ENCEPHALOMYOCARDITIS VIRUS-SPECIFIC POLYPEPTIDE p22 POSSESSING A PROTEOLYTIC ACTIVITY

Preliminary mapping on the viral genome

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1. Introduction

Our recent study has shown that a protein with M_r ~22 000, p22, which is present in cells infected with encephalomyocarditis (EMC) virus, is involved in the cleavage of virus-specific high-molecular-weight polypeptide(s) [1]. This polypeptide was probably first described in [2] and was suggested to represent a product encoded in the host cell genome. Some indirect evidence obtained in our laboratory, however, made it more likely that p22 was a viral genome-coded protein [3,4]. The results reported here clearly establish the viral origin of p22 and suggest that the nucleotide sequence coding for this polypeptide is located in the central part of the viral genome, most likely between the sequences coding for polypeptides F and E. A preliminary account of this work has been published [5] (in which the polypeptide corresponding to p22 was designated p23).

2. Methods

Cell-free synthesis of EMC virus-specific polypeptides was performed in micrococcal nuclease-treated S_{30} extracts from uninfected Krebs-2 cells essentially as in [6] except that dithiothreitol (2 mM) was present in the incubation mixtures. This modification was found to greatly improve the post-translational cleavage of the high-molecular-weight virus-specific precursor polypeptides [5]. Concentrations of K^+ and Mg^{2+} in the incubation mixture were 135 mM and 3.3 mM, respectively. Electrophoretic analysis of the products was performed either in 12.5% or in gradient (8–20%) polyacrylamide slab gels.

A comparison of the products of partial proteolysis of virus-specific proteins was performed as in [7] with some modifications. First-dimension electrophoresis was carried out in SDS-containing 12.5% polyacrylamide slabs. Strips, ~5 mm in width, were cut from the appropriate regions of unstained or Coumassie-stained/destained gels and were soaked in 2 changes of TED buffer (0.09 M Tris–HCl (pH 6.8), 0.001 M EDTA, 0.1% sodium dodecyl sulfate), for ⩾1 h in 20 vol. buffer each time. The strips were closely laid over a concentrating gel (3.3% polyacrylamide) containing a protease from *Streptomyces caespitosus* (300 μg/ml, Sigma). A layer, ~5 mm in height, of TED buffer containing the same protease (300 μg/ml), 10% glycerol and 0.01% bromophenol blue was put over the strip. The second-dimension electrophoresis was carried out in 17.5% polyacrylamide slab gels.

3. Results

A polypeptide comigrating with (in vivo) p22 upon SDS-polyacrylamide gel electrophoresis, is a major product of translation of EMC virus RNA in the cell-free system from Krebs-2 cells (fig.1A). This polypeptide is first detectable after a relatively long translation time, for example, after 70 min in fig.1B, that is later than the majority of the virus-specific in vitro products including polypeptide E appear. Since E is known to be encoded in the 3′-end-adjacent

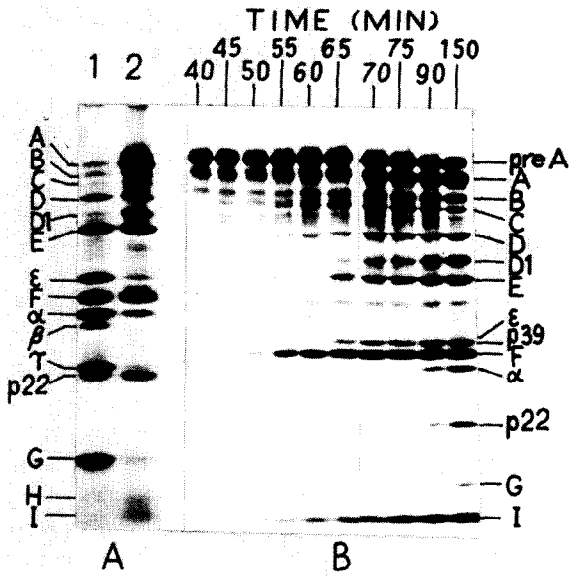

Fig.1. (A) A comparison of EMC virus-specific polypeptides synthesized in vitro and in vivo. In vivo products (slot 1) were labeled between 3.5 and 4.5 h infection at 37°C. Products of the in vitro synthesis (slot 2) were obtained upon incubation of the standard cell-free system for 3.0 h. Electrophoresis was performed in a 12.5% polyacrylamide slab gel. (B) Kinetics of the appearance of distinct polypeptides in extracts from uninfected cells programmed with EMC virus RNA. Incubation was carried out in the presence of 135 mM KCl, 3.3 mM $MgCl_2$ and 75 μg viral RNA/ml at 30°C. At the times indicated, aliquots were withdrawn from the cell-free system and fixed for electrophoresis. Electrophoresis was performed in a gradient (8–20%) polyacrylamide slab gel.

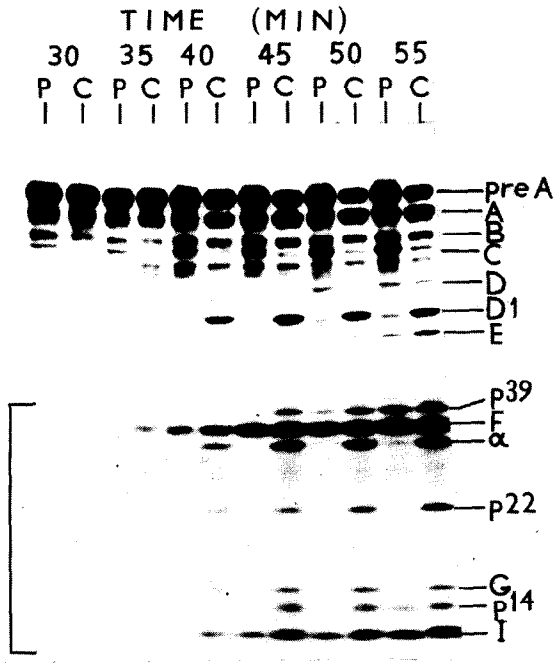

Fig.2. Kinetics of the synthesis of individual polypeptides in extracts from uninfected cells programmed with EMC virus RNA; a pulse-chase experiment. The conditions for cell-free synthesis were the same as in the legend to fig.1. At time intervals indicated, 2 aliquots were withdrawn from the cell-free system; one of them (p, pulse) was immediately fixed for electrophoresis, while the other (c, chase) was additionally incubated in the presence of cycloheximide (200 μg/ml). Total incubation time in the chased samples was 2.5 h. The exposure to the gel of the film used for printing the lower part of the figure was considerably longer than that used for printing the upper part of the figure.

part of the viral genome [8] this fact suggests that either the sequence coding for p22 is located in the terminal portion of the translatable segment of the genome or p22 is not terminal and is cleaved from a precursor polypeptide at a relatively low rate.

To discriminate between these two possibilities, experiments of pulse-chase type were performed. Duplicate samples were withdrawn at different time intervals from the standard protein-synthesizing system programmed with EMC virus RNA; one sample was fixed immediately, while the other was 'chased' in the presence of cycloheximide to allow processing of the precursor polypeptides that were formed by the time the drug was added. The electrophoretic pattern presented in fig.2 indicated that p22 first becomes apparent in a sample that was chased after protein synthesis had been allowed to proceed for 40 min. Polypeptides D1, α and G, the products of the cleavage of the capsid precursor polypeptide, were also formed concurrently with the accumulation of p22. Since the above results indicated that p22 was formed by processing of a precursor polypeptide, we attempted to identify this precursor. As a first step, the products of in vitro translation of EMC virus RNA were subjected to partial proteolysis with *Streptomyces caespitosus* protease and the products obtained were analyzed by electrophoresis. Although the unequivocal interpretation of the pattern obtained (fig.3) is hardly possible, the following results seem

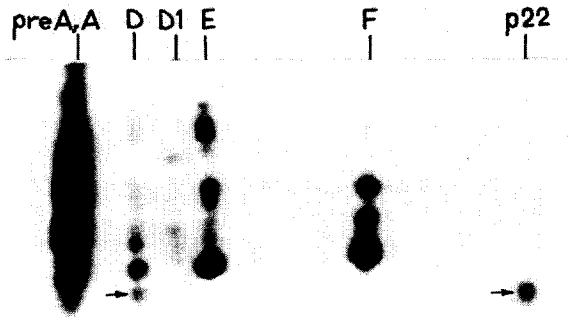

Fig.3. A comparison of products of partial proteolysis of EMC virus-specific polypeptides. For details see section 2.

to be pertinent to our problem. The peptides derived from protein E- and the D-derived peptides overlap almost completely; this finding could well be anticipated since E is known to originate from D by cleavage [8]. However, we would like to point out a low-molecular-weight peptide which is present in the hydrolysate of D but is absent from that of E (indicated by an arrow in fig.3). An apparently similar peptide is formed upon proteolysis of p22. This result is compatible with the assumption that the amino acid sequence of p22 is contained, at least partially, in protein D and that it is the sequence that is cleaved off upon the D → E conversion.

4. Discussion

The above results clearly establish the virus-specific origin of polypeptide p22. The strongest evidence for this notion is the ability of EMC virus RNA to direct the synthesis of p22 in a cell-free system. The identity of p22 formed in vitro to its in vivo counterpart can hardly be questioned. Both proteins not only have the same M_r but also appear to possess a similar enzymatic activity. The protease activity of in vivo p22 has been documented in [1] and a similar activity of the in vitro p22 is suggested by the results presented in fig.2: the times of first appearance of both p22 and the enzyme that cleaves the capsid precursor polypeptide are closely related (at first we had an impression that the appearance of the virus-specific protease might coincide with the synthesis of polypeptides F or I (cf. [5]) but examination of overexposed films prompted us to reconsider this suggestion).

The approximate position of the nucleotide sequence coding for p22 can also be deduced from kinetic experiments. Since all the major picornaviral proteins seem to be translated using a single initiation point on the viral RNA molecule, the time required for translation of a region of the viral genome is roughly proportional to the distance of this region from the site of initiation of translation. The times needed for translation of regions coding for proteins F, I p22, and E seem to increase in the order indicated (see fig.2). Thus, p22 is likely to be encoded in a region which lies between the sequences coding for F (I) and E. It should be noted that it was also suggested [9] that a virus-specific protease is encoded in a central portion of EMC virus genome and quoted their preliminary unpublished observations suggesting that this protease might correspond to p22.

A more precise mapping of p22 on the viral genome requires the identification of its precursor. The analysis of products of partial proteolysis of in vitro synthesized EMC virus-specific polypeptides (fig.3) suggests that the amino acid sequence of p22 may overlap, at least partially, with a portion of protein D, which itself is a cleavage product of C (see fig.4). Nevertheless, for unequivocal mapping of p22 classical fingerprinting analysis of these (and other) virus-specific proteins is desirable.

It should be noted that relatedness of D and p22, does not mean that all p22 molecules are necessarily formed by the cleavage of D. The kinetics of p22 formation and in particular its appearance, when the synthesis of C (and D) is not yet completed, may

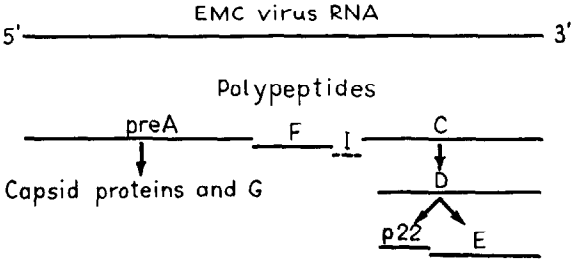

Fig.4. A proposed localization of the region coding for p22 within the genome of EMC virus.

rather indicate that p22 can be cleaved from the nascent uncompleted polypeptide still attached to the ribosome. It is evident that in this case proteins C and D will not be formed at all (cf. [2])

An important question concerns the nature of the protease responsible for the formation of p22 from its precursor. The present experiments are unable to answer this question.

Acknowledgements

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