Formation of subviral particles by in vitro translation of subgenomic poliovirus RNAs

Jan P. M. Jore,1* Gerrit Veldhuisen,1 Peter H. Pouwels,1 Albert Boey6,2 Raf Vrijsen2 and Bart Rombaut2

1Medical Biological Laboratory TNO, P.O. Box 45, 2280 AA Rijswijk, The Netherlands and 2Department of Microbiology and Hygiene, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium

Rabbit reticulocyte lysates were programmed with either RNA extracted from purified poliovirus or a mixture of mRNAs encoding the capsid precursor, P1, and proteinase 3CD. In both cases, 14S subunits were formed at 30 °C and empty capsids at 37 °C. Both the 14S subunits and empty capsids had the expected polypeptide composition and neutralization epitopes. It is concluded that the proteinase 3CD gene is the only viral genetic information needed for the correct processing of P1 and the formation of 14S subunits, and their assembly into antigenically correct empty capsids.

Introduction

The genome of picornaviruses consists of a positive-stranded RNA molecule encoding a single polyprotein. All functional structural, as well as non-structural, proteins are derived from this polyprotein by a cascade of proteolytic cleavages. The common precursor of the structural proteins, P1, is produced by the action of virus-encoded proteinases like 2A in the case of poliovirus (Toyoda et al., 1986). In encephalomyocarditis (EMCV) and foot-and-mouth disease (FMDV) viruses, proteinase 3C is responsible for the cleavage of P1 into the structural proteins VP0, VP1 and VP3 (Parks et al., 1986; Vakharia et al., 1987; Clarke & Sangar, 1988). In poliovirus, however, this function is associated with proteinase 3CD rather than 3C (Jore et al., 1988; Ypma-Wong et al., 1988).

Subviral particles consisting of VP0, VP1 and VP3 are formed in all picornavirus-infected cells (Boege et al., 1986; and references therein). These include 14S subunits (pentamers of the basic structural unit consisting of one molecule of each of the capsid proteins) and procapсидs; the latter particles are made of 60 structural units, and differ from mature virions in lacking RNA and containing VP0 instead of VP4 and VP2.

Mature poliovirus virions express four neutralizing antigenic sites (1, 2, 3A and 3B; Page et al., 1988). Native procapсидs express the same four sites whereas 14S subunits possess only three (the fourth site spans the boundary between two 14S subunits and is formed as a result of capsid assembly; Rombaut et al., 1990a); in addition, 14S subunits have at least one of the epitopes of the H antigen (i.e. heat-denatured virions). This latter epitope is called H1 to distinguish it from H2 epitopes, which are present on heat-denatured virions only (Rombaut & Boey6, 1991).

It has recently been shown that poliovirus procapсидs, when protected against thermal denaturation by chemical stabilizers such as WIN 51711, elicit the production of high neutralizing antibody titres in mice (Rombaut et al., 1990b). Poliovirus 14S subunits spontaneously assemble into empty capsids at 37 °C in vitro (Putnak & Phillips, 1981). When assembly occurs in the presence of cellular components (Rombaut et al., 1983) or WIN 51711 (Rombaut & Boey6, 1991), these empty capsids also possess all the antigenic sites present on native procapсидs and virions.

Obviously 14S subunits, native procapсидs extracted from infected cells and empty capsids assembled in vitro qualify as potential immunogens for use in vaccines, although as long as the subviral particles have to be purified from infected cells the cost would be prohibitive. The solution to this problem is to try and produce immunogenic particles by means of recombinant DNA techniques. In the case of poliovirus, the first question to be answered is which genome elements are required in addition to that which encodes P1, the precursor of the capsid proteins. Since two Gln–Gly bonds within P1 (i.e. those at the VP0–VP3 and VP3–VP1 boundaries) must be cleaved, the 3CD proteinase is also required. We therefore investigated whether a cell-free translation system programmed with a mixture of two subgenomic...
RNAs representing the PI- and proteinase 3CD-encoding regions of the genome could synthesize 14S subunits and empty capsids.

Methods

Preparation of viral RNA. The purification of type 1 (Mahoney) poliovirus (Rombaut et al., 1985) and the extraction of vRNA from purified virions (Phillips & Emmert, 1986) were essentially as described.

Plasmids and in vitro transcription. Plasmid pLOP315 contains the proteinase 3CD coding sequence starting at nucleotide 5438 preceded by the translation start codon and the T7 promoter. Plasmid pLOP324 contains nucleotides 743 to 3386 of the PI coding region followed by a translation stop codon and preceded by the T7 promoter. Both plasmids as well as the conditions for in vitro transcription of the plasmid DNA have been described (Jore et al., 1988). Briefly, DNA was linearized with an appropriate restriction enzyme and transcribed in a reaction mixture composed of 40 mM-Tris-HCl pH 8-0, 15 mM-MgCl₂, 10 mM-MMT, 1 mM each of ATP, CTP and UTP, 0-2 mM-GTP, 1 mM-m-5-Gppp(5')Gp and 100 μg/ml bovine serum albumin (BSA). For each μg of DNA, 10 to 20 units T7 RNA polymerase and RNasin were added. During the transcription reaction (20 min at 37 °C), GTP was added to a final concentration of 1 mM in two steps. The reaction was stopped by extraction with phenol. RNA was purified by gel filtration of the inorganic phase and precipitation with ethanol, and the RNA precipitate was dissolved in sterile water.

In vitro translation. Translation in a rabbit reticulocyte lysate was essentially as described (Jore et al., 1988), but with the substitution of potassium acetate for KCl. Briefly, nuclease-treated rabbit reticulocyte lysate (Promega Biotec) was supplemented with 50 μM-amino acids (minus methionine), 10 mM-DTT, [³⁵S]methionine (10 μCi/25 μl) and RNA as indicated. Incubation was at 30 °C.

Analysis of subviral particles

(i) Sucrose gradients. Samples (25 to 50 μl) from in vitro translation reactions were diluted fourfold in 20 mM-HEPES pH 6-9, 0-2 mM-KCl, 5 mM-MgCl₂, 5 mM-2-mercaptoethanol and loaded onto 12 ml 5 to 30% or 14 ml 15 to 30% sucrose gradients in the same buffer. The 5 to 30% gradients were centrifuged for 8 h at 197000 g and 20 °C to isolate particles < 14S and 2 h for larger particles; the 15 to 30% gradients were centrifuged for 17 h at 64000 g and 4 °C. After centrifugation, the gradients were fractionated and acid-precipitable counts determined according to Palmenberg (1982). To prevent loss of 14S, and possibly larger particles due to stickiness (Onodera & Phillips, 1987), pre-precipitation of tubes with PBS supplemented with 1% foetal calf serum and 0-05% Tween 20 (2 h at 37 °C) proved to be effective.

(ii) Gel electrophoresis. Samples (100 μl) from gradient fractions were diluted with 300 μl sterile water containing 10 μg of BSA, and proteins were precipitated by the addition of 1 ml acetone. After overnight incubation at −20 °C, precipitates were collected by centrifugation and dissolved in 15 μl sample buffer (Laemmli, 1970). The samples were run on 12.5% homogeneous, 0-8 mm thick polyacrylamide gels as described (Jore et al., 1988). When samples from translation mixtures were to be analysed directly, i.e. without prior fractionation on gradients, they were not concentrated by acetone precipitation. Further processing of gels was as described (Jore et al., 1988).

(iii) Immunological characterization. Eight monoclonal antibodies (MAbs) directed against the three different immunodominant, neutralizing sites of poliovirus type 1 were used (Page et al., 1988; Rombaut et al., 1990a). As the MAbs directed against the same site always yielded identical results we used the data from one against each site: MAbs 36-5h2 (site 2), 424 (site 3A) and 35-2b6 (site 3B). The non-neutralizing MAbs 39-5d6 and 39-5b4 were also used; both recognize H antigen, but not native virions or procapsids. The site recognized by MAbs 39-5d6 is called H1, which is expressed equally well on 14S subunits and heated virus (identical immunoprecipitation titres for 39-5d6 against both antigens). In contrast, antibody 39-5b4 recognizes 14S subunits poorly, the immunoprecipitation titre against 14S subunits being 1000-fold lower than against heated virus (Rombaut et al., 1983) and, at a 10⁻² dilution, there is no reaction with 14S subunits. The binding site of 39-5b4 is called H2.

Results

In vitro translation of full-length vRNA

RNA prepared from purified virions was used to programme a messenger-dependent rabbit reticulocyte lysate, which was then incubated at 30 °C. Samples were collected at various times up to 24 h and analysed by sucrose gradient ultracentrifugation (Fig. 1); radioactivity was found in a broad peak around 5S. This material, the radioactivity of which failed to increase after 3 h of incubation, consisted of a complex mixture of polypeptides, some of which migrated close to the position of the capsid proteins (Fig. 1). However, these polypeptides did not exhibit the epitopes of capsid proteins because none was precipitated by an antiserum raised against disrupted virus and able to bind all the capsid proteins and their precursors (Vrijisen et al., 1980) (results not shown).

A second peak of radioactive material with a sedimentation coefficient of 14S was formed slowly. The protein composition of this material was VP0, VP1 and VP3 as expected, with minor admixtures of proteinase 3CD (Fig. 1).

It has been reported that 14S subunits are formed in the infected cell at 30 °C, but only assembled into virions after the temperature is raised to 37 °C (Rombaut et al., 1990d). We therefore examined whether the 14S subunits formed at 30 °C in a vRNA-programmed reticulocyte lysate would assemble similarly after shift-up to 37 °C. One portion of a lysate was shifted up after 3 h, and another portion after 15 h. In both cases, incubation was continued for 1 h at 37 °C. In controls, incubation was continued at 30 °C for the same period. The results are shown in Fig. 2.

As can be seen, only a trace of empty capsid material (peak fraction 17) was formed after 16 h at 30 °C (tracing III), but the amount of empty capsids increased greatly...
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14S
24 h
x 19h
~ ~
10~
10~
3CD
VP0
VP1
VP3
~31h
0 0 20 40 14S 5S
Bottom Top
Fraction
Fig. 1. Formation of subviral particles in a lysate programmed with full-length poliovirus vRNA. (a) A rabbit reticulocyte lysate programmed with 20 μg/ml poliovirus RNA was incubated at 30 °C and 25 μl samples were taken at the times indicated. The samples were centrifuged through 5 to 30% sucrose gradients for 8 h at 197000 g. Fractions (250 μl) were collected, of which 20 μl was used for 35S determination as indicated in Methods. Haemoglobin (4.2S) peaked in fractions 31 and 32. The position of the 14S marker was determined by running a preparation of purified, labelled 14S subunits on a separate gradient. Fractions 17 (14S) and 30 (5S) from the gradient marked 15 h were precipitated with acetone. (b) Polypeptide patterns obtained after PAGE and fluorography.

Bottom Top 14S
Fraction
Fig. 2. Formation of subviral particles and the effect of shift-up from 30 °C to 37 °C. (a) A rabbit reticulocyte lysate was programmed with 20 μg/ml poliovirus vRNA. Samples (25 μl) were taken after 4 h at 30 °C (tracing I), 3 h at 30 °C plus 1 h at 37 °C (II), 16 h at 30 °C (III), or 15 h at 30 °C plus 1 h at 37 °C (IV). The samples were subjected to centrifugation through 5 to 30% sucrose gradients for 2 h at 197000 g. Fractions (250 μl) were collected, of which 20 μl was used for 35S determination as described in Methods. Labelled, purified 14S subunits and 80S particles (i.e. virions heated for 20 min at 56 °C) served as external markers. Aliquots from gradient fractions (tracing IV) were precipitated with acetone and separated on a polyacrylamide gel. (b) Peak fractions 14S and 74S after fluorography. Lane M shows the polypeptide composition of the lysate before fractionation.

after 1 h at 37 °C; simultaneously, the amount of slow sedimenting material decreased (tracing IV). On the other hand, no empty capsids were formed upon incubation at 37 °C without a preincubation at 30 °C (results not shown), or when the temperature was shifted up after only 3 h at 30 °C (tracing II). The results suggest that at 37 °C empty capsids are rapidly assembled from 14S subunits, provided these are allowed to accumulate sufficiently. This requirement presumably reflects the need for a threshold concentration of 14S subunits for assembly to occur (Rombaut et al., 1991). Fig. 2 also shows the polypeptide composition of peak fractions from the gradient shown in tracing IV (incubation for 15 h at 30 °C and 1 h at 37 °C). As can be seen, the presumed empty capsids had the expected VP0, VP1 and VP3 composition, with minor admixtures of proteinase 3CD. Before fractionation the lysate contained a considerable amount of non-structural proteins as well as the capsid proteins. Two observations give some indication as to the fate of these non-structural proteins. First, upon inspection of the complete fluorogram the non-structural proteins appeared to be more or less evenly distributed over the whole gradient, whereas the structural proteins appeared in distinct peaks. Second, a pellet was often present after centrifugation, the polypeptide composition of which was rich in non-structural proteins. However, we cannot explain why non-structural proteins smear or pellet preferentially.

In vitro translation of subgenomic RNAs

Subgenomic RNAs encoding P1 and proteinase 3CD separately were prepared as described in Methods and
cotranslated in a reticulocyte lysate. After 15 h at 30 °C, incubation was continued for 1 h at 37 °C (or at 30 °C in the control). The lysates were analysed by sucrose density gradient centrifugation. As can be seen in Fig. 3(a) and (b), both 5S and 14S material (tracing I), but no empty capsids (tracing III), accumulated at 30 °C. After shift-up to 37 °C, the radioactivity in the 5S and 14S regions decreased (compare tracings I and II), and empty capsids were formed (compare tracings III and IV). Moreover, tracings III and IV show counts at the bottom of the gradient which did not appear on the gel; the identity of this material is not clear. The identification of the 14S subunits and empty capsids was confirmed by their VP0, VP1 and VP3 polypeptide composition (Fig. 3c and d).

Antigenicity of subviral particles synthesized in the reticulocyte lysate

The antigenicity of 14S subunits and empty capsids from in vitro translation experiments was ascertained by micro-immunoprecipitation using MAbs (Vrijsen et al., 1983). The MAbs used were directed against sites 2, 3A, 3B, H1 and H2 (see Introduction); site 1 was disregarded, as it is not very immunogenic in inbred mice (Page et al., 1988). Table 1 shows the results. The 14S subunits, originating from in vitro translation of either poliovirus vRNA or a mixture of P1 and proteinase 3CD RNAs, showed the same (sites 2, 3A and H1) antigenic conformation as native 14S subunits isolated from infected HeLa cells. The empty capsids showed the (2, 3A and 3B) antigenicity of native procapsids (Rombaut et al., 1990a).

Discussion

The formation of subviral particles in reticulocyte lysates programmed with genomic RNA has been reported for several picornaviruses. 14S subunits are formed with the
Formation of subviral poliovirus particles

Table 1. Antigenicity of subviral particles

<table>
<thead>
<tr>
<th>Translation conditions in reticulocyte lysate</th>
<th>Fig. showing sedimentation profile</th>
<th>Particle</th>
<th>Input radioactivity precipitated by MAb (%)</th>
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<tbody>
<tr>
<td>Origin of mRNA</td>
<td>Duration and temperature</td>
<td></td>
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<tr>
<td>vRNA</td>
<td>16 h, 30 °C</td>
<td>1</td>
<td>14S subunits</td>
</tr>
<tr>
<td>vRNA</td>
<td>15 h, 30 °C</td>
<td>2</td>
<td>Empty capsids</td>
</tr>
<tr>
<td>Subgenomic, P1 + 3CD</td>
<td>16 h, 30 °C</td>
<td>(tracing IV)</td>
<td>14S subunits</td>
</tr>
<tr>
<td>Subgenomic, P1 + 3CD</td>
<td>15 h, 30 °C</td>
<td>(tracing I)</td>
<td>Empty capsids</td>
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vRNAs of EMCV, Mengo virus and FMDV (Palmenberg, 1982; Grubman, 1984; Grubman et al., 1985; Boege et al., 1986), and empty capsids with the vRNAs of EMCV and FMDV (Palmenberg, 1982; Grubman, 1984; Grubman et al., 1985). The reason there has not been a similar report for poliovirus is possibly the much lower efficiency with which poliovirus vRNA is translated in a rabbit reticulocyte lysate (Jackson, 1989). However, the high protein synthesis activity of present-day commercially available lysates, like those which have been used here, may, at least partially, have abolished this problem.

In this study we have shown the formation of both 14S subunits and empty capsids in reticulocyte lysates programmed with poliovirus genomic RNA. Although 14S subunits accumulate at 30 °C, empty capsids appear only after shift-up to 37 °C, in agreement with the known temperature requirements of assembly (Putnak & Phillips, 1981). The subviral particles synthesized in the lysates possess all known neutralization epitopes of native 14S subunits and procapsids (Table 1). The presence of antigenic site 3B on the empty capsids shows that the 14S subunits assembled correctly (Rombaut et al., 1990a). It should be emphasized that the acquisition of N antigenicity is not an automatic consequence of assembly; it requires an as yet unidentified 'antigenicity conferring activity', present in poliovirus-infected HeLa cells, which can be mimicked by WIN 51711 (Rombaut & Boeyé, 1991). Our results show that this activity is also present in reticulocyte lysate.

14S subunits and empty capsids were also formed when the reticulocyte lysate was programmed with a mixture of RNA transcripts encoding P1 and proteinase 3CD, and the temperature requirements were the same as when the lysate was programmed with vRNA. Again, the 14S subunits and empty capsids were antigenically correct (Table 1). We conclude that N antigenic particles can be synthesized in a cell-free system programmed with the P1 and proteinase 3CD portions of the genome only. Thus, the system has been reduced to the absolute minimum. Avoiding the simultaneous expression of superfluous poliovirus-encoded proteins is important as they may have adverse effects. It has been reported that attempts to grow poliovirus–vaccinia virus recombinants for vaccine purposes were thwarted by expression of proteinase 2A (Turner et al., 1989; Jewell et al., 1990).

Is a subunit vaccine based on 14S subunits and/or empty capsids feasible? Although full purification of these particles is now possible (Rombaut et al., 1990c), their large-scale production in either infected cells or cell-free lysates would be prohibitively expensive. However, three other systems are being explored, i.e. adenovirus recombinants in mammalian cells, baculovirus recombinants in insect cells (Urakawa et al., 1989), and autonomously replicating expression vectors in the yeast Saccharomyces cerevisiae. In the yeast expression system, we have achieved relatively high levels of expression of poliovirus VP2 (Verbakel et al., 1987), P1 (J. M. A. Verbakel et al., unpublished results), as well as synthesis of active proteinase 3CD (J. P. M. Jore et al., unpublished results). Recently, we have been able to show that joint expression of P1 and proteinase 3CD in S. cerevisiae leads to the production of processed P1; the possible formation of subviral particles is being studied.

Note. After the submission of this manuscript a paper concerning the assembly of virus particles by recombinant vaccinia virus expressing P1 and P3 was published (Ansardi et al., 1991). Although one may presume that the particles described are identical to the particles described here, immunological data to support this assumption are not available.

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References


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