Cell-Free Synthesis of Encephalomyocarditis Virus

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We developed a system for complete replication of encephalomyocarditis virus (EMCV) in a test tube by using an in vitro translation extract from Krebs-2 cells. Efficient virus synthesis occurred in a narrow range of Mg^{2+} and EMCV RNA concentrations. Excess input RNA impaired RNA replication and virus production but not translation. This suggests the existence of a negative-feedback mechanism for regulation of RNA replication by the viral plus-strand RNA or proteins.

Encephalomyocarditis virus (EMCV) is the prototype *Cardiovirus* within the family *Picornaviridae* (31). The genome of EMCV is a ~7,900-nucleotide-long positive-strand RNA. Within cells, EMCV RNA sequentially functions as a template for virus-specific translation and negative-strand RNA synthesis; it is also packaged into the virion at the late stage of infection. EMCV RNA recruits ribosomes with the aid of an internal ribosome entry site (10, 19), which acts as a binding site for translational initiation factor eIF4F (26, 28). Translation proceeds from a single initiation site to yield a large polyprotein. The polyprotein is cleaved in a series of steps by the viral protease 3C[pro] or 3ABC[pro] into mature virus proteins (14, 18, 25). RNA replication involves negative and positive RNA synthesis and requires functions of at least two viral proteins, the RNA polymerase 3D[pol] and VPg. The detailed mechanisms of these processes are controversial with respect to the involvement of particular *cis*-acting replication elements (*cre*) and *trans*-acting cellular factors (1).

Synthesis of an infectious picornavirus in a test tube has been achieved but only for poliovirus (PV) (4, 21, 22). Such a system is paramount for the understanding of mechanisms of virus replication and for screening antiviral drugs. EMCV RNA is translated accurately, with the formation of authentic viral proteins and capsid intermediates, in a nuclease-treated Krebs-2 cell extract (S10) (2, 32, 33). Here, we show that this system also supports other virus-specific processes required for complete virus replication.

Krebs-2 ascites cells (7) were washed twice with Earle's solution ($80 \times g$ for 8 min at 4°C), suspended in methionine-free Dulbecco's minimal essential medium (5×10^6 to 10×10^6 cells/ml), and incubated at 37°C for 2 h on a rotary shaker (20). Incubation of cells in nutritionally rich medium (i.e., Dulbecco's minimal essential medium) increased translation efficiency, in agreement with earlier data (17, 20). After incubation, cells were harvested and washed three times with cold isotonic buffer (35 mM HEPES-KOH [pH 7.3], 146 mM NaCl, 11 mM D-glucose) (4), with a final centrifugation at $750 \times g$. Packed cells were suspended in 2 volumes of hypotonic buffer (25 mM HEPES-KOH [pH 7.3], 50 mM KCl, 1.5 mM MgCl$_2$,

1 mM dithiothreitol), allowed to swell on ice for 20 min, and lysed with ~30 strokes of a tightly fitting Dounce homogenizer (Kontes). Concentrated buffer solution (1/9 volume; 25 mM HEPES-KOH [pH 7.3], 1 M KCH$_3$COO, 30 mM MgCl$_2$, 30 mM dithiothreitol) was then added, and the homogenate was centrifuged at $18,000 \times g$ for 20 min at 4°C. The supernatant (35 to 45 A_{260} U per ml) was stored at −70°C. Before translation, the extract was treated with micrococcal nuclease (150 U/ml; Roche) essentially as described previously (32). EMCV (K2) RNA was extracted from the purified virus (34). Translation reaction mixtures (50 μl) contained 25 μl (50% by volume) of S10, 5 μl of a master mix (10 mM ATP, 2 mM GTP, 2 mM CTP, 2 mM UTP, 100 mM creatine phosphate [dipotassium salt], 1 mg of rabbit muscle creatine phosphokinase [Calbiochem]/ml, 19 unlabeled L-amino acids [without L-methionine; 0.2 mM each], 125 mM HEPES-KOH [pH 7.3]), 5 μl of a salt solution (0.75 M KCH$_3$COO, 10 mM MgCl$_2$, 2.5 mM spermidine), 2.5 μl of [^{35}S]methionine (10 mCi/ml [>1,000 Ci/mmol]), and EMCV RNA (2 to 30 μg/ml). Translation was at 32 to 33°C for the times indicated in the figure legends. Proteins were analyzed by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS–15% PAGE) and autoradiography. RNA replication and virion production were assayed in similarly programmed reactions, except that unlabeled 0.4 mM L-methionine was substituted for [^{35}S]methionine in the reaction cocktail.

In agreement with previous data (32), translation of EMCV RNA in Krebs-2 S10 yielded all the known virus proteins (Fig. 1A). A time course of the appearance of L-P1-2A (the N-terminal portion of the viral polyprotein), 2C (the central portion), and P3 (the C-terminal portion) was consistent with the complete translation of the viral genome over 0.5 h of incubation time. During the subsequent incubation, the L-P1-2A and P3 polypeptides were cleaved and the mature viral structural and nonstructural proteins appeared. The magnesium concentration range for optimum translation was relatively broad, and variations within the 2 to 4 mM concentration range did not significantly affect labeling or the pattern of the products. Polypeptide synthesis was EMCV RNA dose dependent (Fig. 1B). Thus, Krebs-2 S10 prepared by the method described translates EMCV RNA efficiently and accurately.

In vitro translation of EMCV RNA yields, among other viral proteins, high amounts of the RNA-dependent RNA polymerase 3D[pol] (Fig. 1). To demonstrate that 3D[pol] (in combination

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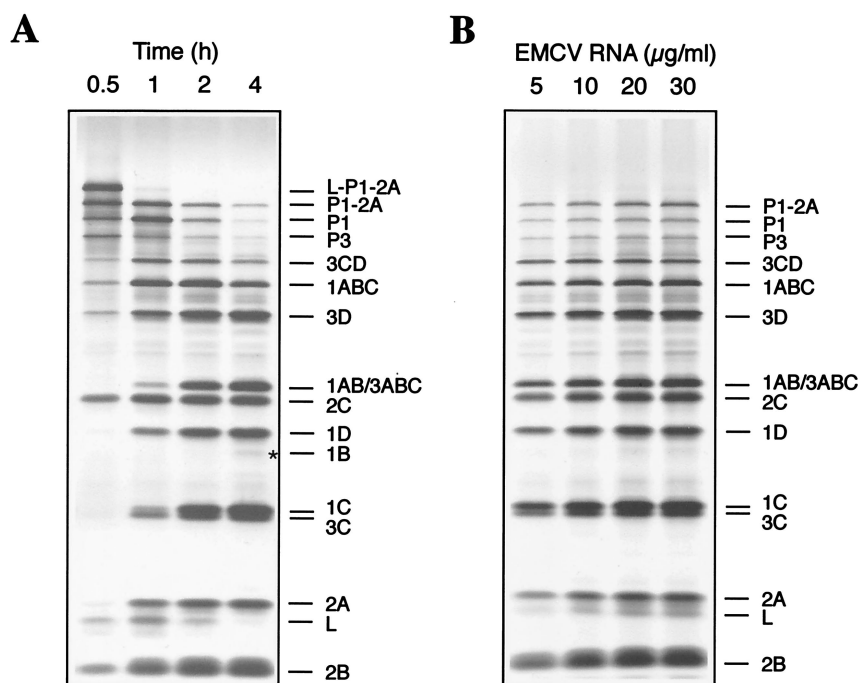
FIG. 1. (A) Time course of synthesis and processing of EMCV-specific proteins in EMCV RNA-programmed Krebs-2 S10. EMCV RNA (20 μg/ml) was translated in the presence of [³⁵S]methionine under standard reaction conditions. At the times indicated, 5-μl aliquots of the reaction mixture were withdrawn, supplemented with SDS sample buffer, and subjected to SDS–15% PAGE. An autoradiogram of the dried gel is shown. The assignment of polypeptides is based on a comparison with proteins synthesized in EMCV-infected BHK-21 cells (34). An asterisk indicates the position of 1B. (B) EMCV RNA dose response of protein synthesis. Reaction mixtures (12.5 μl) were programmed by the indicated concentrations of EMCV RNA at 32°C for 2 h. Conditions for translation and PAGE were as described for panel A.

with other factors) supports RNA replication, we pulse-labeled the reaction mixtures with [α-³²P]CTP at different times after the beginning of incubation. The newly synthesized RNA was extracted and quantified after trichloroacetic acid precipitation. RNA synthesis was first detectable at ~2 h and reached a maximum at 4 to 5 h of incubation (Fig. 2A). On the other hand, pulse-labeling of reaction mixtures with [³⁵S]methionine during the same time course revealed a maximum rate of viral translation at ~1 h of incubation. By the time of maximal RNA synthesis (4 h), the rate of virus translation had declined dramatically (to less than 5% of the maximal rate level). Thus, our in vitro system recapitulates the switch from viral translation to RNA replication characteristic of many picornavirus systems in vivo (12). The major RNA product comigrated with the full-length EMCV RNA in an agarose gel (Fig. 2B). To examine the possibility that this product results from labeling of the input RNA by 3D^pol-associated terminal nucleotidyltransferase activity (29), we performed its digestion with RNase T1. This digestion, similarly to that of virion RNA (8, 35), liberated a large (~150-nucleotide) poly(C) tract-containing fragment (data not shown). This strongly suggests that the vast majority of the in vitro product was uniformly labeled and was represented by the newly synthesized plus-strand RNA. It is noteworthy that small amounts of slowly migrating RNA species were generated in EMCV RNA-programmed but not in mock-programmed reactions (Fig. 2B). This product comigrates with the double-stranded replicative-form RNA (3). The propensity of the system for the synthesis of plus-strand RNA accords well

with the known asymmetry of picornaviral replication in vivo, where the ratio of newly synthesized positive- to negative-strand RNAs is 30 to 70:1 (13, 24). Efficient RNA synthesis required Mg²⁺ in a narrow (3 to 3.5 mM) range of concentrations (Fig. 2C), in sharp contrast to the broad range observed for translation. Lowering the magnesium salt concentration to 2 mM resulted in almost no RNA synthesis (Fig. 2C) while causing only marginal inhibition of translation (data not shown). An EMCV RNA dose-response experiment showed that saturation of RNA synthesis is reached at a surprisingly low level of input RNA (5 μg/ml; Fig. 2D). Higher RNA concentrations (10 to 30 μg/ml) markedly inhibited RNA synthesis. Also surprising was that this inhibition occurred at RNA concentrations which stimulated rather than inhibited viral translation (Fig. 1B). A plausible explanation is that a viral protein(s), when accumulated to a certain level, can inhibit RNA replication. For example, RNA interaction with the structural proteins would engage it in a packaging complex and would preclude replication. Alternatively, EMCV RNA excess may sequester a host factor(s), which would facilitate RNA replication (3, 9). It would be interesting to determine whether the negative-feedback mechanism for regulation of RNA replication by virus-specific products governs the switch from replication to encapsidation of the viral RNA in vivo.

After a 4-h incubation period, a small amount of 1B was evident in a protein-synthesizing reaction (Fig. 1A), indicative of the final cleavage of 1AB, which accompanies virion morphogenesis (15). To determine whether the EMCV RNA-pro-
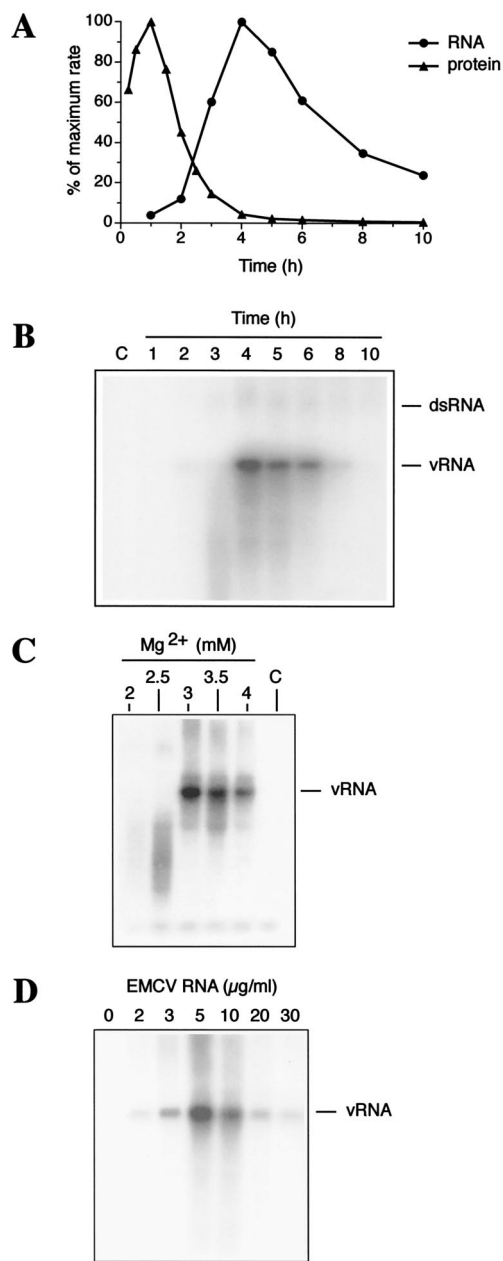
FIG. 2. EMCV RNA replication. (A) Kinetics of EMCV RNA and protein synthesis. EMCV RNA (10 μg/ml)-programmed reaction mixtures (50 μl) were pulse-labeled with 1.5 μl of [α-^{32}P]CTP (10 mCi/ml [3,000 Ci/mmol]) for 1 h (the label was added 30 min before the times indicated in the figure) (4). Samples were treated with proteinase K, and RNA was extracted with a mixture of phenol and chloroform. Viral proteins were pulse-labeled with [^{35}S]methionine for 30 min (15 min before the times indicated in the figure) and dissolved in an SDS sample buffer. Portions of each sample were assayed for trichloroacetic acid-insoluble radioactivity. Data (average of two independent determinations) are given as percentages of maximum incorporation. (B) Products of RNA synthesis (from the reaction described for panel A) analyzed by native Tris-borate-EDTA–1% agarose gel electrophoresis and autoradiography (16). The positions of single-stranded (vRNA) and double-stranded (dsRNA) EMCV RNA are indicated. (C) Mg^{2+} concentration dependence of RNA synthesis. Reaction mixtures contained 10 μg of EMCV RNA/ml and the indicated concentrations of MgCl$_2$. (D) EMCV RNA dose response of RNA synthesis. Reaction mixtures contained 3 mM MgCl$_2$ and the indicated concentrations of EMCV RNA. For panels C and D, RNA was labeled with [α-^{32}P]CTP between 4 and 5 h of incubation and analyzed as described above.
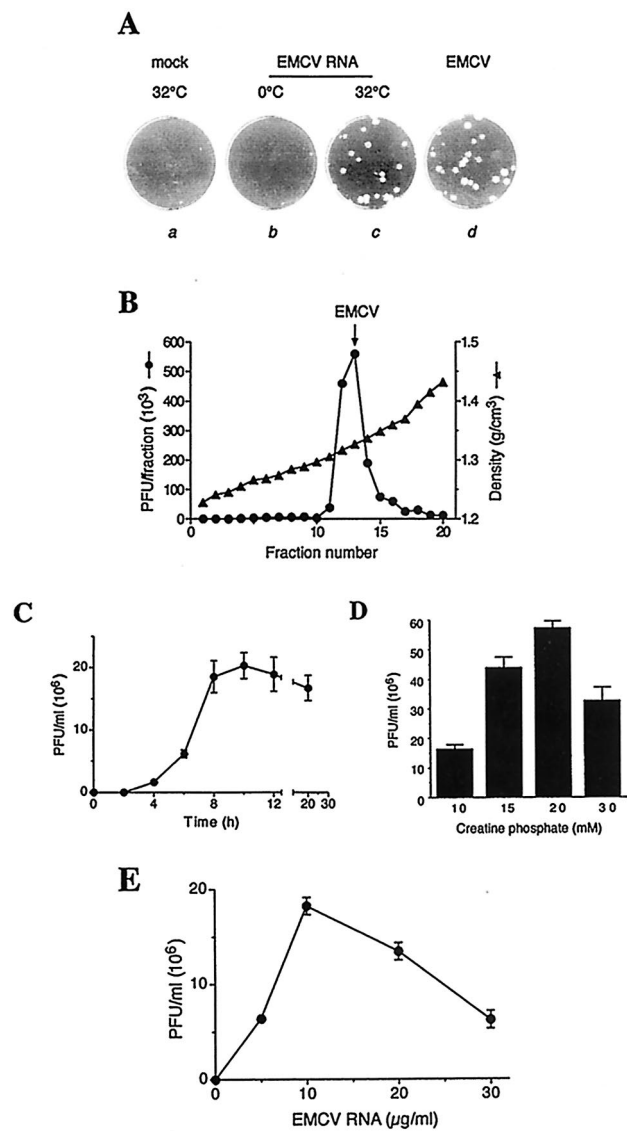

FIG. 3. Synthesis of EMCV in vitro. (A) Krebs-2 S10 extracts were incubated for 20 h either at 32°C (incubations *a* and *c*) or 0°C (incubation *b*) in the absence (incubation *a*) or presence (incubations *b* and *c*) of EMCV RNA (20 μg/ml). The samples were treated with a mixture of RNase A and T1 (21). Using serial dilutions of samples, a plaque assay was carried out on confluent BHK-21 cells (30). Plaques characteristic for EMCV, which was propagated in Krebs-2 cells in vivo, are shown for comparison (incubation *d*). (B) Isopycnic banding of in vitro-synthesized EMCV in a cesium chloride density gradient. A 250-μl translation reaction mixture was programmed with EMCV RNA for 20 h at 32°C and treated with the RNase A/T1 cocktail as described above. Triton X-100 was added to 0.5%, and the mixture was diluted to 5 ml with CsCl/phosphate-buffered saline (1.34 g/cm^3 solution density) (30). The mixture was subjected to centrifugation at 45,000 rpm for 18 h at 6°C in a SW55 rotor. Fractions (0.25 ml) were collected and assayed for infectivity following serial dilutions. The arrow indicates the location of the virus formed in EMCV-infected Krebs-2 cells. (C) Time course of the infectivity titer of in vitro-synthesized EMCV. (D) Effect of creatine phosphate concentrations on EMCV yields. Reaction mixtures containing the indicated concentrations of creatine phosphate and 3.4 mM MgCl$_2$ were programmed with EMCV RNA for 20 h and assayed for infectivity as described above. In panels B, C, and D, programming was with 10 μg of EMCV RNA/ml. (E) EMCV RNA dose response of virus yield. Reaction mixtures were programmed with the indicated concentrations of EMCV RNA for 20 h at 32°C, treated with RNase A/T1, and assayed for infectivity following serial dilutions. In panels C, D, and E, the data are averages (with standard deviations from the means) of three independent titer determinations.

grammed reactions yield infectious virus, aliquots of translation samples withdrawn after 20 h of incubation were assayed for virus infectivity on BHK-21 cell monolayers. The in vitro reactions generated virus, and plaque morphologies were indistinguishable between in vitro- and in vivo-generated virus (Fig. 3A). No plaques were detected in samples that were incubated at 0°C. Thus, infectivity of the agent generated in in vitro reactions is dependent on a temperature-sensitive process and cannot be accounted for by the presence of the infectious input RNA. CsCl gradient analysis demonstrated that the in vitro-generated infectious agent was associated with the characteristic buoyant density of mature cardiovirus particles (1.33 g/cm^3) (Fig. 3B) (30). To conclusively demonstrate the generation of intact virus particles, it is imperative to perform electron-microscopic examination. To this end, the peak fraction from the CsCl gradient (Fig. 3B) was subjected to dialysis and concentration. These procedures, however, resulted in significant loss of virus, probably because of its adsorption to plastic surfaces (30). Thus, electron-microscopic studies are not technically feasible now. Similar to RNA synthesis, EMCV production was more Mg^{2+} concentration dependent than translation and was maximal at 3 to 3.5 mM MgCl$_2$ (data not shown). The infectivity of the in vitro-synthesized virus was already detectable between 2 and 4 h and reached maximum at 8 to 10 h of incubation (Fig. 3C). Subsequent incubation somewhat decreased infectivity. We also found that raising the creatine phosphate level from 10 to 20 mM stimulated virus production more than threefold (up to 5×10^7 to 6×10^7 PFU/ml virus titer; Fig. 3D), apparently by preventing the depletion of the nucleoside triphosphate pool (23). Virus yield reached a maximum at 10 μg of RNA/ml (Fig. 3E). Higher RNA concentrations resulted in reduced virus synthesis, most likely because of the inhibition of RNA replication (Fig. 2D).

PV replication both in vivo and in vitro occurs in association with membranes (6, 11, 22, 37). Although not investigated in this study, it is likely that EMCV replication in vitro is also a membrane-dependent process. Indeed, we failed to show EMCV RNA replication and virus synthesis in a rabbit reticulocyte lysate (unpublished observations), which synthesizes EMCV proteins fairly well (25, 27) but is deficient in membranous structures compared to Krebs-2 S10 (5, 36, 38).

In summary, we have described an efficient system for picornavirus synthesis. This system might be applicable to the studies of replication of pathogens other than EMCV. Indeed, PV synthesis was readily demonstrated in a Krebs-2 extract under conditions optimized for EMCV replication (unpublished observations). The system eliminates an important problem associated with cell membrane permeability and provides a means to study the effects of a wide spectrum of macromolecules and drugs on virus-specific processes.

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