Function of rotavirus VP3 polypeptide in viral morphogenesis

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The phenotype of the rotavirus SA-11 mutant tsB carrying a thermosensitive mutation in gene 3, which encodes VP3, was characterized further from both infected cells and purified viral particles. The mutant phenotype was initially identified as negative for in vitro double- and single-stranded RNA synthesis. Our results show that the in vitro transcriptional properties of the tsB mutant at the restrictive temperature were identical to those of the wild-type strain. Similar results were obtained with respect to the VP3-associated guanylyltransferase activity. Analysis of viral particles made by mutant-infected cells at the restrictive temperature showed that only empty single-shelled particles were assembled. This indicates that viral morphogenesis is halted after the initial viral transcription and before RNA replication, suggesting that VP3 may be required as part of the replicase system but not for subviral particle assembly. These data suggest that such a phenotype is not due to alteration of a VP3 function related to transcription.

Rotaviruses, a genus of the Reoviridae family, are composed of a double-shelled protein capsid surrounding a central core that contains the viral genome consisting of 11 dsRNA segments (Arias et al., 1982; Mason et al., 1983). The outer capsid is made of a glycoprotein, VP7, and dimers of VP4, the haemagglutinin, which has a trypsin-sensitive site (Espejo et al., 1981; Suzuki et al., 1986). The inner capsid is made of VP6 and the viral core consists of polypeptides VP1, VP2 and VP3 and the genome (Estes & Cohen, 1989; McCrae & McCorquodale, 1982).

The function of the viral polypeptides during viral RNA replication and transcription has been studied recently (Sandino et al., 1988; Patton & Gallegos, 1988, 1990; Mansell & Patton, 1990; Valenzuela et al., 1991). Studies on in vitro transcription have allowed the identification of VP1 as the viral RNA polymerase (Valenzuela et al., 1991), VP3 as the guanylyltransferase, and VP2 as a single- and double-stranded RNA-binding protein (Pizarro et al., 1991; Liu et al., 1992; Boyle & Holmes, 1986). VP6, the major inner capsid polypeptide, is strictly required for transcription although the polypeptide seems to lack any enzymatic activity directly involved in transcription. These results suggest that viral transcription is catalysed by a multienzyme complex, present in the single-shelled particle, which requires enzymatic activities other than the viral RNA polymerase VP1 (Sandino et al., 1986; Valenzuela et al., 1991). Viral RNA replication has also been found to occur in precore particles containing polypeptides VP1, VP2 and VP3 and some of the non-structural proteins (Patton & Gallegos, 1988).

The purpose of the present communication is to investigate the involvement of VP3 in RNA replication and transcription, using a temperature-sensitive (ts) mutant of rotavirus strain SA-11 with a lesion in gene 3, which encodes the polypeptide VP3. The mutant, tsB, was obtained from F. Ramig (Ramig, 1982, 1983; Gombold et al., 1985). It was phenotypically characterized in infected cells as an ss- and dsRNA- mutant (Chen et al., 1990; Ramig & Petrie, 1984).

Using the capability of the virus to transcribe RNA products in vitro similar to those in infected cells, the mutant phenotype for transcription was determined at both restrictive and permissive temperatures. In Fig. 1 the mRNA products transcribed by SA-11 and tsB virus particles are shown. Transcription assays were carried out using purified virus particles subjected to a thermal shock (1 min at 55°C) (controls were not subjected) and further incubated at 31°C or 45°C. As seen in Fig. 1, after 30 min of incubation at 31°C there are considerably fewer ssRNA products than at 45°C. Similar results were obtained when the virus was subjected to a thermal shock before the transcription assay, suggesting that in vitro the mutant does not exhibit a ts phenotype and that its in vitro mRNA synthesis is the same as the wild-type strain. These results are obtained when similar amounts of virus were assayed (2 μg of viral protein).

Since the mutation is located in the gene encoding the VP3 guanylyltransferase, the effect of S-adenosyl-
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Fig. 1. Electrophoretic analysis of transcripts synthesized in an in vitro assay by tsB mutant and SA-11 virus particles at 31 °C (lanes 1 to 4) and 45 °C (lanes 5 to 8). Purified SA-11 (lanes 1, 3, 5 and 7) and tsB (lanes 2, 4, 6 and 8) virus particles (2 μg each) were assayed for RNA polymerase activity as previously described but using [α-32P]GTP of a high specific activity (3000 Ci/mmol) (Valenzuela et al., 1991). The mixture was incubated for 30 min at 31 °C or 45 °C; previously the virus particles were incubated, or not, at 55 °C for 1 min. The products were analysed by gel electrophoresis in 8 M-urea-5% polyacrylamide (Sandino et al., 1986). The assays were done at 31 °C, and the particles subjected (lanes 1 to 2) or not (lanes 3 to 4) to a thermal shock (1 min at 55 °C). The assay was also done at 45 °C as shown in lanes 5 to 8, and they were heat-activated (lanes 5 to 6) or not (lanes 7 to 8).

methionine (SAM) addition on viral RNA synthesis was also studied (Pizarro et al., 1991; Liu et al., 1992). The addition of SAM to the transcription mixture at a final concentration of 0.3 mM increases the yield of RNA synthesis, by four- to fivefold for SA-11 and the mutant, at all the temperatures studied. When similar experiments were carried out at 31 °C the yield and the rate of RNA synthesis were less than 20% of that obtained at 45 °C for both the mutant and the wild-type strain (data not shown).

Another assay performed to characterize the guanylyltransferase activity was based on the unique property of the enzyme to form a covalent complex with GMP. This complex is an intermediate during the synthesis of the cap at the 5' end of the mRNA (Shuman, 1982). Assays were carried out at two temperatures, 31 °C and 37 °C. Purified viral particles were assayed for GMP–enzyme complex formation in a 12 μl reaction mixture as previously described using [α-32P]GTP (specific activity of 3000 Ci/mmol) (Pizarro et al., 1991). The formation of a covalent complex between GMP and VP3 was determined by electrophoresis in a 12% polyacrylamide gel (Laemmlli, 1970). Gels were dried, strips of approximately 0.25 cm were cut and the associated radioactivity was determined in a liquid scintillation counter. In Fig. 2 the formation of the covalent complex [α-32P]GMP–enzyme, for both the tsB mutant and the wild-type strain at both restrictive and permissive temperatures are shown. As seen in this figure, at 37 °C there is a peak of radioactivity associated with viral proteins with a relative mobility corresponding to VP3, for both SA-11 and tsB. When the same assay was done at 31 °C, the VP3-associated radioactivity was similar to the background, for SA-11 as well as for tsB. These results imply that the guanylyltransferase properties of the tsB mutant and SA-11 are similar with respect to the formation of the covalent GMP–enzyme complex. These results suggest that once the mutant viral proteins are assembled in vivo at a permissive temperature (31 °C), the mutation in VP3 is not expressed in the viral progeny thus showing similar characteristics to the wild-type strain for in vitro mRNA synthesis.
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*d.o.

500 400 300 200 100 0

1 2

VP1 VP2 VP3 VP4

500 400 300 200 100 0

1 2 3 4 5 6 7

Strips

Fig. 2. Quantification of the formation of the covalent VP3-GMP complex. The virus particles SA-11 (a) and tsB (b) were assayed at permissive temperature (31 °C; hatched columns) and at 37 °C (solid columns). The formation of the covalent complex VP3-GMP was assayed in a 12 µl reaction mixture as previously described using 1 µCi of [α-32P]GTP (specific activity 3000 Ci/mmol) and 1 µg of purified SA-11 or tsB virus particles (Pizarro et al., 1991). The virus particles showed similar transcriptional activity as seen in Fig. 1. The formation of a covalent complex between GMP and VP3 was determined as described in the text. The graphics represent the radioactivity associated with proteins in the different strips. The position of the viral proteins was determined according to the migration of 35S-labelled rotavirus proteins.

Based on the above results and others previously reported (Chen et al., 1990), the mutant phenotype initially described as in vivo ss- and dsRNA− could be accounted for by the following alternatives: (i) VP3 is the viral replicase; (ii) the VP3 mutation affects the assembly of a subviral particle required for the encapsidation of the viral dsRNA; (iii) VP3 interacts with other proteins in a multi-enzyme complex or subviral particle which has the replicase activity. The first theory is unlikely because it has been reported that VP1 is the viral polymerase (Valenzuela et al., 1991). The second is also improbable since at restrictive temperatures no dsRNA is detected in infected cells (Chen et al., 1990; E. Spencer, unpublished results). The last possibility appears most likely, because in isolated subviral particles engaged in viral RNA replication the presence of VP1, VP2 and VP3 and some of the non-structural polypeptides has been detected (Helmburger-Jones & Patton, 1986; Mansell & Patton, 1990).

To study the association between the formation of subviral particles and viral RNA replication, the kinetics of assembly of subviral particles was studied at restrictive and permissive temperatures. For this purpose, MA-104 cells were infected with tsB at both 31 °C and 39 °C and subviral particles were isolated from these cells 8, 12 and 24 h post-infection (p.i.). Fig. 3 shows the autoradiogram obtained after electrophoresis on Tris–glycine agarose gels of the different subviral particles recovered from the cytoplasm of infected cells (Gallegos & Patton, 1989). To identify the different virus particles, markers were prepared from purified virus particles as described elsewhere (Sandino et al., 1986). At 12 h p.i. at the permissive temperature (lanes 1 to 3) the synthesis of single-shelled particles reached a maximum level.

Fig. 3. Electrophoresis of subviral particles recovered from MA-104 infected cells using the tsB mutant at permissive (31 °C) and restrictive (39 °C) temperatures. Viral particles were isolated from cells grown at 31 °C (lanes 1 to 3) and 39 °C (lanes 4 to 6), at 8 h (lanes 1 and 4), 12 h (lanes 2 and 5) and 24 h p.i. (lanes 3 and 6). 35S-labelled subviral particles were obtained as previously described from confluent monolayers of MA-104 cells infected with 5 p.f.u. (Patton & Gallegos, 1990). The pellets containing the subviral particles were suspended in 25 µl of buffer HGD (10 mM-HEPES pH 7.6, 10% glycerol, 2 mM-dithioerythritol) and subjected to electrophoresis on a 0.6% Tris–glycine agarose gel, then dried and analysed by autoradiography. The migration of purified double-shelled, single-shelled and core particles are indicated in the figure.
Double-shelled particles seemed to accumulate after 12 h p.i. during viral morphogenesis. These results were identical to those obtained with the wild-type strain, SA-11 (data not shown). When the same experiment was carried out at the restrictive temperature (lanes 4 to 6), mostly single-shelled particles were synthesized, even at late times p.i. but the assembly of double-shelled particles seemed to be inhibited. Moreover, single-shelled particles appeared to be unstable since their amount decreased during the course of infection. This suggests that the single-shelled particles made at the restrictive temperature may correspond to particles lacking the genome RNA.

To test this hypothesis, the RNA of tsB subviral particles was phenol–chloroform-extracted and then resolved by electrophoresis on a polyacrylamide gel. Fig. 4 shows the electrophoretic analysis of the RNA content of such particles after silver staining. Lanes 1 to 3 correspond to the viral RNA extracted from subviral particles obtained at 31 °C at 8, 12 and 24 h p.i. An increase in the dsRNA content can be observed during the course of infection as expected for viral morphogenesis. On the other hand, when the RNA content of subviral particles obtained at restrictive temperature was analysed (lanes 4 to 6), no dsRNA was detected at any time after infection, suggesting that the defect lies in the minus-strand RNA synthesis, and not in the assembly of subviral particles. Furthermore, PAGE analysis revealed the presence of VP1, VP2, VP3 and VP6 in the purified subviral particle fraction before separation by electrophoresis in Tris–glycine agarose gels (data not shown).

These results make the role of VP3 in RNA replication very difficult to understand, since during transcription VP3 is responsible for the capping of viral mRNA, a reaction not carried out during replication. Furthermore the cap fragment is already present in the template (capped mRNA), suggesting that VP3 may function differently depending on the stage of viral infection, i.e. transcription or replication.

The results also show that there is some level of transcription, which may correspond to mRNA synthesis, catalysed by the originally infecting virions, which may allow the initial synthesis of sufficient structural and non-structural viral polypeptides, since the mutation does not affect that function of VP3. Therefore it is
possible to argue that the mutation affects only the process of RNA replication in such a way that the subviral particles made during the infective cycle would not be able to replicate the genome and therefore the newly made particles will be unable to transcribe.

It is of interest to point out that the formation of the single-shelled particle does not require viral replication. However, this result could be artefactual, not representing a real step in the morphogenetic pathway of the virus, and due only to the high affinity of viral polypeptides that accumulate in the absence of dsRNA and form particles under the restrictive conditions. Formation of virus-like particles had been observed when VP2 and VP6 were coexpressed in the baculovirus system (Tosser et al., 1992). The study of the formation of empty particles by a mutant may be of interest for possible rotavirus vaccine development.

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References


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