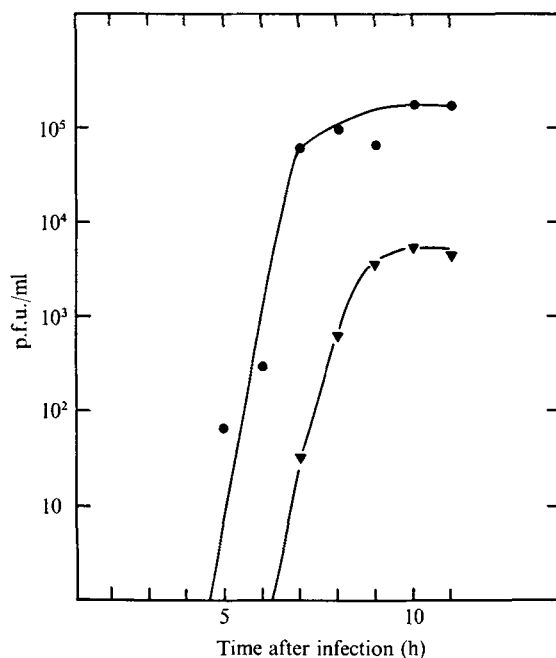


Fig. 3. Virus multiplication after inoculation of infectious RNA. ●—●, MKS; ▼—▼, CV₁.

Virus RNA synthesis in MKS and CV₁ cells infected with EMC virus

Mouse MKS and monkey CV₁ cells were pulse-labelled with [³H]-uridine in the presence of actinomycin D as described in Methods. As shown in Fig. 4, virus RNA was synthesized in MKS and CV₁ cells between 5 and 10 h and the rate of synthesis was highest between 11 and 14 h. In CV₁ cells, much less virus RNA was produced than in MKS.

To characterize this virus RNA, the synthesis of single-stranded and double-stranded RNA was investigated in infected parental and hybrid cells. After labelling with [³H]-uridine between 4 and 9 h after infection in the presence of actinomycin D, RNA was extracted from infected cells and analysed in sucrose gradients. As shown in Fig. 5(a) synthesis of single-stranded RNA (32S) occurred in MKS cells, whereas only a small amount of 32S virus RNA was synthesized in CV₁ cells. In semi-permissive hybrid Cl 4/Cl 3, the amount of virus RNA produced was intermediate between that obtained with the parental cells.

After treatment of the gradient fractions with RNase, double-stranded virus RNA was found in the 18S region (Fig. 5b). An unexpected observation was the large amount of RNase-resistant virus RNA located in hybrid Cl 4/Cl 3, compared with that found in the MKS cells. Thus, an accumulation of double-stranded RNA was observed in the semi-permissive hybrid. In spite of this increased amount of replicative virus RNA present in the cell, no increased amount of mature infectious virus particles was synthesized.

Karyotype analysis of some hybrid clones

Several hybrid clones were studied for their chromosomal content. Since each cell contained the whole mouse genome, the total number of simian chromosomes per cell (for 25 mitoses) was calculated (Table 3). Only a few large chromosomes were found since

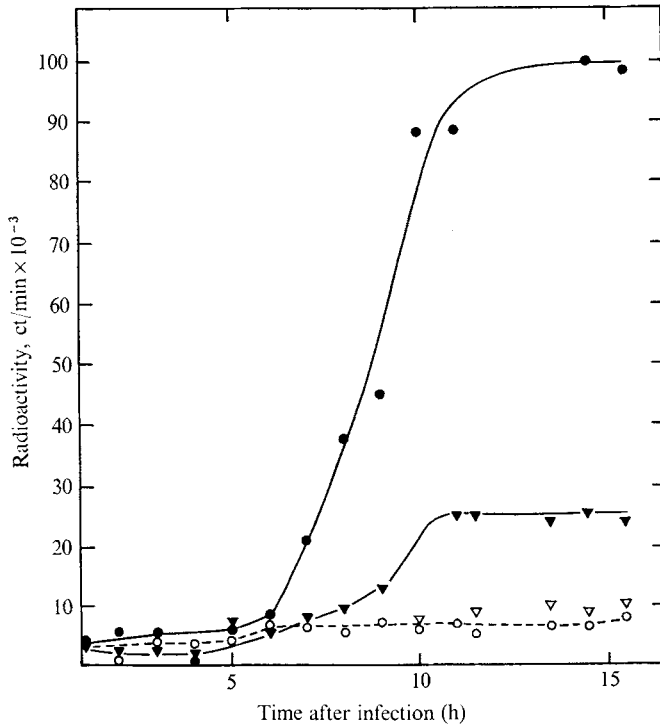


Fig. 4. Virus RNA synthesis in MKS and CV₁ cells infected with EMC virus. ●—●, infected MKS; ▼—▼, infected CV₁; ○---○, uninfected MKS; ▽---▽, uninfected CV₁.

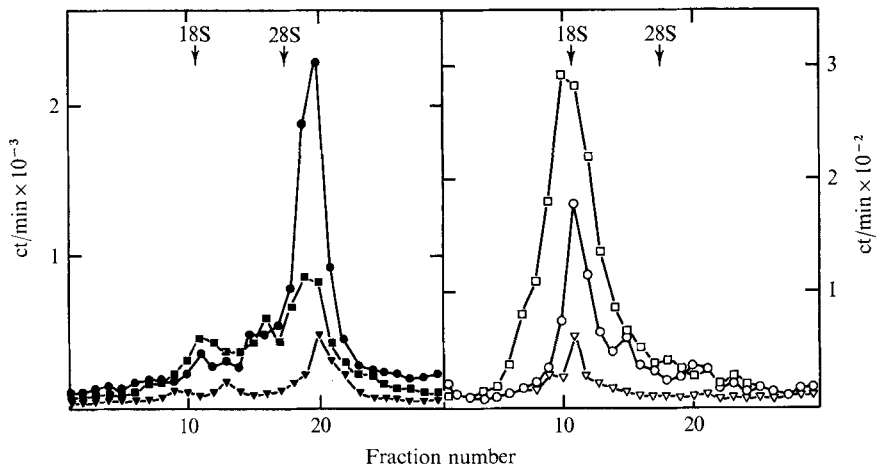


Fig. 5. Sucrose gradient sedimentation of RNA extracted from virus infected parental and hybrid cells. (a) Radioactivity of 10^6 infected cells: ●—●, MKS; ▼—▼, CV₁; ■—■, Cl 4/Cl 3. (b) Radioactivity of 10^6 infected cells after treatment with RNase: ○—○, MKS; ▽—▽, CV₁; □—□, Cl 4/Cl 3.

Table 3. *Karyotype analysis of monkey-mouse hybrid clones*

Hybrid clones	Mean number of monkey chromosomes/cell
Cl 2/Cl M	3.20 ± 1.71
Cl 4	12.8 ± 6.20
Cl 4/Cl 3	11.88 ± 6.18
Cl 4/Cl 33 (12th passage)	13.16 ± 6.27
Cl 4/Cl 33 (54th passage)	7.33 ± 3.32
Cl 4/Cl 33-3	7.33 ± 4.04

they were probably the first to be eliminated during the passages. In contrast, the small chromosomes were left over in most of the hybrids. The hybrid clones which contained the greatest number of simian chromosomes (Cl 4, Cl 4/Cl 3, Cl 4/Cl 33 – early passages) were the least permissive for EMC virus, while Cl 2/Cl M, Cl 4/Cl 33, Cl 4/Cl 33-3, which contained less simian chromosomes, were fully permissive. Thus the semi-permissiveness of some hybrid clones seems to be related to their simian chromosomal content.

DISCUSSION

Restrictive replication of picornaviruses in different cell types has been reported previously (Buck *et al.* 1967; Sturman & Tamm 1966, 1969). Wall & Taylor (1969) observed that Maden's bovine kidney (MDBK) cells synthesize 1 000-fold less EMC virus than productive L cells. In our system, mouse MKS cells are highly permissive to EMC virus, each infected cell producing about 1 000 to 5 000 virus particles; in contrast, monkey CV₁ cells are poorly permissive, each cell synthesizing 10 to 100 virus particles. Like MDBK cells, monkey cells are sensitive to EMC virus. Moreover, the same differences of permissiveness are observed when mouse and monkey cells are infected with virus RNA. Thus the restrictive event in virus multiplication in monkey cells must occur after uncoating.

The permissiveness of different monkey-mouse hybrid clones varies according to the number of monkey chromosomes present in each clone: Cl 4, Cl 4/Cl 3, and Cl 33 (early passages) which possess the greatest number of monkey chromosomes are the least permissive. Thus a virus function could be inhibited by the gene product from the semi-permissive monkey species. This situation was observed in the hamster-mouse hybrids infected with polyoma virus (Green *et al.* 1971). However the monkey chromosomes involved in this restriction of EMC virus replication in hybrid cells are not yet identified.

It was of interest to compare virus RNA synthesis in parental and hybrid cells. In monkey cells, double-stranded and single-stranded virus RNA are synthesized together in small amounts. In the semi-permissive hybrids (Cl 4/Cl 3) the RNase-resistant virus RNA is found in quantities higher than mouse cells, while the yield of single-stranded virus RNA is significantly lower. On the basis of this observation, more than one restrictive event could be responsible for the inefficient replication of EMC virus in monkey cells. It is also possible that the same inhibitory factor acts first on single-stranded and then on double-stranded RNA synthesis.

As shown in the kinetic studies of synthesis of virus RNA, it is unlikely that the small amount of single-stranded RNA detected could result from its rapid degradation by cellular

ribonucleases as reported in the case of MDBK cells infected with Mengovirus (Wall & Taylor, 1969).

In the hybrid system studied here, the restrictive event involves only the RNase-sensitive single-stranded virus RNA; thus the blockage occurs on one of the replicative forms of virus RNA. The mechanism of this blockage is now under investigation, using cell hybrids from different monkey-mouse and human-mouse systems.

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REFERENCES

- BRATT, M. A. & ROBINSON, W. S. (1967). Ribonucleic acid synthesis in cells infected with Newcastle disease virus. *Journal of Molecular Biology* **23**, 1-21.
- BUCK, C. A., GRANGER, G. A., TAYLOR, M. W. & HOLLAND, J. J. (1967). Efficient, inefficient and abortive infection of different mammalian cells by small RNA viruses. *Virology* **33**, 36-46.
- CASSINGENA, R. & TOURNIER, P. (1968). Mise en évidence d'un répresseur spécifique dans les cellules d'espèces différentes transformées par le virus SV 40. *Compte Rendu de l'Académie des Sciences, Paris* **267**, 2251-2254.
- CRAMER, R. (1969). Apparition et persistance d'un répresseur spécifique dans les cellules de hamster syrien infectées par le virus polyome. *Compte Rendu de l'Académie des Sciences, Paris* **268**, 3142-3145.
- DUBBS, D. R., KIT, S., DE TORRES, R. A. & ANKEN, M. (1967). Virogenic properties of bromodeoxyuridine-sensitive and bromodeoxyuridine-resistant simian virus 40-transformed mouse kidney cells. *Journal of Virology* **1**, 968-979.
- GREEN, H., WANG, R., BASILICO, C., POLLACK, R., KUSANO, T. & SALAS, J. (1971). Mammalian somatic cell hybrids and their susceptibility to viral infection. *Federation Proceedings* **30**, 930-934.
- JENSEN, F. C., GIRARDI, A. J., GILDEN, R. V. & KOPROWSKI, H. (1964). Infection of human and simian tissue cultures with Rous sarcoma virus. *Proceedings of the National Academy of Sciences of the United States of America* **52**, 53-59.
- KIT, S., NAKAJIMA, K., KURIMURA, T., DUBBS, D. R. & CASSINGENA, R. (1970). Monkey-mouse hybrid cell lines containing the SV 40 genome in a partially repressed state. *International Journal of Cancer* **5**, 1-14.
- LITTLEFIELD, J. W. (1965). Studies on thymidine kinase in cultured mouse fibroblasts. *Biochimica et Biophysica Acta* **95**, 14-22.
- MONTAGNIER, L. & MACPHERSON, I. (1964). Croissance sélective en gélose de cellules de hamster transformées par le virus polyome. *Compte Rendu de l'Académie des Sciences, Paris* **258**, 4171-4173.
- PAGANO, J. S., MCCUTCHAN, J. H. & VAHERI, A. (1967). Factors influencing the enhancement of the infectivity of poliovirus ribonucleic acid by diethylaminoethyl-dextran. *Journal of Virology* **1**, 891-897.
- STURMAN, L. S. & TAMM, I. (1966). Host-dependence of GDVII virus: complete or abortive multiplication in various cell types. *Journal of Immunology* **97**, 885-896.
- STURMAN, L. S. & TAMM, I. (1969). Formation of viral ribonucleic acid and virus in cells that are permissive or nonpermissive for murine encephalomyelitis virus (GDVII). *Journal of Virology* **3**, 8-16.
- SUAREZ, H. G., SONENSHEIN, G. E., ESTRADA, S., BOURALI, M. F., CASSINGENA, R. & TOURNIER, P. (1972). Activation of the viral genome in simian virus 40-transformed non-permissive cells by permissive cell extracts. *Proceedings of the National Academy of Sciences of the United States of America* **69**, 1290-1293.
- WALL, R. & TAYLOR, M. W. (1969). Host-dependent restriction of mengovirus replication. *Journal of Virology* **4**, 681-687.

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