Localization of the Restrictive Event of EMC Virus Replication in Semi-permissive Monkey and Monkey-Mouse Hybrid Cells

By MARIE-FRANCOISE DUBOIS

Institut National de la Santé et de la Recherche Médicale, U.43,
Hôpital Saint-Vincent de Paul, 74 avenue Denfert-Rochereau, Paris 75014, France

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SUMMARY

Encephalomyocarditis (EMC) virus replication was investigated in permissive mouse MKS cells, semi-permissive monkey CV1 cells, and in somatic monkey-mouse MKCVm hybrid cells whose permissiveness is under the negative control of the simian genome. We found that in CV1 cells the synthesis of both single- and double-stranded virus RNAs was restricted. In contrast, in semi-permissive hybrid C14/3 cells only the single-stranded virus RNA was synthesized in small amounts, whereas the double-stranded virus RNA accumulated late after infection. The synthesis of virus polyribosomes and virus polypeptides was lowered in semi-permissive conditions. In the presence of quaternary ammonium ions, the synthesis of EMC virus was partially relieved in CV1 cells. Thus, it can be postulated that a defective function in the replication complex is involved in the restrictive event.

INTRODUCTION

The yield of encephalomyocarditis (EMC) virus and mengovirus from different cell lines varies over a wide range depending on the host system (Tobey & Campbell, 1965; Buck et al. 1967; Wall & Taylor, 1969). In a previous report (Dubois & Chany, 1976) we have shown that EMC virus replicates to high titre in murine MKS cells, but that its multiplication is restricted in simian CV1 cells where virus yields are only about 6% of those of the permissive hosts. In the case of somatic monkey-mouse hybrid cells (MKCVm), the permissiveness of each clone varies according to its simian chromosomal content. Thus, EMC replication could be under a negative control of the simian genome. We have found that in CV1 monkey cells, single-stranded and double-stranded virus RNAs are synthesized in small amounts, whereas in the semi-permissive hybrid cells, only the synthesis of the single-stranded virus RNA is restricted.

In this paper we study the mechanism of this restriction in EMC virus replication and analyse the kinetics of the synthesis of single- or double-stranded virus RNA, and of virus polyribosomes or polypeptides, in infected cells.

METHODS

Virus. EMC virus was propagated in L cells and the stock used contained 5 × 10^9 p.f.u./ml.

Cells. Mouse L cells are routinely maintained in the laboratory. CV1 is a continuous cell line originating from African green monkey, Cercopithecus aethiops (Jensen, Girardi &
Gilden, 1964). MKS-BU_{100} cells originate from mouse kidney cells transformed by SV40 and resistant to 5-bromodeoxyuridine, thus lacking thymidine kinase activity (Dubbs et al. 1967). The monkey-mouse hybrid cell line MLCV_{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 a previously described technique (Montagnier & MacPherson, 1964). Two hybrid clones were selected for our experiments, permissive MLCV_{III} C1_{1}/C and semi-permissive MLCV_{III} C1_{4/3}. All cell cultures were maintained in Eagle’s medium (MEM) supplemented with 10% calf serum.

**Extraction of virus RNA.** Cell monolayers (about 5x10^7 cells) were infected with EMC virus (20 p.f.u./cell) at 37 °C. One hour later, virus was removed and 30 ml of MEM added. Two hours p.i., actinomycin D was added to the culture medium in one bottle of each cell type (2 µg/ml for MKS and hybrid cells, 10 µg/ml for CV_{1} cells). One hour later, 3H-uridine (5 µCi/ml) was added for 1 h and RNA extracted. Every hour up to 9 h p.i., a series of cell cultures previously treated with actinomycin D for 1 h was labelled with 3H-uridine for 1 h and RNA extracted following the method of Bratt & Robinson (1967). Monolayers were rinsed three times with NTE pH 8.4 buffer (NaCl 0.1 M, tris 0.01 M, pH 8.4, EDTA 0.001 M) and dissociated with the same buffer containing 1% SDS and 1% β-mercaptoethanol. RNA was extracted twice at 4 °C with twice distilled phenol (Merek) saturated with NTE pH 8.4 buffer containing 0.5% β-mercaptoethanol. The third extraction was performed with NTE pH 7.4 saturated phenol. The last aqueous phase was precipitated with ethanol at -20 °C for 12 h or more. The RNA precipitate was dissolved in 1 to 2 ml NTE pH 7.4 and layered on a 5 to 30% sucrose gradient prepared in the same buffer. After centrifugation for 18 h at 20,000 rev/min in a Beckman L 3 ultracentrifuge (SW 25.1 rotor), 1 ml fractions were collected with an ISCO fraction collector and 0.5 ml of each 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 at 4 °C. The precipitates were filtered through a Whatman GF/C filter and thoroughly rinsed with 5% TCA and ethanol. After drying, the filters were counted with toluene PPO-POPOP liquid in a Packard scintillation counter.

**Analysis of virus polyribosomes.** Cell monolayers (10^7 cells) were infected with EMC virus (10 p.f.u./cell) for 1 h at 37 °C. The virus was then removed and MEM added. Four h p.i., actinomycin D was added (2 µg/ml for MKS and hybrid cells, 10 µg/ml for CV_{1} cells) and 1 h later 5 µCi/ml of 3H-uridine was added for 1 h. Virus polyribosomes were extracted as described by Morse, Herrmann & Heywood (1971). Cells were rinsed with 10 ml TKM buffer (tris 0.01 M, pH 7.4, KCl 0.25 M, MgCl_{2} 0.01 M) at 4 °C, scraped and suspended in TKM buffer (10 ml) then centrifuged at 1200 rev/min in a Martin-Christ centrifuge for 10 min at 4 °C. The cell pellet was suspended in 0.2 ml of TKM buffer containing 0.5% Triton X-100. The cell suspension was then drawn in and out of a Pasteur pipette 20 times and centrifuged at 5000 rev/min for 10 min at 4 °C. The supernatant was layered on a sucrose gradient (15 to 40%) in TKM buffer and centrifuged in a Spinco SW 41 rotor at 40,500 rev/min for 70 min at 4 °C. Fractions were collected by puncturing the bottom of the tube and the acid-insoluble radioactivity was measured as previously described.

**Infection of monolayers and preparation of cell lysates of labelled EMC virus-specific proteins.** Confluent monolayers (approx. 5x10^6 cells) were infected with EMC virus at a m.o.i. of 100. After incubation for 1 h at 37 °C to allow the attachment of the virus, the virus suspension was removed and replaced by 4 ml of amino acid-deficient Eagle’s medium.
Table 1. Time course of single-stranded 32S (ssRNA) and double-stranded 18S (dsRNA) virus RNA synthesis in permissive and semi-permissive cells

<table>
<thead>
<tr>
<th>Hours after infection</th>
<th>MKS</th>
<th>CV1</th>
<th>Cl4/a</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssRNA</td>
<td>dsRNA</td>
<td>ssRNA</td>
<td>dsRNA</td>
</tr>
<tr>
<td>3-4</td>
<td>175*</td>
<td>27</td>
<td>18</td>
</tr>
<tr>
<td>4-5</td>
<td>281</td>
<td>19</td>
<td>80</td>
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<td>5-6</td>
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<td>36</td>
<td>356</td>
</tr>
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<td>6-7</td>
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<td>89</td>
<td>486</td>
</tr>
<tr>
<td>7-8</td>
<td>1'446</td>
<td>52</td>
<td>492</td>
</tr>
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<td>1'018</td>
<td>59</td>
<td>80</td>
</tr>
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<td>18</td>
</tr>
<tr>
<td>3-10</td>
<td>5'847</td>
<td>308</td>
<td>1'530</td>
</tr>
</tbody>
</table>

* Ct/min/10^6 cells.

(pre-warmed at 37 °C) containing 1 % agamma-globulin calf serum and actinomycin D (5 μg/ml for MKS and hybrid cells, 10 μg/ml for CV1 cells). At different times after infection, cells were pulse-labelled for 70 min by adding 3H-protein hydrolysate (10 μCi/ml).

Cells were scraped from the glass, washed twice with cold PBS and the pelleted cells covered with 0.2 ml of lysis mixture, 0.01 M-sodium phosphate buffer, pH 7.2, containing 2 % SDS and 5 % β-mercaptoethanol. Lysates were stored at -20 °C until analysed.

Polyacrylamide gel electrophoresis. Gels were prepared according to the procedure described by Maizel (1970). The gel columns (0.7 × 22 cm) contained 0.1 M-sodium phosphate buffer (pH 7.2), 0.1 % SDS, 7.5 % acrylamide, 0.2 % (v/v) methylene bisacrylamide. Polymerization was catalysed by ammonium persulphate (0.5 mg/ml) and 0.05 % N,N,N',N'-tetramethylethylene-diamine. Samples (50 to 100 μl) were prepared by adding 10 % glycerol and 0.002 % bromophenol blue and were immersed in boiling water for 4 min immediately before layering on to the gels. Electrophoresis was carried out at 4 mA/gel for 1 h and 8 mA/gel until the bromophenol blue had migrated to within 2 cm of the end of the gel (20 to 22 h). Gels were fractionated into 1 mm slices and transferred to a scintillation vial with 0.2 ml of ammonia for one night. Two ml scintillation fluid (Instagel, Packard Instrument) was then added and radioactivity measured in a liquid scintillation spectrometer (Packard 3320). The mol. wt. of the virus-specific polypeptides were determined by comparison with marker proteins, bovine serum albumin (mol. wt. = 66500), pepsin (13400), chymotrypsinogen (22500) and cytochrome c (13400). The reference gels were stained with Coomassie brilliant blue (0.2 % in 50 % methanol, 50 % water, containing 7 % acetic acid).

Reagents. Actinomycin D was a gift from Merck, Sharp and Dohme (New Jersey, U.S.A.). 3H-uridine (sp. act. 20 Ci/mmol) and 14C-protein hydrolysate (sp. act. 0.5 mCi/mg) were purchased from the Biology Department of the Centre de l'Energie Atomique (Saclay, France).

RESULTS

Kinetic analysis of virus RNA synthesis

In a previous report Dubois & Chany (1976) showed that virus RNA synthesis was restricted in semi-permissive cells and both single-stranded and double-stranded virus RNAs were synthesized in small amounts in CV1 cells. In contrast, only the synthesis of single-stranded virus RNA was restricted in the semi-permissive Cl4/a hybrid cells. To explore this phenomenon further, EMC virus-infected cells were pulse labelled for 1 h with 3H-uridine
from 4 to 10 h p.i. The $^3$H-uridine incorporation into single-stranded 32S and double-stranded 18S RNAs was measured at different times after infection of permissive MKS cells and semi-permissive CV₁ and MKCV₁ Cl₁/t cells. The results are illustrated in Table 1. In the case of parental MKS cells, the synthesis of single-stranded (32S) and double-stranded (18S) virus RNAs increases up to 7 h p.i. and rapidly declines thereafter. In the semi-permissive CV₁ cells, the rate of synthesis of both virus RNAs is maximal at 8 h p.i. and low compared to MKS cells. In the semi-permissive hybrid Cl₁/t, the synthesis of single-stranded 32S virus RNA is detected at 3 h p.i. (as in MKS cells) but is already decreasing at 5 h p.i. In contrast, the synthesis of double-stranded virus RNA (RNase-resistant) increases until 8 h p.i. and declines thereafter. Moreover, the amount of double-stranded virus RNA synthesized from 4 to 10 h p.i. by this hybrid Cl₁/t is as high as that synthesized in MKS cells.

The difference between the semi-permissive parental CV₁ and hybrid Cl₁/t cells is interest-
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(a)

Fig. 2. Electrophoretic profile of proteins synthesized in Cl2/M cells after infection with EMC virus. Cells were pulse-labelled with 14C-protein hydrolysate for 70 min at 5 h p.i. Whole cell extracts and analysis by gel electrophoresis were performed as described in Methods. (a) Cl2/M cells infected with EMC virus; (b) Cl2/M uninfected control cells.

In the first case, both single- and double-stranded virus RNAs are synthesized in small amounts, whereas in the hybrid Cl4/a cells only the synthesis of the single-stranded virus RNA is restricted.

Virus-specific polysomes

EMC virus-specific polysomes were examined in permissive MKS and hybrid Cl2/M and in semi-permissive CV1 and hybrid MKS and hybrid Cl4/a cells. After labelling with 3H-uridine between 5 and 6 h p.i., polysomes were extracted and analysed in a sucrose gradient as described in Methods. Cells were treated with actinomycin D before labelling so that
the host polysomes were not labelled. Virus-induced polysomes (fractions 7 to 12) appear with a sedimentation coefficient of 350S to 400S in both restrictive and permissive cells (Fig. 1). The $s_{20}$ value was calculated according to the McEwen tables (1967). These 350S to 400S peaks are in fact virus polysomes, since cellular polysomes have a smaller sedimentation constant and virions sediment at 150S (Roizman, Mayer & Rapp, 1958). Moreover, they disappear after treatment with ribonuclease. In permissive Cl$_{12/3}$ cells, where the virus yield is 3 times greater than in MKS cells, the radioactivity incorporated into virus RNA linked to the ribosomes is also three times greater. On the contrary, in semi-permissive Cl$_{14/3}$, where both the synthesis of virions and virus RNA is three to four times less than in MKS cells, the synthesis of polysomes is also three times less. In the case of semi-permissive CV$_1$ cells, the peak of polysomes is not detectable corresponding to the low synthesis of single-stranded RNA.

Thus it seems that the restrictive multiplication of EMC virus in semi-permissive cells is not related to a fault in the linkage of virus RNA to host ribosomes. The increase in the number of ribosomes corresponds to the amount of virus RNA synthesized.

**Synthesis of virus-specific polypeptides**

The synthesis of virus-specific polypeptides in EMC virus-infected cells (MKS, CV$_1$, Cl$_{12/3}$, Cl$_{14/3}$) was investigated using the method of SDS-polyacrylamide gel electrophoresis (based on the conditions outlined in Methods). In these experiments, cells were pulse-labelled with a $^{14}$C-protein hydrolysate for 70 min, the time which corresponds to the mid-logarithmic phase of virus production.

The electropherogram of Cl$_{12/3}$-infected cells (the most permissive hybrid for EMC virus) is presented in Fig. 2 (a). The profile is comparable to that obtained by Paucha, Seehafer & Colter (1974) in L cells infected with mengovirus. The nomenclature used is that employed by Butterworth _et al._ (1970 in their studies with EMC virus. It appears that in permissive Cl$_{12/3}$, where EMC virus RNA and virions are actively synthesized, all the virus precursors and capsid polypeptides are synthesized normally after pulse-labelling for 70 min at 5 h p.i.

To make sure that these polypeptides were not of cell origin, the characteristic electrophoretic pattern obtained with lysates of mock-infected C$_{2/3}$ is shown in Fig. 2 (b). Cellular protein synthesis is decreased markedly by infection with EMC virus. The residual host proteins synthesized do not have their equivalent in the infected cells. In the case of permissive MKS cells (Fig. 3 a), the precursor polypeptides A and C and their cleavage products B, H, C, D and I are present in small quantities after labelling of the infected cells for 70 min at 5 h p.i. In contrast, capsid polypeptides and non-structural polypeptides E, F and G are synthesized in large amounts.

In a further experiment, Cl$_{14/3}$ cells were labelled at 6 h p.i. and CV$_1$ cells at 8 h p.i. for 70 min. Precursor polypeptides were labelled in the hybrid clone (Fig. 3 b) to a very small extent, whereas in CV$_1$ cells (Fig. 3 c) they were not detected. In the two types of cells, capsid polypeptides were labelled but in smaller amounts than in permissive cells.

**Virus replication in the presence of hexamethonium bromide**

It has been found (Prather & Taylor, 1975) that quaternary ammonium ions partially relieve the restricted replication of mengovirus in Madin-Darby bovine kidney (MDBK) cells. These compounds are known to reverse the inhibition of poliovirus replication by guanidine (Caliguiri & Tamm, 1973). Moreover, a resemblance between EMC virus restriction and the guanidine effect on poliovirus replication could be established. Thus, the
Fig. 3. Electrophoretic profile of proteins synthesized in (a) MKS cells; (b) Cl4/3 hybrid cells; (c) CV1 cells after infection with EMC virus. Cells were pulse-labelled with 14C-protein hydrolysate for 70 min at 5 h p.i. in MKS cells, 6 h p.i. in Cl4/3 cells, and 8 h p.i. in CV1 cells. Whole cell extracts and analysis by gel electrophoresis were performed as described in Methods.
One h p.i. (at an m.o.i. of 10) MKS, CV₁ and Cl₁/₁₃ cells were treated with hexamethonium bromide at various concentrations (1 to 120 mM) and incubated at 37 °C for 16 h. The virus yield was then determined by the plaque-forming method in L cells. The dose-response curve for hexamethonium is shown in Fig. 4. In permissive MKS cells, the increase in virus
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yield is very low, irrespective of the concentration of hexamethonium. In contrast, in the presence of an 80 mM solution of hexamethonium, the virus yield is increased up to 6 times in semi-permissive CV₁ cells and two fold in Cl₁/₁₃.

Another quaternary ammonium ion, choline chloride, has a similar effect on virus replication in the parental and hybrid cells. The results of several experiments performed in the presence of optimal concentrations (80 mM) of hexamethonium bromide and choline chloride are summarized in Table 2. The relative increase in virus yields ranges from 2.5 to 5.3 in the presence of hexamethonium and from 2 to 8.2 in the presence of choline.

DISCUSSION

We reported previously that the restricted replication of EMC virus in monkey CV₁ cells was not due to an absence of binding sites for virus attachment or to a decreased uncoating of the virus (Dubois & Chany, 1976). In fact it is not possible to increase the virus yield by infecting cells with virus nucleic acid. In somatic monkey-mouse hybrid cells, the decreased virus yield is probably not due to a lack of cellular factors which depend on the murine genome. Therefore, the simian genome seems to be partly responsible for the restrictive event. In order to localize this event, we have investigated the different stages of virus replication in parental mouse MKS, simian CV₁ and somatic hybrid MKCV₁ cells. We have shown that single-stranded virus RNA synthesis is maximal between 6 and 7 h p.i. in MKS cells, 7 to 8 h p.i. in CV₁ cells and 5 to 6 h p.i. in Cl₁ cells.

From the data reported in Table 1, the radioactivity incorporated in double-stranded virus RNA (RF) can be calculated. It represents 5% of that incorporated in single-stranded virus RNA in MKS cells, 6% in CV₁ cells, but rises to 21% in Cl₁ cells. It can be concluded that, in these hybrids, an accumulation of RF occurs, whereas the synthesis of single-stranded RNA is reduced. The same phenomenon has been observed in MDBK cells infected with mengovirus when virus RNA synthesis was arrested 4 h p.i., whereas RF synthesis continued at a rate comparable to that observed in permissive mouse L cells (Wall & Taylor, 1969, 1976).

Since our pulse-labelling intervals with ³H-uridine were 1 h, we could not detect the replicative intermediary (RI) form which is labelled by brief pulse-labelling. Thus, the double-stranded RNase-resistant RNA detected in our experiments is the RF form sedimenting at 18S which is considered to be an end-product of the replication.

It is known that RNA synthesis in cells infected by picornaviruses is associated with two membrane complexes (Arlinghaus, Syrewicz & Loesch, 1972; Caliguri, 1974). A large replication complex (sedimenting in a 100 to 300S region) contains predominantly single-stranded 35S RNA, while a small replication complex (sedimenting at 70S) contains double-stranded RNA. We postulate that in our system the heavy replication complex would have a disturbed functioning in the semi-permissive simian and hybrid cells, causing a low synthesis of 35S virus RNA. On the contrary, the light replication complex, synthesizing double-stranded RNA, would be non-functional in simian cells but active late after infection in semi-permissive hybrid cells.

Whatever the mechanism of blocking RNA virus synthesis may be in the semi-permissive cells, it can be assumed that the inhibitory step of virus multiplication is located at this stage. Indeed, the virus polyribosomes in murine, simian and hybrid cells have the same sedimentation constant (350 to 400S); thus, the same number of ribosomes is linked to a messenger RNA of the same size. The only difference between virus polysomes in the cells is of a quantitative order. With increasing cell permissiveness, more polyribosomes are synthesized.
From our analysis of polypeptides synthesized in the infected cells at the mid-logarithmic phase of virus production, the semi-permissiveness of simian and hybrid cells cannot be related to a different scheme of polypeptide synthesis.

The experiments performed in the presence of quaternary ammonium ions indicate that the restrictive event of EMC virus replication in semi-permissive cells is located at the level of the replication complex. It is known that the picornavirus RNA-polymerase complex, including enzyme, template and nascent RNA, is associated with a specific cellular membrane fraction (Caliguiri & Tamm, 1970; Ehrenfeld, Maizel & Summers, 1970). We suggest that in our system the disturbed functioning of the replication in semi-permissive simian cells could be related to the RNA polymerase itself. Indeed, its polypeptide composition is not yet well defined for any picornavirus. However, it has been shown with poliovirus that the virus polypeptide X (which corresponds to F polypeptide for EMC virus) might be responsible for organizing the polymerase on the membrane. In addition, several host polypeptides might be components of the polymerase (Butterworth, Shimshick & Yin, 1976).

In our case, these host polypeptides could possibly be found in smaller amounts in simian cells. This hypothesis is however not easy to verify since the RNA polymerase is not functional when isolated from the smooth cytoplasmic membranes and is thus difficult to purify.

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