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Replication of poliovirus in *Xenopus* oocytes requires two human factors

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We described a novel system to study poliovirus replication in *Xenopus* oocytes. Poliovirus RNA microinjected into *Xenopus* oocytes initiates a complete cycle of viral replication, yielding a high level of infectious viruses. Two distinct HeLa cell activities are required, one involved in initiation of translation and the other in RNA synthesis. The translation factor is a large cytoplasmic protein or complex, which is specifically used for initiation of poliovirus translation. The replication factor is required at early stages of RNA synthesis. Formation of infectious poliovirus is highly temperature dependent. At temperatures below 27°C, capsid assembly appears to be impaired. The oocyte system described here could be useful in identifying and characterizing viral and cellular factors involved in virus replication.

Keywords: poliovirus replication factor/translation/ *Xenopus* oocytes

Introduction

Poliovirus replication follows a strategy common to all positive single-stranded lytic RNA viruses (Baltimore, 1984). After entry, the RNA genome is directly translated by the cellular protein synthesis apparatus in order to produce specific viral proteins. The viral genomic RNA is then transcribed into a complementary RNA (negative strand), which in turn is used as a template to synthesize new strands of genomic RNA (positive strand). Translation is directed by an RNA element located at the 5′-end of the genomic RNA, the IRES (Jang *et al.*, 1988; Pelletier *et al.*, 1988a; Trono *et al.*, 1988b), which is presumably recognized by the cellular translation machinery. Artificial dicistronic and circular RNAs have been used to demonstrate that the picornavirus internal ribosomal entry site (IRES) elements allow ribosomes to enter the RNA internally without scanning from the 5′ end (Pelletier and Sonenberg, 1988; Chen and Sarnow, 1995). The mechanism of internal initiation of translation on picornavirus RNAs is still unknown. However, many lines of evidence suggest that besides the known initiation factors, additional *trans*-acting cellular proteins are involved in this process (for review see Jackson *et al.*, 1995). A common approach utilized in the search for such a factor(s) is the examination of the binding of proteins to the IRES elements, using UV cross-linking or gel retardation assays. A growing number of proteins are being identified that interact with the IRES (Najita and Sarnow, 1990; Pestova *et al.*, 1991; Dildine and Semler, 1992; Gebhard and Ehrenfeld, 1992; Haller and Semler, 1995), but it remains to be determined which interactions have a function in translation.

For RNA replication, a virus-encoded RNA-dependent RNA polymerase ($3D^{pol}$) is thought to catalyze the synthesis of both RNA strands. However, because $3D^{pol}$ is a primer-dependent enzyme (Flanegan and Baltimore, 1977; Van Dyke and Flanegan, 1980), several other viral factors (proteins and RNA structures), as well as cellular factors, are likely to participate in the initiation of RNA synthesis. Genetic analysis has implicated most of the non-structural viral proteins in RNA synthesis (Bernstein *et al.*, 1986; Kuhn *et al.*, 1988; Li and Baltimore, 1988; Burns *et al.*, 1989; Giachetti and Semler, 1991), but few cellular components or mechanisms have been identified.

Isolation of the cellular factors involved in poliovirus replication, and studies of the mechanisms involved, have been limited by the lack of an experimental system in which the process is both physiologically relevant and amenable to biochemical analysis. Recently, Molla *et al.* (1991) found that infectious poliovirus is formed during *in vitro* translation of viral RNA in a crude cytoplasmic extract. This observation opened the exciting possibility of biochemical manipulation of poliovirus replication. The extract contains cellular membranes, which are required for authentic initiation of RNA synthesis, and each of the expected products and subproducts of poliovirus replication are produced. Because the extract is prepared from human cells, all the cellular components necessary for viral replication are already present, and identification of cellular factors involved in the virus life cycle can be approached by fractionating and reconstituting the system from purified fractions.

As an alternative and complementary method to the study of poliovirus replication and identification of cellular factors involved in the process, we have started to explore replication of poliovirus in *Xenopus laevis* oocytes. Here, we show that microinjection of viral RNA initiates an authentic and complete replication cycle, though only when a cytoplasmic HeLa cell extract is co-injected with the RNA. We discovered that two independent activities from HeLa cells are essential for replication, one necessary for translation and the other for RNA synthesis. These results demonstrate that human cell factors are indeed utilized by poliovirus at different stages during viral replication.

Results

Cap-independent translation of poliovirus RNA in Xenopus oocytes requires a HeLa cell cytoplasmic factor

Translation of the genomic RNA is the first step in poliovirus replication. Because the genomic RNA is natur-

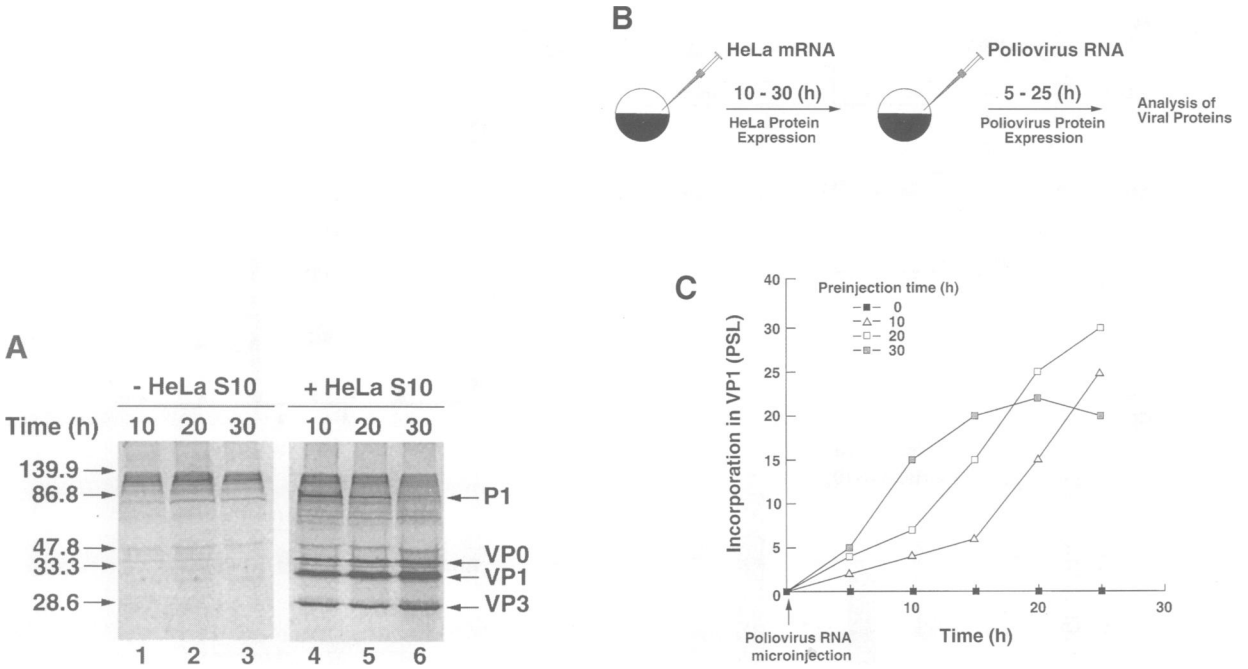

Fig. 1. A HeLa cell cytoplasmic factor is required for initiation of poliovirus translation in *Xenopus* oocytes. (**A**) SDS–polyacrylamide gel analysis of ^{35}S-labeled viral proteins synthesized in oocytes. Oocytes were microinjected with poliovirus RNA in the absence (lanes 1–3) or presence (lanes 4–6) of HeLa cell S10 extract and incubated in [^{35}S]methionine at 22°C for 10 h (lanes 1 and 4), 20 h (lanes 2 and 5) and 30 h (lanes 3 and 6). Cytoplasmic extracts were immunoprecipitated with antiserum directed against capsid proteins and analyzed on gels. The relative migration of poliovirus capsid proteins and molecular weight markers is indicated. (**B**) Schematic diagram of pre-injection of HeLa mRNA and injection of poliovirus RNA. Oocytes were injected with HeLa cell total RNA (50 nl of 2 μg/μl) and incubated at 17°C for 10–30 h. After incubation, oocytes were microinjected a second time with 50 nl of poliovirus RNA alone, and incubated for additional 5–25 h at 22°C in the presence of [^{35}S]methionine and labeled proteins were analyzed as in (A). (**C**) Synthesis of capsid protein VP1 in oocytes pre-injected with HeLa cell mRNA. Oocytes were pre-injected and injected as described in (B), and the amount of radioactivity (PSL) incorporated in the specific band was quantitated by a Bio-Imaging Analyser (Fuji Bas1000) apparatus. [^{35}S]methionine incorporation into VP1 is represented as a function of the incubation time after poliovirus RNA injection. Different curves represent results obtained with different pre-incubation times as indicated in the inset.

ally uncapped and is translated *in vivo* under conditions where cap-dependent translation is impaired, it must be translated using a cap-independent mechanism. *Xenopus* oocytes can efficiently translate capped mRNAs but do not support poliovirus translation (Pelletier *et al.*, 1988b). To confirm and extend this observation, we microinjected poliovirus RNA into *Xenopus* oocytes, either alone or with HeLa S10 extract. Oocytes were incubated in the presence of [^{35}S]methionine for 10, 20 or 30 h at 22°C, and total cytoplasmic extracts were immunoprecipitated with antibodies directed against capsid proteins and analyzed by SDS–PAGE. Efficient poliovirus protein synthesis was detected only when viral RNA was injected together with HeLa cytoplasmic extract (Figure 1). The capsid protein precursor, P1, was made and properly processed into poliovirus structural proteins VP0, VP1 and VP3. Other poliovirus proteins, such as 2BC, 3CD and 3D, were also detected by immunoprecipitation with specific antibodies but only when viral RNA was co-injected with HeLa cell cytoplasmic extract (data not shown). This experiment confirms that oocytes are unable to use poliovirus RNA to initiate translation and suggests that a HeLa cell factor (poliovirus translation factor, PTF) can be provided *in trans* to overcome this block. We presume that the HeLa cell factor directs the efficient internal entry of ribosomes onto the poliovirus RNA.

To determine whether PTF is a protein factor, we pre-treated HeLa extracts with proteinase K–agarose beads or incubated the extract at 80°C for 5 min before microinjection. Both treatments completely eliminated the stimulatory effect of the extract on poliovirus translation (data not shown). On the other hand, RNase A–agarose treatment did not modify the stimulating activity, suggesting that RNA is not an essential component of PTF.

Furthermore, poliovirus protein synthesis was stimulated by injecting HeLa mRNA. Total HeLa cell mRNA was injected into oocytes and incubated for 0–30 h before the injection of poliovirus RNA (Figure 1B). Incorporation of [^{35}S]methionine in capsid protein VP1 was stimulated more than 100-fold by pre-injecting HeLa mRNA (Figure 1C). For maximal stimulation, mRNA must be injected several hours before poliovirus RNA, and longer periods of pre-incubation resulted in higher levels of stimulation (Figure 1C). Co-injection of both RNAs yielded no translation stimulation and is consistent with the idea that the translation factor must be expressed from the HeLa mRNA before it can enhance poliovirus protein synthesis. Thus, this last result suggests that the stimulating effect is not due to residual HeLa protein or nucleic acids present in the mRNA preparation.

The translation factor is specific for poliovirus IRES

To simplify the characterization of PTF, we used a poliovirus subgenomic RNA in which the coding region of the capsid proteins was replaced by the *Photinus pyralis* (firefly) luciferase reporter gene (de Wet *et al.*, 1987). An artificial cleavage site for the viral protease 2A^{pro} was

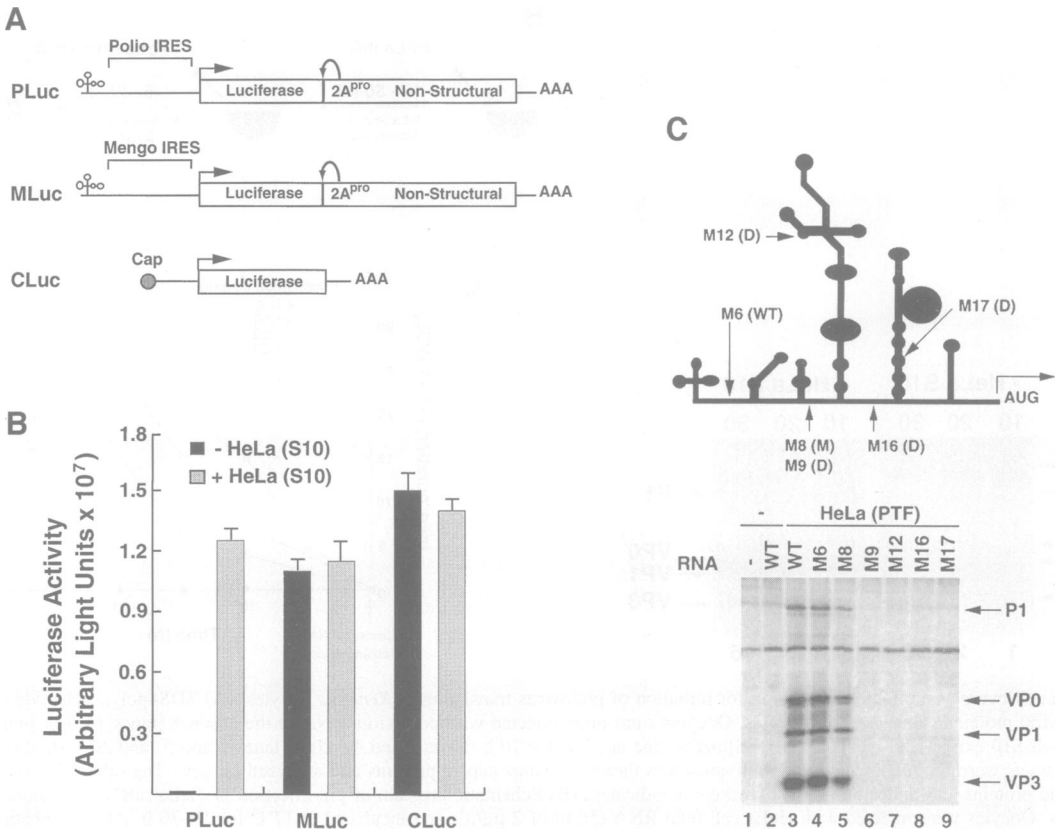

Fig. 2. A HeLa cell cytoplasmic factor is specific for poliovirus internal ribosomal entry. (**A**) Schematic representation of the genome of chimeric poliovirus replicons and capped RNA carrying luciferase reporter gene. A recognition and cleavage site for $2A^{pro}$ has been introduced between luciferase and $2A^{pro}$ and the proteolytic processing at this site is represented by an arrow. An internal-ribosomal-entry-site (IRES) at the 5′-non-coding region is indicated, PLuc carries the complete poliovirus IRES, and in MLuc replicon the nucleotides 109–743 of poliovirus RNA have been replaced by nucleotides 171–718 of mengovirus RNA. An RNA motif implicated in RNA synthesis is represented by a cloverleaf at the 5′-end of the replicon RNAs. (**B**) Luciferase activity following microinjection of replicons and capped RNA in the presence or absence of HeLa cell S10 extract. *In vitro*-synthesized RNAs were microinjected into oocytes alone (black bars) or co-injected together with S10 HeLa extract (gray bars). Injected oocytes were incubated at 22°C for 20 h, and luciferase activity was determined. (**C**) RNA elements required for poliovirus translation in oocytes. Top diagram: computer-predicted secondary structure of the 5′NCR of poliovirus RNA. The arrows indicate the location of six IRES mutants: M6 (11-base insertion in position 108), M8 (four-base insertion in position 224), M9 (12-base insertion in position 224), M12 (three-base insertion in position 325), M16 (11-base insertion in position 443) and M17 (two-base deletion in position 460). The phenotype of poliovirus mutants, as determined in HeLa cells, is indicated between brackets. WT, wild-type (silent mutation); M, mutant (small plaque phenotype); D, dead (lethal mutation). Bottom diagram: SDS–polyacrylamide gel analysis of ^{35}S-labeled viral proteins synthesized in oocytes. Oocytes were microinjected with *in vitro* transcribed RNA of wild-type (lanes 2 and 3) or mutants (lanes 4–9). HeLa extracts were omitted (lanes 1 and 2) or co-injected with the RNA (lanes 3–9). Poliovirus protein synthesis was analyzed as described in Figure 1A. The relative position of poliovirus capsid proteins is indicated.

introduced at the 3′ end of the reporter gene. Translation of this subgenomic RNA (PLuc) generates a polyprotein-containing luciferase at its amino-terminus. Appropriate proteolytic processing by $2A^{pro}$ releases the active enzyme from the rest of the polyprotein (Figure 2A).

No luciferase activity was detected in oocytes micro-injected with PLuc RNA alone (Figure 2B), but co-injection of the poliovirus subgenomic RNA with HeLa S10 extract stimulated translation of luciferase 10 000-fold. Luciferase activity correlated with the amount of HeLa extract injected (data not shown), indicating that PTF stimulates translation in a dose-dependent manner.

To determine whether PTF specifically increases poliovirus translation, a capped RNA encoding the luciferase gene flanked by 5′ and 3′ untranslated regions of *Xenopus* β-globin was used as a control. As expected, this RNA was very efficiently translated in oocytes, and co-injection of HeLa S10 extract did not stimulate translation (Figure 2B), suggesting that PTF is not a general initiation factor used in cap-dependent translation.

Based on sequence homologies of the IRES elements, the picornavirus family can be divided into two major groups: enteroviruses and rhinoviruses (type I), and cardio-viruses and aphtoviruses (type II). There is strong sequence conservation within each group, but little between groups, and it is not known whether translation initiation occurs by similar mechanisms. To determine whether PTF is required for both types of IRES-mediated translation, we microinjected a chimeric subgenomic RNA in which the poliovirus IRES was replaced by the mengovirus IRES (MLuc). MLuc RNA was efficiently translated in oocytes whether or not HeLa proteins were co-injected (Figure 2B), suggesting that PTF is required specifically for poliovirus IRES function and not for cap-dependent or cardiovirus translation. This result is consistent with a previous observation in which it was shown that encephalomyocarditis virus RNA (a closely related cardiovirus) is efficiently translated in *Xenopus* oocytes (Laskey *et al.*, 1972).

We have previously generated mutations within the

Table I. Subcellular localization of PTF in mammalian cells

	Source of protein		Stimulation index[a]
Human	HeLa cells	cytoplasmic	1×10^4
		nuclear	1×10^2
		S100	4×10^3
		ribosomal wash	6×10^3
	La protein[b]		3
Rat	brain	cytoplasmic	5×10^3
		ribosomal wash	3×10^3
Rabbit	reticulocyte lysate		1×10^3
Control[c]			1

[a]Translation stimulation index is the ratio between total luciferase activity in the sample and luciferase activity in the control.
[b]Recombinant La protein (1 μg/ml).
[c]Microinjection with PLuc alone.

poliovirus IRES which disrupt the ability of the RNA to be translated in HeLa cells. To establish whether oocytes utilize the same RNA elements for internal initiation of translation, we measured the translation efficiency of several deletion or insertion mutants having different phenotypes. The M6 mutation does not alter the viral phenotype, the M8 mutation yields viruses with a small plaque phenotype, and M9, M12, M16 and M17 mutations completely disrupt virus production in HeLa cells. Oocytes were microinjected with wild-type or mutated RNAs, and protein synthesis was monitored by [^{35}S]methionine incorporation into capsid proteins (Figure 2C). An excellent correlation was observed between the phenotype in HeLa cells and the ability to translate in oocytes. Wild-type virus and M6 produced high levels of capsid proteins (lanes 3 and 4), the small plaque mutant (M8) produced 5-fold less protein than wild-type (lane 5), and all lethal mutants produced no protein (lanes 6–9). As expected, capsid protein production was dependent on viral RNA and S10 HeLa cell extract (lanes 1 and 2). Thus, it appears that many of the same RNA elements required for translation in HeLa cells are also necessary for efficient translation in oocytes, suggesting that similar mechanisms of translation initiation are likely to be used in both cell types.

PTF is a large polypeptide or a complex

Subcellular fractionation indicated that 99% of the PTF activity is localized in the cytoplasm (Table I). PTF is distributed between a ribosomal fraction and the cytosol, and 60% of the activity can be eluted from the ribosomal fraction by a high-salt wash (Table I).

Among several cellular proteins implicated in internal initiation of poliovirus translation, a 52 kDa protein, identified as La autoantigen, has been shown to be required in rabbit reticulocyte lysate (RRL) (Meerovitch *et al.*, 1989, 1993). To determine whether PTF is the La autoantigen, recombinant La, which was active to stimulate translation in RRL, was co-injected together with PLuc. No translation stimulation was observed (Table I), suggesting that La is not the factor missing in oocytes.

PTF activity was also detected in several other mammalian cells, including rat brain and rabbit reticulocytes (Table I); however, the specific activity from these tissues was lower than in HeLa cell extracts. The amount of

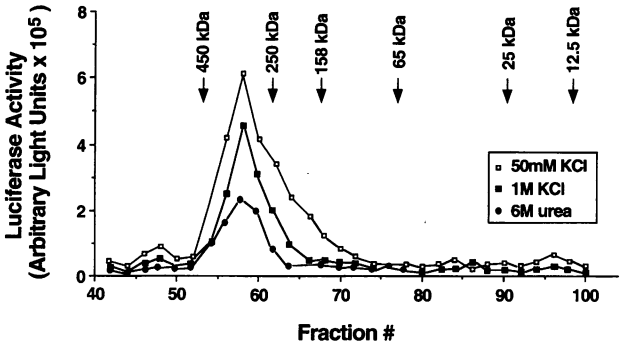

Fig. 3. The HeLa cell factor required for poliovirus translation is a protein or complex of proteins. A highly concentrated PTF was eluted by high salt-wash of a ribosomal pellet and loaded onto Superdex-S200 equilibrated with buffer S (□). Other chromatograms were obtained under dissociating conditions (■, 1 M KCl or ●, 6 M urea). PTF activity was determined by microinjection in oocytes as described in Materials and methods.

PTF activity in rabbit reticulocytes is considerably high, reaching to a 1000-fold stimulation; however, it was 10-fold lower than in HeLa cell extracts. At this time, we do not know whether the difference is due to a lower amount of PTF in rabbit reticulocytes or to a less active factor.

The molecular weight of PTF was estimated by gel filtration (Superdex-S200) to be ~300 kDa (Figure 3). To determine whether PTF is a single large polypeptide or a complex of several proteins, we measured the elution profile of PTF by gel filtration under dissociating conditions. The translation stimulation activity eluted in the same fractions in 6 M urea or 1 M KCl as under native conditions (Figure 3), suggesting that PTF is either an unusually stable complex or a single large polypeptide.

A second factor from HeLa cell is required for poliovirus RNA synthesis in Xenopus oocytes

To determine whether poliovirus RNA is synthesized in oocytes, viral RNA was co-injected with HeLa S10 extract and [α-^{32}P]GTP, and incubated in the presence of actinomycin D to inhibit DNA-directed RNA synthesis. Total cytoplasmic RNA was extracted 10 h post-injection and analyzed by agarose gel electrophoresis. Two major species of labeled RNA were detected, one corresponding to single-stranded poliovirus RNA (ssRNA) and the other to double-stranded replicative form (dsRNA) (Figure 4A). As a control for authentic poliovirus RNA synthesis, we analyzed viral RNA synthesis *in vitro* using poliovirus-infected HeLa cell extracts (crude replication complexes, CRC) (Caliguiri and Tamm, 1970). The RNA species obtained in oocytes were indistinguishable from those obtained in HeLa cell CRC and the molar ratio between ssRNA and dsRNA produced by the oocytes closely resembled that obtained from poliovirus-infected HeLa cells. In addition, brefeldin A, a potent inhibitor of poliovirus RNA replication (Maynell *et al.*, 1992), completely abolished viral RNA production in oocytes (data not shown), providing further evidence that the oocyte system faithfully reproduces the mechanisms used by poliovirus in the natural host.

To determine whether factors in addition to PTF are required for RNA synthesis in oocytes, we employed a chimeric poliovirus (PoMe) RNA in which the original

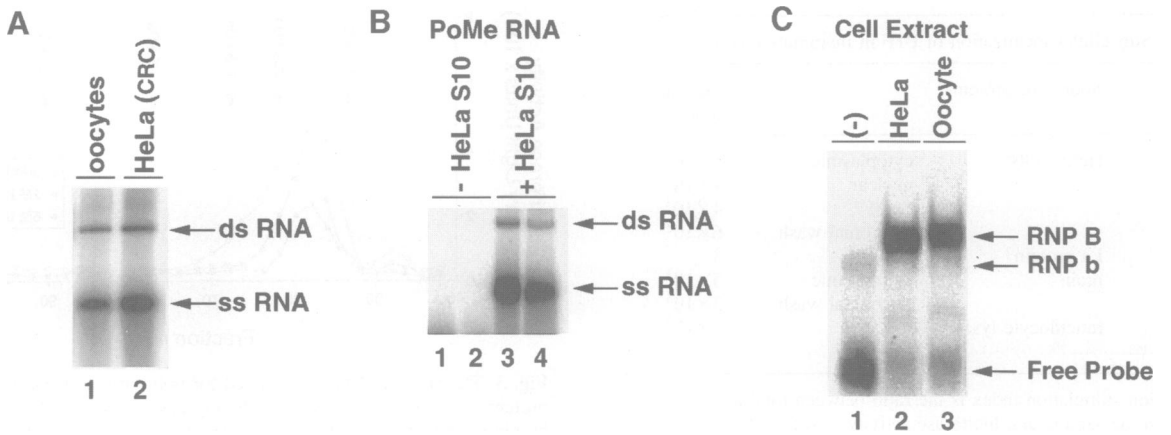

Fig. 4. Poliovirus RNA synthesis in oocytes requires a HeLa cell *trans*-acting factor. (**A**) *De novo* synthesis of poliovirus RNA in oocytes. Oocytes were microinjected with poliovirus RNA, HeLa cell S10 extract and [α-^{32}P]GTP. Injected oocytes were incubated for 10 h at 27°C and total cytoplasmic RNA was analyzed on agarose gel. For comparison, poliovirus RNA was synthesized *in vitro* using viral-infected HeLa cell CRC. dsRNA and ssRNA are indicated. (**B**) Synthesis of poliovirus/mengovirus chimeric RNA in oocytes. Poliovirus RNA, in which the IRES has been replaced by the mengovirus IRES, was injected in the absence (lanes 1 and 2) or in presence (lanes 3 and 4) of HeLa cell S10 extract. Microinjected oocytes were incubated at 27°C for 10 h (lanes 1 and 3) or 15 h (lanes 2 and 4). Total cytoplasmic RNA was obtained and analyzed by agarose gel electrophoresis. (**C**) Cytoplasmic oocyte extract contains an activity that forms a ribonucleoprotein complex previously identified to be involved in RNA synthesis. Uniformly labeled first 100 nucleotides of the poliovirus genome, the cloverleaf RNA (20 000 c.p.m./ng), was incubated with 1 μl of bacterial extract expressing the uncleaved precursor for the viral protease and polymerase (3CD), in the absence (lane 1) or presence of 13 μg/μl of mock-infected HeLa cell extract (lane 2), or 6 μg/μl of cytoplasmic oocyte extract (lane 3). Samples were separated by electrophoresis through a native polyacrylamide gel in 0.5×TBE buffer, and autoradiographed. Complex RNP B, RNP b (containing 3CD and cloverleaf) and 'Free Probe' are indicated at the right.

poliovirus IRES was replaced by the mengovirus IRES. In HeLa cells, this chimeric virus replicates with similar kinetics to wild-type poliovirus (unpublished data). Despite the fact that the mengovirus IRES directs efficient translation in oocytes without additional HeLa factors (Figure 2B), PoMe RNA was unable to amplify its RNA in the absence of HeLa cell S10 extract (Figure 4B). Thus, it appears that additional cellular factor(s) are required for poliovirus RNA synthesis in oocytes.

We have previously shown that the first 90 nucleotides of the poliovirus genome fold into a cloverleaf-like structure and bind the uncleaved 3CD–viral protease–polymerase precursor and a 36 kDa ribosome-associated cellular protein (p36). This complex (RNP-B) appears to participate in the initiation of positive strand synthesis. p36 specifically interacts with one of the stem–loops of the cloverleaf structure and is required for complex formation (Andino *et al.*, 1993). To determine whether p36 is present in oocytes, we performed RNA-binding experiments using a mobility shift assay. Oocyte extracts formed a RNP-B complex indistinguishable from the HeLa complex (Figure 4C) and appeared to contain a similar amount of p36. While the binding assay does not demonstrate that p36 is functional for poliovirus replication in oocytes, its presence in extracts suggests that a different factor required for RNA replication may be absent in oocytes.

Poliovirus replication in Xenopus oocytes produces a high titer of infectious particles

To establish if a complete viral replication cycle can be achieved in oocytes, we microinjected poliovirus RNA in the presence of HeLa cell S10 extract, prepared cell lysates between 0 and 20 h post-injection, and determined the yield of infectious virus by a plaque assay. Infectious virus was detected as early as 6 h post-injection (100 p.f.u. per oocyte) and the amount of virus increased exponentially, reaching a maximum of 5×10^5 p.f.u. per oocyte after 15 h (Figure 5A). The production of virus was strikingly dependent on temperature: no infectious virus was produced at 17, 22, 24 or 37°C, whereas a large amount of virus was made at 27, 30 and 32°C, with a 30°C optimum (Figure 5B).

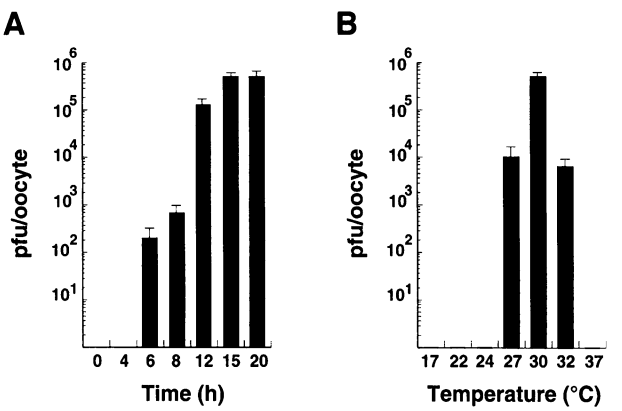

Fig. 5. Microinjection of poliovirus RNA into oocytes yielded newly synthesized infectious virus. (**A**) Poliovirus RNA was injected into oocytes and incubated at 30°C for 0, 4, 6, 8, 12, 15 and 20 h. Infectious poliovirus titers were determined by plaque assay in HeLa cells. Viral titers are expressed as the logarithm of plaque forming units (p.f.u.) per oocyte. Data represents the average of four independent experiments (error bars indicate standard deviations). (**B**) Essentially as described in (A) but the microinjected oocytes were incubated for 20 h at 17, 22, 24, 27, 30, 32 and 37°C.

To determine which step of viral replication was temperature dependent, RNA translation, RNA replication and viral assembly were analyzed at permissive and non-permissive temperatures. Translation of poliovirus RNA was measured by [^{35}S]methionine incorporation into VP1. The oocyte translation apparatus was able to efficiently

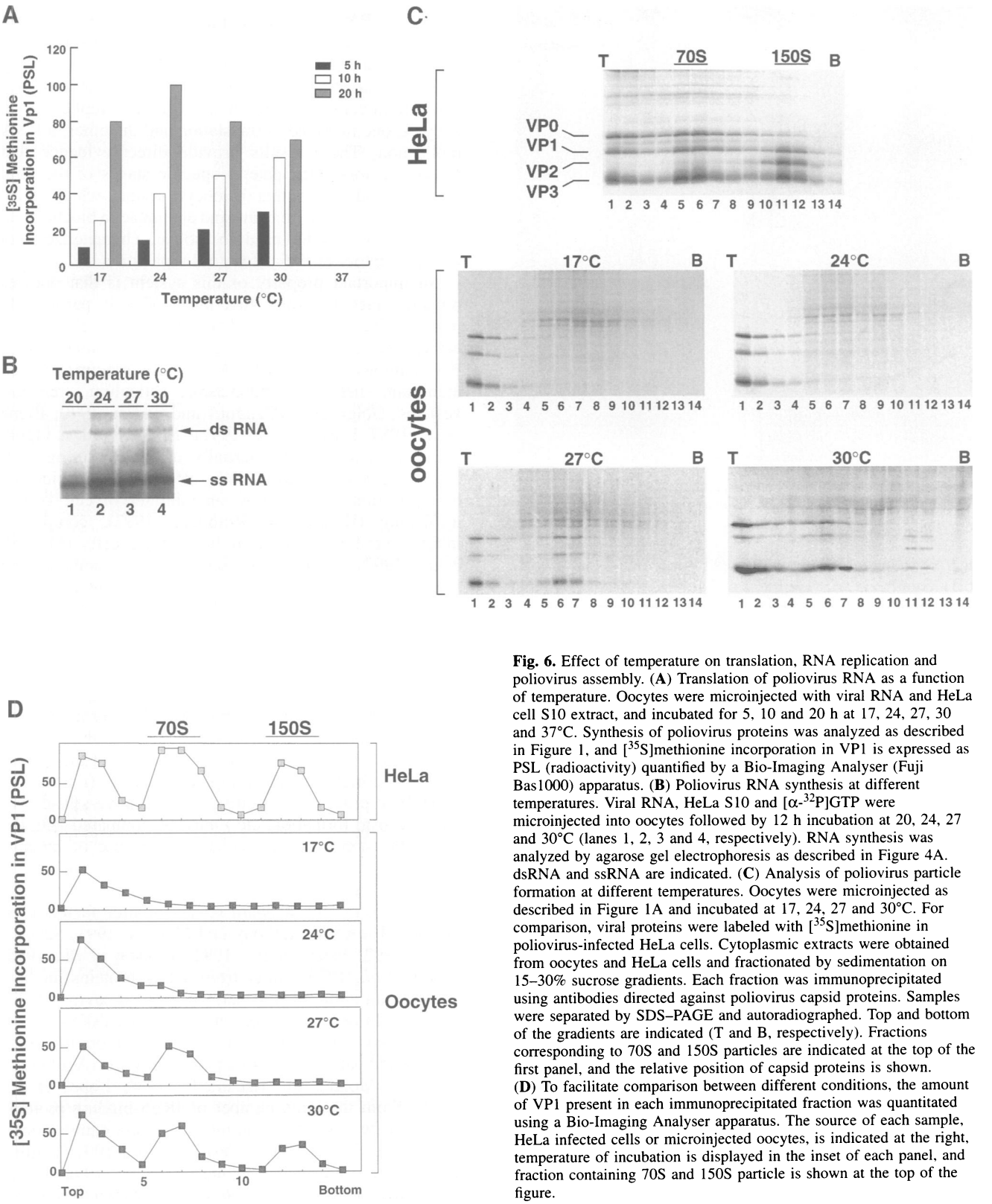

Fig. 6. Effect of temperature on translation, RNA replication and poliovirus assembly. (**A**) Translation of poliovirus RNA as a function of temperature. Oocytes were microinjected with viral RNA and HeLa cell S10 extract, and incubated for 5, 10 and 20 h at 17, 24, 27, 30 and 37°C. Synthesis of poliovirus proteins was analyzed as described in Figure 1, and [^{35}S]methionine incorporation in VP1 is expressed as PSL (radioactivity) quantified by a Bio-Imaging Analyser (Fuji Bas1000) apparatus. (**B**) Poliovirus RNA synthesis at different temperatures. Viral RNA, HeLa S10 and [α-^{32}P]GTP were microinjected into oocytes followed by 12 h incubation at 20, 24, 27 and 30°C (lanes 1, 2, 3 and 4, respectively). RNA synthesis was analyzed by agarose gel electrophoresis as described in Figure 4A. dsRNA and ssRNA are indicated. (**C**) Analysis of poliovirus particle formation at different temperatures. Oocytes were microinjected as described in Figure 1A and incubated at 17, 24, 27 and 30°C. For comparison, viral proteins were labeled with [^{35}S]methionine in poliovirus-infected HeLa cells. Cytoplasmic extracts were obtained from oocytes and HeLa cells and fractionated by sedimentation on 15–30% sucrose gradients. Each fraction was immunoprecipitated using antibodies directed against poliovirus capsid proteins. Samples were separated by SDS–PAGE and autoradiographed. Top and bottom of the gradients are indicated (T and B, respectively). Fractions corresponding to 70S and 150S particles are indicated at the top of the first panel, and the relative position of capsid proteins is shown. (**D**) To facilitate comparison between different conditions, the amount of VP1 present in each immunoprecipitated fraction was quantitated using a Bio-Imaging Analyser apparatus. The source of each sample, HeLa infected cells or microinjected oocytes, is indicated at the right, temperature of incubation is displayed in the inset of each panel, and fraction containing 70S and 150S particle is shown at the top of the figure.

support poliovirus translation at 17, 24, 27 and 30°C, while no capsid protein was synthesized at 37°C (Figure 6A). Thus, the inability to produce virus at 37°C may be attributed to an effect on translation although it is not a major block at low temperatures.

The effect of temperature on RNA synthesis was analyzed by microinjecting poliovirus RNA, HeLa S10 extract and [α-^{32}P]GTP. Microinjected oocytes were incubated at different temperatures for 12 h, and total cytoplasmic RNA analyzed by agarose gel electrophoresis. At all temperatures examined, the accumulation of newly synthesized viral RNA was similar, with a slight increase of

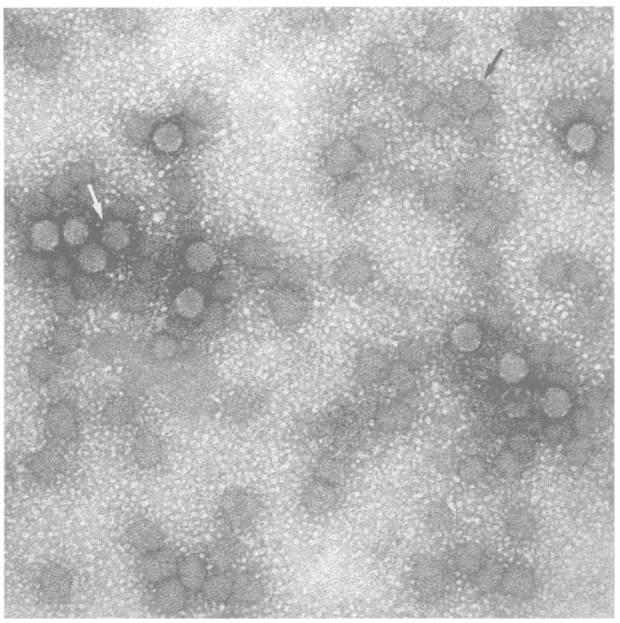

Fig. 7. Electron micrograph showing poliovirus-like particles of about 30 nm produced in microinjected *Xenopus* oocytes.

RNA production at temperatures higher than 24°C (Figure 6B). Synthesis was identical at 24 and 30°C even though virus production was drastically different (Figure 5B).

We next examined viral particle assembly. Oocytes were injected with poliovirus RNA and HeLa cell S10 extract and were incubated at 17, 22, 27 and 30°C for 20 h in the presence of [^{35}S]methionine. Cytoplasmic extracts were fractionated by sedimentation in a 15–30% sucrose gradient. Each fraction was analyzed by immunoprecipitation with anti-capsid antibodies and SDS–PAGE (Figure 6C). As a control, we prepared particles from poliovirus-infected HeLa cell extracts using the same procedure. Extracts from infected HeLa cells yielded unassembled protomers or pentamers at the top of the gradient, 70S empty capsids (fractions 5–7), and virions with a sedimentation coefficient of 150S (fractions 11–12). In oocytes, at 17, 22°C and 24°C, capsid proteins were found only at the top of the gradient, indicating that no viral particles were formed. In contrast, at 27 and 30°C, empty capsids (70S particles) were readily detected in fractions 5–7. More significantly, at 30°C, the optimal temperature for virus production, immunoreactive capsid proteins were detected in fractions which contained the mature 150S particle. In addition, these fractions displayed a polypeptide composition typical of mature virions in which VP2 is detected. This strongly suggests that, at 30°C, virus assembly is completed, therefore yielding infectious virus. In contrast, at temperatures below 27°C assembly is impaired.

Poliovirus particle production was confirmed by transmission electron microscopy. High-magnification views of microinjected oocyte extracts revealed an abundance of poliovirus-like particles (Figure 7).

Discussion

In this study we demonstrated that *Xenopus* oocytes are able to support poliovirus replication. Microinjection of poliovirus RNA initiates a replication cycle, in which RNA translation, genome replication, and virus assembly closely resemble those processes observed in a naturally infected host. We discovered that two different activities from HeLa cells are required for poliovirus replication in oocytes, one involved in translation and the other in RNA replication. These results provide direct evidence that cellular factors participate at specific stages of the viral life cycle and suggest that the oocyte system, which seems both physiologically relevant and amenable to biochemical intervention, can be used to isolate, characterize, and identify factors essential for viral replication.

An important property of this system is that oocytes remain intact and fully functional. This is particularly relevant since poliovirus replication employs cellular processes that may work efficiently only in intact cells. For example, poliovirus RNA synthesis requires the cell membrane network (endoplasmic reticulum, transport vesicles, Golgi, etc.) (Caliguiri and Tamm, 1970; Bienz *et al.*, 1987; Irurzun *et al.*, 1992). This network is highly organized and may be partially disrupted during cell-free extract preparation, thus affecting the efficiency of replication. Brefeldin A, an inhibitor of cell vesicle trafficking (Helms and Rothman, 1992), completely inhibits viral production in both HeLa cells (Maynell *et al.*, 1992) and oocytes, suggesting that some of the fundamental processes involved in recruiting the membranous components for viral replication are faithfully reconstituted in oocytes. Moreover, the biochemical environment in oocytes is expected to more closely resemble that of the intact HeLa cell (pH, salt, protein concentration, other metabolites, etc.); thus, biochemical interventions in this system may provide information that better reflects those processes which occur during viral replication. However, because oocytes are not the natural host for poliovirus, each replication step (translation, proteolytic processing, positive- and negative-strand synthesis, capsid formation, etc.) must be compared with the analogous step in human cells, and the use of mutants with well-defined phenotypes is critical if we are to establish a tight correlation.

In addition to the standard set of initiation factors used by capped mRNA (Anthony and Merrick, 1991; Scheper *et al.*, 1992; Pause *et al.*, 1994; Jackson *et al.*, 1995; Meyer *et al.*, 1995), other *trans*-acting proteins mediate internal initiation of translation by picornavirus IRES elements. In fact, it has been proposed that IRES-specific factors may contribute to the determination of tissue tropism and pathogenesis of a particular virus (Agol *et al.*, 1989; La Monica and Racaniello, 1989; Lopez *et al.*, 1989). From the large number of IRES-binding proteins identified by UV cross-linking or gel retardation assays (Najita and Sarnow, 1990; Pestova *et al.*, 1991; Dildine and Semler, 1992; Gebhard and Ehrenfeld, 1992; Haller and Semler, 1995), only two have been shown to promote internal initiation in a functional assay: the La autoantigen (52 kDa) (Meerovitch *et al.*, 1989, 1993) and a 57 kDa protein identified as polypyrimidine-tract-binding protein (Hellen *et al.*, 1993; Kaminski *et al.*, 1995). La autoantigen appears to be utilized specifically by poliovirus IRES, whereas polypyrimidine-tract-binding protein may be used by both enterovirus and cardiovirus IRESes. Because PTF, the poliovirus translation factor reported here, appears to

be required for poliovirus but not for mengovirus, it seems that PTF is not the polypyrimidine-tract-binding protein. We also present evidence suggesting that La protein is not the HeLa factor absent in the oocytes, since microinjection of recombinant La protein did not stimulate poliovirus translation (see Table I). Furthermore, a protein of 97 kDa (p97), which binds to enterovirus IRES and stimulates rhinovirus RNA translation in rabbit reticulocyte lysates, has recently been reported to form part of a large complex of proteins (>400 kDa) (Borman *et al.*, 1993). This activity is not present in rabbit reticulocyte lysates, and the complex is unstable at 250 mM KCl (Borman *et al.*, 1993). In contrast, 40–70% of PTF activity remained after treatment with 1 M KCl or 6 M urea and considerable activity is present in RRL. These differences suggest that the complex containing p97 and PTF could be different factors; however, further analysis will be required to establish the identity of PTF. Also, it remains to be determined whether or not PTF directly binds to the IRES or participates in internal ribosomal entry by interacting with other parts of the translation machinery.

PTF activity can be provided by pre-injecting oocytes with HeLa cell total RNA. However, the activity was observed only if pre-injected oocytes were incubated for several hours before injection of poliovirus RNA (Figure 1C). This lag must represent the time required for the expression of proteins that constitute PTF. Furthermore, because the activity can be provided by microinjection of mRNA, it may be possible to employ an expression cloning strategy in which *in vitro* transcripts obtained from a cDNA HeLa cell library are microinjected into oocytes, to isolate a molecular clone (Masu *et al.*, 1987; Julius *et al.*, 1988; Brake *et al.*, 1994).

A poliovirus-containing mengovirus IRES (PoMe) could be translated in oocytes without addition of HeLa cell factors. However, none of the poliovirus RNA species observed in infected HeLa cells was detected unless HeLa proteins were also microinjected. This result suggests that oocytes lack a factor(s) required to support RNA replication (poliovirus replication factor, PRF). Because we can detect negative-strand RNA production in the absence of positive-strand synthesis (data not shown), we believe that the HeLa PRF is required at the earliest stages of RNA replication, perhaps during the initiation of negative-strand RNA synthesis. The specific activity of this replication factor was 10-fold higher in S10 cytoplasmic extracts than in a ribosomal salt-wash fraction, in contrast to the translation factor, which was more abundant in the ribosome fraction. Therefore it appears that the translation and replication activities reside on at least two different proteins.

Recently, using a HeLa cell *in vitro* system, it was found that the initiation of poliovirus RNA synthesis was dependent on a soluble HeLa cell cytoplasmic fraction (Barton *et al.*, 1995). Although we do not know whether the factor identified in the *in vitro* system is the same as required in oocytes, the observations are consistent with the idea that cellular factors actively participate in viral RNA synthesis.

Despite the fact that both viral translation and RNA synthesis are very efficient at 24°C, no viral particles were formed at temperatures below 27°C, indicating that a critical step in particle formation or RNA packaging is temperature dependent. We demonstrated that empty capsid assembly proceeds only at temperatures higher than 27°C, which also correlated with the minimal temperature required for virus formation. The poliovirus particle assembly is a poorly understood process, and controversy remains as to whether the 70S empty capsids are direct precursors to mature particles, or whether they are a storage pool of capsid proteins (Rombaut *et al.*, 1987; Hellen and Wimmer, 1995). We propose that manipulation of poliovirus assembly in oocytes, by shifting the temperature between 24 and 27°C, could be used to study the molecular details of these processes.

In summary, a system has been developed in which correct viral translation, protein processing, RNA replication and assembly of capsid protein leads to the production of infectious poliovirus in *Xenopus* oocytes. Because *trans*-acting factors from HeLa cells are required for viral replication, the oocyte system may be used to identify these cellular factors by complementation of the missing function.

Materials and methods

DNA procedures and plasmids

Restriction enzymes, T4 DNA polymerase, T4 DNA ligase, DNA polymerase I (Klenow fragment), T7 and SP6 RNA polymerase and human placenta RNase inhibitor were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN); all enzymes and compounds were used according to the manufacturer's instructions. Plasmids containing the entire infectious poliovirus cDNA, pXpA and the subgenomic replicon carrying the luciferase reporter gene, PLuc (previously named pRLuc), have been described previously (Andino *et al.*, 1993). pPoMe and MLuc were constructed by replacing nucleotides 109–743 of the poliovirus IRES for nucleotides 171–718 of the mengovirus IRES, in poliovirus wild-type cDNA molecular clone and in PLuc, respectively. Briefly, a *Bss*HII–*Eco*RI fragment corresponding to the mengovirus IRES was obtained by PCR using oligonucleotides 1 and 2 (oligonucleotides: 1. CTTAGCGCGCACATTACTGGCCGAA; 2. CCTCCATAGCGGCCG-CTTGTTCCATGGTTG) and as a template we used a mengovirus full-length cDNA plasmid, pM16, generously provided by Ann Palmenberg (Duke and Palmenberg, 1989). The PCR fragment was inserted into the PLuc poliovirus replicon digested with *Bss*HII–*Eco*RI. The chimeric subgenomic replicon was named MLuc. Infectious polio–mengovirus chimeric cDNA, pPoMe, was constructed by replacing the luciferase gene in MLuc by the poliovirus capsid protein coding sequence. A plasmid encoding the luciferase gene flanked by β-globin non-coding regions, pSP64TLuc, which is referred throughout as CLuc, was generously provided by D.Standring (Zhou and Standring, 1991). Mutants in the 5′NCR of poliovirus have been previously described (Trono *et al.*, 1988a). The mutants pPN6, pPN8, pPN9, pPN12, pPN16 and pPN17 were used and each corresponding RNA is indicated by the letter M followed by the respective number (i.e. M6, M8, etc.). The sequence and position of the mutations are as follows:

pPN6: CTTA 108 GGAATTCCTTAG; WT: CTTA 108 G
pPN8: GGATC 224 GATCC; WT: GGATC 224 C
pPN9: GGATC 224 GGAATTCCGATCC; WT: GGATC 224 C
pPN12: GAGT 325 AGTC; WT: GAGT 325 C
pPN16: GAAT 443 GGAATTCCAATC; WT: GAAT443 C
pPN17: GAATG 460 - - GC; WT: GAATG 460 CGGC

Viral RNA was prepared by *in vitro* transcription using the plasmids described above as templates along with T7 RNA polymerase (Maniatis *et al.*, 1989).

HeLa cell extracts

HeLa S3 cells were grown in suspension with 5% fetal calf serum and 5% horse serum. Cytoplasmic extracts (S10) were prepared from 20 l of uninfected HeLa cells. The cells were harvested by centrifugation, washed three times with cold phosphate-buffered saline (PBS), and collected by centrifugation. The pellet was resuspended in 2 vols of hypotonic buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $MgAc_2$, 2 mM DTT), incubated on ice for 20 min and broken with 20 strokes

of a glass Dounce homogenizer. A post-nuclei supernatant was obtained by centrifugation at 5000 *g* for 10 min at 4°C. This supernatant was submitted to a second centrifugation (15 000 *g* for 20 min) in order to obtain S10 cytoplasmic extract. Further centrifugation yielded a post-ribosomal supernatant (S100) and a ribosomal pellet as described previously (Brown and Ehrenfeld, 1979). Briefly, the S10 supernatant was centrifuged at 300 000 *g* for 20 min at 4°C in order to pellet the ribosomes. The supernatant S100 was stored at –70°C and the ribosomal pellet was resuspended in hypotonic buffer in one-tenth of the original volume. The final salt concentration of the ribosome suspension was adjusted to 0.5 M KCl by addition of 4 M KCl, incubated for 20 min on ice, and centrifuged at 300 000 *g* for 20 min. The supernatant (ribosomal-wash fraction) was supplemented with 5% glycerol and stored. Nuclear extracts were prepared as described by Dignam *et al.* (1983). Recombinant La autoantigen protein was generously provided by N.Sonenberg (Svitkin *et al.*, 1994).

Fractionation by gel filtration was performed by FPLC (Pharmacia) using a Superdex-S200 column. The column was equilibrated in buffer S (20 mM HEPES, pH 7.5, 1 mM DTT, 100 mM KCl) or in buffer S containing high salt (1 M KCl) or 6 M urea. Ribosomal-wash fractions (200 µl) were loaded in each case, and 2 ml fractions were collected. The eluted samples containing KCl or urea were dialyzed overnight against buffer S. To analyze the translation stimulation activity of PTF, 25 nl of each fraction were microinjected into oocytes together with 25 nl of PLuc (1 µg/µl) and luciferase activity was measured after 20 h of incubation at 22°C.

HeLa RNA was prepared from 1 l of suspension culture. Cells were harvested by centrifugation and washed three times with cold PBS. The pellet was resuspended and lysed in 5 ml of buffer H [10 mM HEPES, pH 7.5, 50 mM KCl, 0.5% Triton X-100, 1 mM DTT, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. Debris and nuclei were removed in a microcentrifuge at 10 000 *g* for 5 min. SDS was added (to a final concentration of 1%), extracted three times with phenol/chloroform and precipitated with ethanol (Ausubel *et al.*, 1994).

Viral RNA preparation

A suspension culture of HeLa S3 cells (1×10^8 cells in 200 ml) was infected with poliovirus type 1 (Mahoney) or chimeric poliovirus (PoMe) at a multiplicity of infection of 10. The cells were harvested 15 h post-infection and lysed by three cycles of freezing and thawing. The cell lysate was clarified by centrifugation at 5000 *g* and used to infect 2×10^9 cells (4L). Infected cells were incubated at 37°C for 7 h, washed with cold PBS, and lysed in 10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 0.1% Nonidet P-40. Nuclei and debris were removed by centrifugation at 10 000 *g* and SDS was added to 0.5%. Viruses were precipitated by centrifugation 1 h at 200 000 *g*. Precipitated viruses were resuspended overnight in 2 ml TSE buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA) and purified by CsCl. The virus bands were dialyzed against TSE buffer and treated with proteinase K (200 µg/ml). Viral RNA was extracted with phenol/chloroform and precipitated with ethanol.

Translation and replication of poliovirus in Xenopus oocytes

Oocytes were surgically isolated (Marcus Sekura and Hitchcock, 1987) and enzymatically defolliculated by incubation with 2 mg/ml of collagenase (Worthington CSL-1) for 3 h at room temperature. Defolliculated oocytes were washed five times with modified Barth's solution [MBS: 7.5 mM Tris, pH 7.6, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 8.2 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, 100 U/ml penicillin, 100 µg/ml streptomycin, 2% Ficoll-400] and kept in MBS at 17°C. Stage VI sorted oocytes were injected with 25 nl of viral or *in vitro*-transcribed RNA (1 µg/µl), plus 25 nl of HeLa cell extract (13 µg/µl of protein) or buffer control. To determine viral RNA translation, microinjected oocytes were incubated in MBS containing 400 µCi [^{35}S]methionine/ml. At each time point 20 oocytes were lysed mechanically in 200 µl of buffer H. Debris was removed by centrifugation at 5000 *g* and proteins were immunoprecipitated according to standard procedures using antibodies directed against capsid proteins or $3D^{pol}$; immunoprecipitated complexes were washed three times with buffer I (100 mM borate, pH 8.2, 0.1% SDS, 1% DOC, 1% Triton X-100, 500 mM NaCl), resuspended in Laemmli sample buffer, incubated at 100°C for 5 min, and analyzed by electrophoresis through a 12.5% SDS–polyacrylamide gel. After electrophoresis, gels were dried and exposed to X-ray film or PhosphoImager plates. Bands were quantified in a Bio-Imaging Analyser (Fuji Bas1000).

To determine translation of subgenomic replicons carrying the luciferase reporter gene, 10 oocytes were lysed in lysis buffer (20 µl per oocyte; Promega) and spun 5 min at 10 000 *g*. The supernatant (5 µl) was assayed using a luciferase system as recommended by the manufacturer (Promega) and quantified using a Optocomp I luminometer.

Poliovirus RNA synthesis in oocytes was measured by incorporation of [α-^{32}P]GTP into newly synthesized RNA. Briefly, viral RNA (1 µg/µl) was mixed with HeLa extracts and co-microinjected (50 nl) into oocytes. After 30 min, the oocytes were microinjected a second time with 20 nl of [α-^{32}P]GTP (10 mCi/ml), and incubated in MBS buffer containing 50 µg/ml of actinomycin D (Buller and White, 1990). Thirty oocytes were lysed at various times in 400 µl of TENSK buffer (50 mM Tris–HCl pH 7.5, 5 mM EDTA, 100 mM NaCl, 1% SDS, 200 µg/ml proteinase K), incubated at 37°C for 1 h, extracted with phenol/chloroform and precipitated with ethanol. Samples were resuspended in 50 µl of TE and analyzed by electrophoresis through 0.7% native agarose gels and autoradiographed. Ribosomal RNA, visualized by ethidium bromide, was used as an internal control for RNA extraction. Crude replication complexes were prepared as previously described (Takeda *et al.*, 1986) and analyzed by the same method as for RNA analysis obtained from oocytes.

Analysis of poliovirus assembly intermediates in oocytes

Microinjected oocytes were incubated for 20 h at 20, 24, 27, 30, 32 and 37°C in MBS containing [^{35}S]methionine. [^{35}S]methionine-labeled oocyte extracts prepared from 20 oocytes were loaded directly on 5 ml gradients containing 15–30% sucrose in 10 mM Tris, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$. The particles were sedimented through the sucrose gradient by centrifugation for 3 h at 150 000 *g* at 4°C. Gradient fractions were immunoprecipitated using anti-capsid antibodies as described above. Immunoprecipitated samples were analyzed on 12.5% SDS–polyacrylamide gels. To identify the position of 150S and 70S particles in the gradient, [^{35}S]methionine-labeled cytoplasmic extracts prepared from 5×10^6 infected HeLa cells were analyzed in parallel.

For electron microscopy, oocytes were microinjected with viral RNA and HeLa S10 extract, incubated at 30°C for 20 h, and lysed in PBS. The extract was clarified by low-speed centrifugation and viruses concentrated by centrifugation through 20% sucrose cushion at 150 000 *g* for 3 h. The viral pellet was resuspended in 100 µl of H_2O, prepared and visualized essentially as described by Dubochet *et al.* (1971).

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