Rotavirus RNA-binding protein NSP3 interacts with eIF4GI and evicts the poly(A) binding protein from eIF4F

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Most eukaryotic mRNAs contain a 5' cap structure and a 3' poly(A) sequence that synergistically increase the efficiency of translation. Rotavirus mRNAs are capped, but lack poly(A) sequences. During rotavirus infection, the viral protein NSP3A is bound to the viral mRNAs 3' end. We looked for cellular proteins that could interact with NSP3A, using the two-hybrid system in yeast. Screening a CV1 cell cDNA library allowed us to isolate a partial cDNA of the human eukaryotic initiation factor 4GI (eIF4GI). The interaction of NSP3A with eIF4GI was confirmed in rotavirus infected cells by co-immunoprecipitation and *in vitro* with NSP3A produced in *Escherichia coli*. In addition, we show that the amount of poly(A) binding protein (PABP) present in eIF4F complexes decreases during rotavirus infection, even though eIF4A and eIF4E remain unaffected. PABP is removed from the eIF4F complex after incubation *in vitro* with the C-terminal part of NSP3A, but not with its N-terminal part produced in *E.coli*. These results show that a physical link between the 5' and the 3' ends of mRNA is necessary for the efficient translation of viral mRNAs and strongly support the closed loop model for the initiation of translation. These results also suggest that NSP3A, by taking the place of PABP on eIF4GI, is responsible for the shut-off of cellular protein synthesis.
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Introduction

Rotaviruses are members of the Reoviridae family and their genome is composed of 11 molecules of double-stranded RNA (dsRNA), ranging from 3.3 to 0.6 kb in size, which encode six structural proteins and six non-structural proteins (Estes, 1990; Mattion *et al.*, 1994). The virus replication cycle occurs entirely in the cytoplasm; as the virus enters the cell, the viral transcriptase is activated and synthesizes capped but non-polyadenylated mRNAs (Imai *et al.*, 1983). These viral mRNAs are translated or used as templates for the synthesis of genomic dsRNAs. Replication is non-conservative; mRNAs are copied in their negative strand and the dsRNAs thus formed are encapsidated in new viral particles. The replication of viral mRNAs occurs in specialized regions of the cell

cytoplasm, called viroplasms, where structural (VP2 and VP6) proteins as well as some non-structural proteins (NSP2 and NSP5) are concentrated (Petrie *et al.*, 1984; Aponte *et al.*, 1996). Other non-structural proteins NSP1 and NSP3 (Hua *et al.*, 1994; Mattion *et al.*, 1994) are not localized in the viroplasms but are, instead, spread in the cytoplasm.

In the Reoviridae, the viral mRNAs bear 5' and 3' untranslated regions (UTR) of variable length and are flanked by two different sequences common to all genes. In the case of group A rotaviruses, the 3' end consensus sequence UGACC is strictly conserved in the 11 genes. The 3' and 5' consensus sequences differ from one rotavirus serogroup to another. For example, in group C rotavirus, the 3' end consensus sequence is UGGCU (Qian *et al.*, 1991). Reassortment between rotavirus strains of different serogroups has not been observed and is thought to be restricted by these different consensus sequences.

We have shown previously that, in group A rotavirus-infected cells, the non-structural protein NSP3 (NSP3A) is bound to the 3' conserved sequence of rotavirus mRNAs (Poncet *et al.*, 1993). Furthermore, we have shown that recombinant NSP3A and NSP3 from group C rotavirus (NSP3C) recognize their cognate 3' end RNA *in vitro* (Poncet *et al.*, 1994). Unlike the rotavirus mRNAs, the vast majority of cellular mRNAs possess a 3' terminal poly-adenylated [poly(A)] sequence. Poly(A) sequences have been involved in translation regulation, RNA stability and degradation (Caponigro and Parker, 1996; Jacobson, 1996; Jacobson and Peltz, 1996; Ford *et al.*, 1997). The 3' non-coding sequences of mRNA have also been involved in RNA localization (Singer, 1996; Ross *et al.*, 1997). By analogy, we have suggested that the binding of NSP3 on the 3' end of viral mRNA was involved in RNA transportation and/or translation (Poncet *et al.*, 1993). Since both these functions may require a cellular partner, we looked for interaction of cellular proteins with NSP3A using the two-hybrid system in yeast. This study reports on the isolation of such a cellular protein.

Results

NSP3A interacts with the N-terminal part of the mammalian eukaryotic initiation factor eIF4GI in yeast

To investigate the possible interaction of NSP3A with a cellular protein, we used the two-hybrid test in yeast originally described by Fields and Song (1989). The use of the entire NSP3A coding sequence fused in-frame with the DNA-binding domain of GAL4 results in trans-activation of the HIS3 reporter gene in the two-hybrid test. Deletion of the first 88 amino acids of NSP3A (NSP3A-met88) abolishes this phenotype but maintains the capacity of NSP3 to multimerize (Poncet *et al.*, 1997).

Therefore, NSP3A-met88 was used to screen a cDNA library of mRNAs from a cell line susceptible to rotavirus infection (monkey CV1 cells). From the 35 HIS⁺ yeast clones obtained, one cDNA (p4/16) was isolated repeatedly. To ensure that p4/16 was a true interactor of NSP3A and not an artefact of the two-hybrid system (Bartel *et al.*, 1993), it was tested against plasmids encoding human p53, lamin or rotavirus proteins NSP1 and NSP2 fused to the activating domain of GAL4. No interactions were observed using either the HIS3 or the β -galactosidase reporter genes (data not shown).

The nucleotide sequence of the 3' end of p4/16 was identical to the published sequence of the human eukaryotic translation initiation factor eIF4GI (or eIF4 gamma or p220; Yan *et al.*, 1992). The cDNA resulted from the internal initiation of the reverse transcription reaction at nucleotide 2302 of eIF4GI mRNA (AF012088; Imataka *et al.*, 1997). The protein encoded by p4/16 was in-frame with the GAL4-activating domain and was 37 amino acids longer at its N terminus than the previously published protein sequence of eIF4GI (Figure 1), but was identical to the eIF4GI sequence and highly homologous to the eIF4GII sequence, published recently (Gradi *et al.*, 1998). Since the initiator methionine of eIF4GI has not yet been identified, we will refer to the published human sequence (amino acid or nucleotide) by using plain numbers and to the new eIF4GI sequence by adding + to the numbering (Figure 1, top).

We next used the yeast two-hybrid system to map the domain of eIF4GI that interacts with NSP3A. The coding sequence of eIF4GI from amino acids 1–770 was amplified by polymerase chain reaction (PCR), subcloned in pGAD424 and several deletions were introduced by restriction digests and religation. The resulting plasmids were introduced into yeast strain HF7C and the transactivation of the β -galactosidase reporter gene and the growth on medium lacking histidine were tested (Figure 1A). None of the constructs that encoded a fragment of eIF4GI downstream of Met1 was able to interact with NSP3A-met88 (Figure 1A, lines 2–5 and 8). In contrast, when the fragments containing the sequence for amino acids +37 to 3 were used, interaction with NSP3A-met88 was observed (Figure 1A, lines 1,6,7,9 and 12). It is interesting to note that the yeast clones containing p4/16 grew well in the absence of histidine, but gave a lighter blue colour than the clones deleted from the 4th to 770th amino acid (Figure 1A, lines 1 and 7). If the yeast two-hybrid system is considered to be roughly quantitative (Estojak *et al.*, 1995), judging by the intensity and time that was taken for the blue colour in the β -galactosidase assay to appear, the interaction was thus stronger with shorter fragments than with longer ones. This observation is not unusual (for an example, see Ikeshima-Kataoka *et al.*, 1997) and can result from a change of protein conformation rendering the protein-interacting domains more accessible to each other. Further deletions in the N-terminal coding region of p4/16 abolished eIF4GI–NSP3A interaction (Figure 1A, lines 10 and 11). To ensure that the interaction was not due to the truncation of NSP3A, the eIF4GI insert +37:174 was fused to the GAL4-DNA-binding domain of pGBT9 and tested with the full-length NSP3A protein expressed in GPD424. Reversing the genes did not abolish the interaction (Figure 1A, lines 7 and 12).

Thus, NSP3A interacts in the yeast two-hybrid system, with 40 amino acids within the N-terminal part of eIF4GI.

NSP3 from group C rotavirus also interacts with eIF4GI

Despite sequence divergence, mainly in the C-terminal half of the protein (Mattion *et al.*, 1994), NSP3 from group C rotavirus presents the same kind of RNA-binding specificity for the 3' end of group C rotavirus mRNAs (Poncet *et al.*, 1994) and probably plays a similar role. We tested the interaction of the entire NSP3C protein with p4/16 product and with the eIF4GI region +37:174. The interaction with eIF4GI was detected with NSP3C (Figure 1B, lines 2–4) and, as with NSP3A, the interaction was significantly stronger with a truncated eIF4GI (Figure 1B, compare lines 3 and 4).

The latter result shows that the interaction between NSP3 and eIF4GI is not restricted to group A rotavirus.

NSP3A interacts with eIF4GI during rotavirus infection

To establish that NSP3A interacts *in vivo* with eIF4GI, infected cell lysates were prepared in a gentle lysis buffer at different times post infection, and NSP3A was immunoprecipitated with a monoclonal antibody. After SDS–PAGE and transfer, eIF4GI was revealed by Western blotting with a rabbit polyclonal antiserum (Figure 2). As NSP3A accumulates in the cells during infection, an increasing amount of eIF4GI was co-immunoprecipitated (Figure 2A). When eIF4GI was immunoprecipitated from the lysates under the same gentle conditions, NSP3A can be detected in the co-immunoprecipitated material by Western blot (Figure 2B). In the latter case, the quantity of co-immunoprecipitated NSP3A increased as infection progressed (Figure 2B), even though the overall quantity of eIF4GI was almost stable during infection (see below and Figure 6). Controls showed that the observed interaction was specific; NSP3A was not detected when a rabbit antiserum unrelated to eIF4GI was used for immunoprecipitation (data not shown) and the cellular protein α -tubulin was not detected after immunoprecipitation with the anti-eIF4GI antiserum or anti-NSP3A monoclonal antibody (Figure 2C). Therefore, NSP3A is interacting with eIF4GI during infection of mammalian cells by rotavirus and an increasing amount of NSP3A is linked to eIF4GI as infection proceeds.

NSP3A interacts with eIF4GI *in vitro*

A fragment of eIF4GI (amino acids 457–932) can be cross-linked to RNA by UV irradiation (Pestova *et al.*, 1996) and a ribonucleoprotein consensus sequence motif has been identified between amino acids 698–705 (Goyer *et al.*, 1993). Since p4/16 contains these regions of eIF4GI and NSP3A has been shown to be a sequence-specific RNA-binding protein (Poncet *et al.*, 1994), we wanted to rule out the possibility that the interaction between NSP3A and eIF4GI was mediated by an RNA intermediate in a manner similar to the three-hybrid test developed recently (Sen Gupta *et al.*, 1996). With this aim, a subclone of the p4/16 cDNA containing only the first 700 nucleotides, and thus lacking the ribonucleoprotein motif, was used to produce a 30 kDa truncated ³⁵S-labelled eIF4GI (+37:176) by *in vitro* coupled transcription–translation (Figure 3A).

Fig. 1. Mapping the interaction between eIF4GI and NSP3 with the two-hybrid assay in yeast. Yeast clones co-transfected with the plasmids encoding NSP3A (A) or NSP3C (B) fused to the GAL4-activating (pGAD424) hybrid or the GAL4 DNA-binding hybrid (pGBT9), were tested for the β -galactosidase assay (LacZ reporter gene; two clones per assay and one clone for the positive control) or for growth in the absence of histidine (HIS3 reporter gene). Left: NSP3A fragments used with the plasmid vector (in parentheses). Right: a schematic representation of eIF4GI is presented with emphasis on the 5' coding sequence of the p4/16 clone. Numbers indicate the amino acid position in the sequence. * indicates an S→A mutation at position 2 due to the introduction of a *Nco*I site into the initiation codon.

NSP3A and its N- and C-terminal halves, expressed in *Escherichia coli*, were purified and then bound through their histidine-tags to beads of nickel chelate Sepharose. The whole NSP3A or its N- and C-terminal fragments were then incubated with the *in vitro*-translated eIF4GI (+37:176), washed, and the labelled material bound to the beads was analysed on SDS-PAGE. Under these conditions (Figure 3), the eIF4GI (+37:176) *in vitro* translation 30 kDa product was recovered with the entire NSP3A expressed in *Escherichia coli*, but not with beads incubated with a lysate prepared from an *E.coli* clone expressing no recombinant protein (Figure 3, lanes

'mock'). The eIF4GI (+37:176) fragment was also recovered with the *E.coli*-expressed C-terminal fragment (despite a lower quantity of material bound to the nickel chelate Sepharose; Figure 3B), but not with *E.coli*-expressed N-terminal fragment (Figure 3A). When the same experiment was conducted in the presence of RNase A, and after treatment of the *in vitro*-translated eIF4GI (+37:176) with RNase A, the same result was obtained (Figure 3A). In addition to the protein migrating at 30 kDa, the *in vitro* translation reaction produced a smaller protein of 25 kDa that was unable to bind to the *E.coli*-expressed NSP3A or derived fragments (Figure

Fig. 2. NSP3A and eIF4GI interact in infected cells. Western blots of eIF4GI co-immunoprecipitated with NSP3A (A) and of NSP3A co-immunoprecipitated with eIF4GI (B) from the lysate of $\sim 3 \times 10^6$ cells infected for 3, 4.5 and 6 h or mock-infected, are shown. Recombinant NSP3A (rNSP3) and a lysate of 10^5 mock-infected cells were used as Western blot controls in (A) and (B). Co-immunoprecipitation controls (C) are provided by Western blot, with anti α -tubulin, of the material immunoprecipitated with the rabbit anti-eIF4GI or the NSP3A-monoclonal antibody; the positions of tubulin (t) and of mouse immunoglobulin heavy chain (H) are indicated. Lysates from cells infected for 6 h (6) or mock-infected (m.) were used as Western blot controls. Western blots (A) and (B) were made on the same gel.

3A). This 25 kDa protein might be synthesized by internal initiation at Met1 (Figure 1) and hence does not have the region of eIF4GI that interacts with NSP3A.

These results show that the N-terminal part of eIF4GI is able to interact *in vitro* specifically with the C terminus of NSP3A. This interaction can occur in the absence of 3' end viral mRNA, and most probably in the absence of any kind of RNA bound to NSP3A and eIF4GI.

Rotavirus infection induces a shut-off of cellular protein synthesis in the absence of eIF4GI degradation

We evaluated the capacities of our RF strain of rotavirus to induce a shut-off of the cellular protein synthesis. Infected or mock-infected cells were pulse-labelled with [35 S]methionine for 15 min at different times post infection, and total proteins were separated on SDS-PAGE (Figure 4). Since rotavirus infection proceeds, in cell culture, in the absence of serum and that serum starvation can deeply modify protein synthesis (Gallie and Traugh, 1994; Shama *et al.*, 1995), uninfected serum-starved cells were used as control (Figure 4, lane 'mock'). At 3 h after rotavirus infection, cellular protein synthesis was greatly inhibited, whereas serum starvation had little effect on MA104 protein synthesis (Figure 4). Thus, the cellular protein synthesis shut-off was due to rotavirus infection, and not to serum starvation.

Various interactions of viral proteins with cellular proteins involved in the translation process have been

Fig. 3. The C-terminal half of NSP3A binds to eIF4GI *in vitro* in the absence of viral RNA. NSP3A, N- and C-terminal fragments expressed in *E. coli* were bound to nickel chelate beads, mixed with an *in vitro*-translated and -labelled (TV) eIF4GI fragment (+37:176), washed and then eluted with imidazole, and analysed by SDS-PAGE and fluorography (A). Coomassie Blue staining of the gel (B) shows the recovery of the different recombinant proteins (indicated by ●). RNase A treatments: the *in vitro*-translated and labelled (TV) eIF4GI fragment (+37:176) was incubated with 20 μ g/ml of RNase A and incubated with the N- and C-terminal fragments of NSP3A (in the presence of 25 μ g/ml RNase A) before washing. Control consists of beads incubated without recombinant protein (mock). An aliquot of the *in vitro*-translated eIF4GI fragment is shown (TV). Numbers on the left indicate the molecular weight (kDa) of the standards (MW). The size (kDa) of the two main *in vitro* translation products are indicated on the right.

described (Ehrenfeld, 1996; Katze, 1996; Mathews, 1996; Schneider, 1996; Wickner, 1996; Hardwick, 1997). For example, during poliovirus infection, a viral protease cleaves eIF4GI (Ehrenfeld, 1996). The consequences of this cleavage is a shut-off of cellular protein synthesis and a more efficient translation of the viral uncapped mRNA. It has been advanced that, late in reovirus infection, a shift from capped- to uncapped-dependent translation occurs (Skup and Millward, 1980; Lemieux *et al.*, 1984). Despite being controversial (Lemay, 1988), this observation prompted us to investigate whether eIF4GI underwent qualitative or quantitative changes during rotavirus infection. A Western blot with a rabbit antiserum against eIF4GI peptide 403–416 was performed on whole-cell lysates obtained at different times post infection

Fig. 4. Shut-off of cellular protein synthesis in rotavirus-infected cells. MA104 cells infected (or mock-infected) by rotavirus were pulse-labelled (15 min) with [35 S]methionine at the indicated times post infection. Whole-cell lysates were analysed on 10% SDS-PAGE. The position of viral proteins is indicated on the right; molecular weight markers (kDa) are shown on the left.

Fig. 5. Translation initiation factor eIF4G1 is not degraded in rotavirus-infected cells. MA104 cells infected by rotavirus were recovered at the indicated times post infection. Mock-infected cells starved of serum for the indicated time were used as controls. Whole-cell proteins were separated on 10% SDS-PAGE and eIF4G1 was revealed by Western blot with anti-eIF4G1(403–416) (Lamphear *et al.*, 1995).

(Figure 5). eIF4G1 was neither cleaved nor extensively degraded during rotavirus infection. The 90 kDa protein appearing at 4 h post infection is the rotavirus structural protein VP2; the presence of antibodies against rotavirus structural proteins is a common observation in non-highly protected rabbit breeding. We concluded that rotavirus infection induces a shut-off of cellular protein synthesis which is not related to eIF4G1 degradation.

Rotavirus infection induces the release of poly(A) binding protein from eIF4F

The functional similarity between NSP3A and poly(A) binding protein (PABP) is quite striking; NSP3 binds to the 3' end consensus sequence of rotavirus mRNA and the PABP binds to the 3' poly(A) of cellular mRNA. PABP from yeast interacts with the eIF4G1 equivalent from yeast (Tarun and Sachs, 1996) and wheat PABP interacts with plant eIF4G (Wei *et al.*, 1998). The synergistic effect of the cap and poly(A) structures on translation

Fig. 6. NSP3A replaces PABP on eIF4F during rotavirus infection. eIF4F complexes present in mock- and rotavirus-infected cells (numbers indicate hours post infection) were immunoprecipitated with the anti-eIF4G1 antiserum and the proteins co-immunoprecipitated were revealed by Western blot with monoclonal antibodies directed against PABP, NSP3A, eIF4E and eIF4A. Aliquots of the whole-cell lysates used for immunoprecipitation were also analysed (right panels). Western blots (A) and (B) were made on the same gel; Western blots (C) and (D) were made with the same samples as for (A) and (B), but on another gel.

has long been suspected to be the result of such an interaction, but a direct interaction between PABP and eIF4G1 has not been observed in metazoan animal cells (Craig *et al.*, 1998). Nevertheless, we looked for PABP in the proteins immunoprecipitated with eIF4G1 from mammalian cells, using the same conditions of immunoprecipitation that allowed us to detect eIF4G1–NSP3A interaction. After immunoprecipitation of mock-infected cell lysate with the anti-eIF4G1, PABP could be readily detected by Western blot (Figure 6A, lane 'mock'). Since α -tubulin was not co-immunoprecipitated under these conditions (see Figure 2), the interaction (direct or indirect) of PABP with eIF4G1 detected in mammalian cells is specific. Moreover, when the same experiment was done with rotavirus-infected cells, the quantity of NSP3A pulled down with eIF4G1 increased with time after infection (Figure 6B), but in the same samples the quantity of PABP decreased (Figure 6A, left), although its overall quantity remained constant in infected cells (Figure 6A, right). Furthermore, the quantity of eIF4A and eIF4E (which, together with eIF4G form the eIF4F complex) pulled down with eIF4G1 or present in the cells lysates (Figure 6C and D, right), also remained constant during infection. These last results confirm that PABP is specifically co-immunoprecipitated with eIF4G1; if the PABP detected was a carry-over, then its quantity should not vary regularly with time post infection. Thus, the decrease in the quantity of PABP co-immunoprecipitated with eIF4G1 is particular to PABP and is induced by rotavirus infection.

To confirm further that PABP and NSP3A cannot be present simultaneously *in vivo* on the same eIF4F complexes, immunoprecipitations were conducted with an anti-NSP3A antibody and the presence of PABP, eIF4A and eIF4E was checked by Western blotting. As expected, PABP was not detected on eIF4G1–NSP3A complexes at any time post infection, although eIF4A and eIF4E were present on the same complexes throughout infection (data not shown).

Altogether, these results show that, following

Fig. 7. The C-terminal part of NSP3A evicts PABP from eIF4F. The experiment is represented schematically in (A). eIF4F complexes present in MA104 cells were immunoprecipitated with the anti-eIF4G antiserum bound to protein A-Sepharose and the beads were incubated with the N- or C-terminal fragments of NSP3A purified from *E.coli* (rNSP3A). The presence of PABP, eIF4GI and eIF4E was then assessed (B) by Western blotting after SDS-PAGE. Controls are provided by complexes incubated with buffer alone (none) and by an aliquot of the cell lysate used for immunoprecipitation. Western blots were made on the same gel.

rotavirus infection, the composition of the eIF4F translation initiation complex is modified; PABP is removed and NSP3A takes its place, but other translation initiation factors (eIF4A and eIF4E) that interact directly with eIF4GI (Lamphear *et al.*, 1995; Mader *et al.*, 1995; Imataka *et al.*, 1997) remain in the complexes.

The C-terminal part of NSP3A evicts PABP from eIF4F in vitro

From the results described above, it could be concluded that NSP3 takes the place of the PABP on eIF4GI. However, it should be noted that viral infection could have a pleiotropic effect and the removal of PABP from eIF4F could be an indirect consequence of infection. To demonstrate that NSP3A was directly involved in the release of PABP from eIF4F, eIF4F complexes were immunoprecipitated from uninfected cell lysate with the rabbit anti-eIF4G antiserum, and the immune complexes bound to protein A-Sepharose were incubated either with the N-terminal part of NSP3A (amino acids 3–178) or the C-terminal part of NSP3A (amino acids 163–315) produced in *E.coli*. After three washes in binding buffer, the presence of PABP still bound on eIF4F was assessed by Western blot (Figure 7). When eIF4F was incubated with the N-terminal part of NSP3A or with renaturation buffer only, the PABP remained anchored to the eIF4F complex (Figure 7B). However, PABP was greatly reduced after incubation with the C-terminal part of NSP3A (Figure 7B). A control Western blot showed that, under these conditions, eIF4GI remained associated with the antibody and comparable quantities of eIF4GI were present in each sample (Figure 7B); hence the loss of PABP was not due

to the loss of immunocomplexes. It is also important to emphasize that, in the same samples, eIF4E remained associated with eIF4GI (Figure 7C), showing that the release of PABP was highly selective. Altogether, these results show that the interaction of NSP3A with eIF4GI precisely evicts PABP from eIF4F, but leaves the other translation factors untouched.

Discussion

The C-terminal part of NSP3 interacts with eIF4GI in the absence of mRNA

Using the two-hybrid system in yeast, we were able to isolate the cDNA of the eukaryotic translation initiation factor eIF4GI as an NSP3A interactor. With the same test, we precisely delineated the interaction on a 40-amino acid sequence of eIF4GI. The region of NSP3A that interacts with eIF4GI has been localized on the C-terminal half of the protein by an *in vitro* interaction assay. The interaction between NSP3A and eIF4GI was then confirmed *in vivo* during rotavirus infection by co-immunoprecipitation and *in vitro* by a pull-down assay. The region by which eIF4GI interacts with NSP3A is part of the N-terminal coding sequence of eIF4GI identified recently (Gradi *et al.*, 1998). This region is also present in the newly described initiation factor eIF4GII (Gradi *et al.*, 1998) and NSP3A might interact with it.

Many arguments favour an eIF4GI–NSP3A interaction independent of a prior binding of RNA on eIF4GI and/or NSP3A: (i) NSP3A is highly specific for the 3' end of rotaviral mRNA (Poncet *et al.*, 1994), but the cDNA constructs used to express NSP3A in yeast and in *E.coli* do not possess the 3' viral UTR; thus, no mRNA with an authentic viral 3' end could interfere in the two-hybrid test in yeast or could co-purify with NSP3A expressed in *E.coli*; (ii) the C-terminal domain of NSP3A binds to eIF4GI in the *in vitro* test (Figure 3) whereas the N-terminal half which contains the RNA-binding domain does not (Piron and Poncet, 1997); (iii) the RNA-binding motif of eIF4GI was deleted from the *in vitro*-translated product used for the *in vitro* pull-down assay (Figure 3); (iv) it is very unlikely that an RNA could remain bound on the recombinant protein because NSP3A and the NSP3A fragments used in this assay were denatured with urea before being bound on nickel beads and then renatured in the absence of added RNA; and (v) in the same test, identical results were obtained after RNase A treatment of the *in vitro*-translated eIF4GI (+37:176) before and during binding with NSP3A (Figure 3).

NSP3 shares many functional characteristics with PABP: it binds to the 3' end of the viral mRNA and, like yeast (Tarun and Sachs, 1996) or wheat (Le *et al.*, 1997) PABP, it interacts with the N-terminal half of eIF4GI in a region close to the eIF4E-binding domain (Mader *et al.*, 1995). Results obtained *in vivo* and *in vitro* with yeast cells (Tarun and Sachs, 1996; Tarun *et al.*, 1997) or *in vitro* with purified wheat initiation factors (Le *et al.*, 1997; Wei *et al.*, 1998) have shown a direct interaction between eIF4G and PABP. In yeast, this interaction requires the binding of poly(A) RNA sequences on PABP (Tarun and Sachs, 1996) and occurs within the second RNA recognition motif (out of the four motifs present on PABP; Kessler and Sachs, 1998). With plant factors, the RNA–

PABP interaction is dispensable, although the binding of PABP on eIF4G enhances the affinity of the cap binding protein (eIF4E) for eIF4G (Wei *et al.*, 1998) and decreases the rate of PABP dissociation from poly(A) (Le *et al.*, 1997). Here, we found that unlike the PABP–eIF4G interaction in yeast, the interaction of NSP3A with eIF4GI does not require the binding of an RNA to occur and, from this point of view, is more similar to the PABP–eIF4G interaction described in plants (Le *et al.*, 1997). However, the absence of sequence homology between PABP and NSP3A precludes the precise identification of the NSP3A region that interacts with eIF4GI.

Role of NSP3 in viral mRNA translation

The 3' poly(A) sequence of mRNA plays a major role in many aspects of cellular metabolism; it has been shown to enhance translation and stability of mRNA together with PABP (Beelman and Parker, 1995; Tanguay and Gallie, 1996; Ford *et al.*, 1997). In metazoans, changes in the length of the poly(A) tail are observed during development and cellular differentiation in relation to regulation of specific protein translation (Richter, 1996).

We have shown previously that NSP3A can be cross-linked in infected cells to the 3' end of rotaviral mRNA (Poncet *et al.*, 1993). Here we show that NSP3A interacts with eIF4GI and is present in a complex with eIF4A and eIF4E. The results we describe lead us to propose that the 3' end consensus sequence of rotaviral mRNAs and NSP3A fulfil, on translation initiation, a role similar to the 3' terminal poly(A) sequences of cellular mRNA and to PABP, respectively. The binding of NSP3A on eIF4GI and its specific interaction with the 3' end of viral mRNA (Poncet *et al.*, 1993, 1994) brings the viral mRNA and the translation initiation machinery in contact, thus certainly favouring the synthesis of viral proteins. The position of NSP3A on eIF4GI, upstream of the eIF4E-binding site (Lamphear *et al.*, 1995; Mader *et al.*, 1995) allows viral mRNAs and cap-binding protein eIF4E to come close together. The proximity of the 5' and 3' ends could ensure efficient reinitiation of the translation of the viral mRNA held by NSP3A and is reminiscent of the role of PABP and poly(A) on translation initiation of cellular mRNAs (Munroe and Jacobson, 1990; Tarun and Sachs, 1995; Preiss and Hentze, 1998). mRNAs of replication-dependent histones are the only cellular cytoplasmic mRNAs that do not have a poly(A) tail, but their translation requires the presence of a specific RNA-binding protein on a stem-loop structure at their 3' end (Gallie *et al.*, 1996; Wang *et al.*, 1996; Martin *et al.*, 1997). Indeed, the results described here suggest that the presence of an RNA-binding protein, linking the 3' end of mRNAs to eIF4G, seems indispensable for an efficient translation initiation and they strongly support the closed-loop model of mRNA translation (Jacobson, 1996).

PABP is part of eIF4F

The functional similarity between NSP3 and PABP led us to consider that the two proteins were in fact antagonists and prompted us to study the interaction of PABP with eIF4F during rotavirus infection. Initially, we showed that PABP can be detected after immunoprecipitation of eIF4GI in uninfected cells. The interaction of eIF4F with PABP is specific because, under the same conditions of immuno-

precipitation, we observed a decreasing quantity of PABP bound to eIF4F as rotavirus infection proceeded, even though the overall quantity of PABP in the cells did not vary with infection (Figure 6). This is the first time that PABP has been detected in eIF4F complexes from mammalian cells. It should be underlined that eIF4GI could not be co-immunoprecipitated with PABP using PABP antibody and HeLa cell extracts (Craig *et al.*, 1998). In that case, the large quantity of free PABP versus PABP bound on eIF4F presumably precluded the co-immunoprecipitation of eIF4GI with anti-PABP (Görlach *et al.*, 1994).

Role of NSP3 in the shut-off of cellular protein synthesis

We showed that incubation of eIF4F complexes with the C-terminal half of NSP3A (but not with the N-terminal half) evicts PABP from eIF4F, while eIF4A and eIF4E remain bound on eIF4GI. It is precisely the C-terminal half of NSP3A that interacts with eIF4GI in our *in vitro* assay (Figure 3). The decreasing amount of PABP bound to eIF4F during rotavirus infection was not the consequence of a specific degradation of PABP induced by infection. Nor was it linked to a specific degradation of poly(A) mRNA in infected cells (as described for herpes simplex virus; Zelus *et al.*, 1996), because no decrease in mRNA encoding β -actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been observed during rotavirus infection (Fuentes *et al.*, 1997). Altogether, our data point to a direct role of NSP3A in the shut-off of cellular poly(A) mRNAs translation, probably by competition with PABP for binding on eIF4F. They underline the absolute requirement of a PABP–eIF4F interaction for the efficient translation of poly(A) mRNA.

In yeast, the poly(A) tail and the PABP complex recruit ribosomal subunits (Tarun and Sachs, 1995), and deliver them to the 5' end (Preiss and Hentze, 1998) of the mRNA and thus enhance translation. The molecular mechanism of recruitment is not known precisely, but has been shown recently to be mediated by the binding of PABP to the yeast equivalent of eIF4GI (Tarun and Sachs, 1996). In the case of NSP3A, the binding of a viral RNA is not required as the interaction between NSP3A and eIF4GI occurs in the absence of viral mRNA. The stability of the eIF4GI–NSP3A interaction, even in the absence of viral mRNA, could allow NSP3A progressively to titrate out the eIF4GI present in the cytoplasm. If this were the case, expression of the NSP3A C-terminal fragment in cells should impair poly(A)-dependent translation.

Possible interaction of PABP with eIF4GI

Our observation that NSP3A can dethrone PABP from eIF4F complexes through a direct interaction with eIF4GI argues for a direct interaction between PABP and eIF4GI. Such a direct protein–protein interaction has not yet been reported for mammalian proteins. The identification of the 5'-terminal sequences of eIF4GI or the sequence of eIF4GII (Gradi *et al.*, 1998) might allow characterization of the PABP interaction site on the translation factor. However, our results do not rule out the presence of a yet unknown protein making the intermediate between PABP and eIF4GI (see below). In the case of a direct interaction of PABP with eIF4GI in mammalian cells (Figure 8A),

Fig. 8. Various models of translation initiation. Models of translation initiation in uninfected [(A) and (B), top, and (C) and (D)] or rotavirus-infected cells [(A) and (B) bottom] as discussed in the text. Model (C) is redrawn from Craig *et al.* (1998). Model (D) is modified from Craig *et al.* (1998) and Imataka and Sonenberg (1997).

the sites of interaction of PABP and NSP3A on eIF4GI can be very close and even overlap. A high affinity of NSP3A for eIF4GI can allow NSP3A to remove PABP and then to hinder binding of new PABP molecules. The concentration of PABP in the cytoplasm of the cell is very high (Görlach *et al.*, 1994). As a consequence, the affinity of NSP3A for eIF4GI should be much higher than the affinity of PABP in order to allow NSP3A to hinder the binding of PABP at the beginning of the infection when the concentration of NSP3A is still low. Alternatively (Figure 8B), the binding of NSP3A on eIF4GI could induce a change in the conformation of eIF4GI that impairs the binding of PABP. It is interesting to note that the interaction between NSP3A and eIF4GI was stronger when a fragment of 40 amino acids was used in place of the longer fragment present in the cDNA clone originally isolated. A conformational change of eIF4GI upon binding of eIF4E has already been suspected in animal cells to facilitate the cleavage of eIF4G by picornavirus protease (Ohlmann *et al.*, 1997) and, in wheat, the increased affinity of eIF4E for eIF4G has been suggested to result from a conformational change of eIF4G upon PABP binding (Wei *et al.*, 1998).

Recently, the identification of a PABP interacting protein (PAIP-1) (Craig *et al.*, 1998) which presents some homology with eIF4GI (but not with the 40-amino acid domain interacting with NSP3A) has provided a different model for translation initiation in mammalian cells (Figure 8C). It has been proposed that PABP does not interact directly with eIF4GI, but via a ternary complex involving PAIP1–

eIF4A and the mRNA. It should be underlined that eIF4GI could not be co-immunoprecipitated with PAIP-1 (Craig *et al.*, 1998). With such a model, the removal of PABP from the multi-ribonucleoprotein complex should be induced by an effect of NSP3A on the cellular mRNA. The co-immunoprecipitation of PABP with eIF4GI, described here, does not rule out this model but shows that the interaction between PABP and eIF4F can withstand conditions of immunoprecipitation in which RNA is generally extensively degraded (Poncet *et al.*, 1993, 1994). An alternative model can be proposed (Figure 8D) in which the interaction between PABP and eIF4GI is mediated by eIF4A. PAIP-1 and eIF4GI both contain eIF4A-binding domains (Imataka and Sonenberg, 1997; Craig *et al.*, 1998). A release of PABP from eIF4F by conformational changes of eIF4GI could fit this model. Another likely hypothesis is that direct and indirect interactions occur in distinct initiation complexes with different protein composition, or transiently during the first steps of complex assembly.

A new kind of virus–cell interaction

Viruses have frequently evolved to turn cellular processes to their own profit (Mathews, 1996; Wickner, 1996; Hardwick, 1997). Until now, the picornaviruses were the only viruses known to interfere with eIF4GI (Ehrenfeld, 1996). The cleavage, by the poliovirus-encoded 2A protease, of the eIF4GI N-terminal part that interacts with eIF4E, from the rest of eIF4GI, impairs the translation of cellular capped mRNAs, but still allows the translation of

uncapped poliovirus mRNAs bearing an internal ribosome entry site (IRES). The interaction of NSP3A with eIF4GI reported here belongs to a new viral strategy on the protein translation battlefield. The fact that NSP3A or NSP3C can interact with eIF4GI, despite a low homology in their C-terminal halves, shows that the interaction described here is conserved in the rotavirus genus. Further experiments are needed to establish whether this interaction is conserved in the whole Reoviridae family and if it exists in other families of non-poly(A) RNA viruses.

Materials and methods

Cells and viruses

The RF strain of group A rotavirus was propagated in monkey kidney cells, MA104, in Eagle's minimal essential medium (MEM) in the presence of trypsin (0.44 µg/ml; type IX, Sigma). Infections were carried out at a multiplicity of infection (m.o.i.) of 5–10 p.f.u./cell. *In vivo* pulse labelling was performed for 15 min from 0 to 6 h post infection with 0.7 MBq/ml of Trans³⁵S-label (ICN: 38 TBq/mmol) as described elsewhere (Poncet *et al.*, 1993).

Serum and monoclonal antibodies

The rabbit anti-peptide antiserum ZP2 directed against amino acids 403–416 of eIF4GI has been described (Lamphear *et al.*, 1995). The monoclonal antibody against NSP3A (ID3) has been described previously (Aponte *et al.*, 1993), monoclonal against eIF4A was a gift of Dr Trachsel (University of Bern, Switzerland), rabbit antiserum against eIF4GI (amino acids 1–480) was a gift of Drs A. Gradi and N. Sonenberg (McGill University, Canada) and monoclonal 10E10 against human PABP was a gift of Dr M. Görlach (Görlach *et al.*, 1994). Mouse monoclonal antibody against eIF4E was purchased from Transduction Laboratories (Lexington, USA).

Co-immunoprecipitation and Western blotting

Confluent MA104 cells in culture plates were washed with 2 ml of cold Eagle's MEM and lysed in 1 ml of TMGK buffer (20 mM Tris pH 8, 20 mM MgCl₂, 110 mM KCl, 1% Triton X-100, 2 µg/ml aprotinin). Cell debris was pelleted by centrifugation and supernatants were precleared with protein A–Sepharose (Pharmacia) for 1 h before immunoprecipitation. To immunoprecipitate the proteins, 1 µl of mouse monoclonal ascitic fluid or 1 µl of rabbit antiserum were added to 1 ml of precleared cell lysate supernatant and incubated overnight at 4°C. Then, 30 µl of a 50% suspension of protein A–Sepharose in TMGK buffer was added and the incubation continued for 1 h at 4°C with end-over-end rotation. Protein A–Sepharose beads were spun down (13 000 g, 10 s), washed three times with TMGK buffer and the beads processed for SDS–PAGE. Proteins were resolved by PAGE after being boiled in loading buffer (10 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 150 mM 2-mercaptoethanol). Gels containing ³⁵S-labelled samples were fixed in 20% ethanol, 10% acetic acid, and treated with Amplify (Amersham) before drying and fluorography.

For immunoblotting, proteins separated by SDS–PAGE were transferred to polyvinylidene difluoride membranes by transverse electrophoresis in 10 mM CAPS pH 11, 10% methanol buffer. Membranes were saturated for 30 min at room temperature in SuperBlock (Pierce) blocking reagent solution, washed three times with Tris 20 mM pH 7.4, NaCl 150 mM, 0.1% Tween 20 (TBS), incubated for 1 h at 37°C with the appropriate dilution of primary antibody [1/10 000 for anti-NSP3A, 1/4000 for anti-eIF4GI, 1/500 for anti-eIF4E, 1/20 for anti-eIF4A and 1/500 for anti-PABP, 1/5000 for anti-α-tubulin (Amersham)], washed again three times with TBS and then incubated for 1 h at 37°C with a 1/20 000 dilution of goat anti-mouse IgG or 1/10 000 goat anti-rabbit IgG H+L (Jackson Laboratories) coupled to horseradish peroxidase. Membranes were washed again five times with TBS and proteins were revealed by enhanced chemiluminescence (Pierce).

In some cases, membranes were stripped of the antibodies by incubation in 2% SDS, 62.5 mM Tris pH 6.8, 100 mM 2-mercaptoethanol for 30 min at 70°C. The Western blot protocol was then resumed with another antibody.

Plasmid constructions

The cDNAs of the RF gene 7 encoding NSP3A, and NSP3C (of the Cowden strain of group C rotavirus) have been described previously

(Poncet *et al.*, 1994). They were subcloned in-frame with the GAL4-activating domain in pGAD424 and the GAL4 DNA-binding domain in pGBT9. The coding sequence of NSP3A starting at Met88 was obtained by PCR using appropriate primers on RF7 cDNA, subcloned in pBS/SK⁺ (Stratagene) and then subcloned in pGBT9, to give NSP3A-met88–pGBT9.

The p4/16 clone contains the monkey eIF4GI cDNA from nucleotide +112 to 3283 cloned in pGAD10 (Clontech). eIF4GI/1:768 was obtained by PCR using oligonucleotides 5'-TAT AGG ATC CTG ACC ATG GCT GGG GC (which introduces a *Nco*I site on the first AUG) and oligonucleotide 5'-CAG GGA TCC TTA CAT CTG GTT GAA ATA C (which introduces a stop codon immediately after amino acid 770) and cloned, in-frame with the DNA-binding or the activating domain, at the *Bam*HI site of pGBT9 and pGAD424 to generate pGBT9–eIF4GI/1:768 and pGAD424–eIF4GI/1:768. In-frame deletions that can be generated by restriction digest and religation, were detected by using the computer program 'Clone-It!' (Lindenbaum, 1998). The C-terminal deletions were introduced in pGAD–eIF4GI/1:768 by hydrolysis with restriction enzymes followed by religation; digestion by *Pst*I gave pGBT9–eIF4GI/1:353, digestion by *Bgl*II gave pGBT9–eIF4GI/1:174. N-terminal deletion was introduced by digestion with *Nco*I and *Bgl*II, treatment with T4 DNA polymerase and ligation to give pGBT9–eIF4GI/174–768. pGAD–GAL4–eIF4GI/+37:353 was obtained by subcloning a *Xho*I–*Pst*I fragment from p4/16 in the *Sal*I–*Pst*I sites of pGAD–GAL4 vector (Stratagene).

The subcloning of the 700 bp *Bgl*II fragment of p4/16 in the *Bam*HI site of pGBT9 and pGAD424 gave plasmids pGBT9–eIF4GI/+37:174 and pGAD424–eIF4GI/+37:174, respectively. Deletions were introduced in pGBT9–eIF4GI/+37:174 by digestion with *Bsp*120 and *Pst*I, or *Bam*HI and *Bsp*120, or *Syl*I and *Pst*I, then treated with T4 DNA polymerase and ligation to give plasmids pGBT9–eIF4GI/+37:4, pGBT9–eIF4GI/4:174 and pGBT9–eIF4GI/+37:+9, respectively. Digestion of pGBT9–eIF4GI/+37:4 with *Eco*RI followed by a treatment with the Klenow fragment of DNA polymerase gave plasmid pGBT9–eIF4GI/+15:4.

The pBS–eIF4GI/+37:176 used for *in vitro* translation, was obtained by subcloning the amplification product of p4/16 with oligonucleotides 3308 (CAG GGA TCC ATG GAC CCC GCC CCA GTT TTG ATG AA; which introduced a methionine and an aspartic acid at the N terminus coding sequence of p4/16) and 3309 (CAC CTG CAG CTC GAG ATC TTC AGA TGG GAC CAT GC; which introduced a *Xho*I site after amino acid 176 of eIF4GI) in pBS-SK(+) (Stratagene). Each construct was sequenced at both ends to control the correctness of the fusion and/or deletions.

Two-hybrid assay

The two-hybrid assay in *S.cerevisiae* (Fields and Song, 1989; Bartel and Fields, 1995), was used to detect the interactions between proteins. The Matchmaker kit (Clontech) was used with a CV1 cell mRNA library in pGAD10 (Clontech). Control plasmids pVA3 (human p53 gene in pGBT9), pTD1 (SV40 large T antigen in pGAD3F) and pLam5' (human lamin gene in pGBT9) were from Clontech. The HF7C yeast strain was first transfected by pGBT9–NSP3A-met88, then by the cDNA library from CV1 cells (Clontech) in pGAD10. 2 × 10⁶ potential yeast transformants were plated on medium lacking tryptophan, leucine and histidine. Plasmids were extracted from yeast clones that grew on triple dropout medium and the plasmids from the library were selected by electroporation in *E.coli* HB101 and plating on selective medium lacking leucine. Plasmids were tested again for their capacity to give the His⁺ phenotype when transfected together with pGBT9–NSP3A-met88. One of the positive plasmids (p4/16) was isolated from at least 10 independent yeast clones and was identified by DNA sequencing as a partial cDNA copy of the eIF4GI mRNA.

The interaction between NSP3 (A or C) and the protein encoded by p4/16 was monitored by co-transfection of DNA-binding (pGBT9) and activating (pGAD) plasmids in the HF7C yeast strain followed by selection on a medium without tryptophan and leucine to monitor the co-transfection efficiency, and on medium without tryptophan, leucine and histidine to assess the interaction between the two fusion proteins. Negative controls for interaction between p4/16 and murine p53, SV40 T antigen, and other rotaviral proteins were included. The transactivation of the β-galactosidase gene was monitored both in HF7C and SFY526 yeast strains. Clones grown on triple dropout medium (HF7C) or double dropout medium (SFY526) were placed on nitrocellulose filters, lysed by immersion in liquid nitrogen, and then incubated on a filter paper moistened with 5-bromo-4-chloro-3-indolyl-2-acetamido-2-deoxy-β-D-glucosamide (X-gal) at 37°C for 6 h.

Expression in *E.coli* and site-directed mutagenesis

NSP3A was expressed in *E.coli* using the T7 expression system (Studier, 1991). The coding sequence of NSP3A starting at Met4 was fused to a track of six histidine residues after PCR and cloning in pET22B+ (Novagen) as a *NdeI*-*XhoI* fragment. The signal peptide from pET22b+ was then removed by site-directed mutagenesis (Kunkel *et al.*, 1991). N- and C-terminal deletions (amino acids 3–178 and 163–315, respectively) were obtained by introducing *XhoI* and *NcoI* sites in the middle of NSP3A by site-directed mutagenesis and then digestion and ligation. The resulting plasmids were transfected in *E.coli* BL21/DE3 and the expression of NSP3A was induced by addition of isopropyl thio- β -D-galactoside (IPTG; final concentration 1 mM). After 3 h, cells were collected and lysed by two rounds of sonication. Insoluble proteins were washed twice in binding buffer A (20 mM Tris pH 8, 500 mM NaCl, 5 mM imidazole) and then dissolved overnight at 4°C in binding buffer A with 6 M urea. Full-length or deleted NSP3A proteins were bound to a metal-chelating Sepharose column (Pharmacia) loaded with Ni²⁺, eluted with 200 mM imidazole (in binding buffer A containing 6 M urea), then renatured by slow dialysis against 50 mM Tris pH 8, 150 mM NaCl, 10% glycerol, 0.5 mM oxidized and 5 mM reduced glutathione.

eIF4G1-NSP3A interaction *in vitro*

Two to five micrograms of renatured, full-length or deleted NSP3A, were immobilized on metal-chelating Sepharose beads by incubation in 1 ml of binding buffer B (50 mM Tris pH 7.4, 150 mM NaCl, 0.4% NP40, 5 μ g/ml leupeptin, 10 μ g/ml aprotinin) plus 1 μ g/ml bovine serum albumin (BSA), for 1 h at 4°C. Unbound protein was removed by three washes with binding buffer B. Then, 2 μ l of the TnT (Promega) *in vitro*-transcribed and -translated pBS-eIF4G1/+37:176 diluted in 1 ml of binding buffer B was added to the resin and incubation continued for 1 h at 4°C. For RNase treatment, the *in vitro* translation products were incubated with 25 μ g/ml RNase A for 45 min at 30°C and incubations with the recombinant NSP3A continued in the presence of 20 μ g/ml RNase A. After three washes in binding buffer B, NSP3A and the proteins bound to it was eluted in 40 μ l of buffer B with 1 M imidazole. Samples were analysed on SDS-PAGE by Coomassie Blue staining and by fluorography.

Eviction of PABP from eIF4F

The eIF4F complex from a MA104 cell lysate in TMGK buffer, was immunoprecipitated with rabbit anti-eIF4G1 antibody, washed three times with TMGK buffer, and incubated with ~5 μ g of purified NSP3A N- or C-terminal fragments in 350 μ l of TMGK buffer. After incubation for 1 h at 4°C with end-over-end rotation, immunocomplexes bound to Sepharose beads were washed three times with TMGK buffer before SDS-PAGE followed by Western blot analysis.

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