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Replicase activity of purified recombinant protein P2 of double-stranded RNA bacteriophage ϕ6

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In nature, synthesis of both minus- and plus-sense RNA strands of all the known double-stranded RNA viruses occurs in the interior of a large protein assembly referred to as the polymerase complex. In addition to other proteins, the complex contains a putative polymerase possessing characteristic sequence motifs. However, none of the previous studies has shown template-dependent RNA synthesis directly with an isolated putative polymerase protein. In this report, recombinant protein P2 of double-stranded RNA bacteriophage ϕ6 was purified and demonstrated in an *in vitro* enzymatic assay to act as the replicase. The enzyme efficiently utilizes phage-specific, positive-sense RNA substrates to produce double-stranded RNA molecules, which are formed by newly synthesized, full-length minus-strands base paired with the plus-strand templates. P2-catalyzed replication is also shown to be very effective with a broad range of heterologous single-stranded RNA templates. The importance and implications of these results are discussed.

Keywords: bacteriophage ϕ6/double-stranded RNA virus/purified RNA-dependent RNA polymerase/replication *in vitro*

Introduction

Double-stranded RNA viruses are known to infect different hosts from prokaryotes to higher eukaryotes. Some of these viruses cause severe infectious diseases affecting humans and economically important animals and plants (Fields and Knipe, 1990). In spite of notable variations in structural organization and host specificity, practically all dsRNA viruses share a common replication strategy. Upon entry, the virion in most cases is converted into a core particle that functions as a transcriptase producing positive-sense, single-stranded RNAs using the genomic dsRNAs as templates. The ssRNAs formed in the viral core are extruded into the cytoplasm where they serve as the messengers directing protein synthesis. The same ssRNAs are also fully active as templates for the synthesis of complementary minus-strands (replication). This process occurs inside the newly assembled core particles and is driven by the viral polymerase. After replication, the minus-strand RNA replica remains associated with the plus-strand template reconstituting the genomic dsRNA. The core particles bearing the dsRNA can either support additional rounds of transcription or alternatively undergo further maturation to form infectious progeny particles. Both replication and transcription of dsRNA viruses thus depend on the virus-encoded polymerase activities and occur in the interior of a large protein complex. Curiously, among several proteins building up the polymerase complex of any dsRNA virus, only one polypeptide species can be found that contains several characteristic sequence motifs conserved across RNA polymerases (Koonin *et al.*, 1989; Bruenn, 1991, 1993). The putative polymerase subunit is believed to be responsible for the catalysis of both minus- and plus-strand synthesis.

Several experimental systems have so far been developed to shed light on the molecular principles that govern the RNA metabolism within the polymerase complexes of dsRNA viruses. The first of those systems was *in vitro* transcription based on purified viruses or core particles derived from virus preparations and thus already containing dsRNA templates. Such systems have been reported for reovirus (Joklik, 1974), bacteriophage ϕ6 (Van Etten *et al.*, 1973; Partridge *et al.*, 1979), infectious pancreatic necrosis virus (Cohen, 1975), yeast virus-like particles (VLPs) (Herring and Bevan, 1977) and many others. These approaches have given detailed information on the mechanisms and regulation of ssRNA synthesis. However, the particle-based reaction did not allow one to address questions on replication.

This was approached using isolated virus intermediates containing packaged ssRNA (see, for example, Fujimura *et al.*, 1986) and empty polymerase particles. In the case of phage ϕ6, empty recombinant polymerase complex particles (PC) were found to be active in the RNA packaging, replication and transcription *in vitro* (Gottlieb *et al.*, 1990; Olkkonen *et al.*, 1990; Van Dijk *et al.*, 1995). Two other systems, the yeast VLPs and rotavirus open-core particles, were demonstrated to support replication of exogenous virus-specific ssRNA templates (Fujimura and Wickner, 1988; Chen *et al.*, 1994).

Considerable progress has been made recently in dissection of the dsRNA virus RNA-synthesizing machinery using recombinant technology. One of the most striking examples is the bacteriophage ϕ6, a complex dsRNA virus of *Pseudomonas syringae* (Vidaver *et al.*, 1973). The ϕ6 genome consists of three dsRNA segments: large (L), medium (M) and small (S) (Semancik *et al.*, 1973; Van Etten *et al.*, 1974). The entire polymerase complex of ϕ6 phage is composed of four protein species P1, P2, P4 and P7, all encoded on the L segment (Mindich *et al.*, 1988). P1 is the major structural protein assembled into a dodecahedral shell with the rest of the protein subunits most probably being located at the 5-fold symmetry positions (Butcher *et al.*, 1997; de Haas *et al.*, 1999). Studies on individual recombinant proteins and genetically engineered incomplete PC particles have contributed to understanding of the functions of P4 and P7. P4 is a

hexameric NTPase responsible for the plus-strand RNA packaging (Gottlieb *et al.*, 1992a; Frilander and Bamford, 1995; Paatero *et al.*, 1995; Juuti *et al.*, 1998; Paatero *et al.*, 1998), while P7 serves as a protein cofactor necessary for the efficient packaging reaction (Juuti and Bamford, 1995, 1997). P2, thus far the least studied PC protein, has been identified as a putative polymerase subunit using computer analysis of the protein sequence (Koonin *et al.*, 1989; Bruenn, 1991). This conclusion was further supported by the biochemical studies on different protein-deficient PC particles (Gottlieb *et al.*, 1990; Casini *et al.*, 1994; Juuti and Bamford, 1995).

One of the fundamental questions still remains unanswered. Can the isolated putative polymerase of a dsRNA virus catalyze template-dependent RNA synthesis alone or is the synthesizing activity strictly associated with the particle-bound polymerase protein? For two putative polymerases, those from the bluetongue virus (BTV) and the infectious bursal disease virus, some polymerase activity has been found in the crude extracts of the cells producing the corresponding recombinant proteins (Urakawa *et al.*, 1989; Macreadie and Azad, 1993). However, these reports have not provided any evidence for direct association of the observed polymerase activities with the proteins of interest. On the other hand, the purified rotavirus putative polymerase VP1 failed to replicate RNA (Patton, 1996) unless supplemented with at least one additional protein VP2 (major structural protein) (Zeng *et al.*, 1996; Patton *et al.*, 1997).

This report addresses the question of the RNA-synthesizing activity of the isolated putative polymerase P2 of the bacteriophage ϕ6. Using an *in vitro* assay system we show that the highly purified recombinant P2 exhibits RNA-dependent RNA polymerase activity catalyzing *de novo* initiation and elongation of replication and producing full-length copies on a range of ssRNA templates. These findings represent the first direct demonstration of the RNA polymerase activity catalyzed by an individual protein derived from the polymerase complex of a dsRNA virus.

Results

Expression and purification of the recombinant P2 protein

To produce recombinant protein P2 in *Escherichia coli* cells, the expression plasmid pEM2 was constructed, which contained a PCR copy of the P2 gene cloned into a vector downstream of the T7 promoter and a strong ribosome-binding site. The sequence of the entire P2 insert was determined. A single amino acid change, Ile457 to Met, was found when compared with the published protein sequence (DDBJ/EMBL/GenBank accession No. AAA32355). The methionine codon at this position was also found in the plasmid pLM687, which had been used as a template for gene amplification. This plasmid contains the cDNA copy of the entire large genomic segment of the ϕ6 phage and it has been employed previously in reverse genetics experiments to obtain viable virus particles (Mindich *et al.*, 1994). Thus, the observed change does not impair P2 activity in the virus.

Strain BL21(DE3/pEM2) bearing the constructed plasmid produced a detectable amount of the soluble P2 protein at 15°C as judged by SDS–PAGE and immunoblotting analysis (Figure 1, lanes 1–3). Expression at 37°C led to much higher yield of P2, with almost all of the synthesized protein in an insoluble form (not shown). The protein purification strategy was based on the assumption that P2 being a putative polymerase might have affinity to resins routinely used for purification of the enzymes involved in nucleic acid metabolism. Indeed, P2 was retarded by Cibacron Blue agarose and heparin agarose eluting from the columns at ~500 and 400 mM NaCl, respectively (Figure 1, lanes 4–5). The final purification step was carried out on an anion-exchange column (Resource Q), from which P2 eluted as a single peak at ~90–100 mM NaCl (Figure 1, lane 6). The estimated yield of the purified protein was ~1 mg per liter of the bacterial culture.

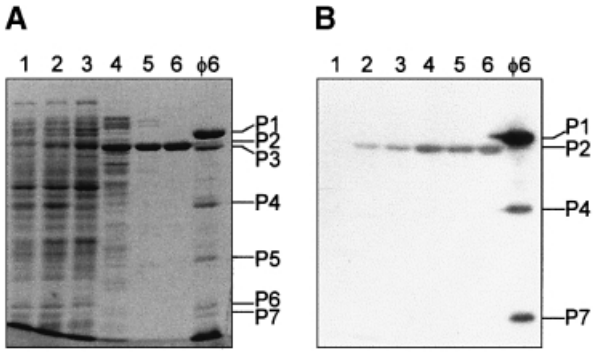

Fig. 1. Purification of the recombinant P2 produced in *E.coli* cells. (**A**) SDS–PAGE gel stained with Coomassie Blue G-250. Lanes: protein composition of bacterial cells BL21(DE3/pEM2) before (1) and after (2) induction of P2 synthesis with IPTG; cleared cell lysate (3); samples after successive purification on Cibacron Blue agarose (4), heparin agarose (5) and the Resource Q column (6). Proteins of the wild-type ϕ6 are marked on the right. (**B**) Immunoblot analysis of the same protein samples using antibodies raised against the entire ϕ6 polymerase complex (proteins P1, P2, P4 and P7). Lane designation is as in (A).

Purified P2 catalyzes RNA-dependent RNA polymerization in vitro

The isolated P2 was directly tested for possible RNA polymerase activity. The assay mixture, besides purified P2, contained single-stranded m^+ RNA substrate (positive-sense m segment of the ϕ6 phage), four nucleoside triphosphates (NTPs) including [α-^{32}P]UTP and the same buffer as described for the RNA synthesis in the recombinant procapsid system (Van Dijk *et al.*, 1995). Analysis of the reaction products showed that the presence of P2 protein correlated with the appearance of a new RNA band migrating as a double-stranded form (M) of the m^+ RNA substrate and visible both on ethidium bromide (EtdBr)-stained gel and autoradiogram (Figure 2). The band intensity was proportional to the amount of added P2 in the tested range (Figure 2, lanes 2–4). No band appeared if P2 was substituted with bovine serum albumin (BSA) or the reaction mixture lacked the RNA substrate (Figure 2, lanes 2 and 1). The enzymatic activity was dependent on the concentration of unlabeled NTPs in the mixture. Two types of final NTP concentrations were tested, those previously employed in the procapsid-based RNA synthesis (Van Dijk *et al.*, 1995): (i) to selectively support RNA replication restricting transcription (0.2 mM

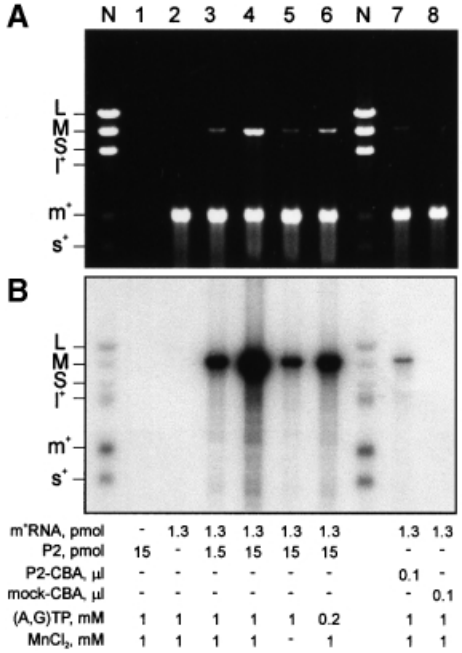

Fig. 2. Recombinant P2 catalyzes RNA-dependent RNA synthesis *in vitro*. Agarose gel analysis of aliquots from the standard 10 µl polymerase assay mixtures containing (except lane 8) the synthetic single-stranded positive-sense m-segment of the ϕ6 phage (m^+ RNA; 1 µg or 1.3 pmol per reaction). The critical additives are indicated below the panels. P2 refers to the purified P2 protein (lane 6 in Figure 1). P2-CBA is partially purified P2 after the Cibacron Blue agarose column (Figure 1, lane 4), and mock-CBA is the analogously prepared protein fraction derived from IPTG-induced BL21(DE3) cells containing pET32b(+) plasmid. The position of the labeled ϕ6 segments produced in the nucleocapsid transcription (N) is shown on the left. Double-stranded segments are marked with capital letters (L, M and S), and the plus-sense single-stranded segments are shown in lowercase (l^+, m^+ and s^+). (**A**) EtdBr-stained gel; (**B**) autoradiogram of the same gel.

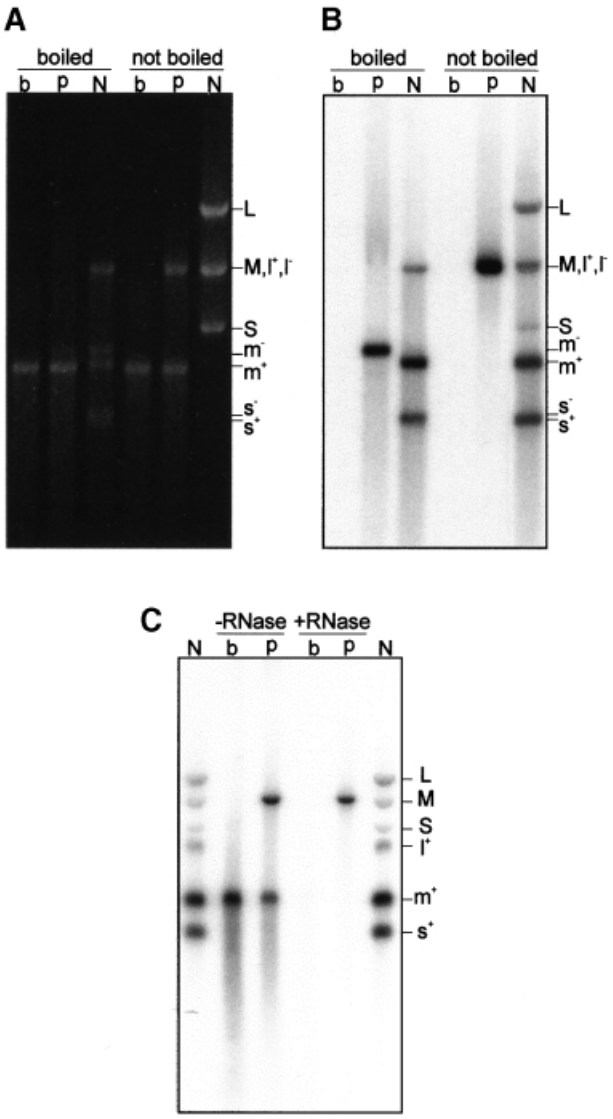

Fig. 3. The product of the RNA synthesis is dsRNA formed by the template and the complementary newly produced strand. Products of the m^+ RNA replication assay analyzed in a strand-separating gel. Lanes marked with p contained P2 protein in the assay (same conditions as in lane 4 of Figure 2); those marked with b were supplemented with an equal amount of the P2 control buffer (same as in lane 2 of Figure 2). Lanes marked with N contained labeled ϕ6 segments produced in the nucleocapsid transcription. Double-stranded RNA segments were heat-denatured (boiled) to yield individual plus (l^+, m^+ and s^+) and minus (l^-, m^- and s^-) RNAs. No strand separation occurred if the boiling step was omitted (not boiled). (**A**) EtdBr-stained gel; (**B**) autoradiogram of the same gel. (**C**) RNase protection assay. Reaction products purified from the P2 (p) or the control (b) replication mixtures containing [α-^{32}P]UMP-labeled m^+ RNA template and no labeled nucleotide triphosphates were incubated with (+RNase) or without (–RNase) addition of RNase I and analyzed in the standard agarose gel.

each of ATP, GTP, CTP and UTP); and (ii) optimal for both replication and transcription (1 mM each of ATP and GTP, 0.2 mM each of CTP and UTP). In our experiments, elevated concentrations of ATP and GTP resulted in a significant increase in the product yield (Figure 2, lanes 4 and 6). No product band appeared when the unlabeled nucleotides were completely omitted from the mixture (not shown). Furthermore, addition of 1 mM $MnCl_2$ (the optimal concentration; not shown) was found to cause a strong stimulatory effect on the P2-related activity (Figure 2, lanes 4 and 5). No stimulation was observed when Mn^{2+} was substituted with Mg^{2+} or Ca^{2+} (not shown). These data show that the tested P2 preparation contains NTP and Mn^{2+} stimulated enzymatic activity catalyzing *de novo* RNA synthesis in a template-dependent manner.

The reaction product is a dsRNA molecule formed by the template and the complementary strand of the newly synthesized RNA

In order to reveal the nature of the newly synthesized RNA product, the P2 reaction mixture was subjected to strand separation analysis (Pagratis and Revel, 1990). Unless heat-treated, the radioactive product migrated at the position of the double-stranded M segment in the strand-separating gel (Figure 3A and B), as was found in the previous experiment. The product mobility, however, changed after the heat denaturation step to that of the minus-strand (m^-) of the M segment. Thus, we conclude that the P2 protein catalyzed the synthesis of the minus-strand complementary to the input plus-strand template, i.e. the replication reaction. As the complementary strands are capable of duplex formation, it was reasonable to check whether the m^+ substrate and the newly synthesized m^- exist as a dsRNA molecule. The corresponding experiment was based on the fact that the RNase I of

E.coli readily hydrolyzes single-stranded and partially double-stranded RNA but not the perfect RNA duplexes (Brewer *et al.*, 1992). As evident from the results shown in Figure 3C, the replication product was almost fully resistant to the RNase digestion, whereas the m^+ RNA substrate was completely degraded under the same conditions. Thus, the replication product represented the double-stranded RNA segment composed of complementary m^+ and m^- strands. In addition to the highly purified P2, RNA polymerase activity was also found in the partially purified extract from the P2-expressing bacteria but not in the similarly prepared fraction of a mock extract (Figure 2, lanes 7 and 8). This showed that the activity was due to P2 protein production. The following experiment was set up to ascertain direct association of the replication catalysis with P2.

Replicase activity is associated with the monomer of P2

Purified P2 was subjected to gel-filtration chromatography and replicase activity was determined in the collected fractions. P2 eluted from the column as a single peak as judged by the optical profile and SDS–PAGE (Figure 4A). The peak elution volume was close to that of a 45 kDa protein, suggesting a monomeric form of P2 in solution. The replicase assay performed with the m^+ RNA substrate showed that the activity was only present in the fractions containing P2 (Figure 4B). Furthermore, the amount of newly produced dsRNA product in the reaction mixtures strongly correlated with the concentration of P2 in the corresponding protein fractions. These findings indicate that P2 possesses the RNA polymerase activity by itself and the activity is associated with the P2 monomer.

P2 is a non-specific replicase

The next experiment was carried out to test the RNA substrate specificity of the P2 RNA replicase. For this purpose, a set of various ϕ6-specific ssRNAs was synthesized and tested in the P2 polymerase assay (Figure 5, lanes 1–8). Exact copies of both large (l^+) and small (s^+) ssRNA segments of the ϕ6 phage gave rise to the labeled dsRNA products migrating in the gel at the positions of L and S, respectively. The replication efficiency of these two substrates was very close to that of the m^+ RNA (Figure 5, lanes 1–3). Comparable replication efficiency was found for the natural single-stranded segments isolated from the ϕ6 nucleocapsid transcription mixture (Figure 5, lane 8). Another substrate tested was the 13.5 kb long transcript consisting of fused s^+, m^+ and l^+ segments (Qiao *et al.*, 1997). The double-stranded product in this case migrated notably more slowly than the L segment, indicating complete or almost complete replication. It is worth noting that the 70–80 base long 3′-terminal part of all three ϕ6 segments is conserved and believed to form extensive secondary structure (Mindich *et al.*, 1994). All the RNA substrates mentioned above contained this feature and were replicable. It was thus interesting to study the possible effect of the 3′-proximal sequence on the RNA replicability *in vitro*. A truncated s^+ segment lacking 158 nucleotides at the 3′ terminus was synthesized and used as a substrate in the replication reaction (Figure 5, lane 4). Surprisingly, no reduction in the product yield was observed. On the contrary, the replication efficiency was somewhat higher than that of the unmodified s^+. Even more efficient replication was detected for s^+ RNA extended with 13 extra nucleotides originating from the plasmid polylinker (Figure 5, lane 5). However, addition of 31 polylinker nucleotides to the s^+ segment significantly reduced the yield of dsRNA product (lane 6). Thus, we conclude that: (i) neither the conserved secondary structure nor the ϕ6-specific sequence at the very 3′ terminus of an RNA substrate are critical for P2-directed replication *in vitro*; however, (ii) replication efficiency does depend on the substrate 3′-terminal sequence. These conclusions encouraged our attempts to replicate heterologous ssRNAs. Indeed, all of the ssRNAs tested turned out to be suitable substrates for the replication reaction, although the yield of dsRNA produced depended on the nature of the input template (Figure 5, lanes 9–12). The mixture of T7 phage transcripts resulted in a very effective synthesis of several dsRNA species (Figure 5, lane 9). Effective templates were the firefly luciferase messenger RNA and the plus-sense transcripts of the BTV (Figure 5, lanes 10 and 12).

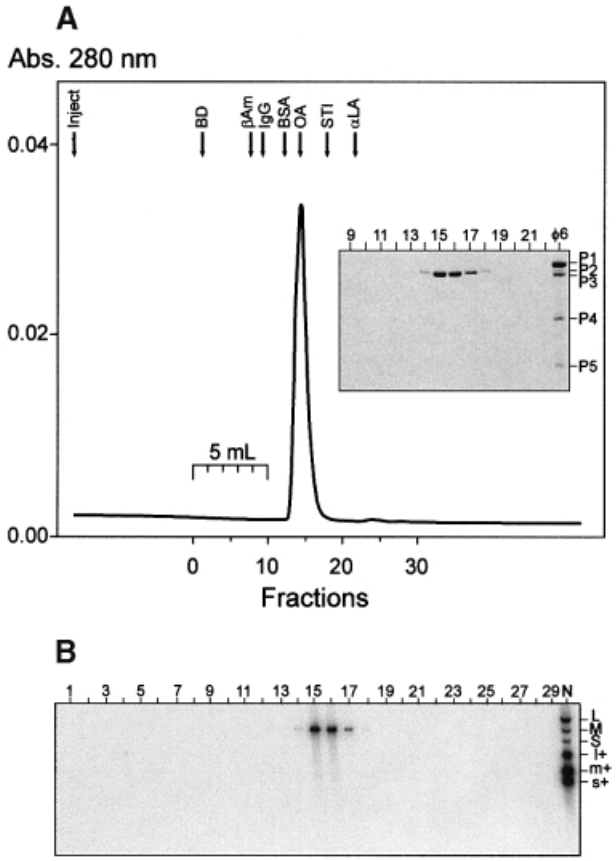

Fig. 4. Replicase activity is associated with the monomer of P2. Purified P2 was analyzed in the Superdex 75 gel-filtration column and the replicase activity was determined in the collected fractions. The peak of the replicase activity coincides with the P2 protein peak. (**A**) Absorbance (280 nm) profile of the eluate from the column. Arrows indicate the P2 injection time (inject) and position of the molecular mass standards: BD, Blue Dextran (2000 kDa); βAm, β-amylase (200 kDa); IgG, mouse immunoglobulin G (150 kDa); BSA, bovine serum albumin (67 kDa); OA, ovalbumin (45 kDa); STI, soybean trypsin inhibitor (20.1 kDa); αLA, α-lactalbumin (14.2 kDa). Inset, SDS–PAGE analysis of the protein content in fractions 9–22. (**B**) Autoradiogram of the agarose gel showing replicase activity in fractions 1–29. Lane N is as defined in Figure 2.

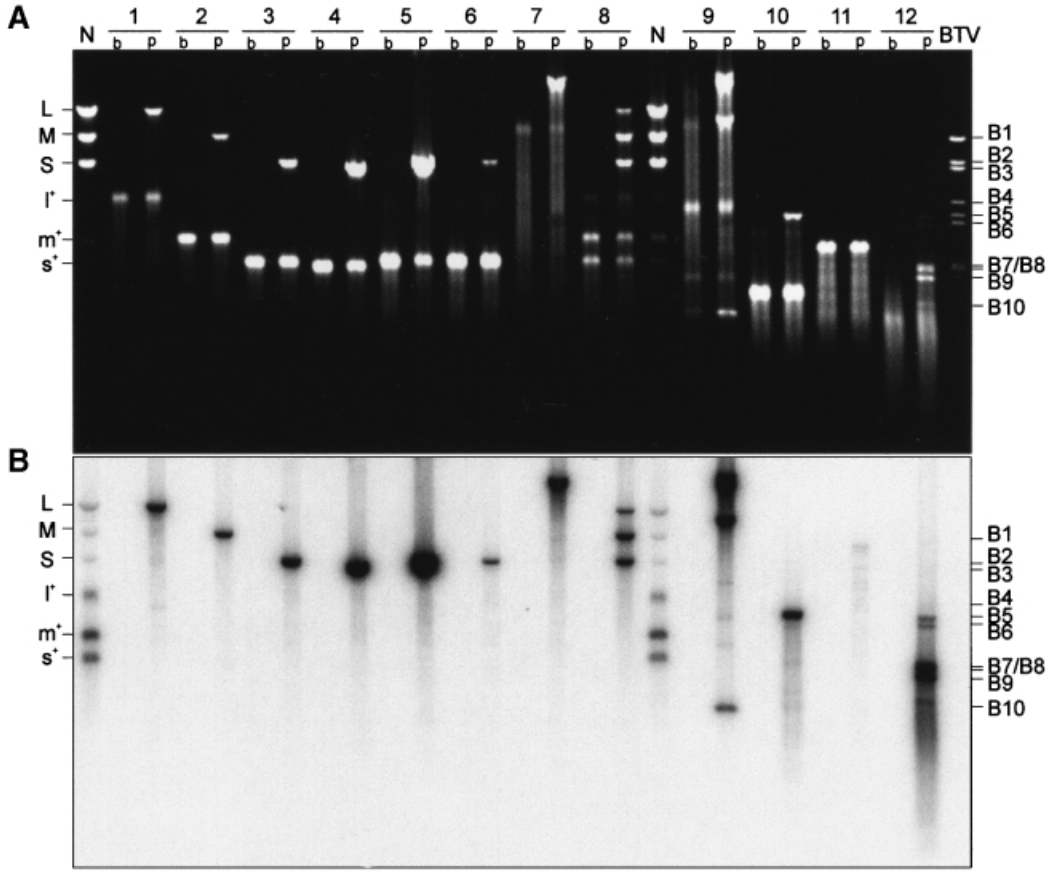

Fig. 5. RNA substrate specificity of the P2 catalyzed replication. (**A**) EtdBr-stained gel showing replication products of the reactions containing the purified P2 protein (p) or the control buffer (b). Single-stranded RNA substrates used to program reactions were as follows. 1, l^+ RNA (synthetic positive-sense large segment of the ϕ6 phage produced with T7 transcription of pLM687 treated with *Xba*I and MBN); 2, m^+ RNA (medium segment, same as in Figure 2); 3, s^+ RNA (small segment; T7 transcript of pLM659 treated with *Xba*I and MBN); 4, shortened s^+ RNA (T7 transcript of pLM659 cut with *Eco*47III); 5, extended s^+ RNA (T7 transcript of pLM659 cut with *Sma*I); 6, extended s^+ RNA (T7 transcript of pLM659 cut with *Eco*RI); 7, 13.5 kb long RNA containing fused s^+, m^+ and l^+ segments (T7 transcript of pLM1809 treated with *Xba*I and MBN); 8, mixture of natural s^+, m^+ and l^+ segments purified from the ϕ6 nucleocapsid-directed transcription; 9, RNA mixture produced by T7 transcription of the entire DNA of bacteriophage T7; 10, firefly luciferase mRNA (SP6 transcript of pGEMluc cut with *Stu*I); 11, genome RNA of the coliphage MS2 (Boehringer Mannheim); and 12, mixture of bluetongue virus (BTV1) ssRNA segments LiCl precipitated from the BTV nucleocapsid transcription. ϕ6 segments from nucleocapsid transcription (N) are marked on the left. Positions of the ten genomic dsRNA segments phenol-extracted from the BTV are shown on the right (B1–B10). (**B**) Autoradiogram of the same gel.

Replication of genomic RNA of the coliphage MS2 was reproducibly inefficient, leading to a barely visible dsRNA product in the original EtdBr-stained gel. Even in this case the product band was clearly detectable on the autoradiogram (Figure 5, lane 11). Additionally, some other RNAs, namely mRNAs encoding thioredoxin [T7 transcript of pET32b(+) cut with *Xho*I], green fluorescent protein (T7 transcript of pTU58 cut with *Eco*RI) and firefly luciferase fused with neomycin phosphotransferase II [T7 transcript of pTZluc(NPT2) cut with *Xho*I], and a mixture of 16S and 23S ribosomal RNAs of *E.coli* (Boehringer Mannheim) were also replicable with the P2 protein (data not shown).

Rate of RNA chain elongation

A kinetic experiment was designed to determine the elongation rate of the replicating P2 polymerase. Replication of the natural ϕ6 transcripts was initiated by adding P2 protein to the mixture, and aliquots were sampled at different time points for subsequent electrophoretic analysis. As evident from the autoradiogram shown in Figure 6A, the full-length S product appeared first after a short lag period, followed successively by M and L. The band intensities then increased at least to the 1 h (3600 s) time point. The accumulation of individual dsRNAs over time was also plotted as time course curves (Figure 6B–D). Extrapolating the linear phases of the curves to the time axis, we obtain characteristic times necessary for the complete synthesis of each double-stranded product. Assuming an even initiation on all three ssRNA species, the average elongation rate (V_{av}) can be calculated as:

$$V_{av} = [(L–M)/(\tau_L–\tau_M) + (M–S)/(\tau_M–\tau_S) + (L–S)/(\tau_L–\tau_S)]/3$$

where L, M and S are the lengths of the corresponding segments (S = 2948 bp, M = 4063 bp and L = 6374 bp; McGraw *et al.*, 1986; Gottlieb *et al.*, 1988; Mindich *et al.*, 1988); τ_L, τ_M and τ_S are the observed characteristic times (Figure 6B–D, insets). Consequently, the elongation rate of P2 under tested conditions was ~120 bp/s.

P2 initiates RNA synthesis at the very 3′-terminal nucleotide of a template

Another experimental question was whether (i) the enzyme initiates minus-strand synthesis from the

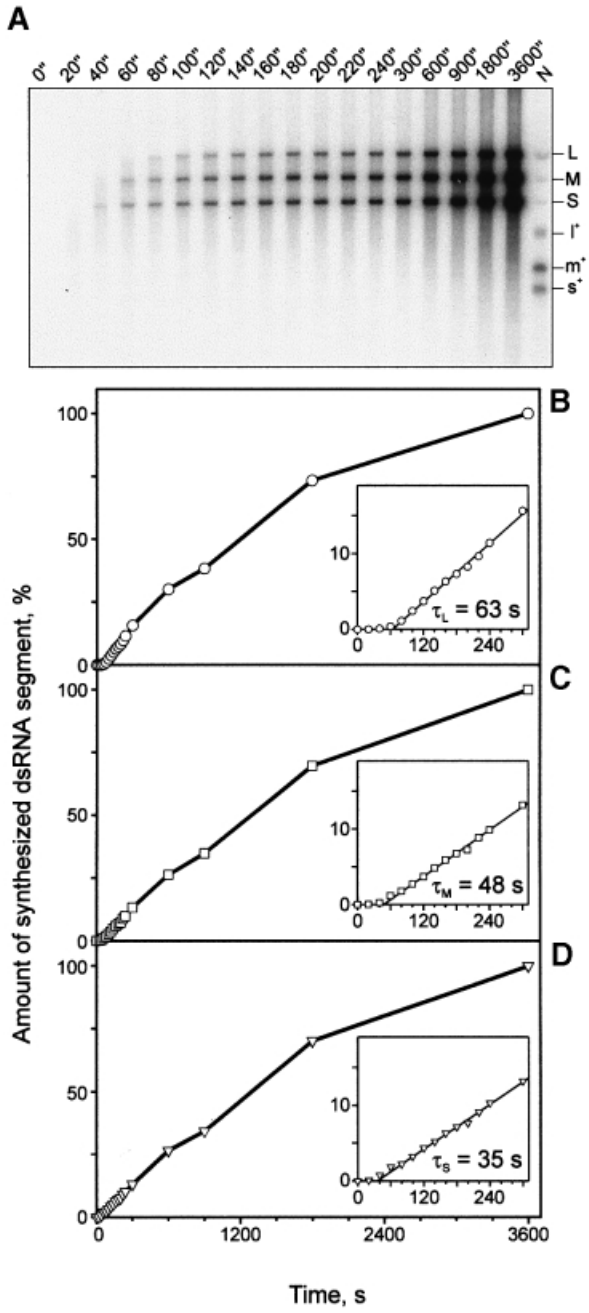

Fig. 6. Time course of P2-directed replication. (**A**) The 100 µl replication mixture programmed with the three natural positive-sense segments was incubated at 28°C in the presence of the P2 protein. Five microliter aliquots, sampled at the time points indicated, were analyzed in the standard agarose gel and autoradiographed. Lane N is as in Figure 2. (**B**, **C** and **D**) Phosphoimager (Fuji BAS1500) analysis of the time-dependent accumulation of replication products L, M and S, respectively. The graphs are normalized so that the highest observed value within each panel is set to 100%. Insets in (B, C and D) show the first 300 s of the time courses. Lines extrapolate linear parts of the plots to the time axis. τ_L, τ_M and τ_S indicate the duration of the lag phases prior to the appearance of relevant full-length dsRNA segments.

nucleotide complementary to the very 3′-terminal nucleotide of the plus-strand template, or (ii) the initiation occurs internally. The primer extension approach was employed to distinguish between these two possibilities. In this experiment, a mixture of natural ssRNAs (l^+, m^+ and s^+) was first replicated and then, after the heat denaturation

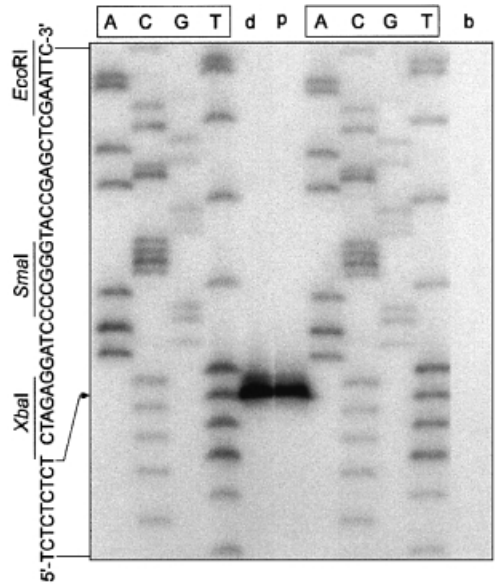

Fig. 7. Replication initiation site. RNA products of the replication reactions programmed with the mixture of natural ssRNA segments (s^+, m^+ and l^+) and containing P2 protein (p) or buffer (b) were assayed in the primer extension experiment with a labeled primer complementary to the minus-strand (s^-) of the small ϕ6 segment. As a control, primer extension was also done on the dsRNA (d) extracted from the wild-type ϕ6. Dideoxynucleotide termination sequencing lanes (A, C, G and T) are boxed. They were produced with the same primer and T7 Sequenase 2.0 (Amersham) using cloned cDNA of the s^+ segment (pLM659) as a template. Sequence reading is shown on the left. The 3′-terminal 'T' of s^+ is marked with the arrow and the unique restriction sites mentioned in Figure 5 are underlined.

step, used to synthesize cDNA from the labeled primer complementary to the s^- strand of the S segment. The heat-denatured mixture of genomic dsRNA extracted from the bacteriophage particles was the template for the cDNA synthesis in a control reaction. Both reactions (Figure 7, p and d) resulted in the appearance of the same cDNA product terminated at the very 3′-terminal nucleotide of the s^+ strand compared with adjacent sequencing lanes. No cDNA synthesis occurred on the ssRNA mixture pre-incubated with buffer instead of P2 (Figure 7, lane b). These data are in accordance with the model of initiation of the P2-directed replication *in vitro* from the first nucleotide of the template.

Discussion

One of the most intriguing features of the double-stranded RNA viruses is that both their replication (minus-strand synthesis) and transcription (plus-strand synthesis) occur inside a large protein complex. A protein with RNA polymerase sequence motifs is believed to be the only constituent of the complex immediately responsible for the catalysis of these two reactions. In prior studies of replication and transcription in dsRNA viruses, RNA synthesizing activity was always associated with complexes including the putative polymerase as a minor protein. This report provides the first direct evidence that the isolated polymerase of a dsRNA virus alone is capable of RNA synthesis *in vitro*. The replication system described here is based on the purified recombinant protein P2 of the dsRNA bacteriophage ϕ6. P2 is shown to initiate *de*

novo and further catalyze the synthesis of a full-length complementary strand on an ssRNA substrate yielding a dsRNA product of the appropriate size. The enzyme demonstrates high processivity, being able to replicate RNA templates up to 13.5 kb in length (Figure 5, lane 7) and probably even longer ones (Figure 5, lane 9). The effective dsRNA synthesis *in vitro* using purified P2 can be considered the first model of bona fide replication established for ϕ6, because the ϕ6 procapsid-based system reported previously (Gottlieb *et al.*, 1990; Olkkonen *et al.*, 1990) does not support replication unless RNA packaging is completed (Frilander *et al.*, 1992).

During optimization experiments, Mn^{2+} and increased concentrations of ATP and GTP were found to enhance dsRNA synthesis considerably (Figure 2). The stimulatory effects of manganese and purine NTPs on the RNA-dependent RNA synthesis have been reported for both the ϕ6 polymerase complex (Van Dijk *et al.*, 1995) and some other viral polymerases (Blumenthal, 1980, and references therein). According to the latter study carried out on Qβ replicase, Mn^{2+} acts by decreasing the concentration of a purine nucleotide required for the effective initiation of RNA synthesis. The same reason might be responsible for the effects found in the P2-catalyzed reaction given that all three ϕ6 plus-strand RNA segments end with a polypyrimidine sequence ...CTCTCTCTCT-3′ (McGraw *et al.*, 1986; Gottlieb *et al.*, 1988; Mindich *et al.*, 1988).

In the non-denaturing gel-filtration experiment (Figure 4), P2 was found to migrate as a monomer with an apparent molecular mass of 45 kDa, whereas the actual molecular mass of P2 is 75 kDa. This difference could not be explained by protein degradation (see SDS–PAGE in Figures 1 and 4A). Possible interaction of the protein with the gel-filtration matrix (Sephadex) also seemed an unlikely explanation, because a similarly low apparent molecular weight was obtained with a different column (Ultrahydrogel 500, Waters; R.Tuma, unpublished observation). Therefore, it is reasonable to propose that the protein is a very compact spherical monomer in solution. This conclusion was further confirmed by preliminary light-scattering data (R.Tuma, unpublished results).

Important implications follow from the fact that the 3′-proximal structure common to all three ϕ6 RNA segments was dispensable for successful replication. Indeed, single-stranded s^+ RNA completely lacking this structure or containing 3′-terminal extensions in some cases was an even more effective substrate than the full-sized segment (Figure 5, lanes 3–6). The observed phenomenon can not be explained as an abnormal initiation on the defective RNAs due to the presence of manganese ions. Omission of Mn^{2+} from the reaction mixture resulted in a proportional decrease in the synthesis of dsRNA products for both truncated and full-length RNA templates not changing their relative replication efficiency (not shown). The results on the replication of s^+ lacking its native 3′-terminal sequence are consistent with the previously published data showing detectable replication of similarly modified m^+ RNA in the ϕ6 procapsid-based system (Mindich *et al.*, 1994). We share the conclusion drawn in the latter report that the role of the secondary structure at the 3′ ends of ϕ6 ssRNAs is distinct from simply promoting replication. In addition to the proposed RNase protection role (Mindich *et al.*, 1994), the structure may regulate minus-strand synthesis rather than ensuring the most effective initiation. Such control might prevent undesirable initiation of the minus-strand synthesis immediately after transcription when the 3′ end of the newly synthesized positive-sense RNA is in close proximity to the P2 polymerase. Another intriguing speculation would be a possible role for the conserved secondary structures in docking the 3′ termini of the packaged ssRNA segments to the P2 protein inside the polymerase particle.

In addition to the full-sized, truncated and extended ssRNA segments of ϕ6, P2 was demonstrated to replicate a number of unspecific templates effectively (Figure 5, lanes 9, 10 and 12; and data not shown). On the contrary, the complete ϕ6 procapsid is rather restrictive in the replication of heterologous ssRNAs (Gottlieb *et al.*, 1990). This suggests that selection of the phage-specific ssRNAs *in vivo* occurs at the RNA packaging step independently of the P2 polymerase. This assumption is in agreement with the specific RNA packaging into the P2-deficient polymerase complex (Juuti and Bamford, 1995).

The observed replicase activity of the purified ϕ6 P2 protein encourages studies on the isolated polymerases from other dsRNA viruses. Notably, recent experiments carried out in the laboratory of Dr P.Roy indicate that the purified putative polymerase from the BTV possesses replicase activity. Unlike its ϕ6 analog, isolated BTV polymerase seems to have pronounced substrate specificity, being able to replicate only BTV ssRNAs (P.Roy, personal communication).

The unspecific nature of the ϕ6 replicase suggests a possible application of the enzyme in molecular biology as a general tool for producing dsRNA from virtually any given ssRNA template. Recently, dsRNA has become the subject of considerable interest as it has been shown to trigger general and sequence-specific suppression mechanisms in both animal and plant cells (reviewed in Sharp, 1999). The availability of the ϕ6 polymerase opens up new avenues for further studies on dsRNA biology.

Materials and methods

Bacterial strains and plasmids

Escherichia coli DH5α (Gibco-BRL) was the host for plasmid propagation and molecular cloning. Plasmids pLM659 (Gottlieb *et al.*, 1992b), pLM656 (Olkkonen *et al.*, 1990) and pLM687 (Mindich *et al.*, 1994) allowed production of positive-sense ssRNA copies of the bacteriophage genomic segments s^+, m^+ and l^+, respectively. Plasmid pLM1809 (Qiao *et al.*, 1997) was used for synthesis of a long RNA containing fused s^+, m^+ and l^+ segments. Plasmid pGEMluc (Promega) was employed to produce *Photinus pyralis* luciferase mRNA. Plasmids pTU58 (Chalfie *et al.*, 1994) and pTZluc(NPT2) (Makeyev *et al.*, 1996) were the templates for production of mRNAs encoding green fluorescent protein and translational fusion of firefly luciferase and neomycin phosphotransferase II. To construct a plasmid for P2 protein expression, P2 gene was PCR-amplified from pLM687 template with the recombinant *Pfu* DNA polymerase (Stratagene) and the oligonucleotides 5′-GGTAAGCGCCATATGCCGAGGAGA-3′ and 5′-TACGAATTC CGGCATGATTACCTAGGCATTACA-3′ serving as upstream and downstream primers, respectively. The PCR fragment digested with *Nde*I and *Eco*RI (underlined sites in the primer sequences) was gel-purified and ligated with the large fragment of the *Nde*I–*Eco*RI cut vector pET32b(+) (Novagen). *Escherichia coli* BL21(DE3) (Studier and Moffatt, 1986; purchased from Novagen) was transformed with the resultant plasmid pEM2 to give P2 producing strain BL21(DE3/pEM2).

Expression and purification of recombinant P2 protein

Purification of P2 protein was monitored by SDS–PAGE in 12.5% acrylamide gel (Olkkonen and Bamford, 1989) and by immunoblotting

with rabbit polyclonal antibodies raised against recombinant PC (Frilander and Bamford, 1995). To achieve expression of soluble P2, a starter culture of BL21(DE3/pEM2) in Luria–Bertani medium containing 150 mg/ml ampicillin was grown at 37°C with shaking until the OD_{540} reached 0.5. This was then diluted 50-fold into 3 l of the same medium. The diluted culture was further grown at 37°C to an OD_{540} of 1.0. The culture was chilled on ice and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). IPTG-induced cells were then transferred to 15°C where they were shaken for 18 h. All the following steps unless otherwise indicated were performed at 4°C. Bacteria were collected by centrifugation and resuspended in 30 ml of buffer A1 (100 mM NaCl, 50 mM Tris–HCl pH 8.0, 1 mM EDTA). The suspension was passed three times at ~105 MPa through a pre-cooled French pressure cell. Phenylmethylsulfonyl fluoride was added to 1 mM after the first passage. The lysate was centrifuged at 120 000 *g* for 2.5 h. The supernatant fraction was loaded onto a dye affinity column (Cibacron Blue 3GA; Sigma). Proteins bound to the column were eluted with buffer A5 (500 mM NaCl, 50 mM Tris–HCl pH 8.0, 1 mM EDTA). Pooled fractions containing P2 were diluted 5-fold with ice-cold distilled water and applied onto a heparin agarose column (Sigma). Proteins were eluted with a linear 0.1–1 M NaCl gradient buffered with 50 mM Tris–HCl pH 8.0 and 1 mM EDTA. Fractions containing P2 were pooled and diluted 10-fold with 20 mM Tris–HCl pH 8.0, filtered and injected onto a Resource Q column (Pharmacia; room temperature). Elution of the bound proteins was performed with a 0–0.5 M NaCl gradient buffered with 50 mM Tris–HCl pH 8.0 and 0.1 mM EDTA. The concentration of the purified P2 protein was determined by the absorbance at 280 nm in 6 M guanidine hydrochloride (based on the value of 1.39 per 1 mg/ml calculated for the unfolded protein; Edelhoch, 1967). Purified P2 was stored on ice for up to 1 month without detectable loss of activity or protein integrity.

Preparation of RNA substrates

Synthetic single-stranded RNA substrates were prepared by *in vitro* transcription with SP6 (for pGEMluc) or T7 (for the rest of DNA templates) RNA polymerases. The unlabeled RNAs were produced in 50 µl transcription mixtures in principle as described in Makeyev *et al*. (1996). Reactions were initiated by the addition of 80 U of either T7 or SP6 RNA polymerases (Promega). The mixtures were incubated at 37°C for 2 h and then stopped by the addition of 1 U of DNase RQ (Promega) per 1 µg of input DNA template. Incubation was continued for a further 15 min at 37°C. RNA preparations were successively extracted with phenol/chloroform (1:1) and chloroform, precipitated with 3 M LiCl and dissolved in sterile water. Labeled m^+ RNA was synthesized as recommended by Promega. The mixture (25 µl) contained 1 mCi/ml of [α-^{32}P]UTP (Amersham; 3000 Ci/mmol), 20 U of RNasin, 4 µg of pLM656 treated with *Xba*I (NEB) and mung bean nuclease (MBN; Promega), and 40 U of T7 RNA polymerase. The reaction was carried out for 1 h and then processed as described for unlabeled transcripts with the only exception that the labeled RNA was additionally purified by passing through a Sephadex G25 spin column (Pharmacia) after the LiCl precipitation step. A mixture of the natural ϕ6 transcripts (single-stranded segments s^+, m^+ and l^+) was prepared using nucleocapsid-directed transcription (Bamford *et al*., 1995) followed by phenol extraction and three successive LiCl precipitations. The RNA concentration was measured by optical density at 260 nm. The quality of the RNAs was determined by electrophoresis either in 5% polyacrylamide gel (PAAG) containing 7.5 M urea or in the standard 1% agarose gel (Pagratis and Revel, 1990; and see below).

P2 polymerase assay

The polymerase activity of P2 protein was typically assayed in a 10 µl reaction mixture containing 50 mM Tris–HCl pH 8.9, 80 mM ammonium acetate (NH_4OAc), 6% (w/v) PEG4000, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM dithiothreitol (DTT), 0.1 mM EDTA, 1 mM each of ATP and GTP, 0.2 mM each of CTP and UTP (all four nucleotide triphosphates from Pharmacia), 0.2 mg/ml BSA (nuclease free; NEB) and 0.8 U/µl RNasin. The final concentration of the added RNA substrates ranged from 40 to 300 µg/ml. Unless otherwise indicated, the mixture was supplemented with 0.25 mCi/ml of [α-^{32}P]UTP (Amersham; 3000 Ci/mmol). Reactions were initiated by addition of 0.2–2 µl of the P2 protein preparation. In the control reactions ('buffer only'), P2 was replaced with an equal volume of the P2 buffer (50 mM Tris–HCl pH 8.0, 90 mM NaCl, 0.1 mM EDTA, 0.2 mg/ml BSA). The mixtures were incubated at 28°C for 1 h and processed for further analysis as described below.

Agarose gel electrophoresis

Two types of agarose gel electrophoresis, both originally described by Pagratis and Revel (1990), were employed in this study for RNA analysis. The first, or standard, type of electrophoresis used to achieve separation of the positive-sense ssRNA and the corresponding dsRNA segments, was carried out in 1% agarose gels containing 0.25 µg/ml EtdBr and buffered with 1× TBE (50 mM Tris–borate pH 8.3, 1 mM EDTA). For analysis of the P2 polymerization products, the reaction was stopped by the addition of an equal volume of U2 buffer [8 M urea, 10 mM EDTA, 0.2% SDS, 6% (v/v) glycerol, 0.05% bromophenol blue and 0.05% xylene cyanol FF]. After the RNA separation (5 V/cm), gels were irradiated with UV light and photographed. To determine the position of the radioactively labeled bands, gels were dried and exposed with Fuji Super RX film. The second technique was the strand-separating gel analysis. In this case, electrophoresis was done in 1% agarose buffered with 1× TBE and containing no EtdBr. Samples for the analysis were prepared by stopping P2 reaction mixtures with 4 vol of 100 mM EDTA, followed by phenol/chloroform (1:1) and chloroform extractions. The aqueous phase was made 2.5 M in NH_4OAc and precipitated with 2.5 vol of ethanol. The pellets were dissolved in U2 buffer diluted 2-fold with sterile water. When appropriate, the samples were boiled for 3 min and then placed on ice for another 3 min. After the RNA separation (5 V/cm), gels were stained with EtdBr and processed as indicated for the standard gels.

RNase protection assay

The assay was performed in 10 µl reaction mixtures containing 10 mM Tris–HCl pH 7.5, 200 mM NH_4OAc, 5 mM EDTA, 1 U of RNase I (RNase ONE; Promega) and the RNA sample purified with phenol/chloroform extraction and ethanol precipitation from the P2 polymerase assay mixture. The reaction was carried out for 1 h at 28°C and stopped by the addition of 0.1% SDS and 10 µg of *E.coli* tRNA (Sigma). The products of the reaction were analyzed by standard electrophoresis in agarose gel.

Analytical gel filtration

Chromatography was performed at room temperature on a Superdex 75 HR 10/30 column (Pharmacia) using buffer containing 50 mM Tris–HCl, 100 mM NaCl and 0.1 mM EDTA, and a flow rate of 0.5 ml/min. The proteins and the Blue Dextran used for calibration were from Sigma except for the purified mouse IgG (Zymed) and soybean trypsin inhibitor (Boehringer Mannheim). Typically, 200 µg of purified P2 was injected onto the column and 0.5 ml fractions were collected. One microliter aliquots from each of the fractions were assayed for replicase activity as described above.

Primer extension

The primer extension assay was done in 10 µl reaction mixtures containing 50 mM Tris–HCl pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM spermidine, 0.6 mM each of the four deoxynucleotide triphosphates, and 5 U of AMV reverse transcriptase (Promega). As a primer, the reaction contained 0.5 pmol of oligonucleotide (5′-GGATAAACAAGTCCTTGTATAAC-3′) terminally labeled with polynucleotide kinase (Promega) and [γ-^{32}P]ATP (Amersham, 3000 Ci/mmol). The primer was designed to be complementary to the minus-strand of the small ϕ6 genome segment (s^-). Denatured RNA for the assay was prepared as follows: the standard 10 µl replication mixtures containing P2 polymerase or the P2 control buffer, and lacking labeled nucleotides, were extracted with phenol/chloroform (1:1) and chloroform, brought to 2.5 M NH_4OAc and precipitated with ethanol. The RNA pellets were dissolved in sterile water, heated at 100°C for 3 min, chilled on ice for another 3 min, and transferred to room temperature. The RNA samples were mixed with the rest of the assay components and the mixtures were incubated at 42°C for 10 min. The reaction was stopped by adding 7.5 µl of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF. The stopped mixtures were then incubated at 80°C for 5 min and analyzed in a 6% PAAG containing 7.5 M urea.

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