Cell Cycle Position and Expression of Encephalomyocarditis Virus in Mouse Embryo Fibroblasts

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SUMMARY

Infection of mouse embryo fibroblasts in G\(_1\) or S phase with encephalomyocarditis virus gave different kinetics of viral RNA synthesis. In S phase cells, RNA synthesis was faster and reached higher levels than in G\(_1\) cells. Virus-specified proteins were fewer in G\(_1\) cells than in S cells during the early stage of the infection and c.p.e. in G\(_1\) cells appeared about 4 h later than in S cells. Addition of a cellular factor with ability to affect cell conformation had an inhibitory effect on viral RNA synthesis.

INTRODUCTION

It has been shown that macromolecular synthesis in intact cells can be affected by changes of cell form (Farmer et al., 1978; Benecke et al., 1978; Ben-Ze'ev et al., 1980; Wittelsberger et al., 1981). In virus-infected cells alteration of cellular structures that contribute to the conformational state of the cell can cause changes in virus expression (Rott et al., 1975; Reeve et al., 1975; Panem, 1977; Mallucci & Edwards, 1982). The structural organization of the cell can also be of significance in the establishment, maintenance and amplification of the antiviral state induced by interferon (Bourgeade & Chany, 1976, 1979). Alteration of the cell’s relationship to the substrate, changes affecting the cytoskeleton and cross-linking agents have been used to investigate these aspects (Folkman & Moscona, 1978; Rott et al., 1975; Reeve et al., 1975; Joseph & Oldstone, 1974; Menna et al., 1975). On the other hand, changes in the cell’s conformational and physiological state are naturally occurring events in cells which progress throughout the cycle.

In the work reported here we have examined aspects of encephalomyocarditis (EMC) virus infection in cells synchronously undergoing one cycle of division. We found that virus macromolecular syntheses were greater during S phase than at the G\(_1\) stationary stage and that viral RNA synthesis was decreased in the presence of a cell-produced protein factor with the ability to affect cell conformation.

METHODS

Cells. Fibroblasts from 14-day-old embryos from mice of the C57BL/6 strain (C57) were grown in Eagle’s medium (Wellcome) supplemented with foetal calf serum (Gibco) and synchronized during secondary and tertiary cultivation by seeding cells at half the density expected at confluence and by varying nutritional conditions according to a protocol described previously (Mallucci et al., 1983). Cultures of quiescent secondary cells and tertiary cells undergoing one cycle of division were grown in scintillation vials incubated in a 37°C water-bath to allow studies of kinetics. Progression through S phase was assessed by \(^{[3H]}\)thymidine pulse labelling (5 µCi/ml \([\text{Me}^{3}\text{H}]\text{TdR}, 18 \text{ to } 25 \text{ Ci}/\text{mmol}; \text{Amersham})\), and position in the cell cycle was assessed by measuring DNA content in a fluorescence-activated cell sorter after mithramycin staining. Cell number was assessed using a Fuchs-Rosenthal haemocytometer. Details of these methods and cell cycle kinetics have been reported elsewhere (Mallucci et al., 1980).

Preparation of factor. In some experiments cells were exposed soon after infection to a protein factor with the property of affecting cell conformation and of impeding progression through the cell cycle. Details of the methods involved in the preparation of the factor have been reported recently (Wells & Mallucci, 1983). Briefly, serum-free conditioned medium from C57 fibroblast cultures was passed through an XM50 Amicon ultrafiltration membrane to remove components greater than 50K mol. wt. and the filtrate was concentrated 100-fold by volume above a
PM10 membrane (mol. wt. cut-off 10K). The preparation was then dialysed against serum-free medium (SFM) to remove components smaller than 10K to 12K, adjusted to a protein concentration of 1 mg/ml and tested for physiological effects as reported elsewhere (Wells & Mallucci, 1983).

**Virus infection and labelling.** Purified EMC virus, a gift from Dr I. Kerr, was added at a multiplicity of infection of 10 p.f.u./cell, allowed to adsorb for 40 min and removed. After two washings, the cells were overlaid with medium containing 0-5% foetal calf serum and 5 μg/ml actinomycin D (AMD) (Calbiochem), a dose which reduced incorporation of exogenous uridine to levels close to background values within 10 min. The cultures were then returned to the water-bath to be pulse-labelled with 5 μCi/ml [5-3H]uridine (sp. act. 25 to 30 Ci/mmol; Amersham). Uptake into trichloroacetic acid (TCA)-soluble and acid-precipitable fractions was assessed as previously described (Wells & Mallucci, 1978). Antibodies to EMC virus, raised in guinea-pig, were a gift from Dr F. Brown. Anti-guinea-pig fluorescein conjugate was purchased from Nordic Immunological Laboratories (Maidenhead, U. K.).

In some experiments, cells were incubated in methionine-free medium and pulse-labelled for 30 min with 10 μCi/ml L-[35S]methionine (sp. act. 3600 Ci/mmol). The cells were then solubilized in 8 M-urea, 1% SDS, 4% 2-mercaptoethanol and samples from 10^6 cells were electrophoresed in SDS-polyacrylamide slab gels with a 10 to 15% gradient of acrylamide (acrylamide : bisacrylamide, 30:0.4) using the Laemmli (1970) buffer system. The gels were fixed, stained with Coomassie Brilliant Blue to visualize markers, dried under vacuum and exposed to X-ray film at −70 °C.

**RESULTS**

**Demonstration of cell cycle progression**

Incorporation of exogenous thymidine, fluorimetric quantification of DNA and cell number (see Methods) were used to assess cell progression through one cycle of division in uninfected cells. The data of Fig. 1 show that cells released from the G1 quiescent state entered S phase after a period of about 12 h, reached the G2 stage at about 22 h and divided by 28 to 30 h. The arrows in Fig. 1 indicate two positions of critical diversity within the cell cycle, during which we have investigated virus expression.

**EMC virus macromolecular syntheses**

Fig. 2 shows that the curve of uridine incorporation in EMC virus-infected cells during S phase was characterized by a sharp rise and a rapid decline while, by contrast, the curve of uridine incorporation during G1 evolved at a slower rate and continued over a longer period, attaining lower levels. Assessment of [3H]uridine in soluble pools in S and G1 cells during virus infection showed differences in the size and in the rate of increase (Fig. 3a). Fig. 3(b) shows, however, that these differences could not account for the differences in the incorporation of the labelled precursor into viral RNA, as the curves obtained by plotting the ratios of the soluble pools in S and G1 cells and the ratio of the incorporation in S and G1 differed. The differences in viral RNA synthesis during the two periods of the cell cycle were paralleled by differences in the time of appearance of virus-specified polypeptides. These were fewer in G1 than in S cells during the early stages of the infection (Fig. 4) but became expressed in equal number at a later stage (data not shown). The autoradiographic detection of viral proteins could be taken as a true representation of the events, as no differences in [35S]methionine pool sizes were found (data not shown). In accordance with the differences in the extent of viral RNA synthesis and in the expression of viral proteins, there was a time difference of about 4 h in the appearance of c.p.e. between cells in S phase and cells at the G1 stage (arrows in Fig. 2).

**Effect of factor**

The experiments of Fig. 5 show that addition of the 10K to 50K mol. wt. macromolecular fraction after the virus inoculum had been removed caused a marked reduction of incorporation of exogenous uridine into the acid-insoluble fraction both in S phase and in randomly growing cells, with minor effects in cells arrested in G1. The lower values of uridine incorporation attained by cells treated with the growth inhibitor were not due to an effect on either transport or uptake of the nucleoside (data not shown) and were therefore consistent with a reduction of viral RNA synthesis.
Cell cycle expression of EMC virus

Fig. 1. Progression of cells through one cycle of division. (a) Distribution of cells in G₁ (○); incorporation of [³H]thymidine into the TCA-insoluble fraction (30 min pulses) (▲). (b) Distribution of cells in S and G₂ (○); cell number (▲).

Fig. 2. Incorporation of [³H]uridine (30 min pulses) into the TCA-insoluble fraction in (a) G₁ and (b) S cells infected with EMC virus.

Fig. 3. (a) Size of TCA-soluble [³H]uridine pools in infected S (○) and in infected G₁ (▲) cells. Accumulation of [³H]uridine in the TCA-soluble fraction was measured from 2 to 3, 2 to 4, 2 to 5 and 2 to 6 h. (b) Ratios of TCA-soluble pools in infected cells in S and G₁ (○) and ratios of incorporations into the TCA-insoluble fraction (from incorporation data similar to those of Fig. 2) in infected cells (▲).

DISCUSSION

There have been several reports on virus expression during the cell cycle. In the case of DNA viruses it has been shown that replication of herpes- (Lawrence, 1971), adeno- (Kraft & Tischer, 1978), papova- (Pages et al., 1973; Thorne, 1973), and parvoviruses (Rhode, 1973; Siegl &
Gautschi, 1973) is increased during S phase, a time when there is greater availability of DNA polymerase enzymes. In the case of RNA viruses, while it is well established that integration of those which use the reverse transcriptase pathway requires cellular DNA synthesis (Temin, 1967; Weiss et al., 1982) little is known of the behaviour of the other viruses. Available information shows that the production of influenza virus antigens during abortive replication is not affected by the stage of the cell cycle (Long & Cooper, 1974; Lomniczi et al., 1977) and an extensive study on the full replicative cycle of fowl plague virus has shown that cell cycle variations of RNA polymerase II do not significantly affect the outcome of the infection (Brownson & Mahy, 1979). In contrast to these observations, there are reports that in HeLa cells the yield of poliovirus (Eremenko et al., 1972) and the yield of Coxsackie virus (Suarez et al., 1973) are higher in S phase than at other times of the cell cycle. Also, it has been reported that poliovirus RNA synthesis follows bimodal curves in late G2, M and during most of the G1 period (Eremenko et al., 1972).

In the study presented above, virus expression was investigated adopting a new approach to obtain cell synchrony. The use of mouse embryo fibroblasts was preferred to that of immortalized cells where the pre-replicative period is variable. Also, control of synchrony by
imposing a seed to yield ratio of 1:2 and by timing changes of nutritional conditions (Mallucci et al., 1983) was preferred to either serum starvation, which favours passage from G1 to G0, further increasing differences in duration of the pre-replicative period, or to the use of a thymidine block and metabolic drugs which perturb the biochemistry of the cell without abolishing G1 differentials after cells have divided. The result of this approach was a cell cycle system where a high degree of uniformity was maintained during each stage of the cycle without knowingly altering the physiological state of the cell. Infection with EMC virus of cells stationed in G1 and of cells in S phase showed clear differences in virus replication. In S cells viral RNA was synthesized faster and to a higher level than in G1 cells, and virus-specified proteins and c.p.e. were promptly expressed. Specific immunofluorescent staining showed that 61% of S phase cells and 68% of G1 cells contained viral antigens.

Although there is no information on the species of viral RNA being synthesized, our findings are in accord with observations that yields of poliovirus and Coxsackie virus in HeLa cells are higher during S phase (Eremenko et al., 1972; Suarez et al., 1973). Our data on viral RNA synthesis show that the differences during S and G1 concerned the extent but not modality of expression of the virus RNA curve. This is in contrast to the report that during the G1 phase that follows release from thymidine block, synthesis of poliovirus RNA in HeLa cells follows a bimodal curve (Eremenko et al., 1972), which in our view can be simply explained by the G0-G1 differentials of tumour cell lines and the presence in the post-mitotic population of both G1 and S cells synthesizing viral RNA at different rates.

It is not understood why virus replication varies with cell cycle position. Differences in biochemical state during the cell cycle may account for changes in virus expression, although different viruses may not be favoured (or impeded) by the same conditions. In the case of EMC virus, our results would point to differences of the transcriptional and translational system available in G1 and in S cells after an actinomycin D block. However, structural variations during the cell cycle, especially those concerning the cytoskeletal framework, should also be considered as there is evidence that the cytoskeleton plays a role in the translation of vesicular stomatitis virus (Cervera et al., 1981). We have not investigated whether the same holds true for EMC virus, but one point of interest from our results is that viral RNA synthesis was affected when cells were exposed to a cell-produced factor with ability to affect cell conformation and with a role, possibly, in the physiology of the cell (Wells & Mallucci, 1983).

In previous work (Mallucci & Edwards, 1982), we have discussed changes of virus expression when cellular parameters are experimentally altered. The evidence presented here shows that the outcome of a virus infection can be influenced by the physiological state of a cell as determined by cell cycle position and also by naturally occurring conditions that have an effect on such states, such as variations of levels of cellular factors which can affect cell conformation.
REFERENCES


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