The P2 and P3 Regions of the Poliovirus Genome are Preferentially Translated at Alkaline pH in Infected HeLa Cells

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(Accepted 16 November 1987)

SUMMARY
HeLa cells infected with poliovirus exclusively synthesized proteins coded by the P2 and P3 regions of the viral genome when they were placed in an alkaline medium lacking sodium ions. The amount of viral protein synthesized was augmented by increasing the concentration of KCl. Also in the presence of KCl, the cells continued synthesizing this altered pattern of proteins for longer times. This effect occurred in the presence of guanidine, it was reversible, and the normal pattern of poliovirus proteins reappeared when control medium was added, even when guanidine was present. These findings suggest that truncated viral RNA is not made under these conditions. Pactamycin and sodium fluoride, two known inhibitors of the initiation of translation, blocked protein synthesis in cells placed in alkaline medium, indicating the possibility that initiation at internal sites of the viral mRNA may take place under these conditions. Finally, the proteins synthesized were analysed by two-dimensional gel electrophoresis. The proteins migrated as authentic viral proteins indicating that if internal initiation takes place, at least some of these initiation events occur in phase with the initiation codon present in the poliovirus genome.

INTRODUCTION
Picornaviruses possess a single, long single-stranded RNA as their genome. This RNA functions as the only known mRNA from which all virus-coded proteins are translated (Carrasco & Castrillo, 1987); it is functionally monocistronic and has a single initiation site for translation located 743 nucleotides from the 5' end of the RNA (Dorner et al., 1982). The mRNA codes for a large polypeptide of $M_r$ 246000 (246K) that is cleaved to the mature proteins (Pallansch et al., 1984).

Translation of poliovirus RNA in vitro has been achieved and authentic viral polypeptides are synthesized under cell-free conditions (Smith & Carrasco, 1978). Analysis of the initiation sites involved in the translation of poliovirus RNA has led to the conclusion that in addition to the physiological initiation site used in infected cells, other initiation sites that are possibly artefacts can be used in vitro. Thus, using N-formyl-$\text{[35S]}$methionine, two initiation sites on the poliovirus genome were identified in HeLa cell lysates that were dependent on the magnesium concentration present (Celma & Ehrenfeld, 1975). Translation of poliovirus RNA in reticulocyte lysates also utilizes two initiation sites. In addition to the initiation site located near the 5' end of the RNA, a site located in the P3 region is used (Dorner et al., 1984). The use of this site is not a consequence of RNA degradation (Ehrenfeld & Brown, 1981; Dorner et al., 1984) and its functional significance, if any, remains unknown. Two initiation sites have also been identified in mengovirus RNA using L cell lysates (Degener et al., 1983).

In addition, many naturally occurring cellular (Perler et al., 1980; Vogeli et al., 1981; Yoo et al., 1982) and viral (Mardon & Varmus, 1983; Marsden et al., 1983; Herman, 1986; Shaw et al., 1983) mRNAs are known to initiate translation at internal AUG codons. Moreover, internal initiation has been demonstrated in mRNAs constructed artificially by recombinant DNA methods (Lomedico & McAndrew, 1982; Subramani et al., 1982; Peabody & Berg, 1986).
A large body of evidence indicates that the translation of picornavirus RNA in infected cells is modulated by the ionic concentrations present in the external medium (Carrasco & Castrillo, 1987; Castrillo et al., 1987). These conditions influence the initiation rates but the site of initiation is not altered and thus the proportion of translation of each region of the genome remains unaltered. We describe in this work how alterations of pH and the ionic conditions lead to an altered pattern of protein synthesis in poliovirus-infected cells. Analysis of the polypeptides synthesized suggests that internal initiation sites can be used in vivo under these conditions, giving rise to authentic viral proteins. Out of phase internal initiation would generate short peptides because of the probability of finding a termination codon, and these would not be easily detected by PAGE analyses.

METHODS

Cells and virus. HeLa cells were propagated in Petri dishes (Falcon) containing 10 ml Eagle’s medium as modified by Dulbecco (E4D) supplemented with 10% newborn calf serum (Gibco) and incubated at 37 °C in a 5% CO₂ atmosphere.

Poliovirus type 1 (Mahoney strain) was grown on HeLa cells in E4D medium supplemented with 2% newborn calf serum (E4D2). Cells and medium were collected and sedimented at 4000 r.p.m. for 20 min. The pellet was resuspended in distilled water, frozen and thawed three times and centrifuged at 4000 r.p.m. for 15 min. The supernatants from both centrifugations were mixed, and the concentration of virus was estimated by plaque assay.

Conditions of infection. HeLa cells grown in 24-well Linbro plates were infected with virus at the m.o.i. described in each experiment. After 30 min incubation at 37 °C, the medium was removed and 0-2 ml E4D2 without NaHCO₃ (E4D2–NaHCO₃) with 20 mM-HEPES pH 7-2 was added. Time of virus addition was considered as −30 min and zero time was taken to be when the virus was removed. At the times indicated in each experiment, E4D2 without NaHCO₃ and NaCl (E4D2–NaHCO₃–NaCl) medium (i.e. without sodium ions), plus 20 mM-HEPES pH 8-4 (8-4/− medium) was added to the cells. Incubation at 37 °C was continued until the end of the labelling period.

Analysis of proteins by SDS–PAGE. At the times post-infection (p.i.) indicated, 0-2 ml methionine-free medium (pH 7-2 or 8-4) and 5 μCi [³⁵S]methionine (1450 Ci/mmol; Amersham) were added to the cells during 1 h of labelling. At the end of the labelling periods, cell monolayers were washed with 1 ml phosphate-buffered saline and dissolved in 100 ml 0-02 m-NaOH plus 1% SDS and 200 μl sample buffer (62.5 mM-Tris-HCl pH 6-8, 2% SDS, 0-1 M-dithiothreitol, 17% glycerol and 0-024% bromophenol blue as indicator). Each sample was sonicated to reduce its viscosity and heated to 90 °C for 5 min. Five μl was applied to a 15% polyacrylamide gel and run overnight at 100 V.

The two-dimensional gel electrophoretic analyses using isoelectric focusing (IEF)/SDS–PAGE and non-equilibrium pH gel electrophoresis (NEPHGE)/SDS–PAGE in the first dimension followed the procedures described by O’Farrell et al. (1977), with some modifications (Bravo & Cells, 1982). [³⁵S]Methionine (0-5 mCi/ml) was added to the cells during 1 h of labelling. Fluorography of the gels was carried out with 2,5-diphenyloxazole/dimethyl sulphoxide (20% w/v).

The gels were placed in a microwave oven (Sharp R-8000E) and dried in only 6 min as described previously (Castrillo et al., 1986). The dried gels were exposed using RP X-ray films (MAFE). Densitometric profiles of the gel were made using a Chromoscan 3 (Joyce Loebi) microdensitometer.

Five μl aliquots of each sample were also precipitated by 1 ml TCA and filtered through GF/C glass fibre filters to determine the total protein synthesized. Dried filters were counted in an LKB 1219-Rackbeta liquid scintillation counter.

RESULTS

In previous experiments we have examined the effects of external cations on translation in poliovirus-infected HeLa cells (Castrillo et al., 1987). When a medium that lacks sodium ions is combined with elevated pH, the pattern of proteins synthesized in poliovirus-infected HeLa cells is drastically altered. Fig. 1 shows that under these conditions HeLa cells synthesized mainly proteins encoded by the P2 and P3 regions of the poliovirus genome. The synthesis of short peptides that would result from out of phase internal initiation would not be detected in these gels. Thus, the viral proteins VP0 and VP3 were virtually absent, whereas polypeptides migrating as 2C, 3 and 3CD were clearly recognized. Quantification of each polypeptide is shown in Table 1. The ratio of polypeptides coded in the P3 region increased as compared with those of the P2 region, suggesting that the P3 region was translated more frequently than the P2
Fig. 1. (a) Analysis by SDS–PAGE of the proteins synthesized in poliovirus-infected HeLa cells in different media. The m.o.i. was 20 p.f.u./cell 8.4/– medium was added 3 h p.i. The infected cells were incubated at 37 °C for 30, 60 or 90 min until the labelling period (lanes 2 to 4, respectively). Then, 5 μCi [35S]methionine was added for 1 h. The proteins were analysed as described in Methods. Lane 1, control poliovirus proteins 3 to 4 h p.i. in E4D2–NaHCO₃ plus 20 mM-HEPES pH 7.2. Poliovirus proteins are marked. (b) Processing map of the poliovirus polyprotein. The polyprotein (heavy line) is divided into three regions (P1, P2 and P3). The shaded area and the arrows show the regions of the genome in which internal initiation of protein synthesis might occur. Densitometric scans of the proteins synthesized in control medium (c) or 8.4/– medium (d) 4 to 5 h p.i. (1 to 2 h after change to the basic pH medium). The arrows show the poliovirus cleavage products in both media.

Table 1. Distribution of poliovirus proteins from the three regions of the poliovirus genome, synthesized in control and alkaline pH medium

<table>
<thead>
<tr>
<th>Region</th>
<th>Control</th>
<th>8.4/–</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>36%</td>
<td>–</td>
</tr>
<tr>
<td>P2</td>
<td>24%</td>
<td>28%</td>
</tr>
<tr>
<td>P3</td>
<td>40%</td>
<td>72%</td>
</tr>
</tbody>
</table>

* The m.o.i. was 20 p.f.u./cell. 8.4/– medium was added 3 h p.i. The cells were incubated at 37 °C for 1 h until the labelling period, 5 μCi [35S]methionine was then added for 1 h. The proteins were analysed as described in Methods. Control medium was E4D2–NaHCO₃ plus 20 mM-HEPES pH 7.2. The numbers are a relative quantification of densitometric profiles of the gel shown in Fig. 1(c).
Fig. 2. Analysis by SDS-PAGE of the proteins synthesized in poliovirus-infected HeLa cells in media without sodium ions, 8-4/- medium supplemented with different concentrations of KCl or NaCl. The m.o.i. was 20 p.f.u./cell. These media were added 3 h p.i. and the cells were incubated at 37 °C until the labelling period. Five μCi of [35S]methionine was added at 4 h p.i. for 1 h of labelling. The proteins were analysed as described in Methods. Lane 1, 8-4/- medium; lanes 2 to 6, 8-4/- medium with 30 mM-KCl, 60 mM-KCl, 10 mM-NaCl, 30 mM-NaCl and 60 mM-NaCl added, respectively.

region. The effect of monovalent cations on this phenomenon is shown in Fig. 2. Addition of NaCl above 30 mM reversed this effect and thus infected cells synthesized the normal ratios of viral proteins, whereas KCl did not have this effect. It is of interest to note that 60 mM-KCl increased the synthesis of the proteins observed with low pH medium without sodium. Cleavage of the precursor proteins to mature components seemed also to be partially inhibited by this medium.

To test whether RNA degradation was involved in the observed behaviour of translation we performed the experiment shown in Fig. 3. Cells were treated with guanidine, a known inhibitor of poliovirus RNA replication. Three h after infection, the cells were placed in medium of elevated pH without sodium or in control medium. The results indicate that even in the presence
Internal initiation in poliovirus mRNA

Medium  
G  
Time (h p.i.)

(a)  
7.2  
3  
(b)  
8.4/−  
3  
(c)  
7.2  
3

Guanidine (G) on the synthesis of poliovirus proteins in 8.4/− medium. The m.o.i. was 20 p.f.u./cell. 8.4/− medium was added at 3 h p.i. in the presence (c) or absence (b) of 3 mM-G. After 3 h (6 h p.i.), the medium was removed and the cells were incubated in control medium (pH 7.2 plus sodium ions). Controls were treated as indicated in (a). (d) Illustrates the order of treatments used in the experiment.

Fig. 3. Effect of guanidine (G) on the synthesis of poliovirus proteins in 8.4/− medium. The m.o.i. was 20 p.f.u./cell. 8.4/− medium was added at 3 h p.i. in the presence (c) or absence (b) of 3 mM-G. After 3 h (6 h p.i.), the medium was removed and the cells were incubated in control medium (pH 7.2 plus sodium ions). Controls were treated as indicated in (a). (d) Illustrates the order of treatments used in the experiment.

of guanidine the cells still synthesized the proteins coded by the P2 and P3 regions, but not those coded by P1. This suggests that no new viral RNA synthesis was necessary for this phenomenon to occur. Furthermore, if cells treated with guanidine and medium were replaced in control medium still containing guanidine, they restarted the normal pattern of protein synthesis and continued synthesizing normal poliovirus proteins. This suggests that the preexisting viral mRNA was not cleaved in basic pH medium because even in the presence of guanidine the normal pattern of poliovirus protein synthesis was re-established, suggesting that viral mRNA remained intact during the treatment period.
Fig. 4. Two-dimensional gel electrophoresis of the proteins synthesized in poliovirus-infected HeLa cells treated with 8-4/- medium. The m.o.i. was 20 p.f.u./cell. The 8-4/- medium was added 3 h p.i. One h later, [35S]methionine (0.5 mCi/ml) was added for 1 h of labelling. The proteins were analysed as described in Methods. The spots corresponding to the poliovirus proteins are indicated. Assignments for proteins 2B1, 2 and 3B1D are tentative (Wiegers & Dernick, 1981). Ac, cellular protein actin; the locations of the spots that appear only in control media are indicated: VP0, VP1, VP2, VP3, 2A and 2B.

Table 2. Effect of different inhibitors on protein synthesis in poliovirus-infected HeLa cells*

<table>
<thead>
<tr>
<th>Additions</th>
<th>Concentration</th>
<th>Control</th>
<th>8-4/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Pactamycin</td>
<td>10⁻⁷ M</td>
<td>20</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>10⁻⁶ M</td>
<td>4</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>10⁻⁵ M</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>NaF</td>
<td>20 mM</td>
<td>74</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>29</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>2 mM</td>
<td>13</td>
<td>34</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>5 × 10⁻⁷ M</td>
<td>102</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>10⁻⁶ M</td>
<td>78</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>10⁻⁵ M</td>
<td>8</td>
<td>18</td>
</tr>
</tbody>
</table>

* The m.o.i. was 20 p.f.u./cell. 8-4/- medium was added 3 h p.i. and the inhibitors were added 20 min later. Protein synthesis was determined as described in Methods between 4 and 5 h p.i. Radioactivities in poliovirus-infected HeLa cells in control medium and in 8-4/- medium, were 112796 and 37460 c.p.m., respectively.
Both pactamycin and sodium fluoride inhibited protein synthesis in poliovirus-infected HeLa cells in basic medium, suggesting that initiation was taking place under those conditions (Table 2). However, the extent of inhibition was higher in cells placed in normal medium. Since the affinity at which translation inhibitors bind to ribosomes can vary with different ionic conditions and pH, it might be that these differences reflected modifications of cell permeability to these compounds or modifications in their affinity constants for ribosome binding. Nevertheless, inhibition of translation by pactamycin and sodium fluoride does suggest, but does not definitively prove, that initiation takes place under alkaline pH.

To identify more accurately the proteins synthesized in poliovirus-infected HeLa cells, they were analysed by two-dimensional gel electrophoresis. Fig. 4 indicates that the proteins synthesized in basic pH medium migrated as authentic viral proteins (Wiegers & Dernick, 1981). Furthermore, it is now possible to conclude that proteins encoded in the P2 and P3 regions were synthesized under those conditions. As stated above it is possible that out of phase internal initiation occurs under these conditions, to generate short peptides that are not detected by these gels.

DISCUSSION

Previous experiments from our laboratory have examined in detail the effect of external cations in picornavirus-infected HeLa cells (Castrillo et al., 1987). We found that a decreased concentration of sodium in the culture medium leads to the suppression of viral protein synthesis. Other cations or modifications in pH had much less influence on translation. The combination of a modified external pH, plus different concentrations of monovalent cations leads to the preferential translation of the P2 and P3 regions.

There are several possible explanations for these findings. One is that when cells are placed in basic pH medium the viral RNA is fragmented, or truncated viral mRNA is newly synthesized and only this new RNA participates in translation. Another possibility is that this medium specifically inhibits initiation of translation and only polysomal run-off takes place. Finally, it could be possible that under these conditions the initiation site(s) utilized for translation on viral mRNA varies and one (or more) internal initiation site(s) is now used. RNA degradation does not seem to be involved in this phenomenon, because it is reversible even in the presence of guanidine.

The possibility that new rounds of translation do not occur in low pH medium and only polysomal run-off takes place has been investigated by means of translation inhibitors. We made use of two known inhibitors of the initiation phase of protein synthesis, i.e. pactamycin and sodium fluoride, and also an inhibitor of elongation, cycloheximide. However, the results obtained with these inhibitors must be interpreted with caution because we do not know whether their ability to block translation under the altered ionic conditions remain similar to the control. It is possible that both polysomal run-off and internal initiation are taking place in alkaline medium.

An argument against run-off taking place exclusively under low pH conditions comes from the kinetics of this phenomenon; i.e. after 30 min incubation in this medium, for the next 30 min the infected cell still makes the same amount of polypeptide 2C, which is encoded by the P2 region (see Fig. 1). After 1 h of incubation, protein 3CD (encoded in the P3 region) is almost exclusively synthesized, suggesting that at later times only the internal site located in the P3 region might operate. It is possible that the poliovirus initiation site is closed under those ionic conditions and that other internal AUG codons are used as initiation sites. In fact, experiments in vitro on the translation of foot-and-mouth disease virus genome have indicated that magnesium ions play a major role determining which of two different AUG codons is used for the initiation of translation (Sangar et al., 1987).

The excellent technical assistance of Ms M. A. Ramos is acknowledged. F.I.S.S. and CAICYT are acknowledged for financial support. J.L.C. is the holder of a CSIC post-doctoral fellowship. A.U. is holder of a Gobierno Vasco fellowship.
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(Received 3 June 1987)