Roles of the influenza virus polymerase and nucleoprotein in forming a functional RNP structure

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Influenza virus transcription and replication is performed by ribonucleoprotein particles (RNPs). They consist of an RNA molecule covered with many copies of nucleoprotein (NP) and carry a trimeric RNA polymerase complex. RNA modification analysis and electron microscopy performed on native RNPs suggest that the polymerase forms a complex with both conserved viral RNA (vRNA) ends, whereas NP binding exposes the RNA bases to the solvent. After chemical removal of the polymerase, the bases at the vRNA extremities become reactive to modification and the vRNPs behave as structures with free ends, as judged from the observation of salt-induced conformational changes by electron microscopy. The vRNA appears to be completely single-stranded in polymerase-free RNPs despite a partial, inverted complementarity of the vRNA ends. The absence of a stable double-stranded panhandle structure in polymerase-free RNPs has important implications for the mechanism of viral transcription and the switch from transcription to replication.
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Introduction

The genome of influenza A viruses consists of eight negative sense, single-stranded RNA segments encoding a total of 10 genes. The viral RNAs (vRNAs) are associated with the polymerase protein subunits (PA, PB1 and PB2) and packed by the nucleoprotein (NP) into structurally distinct ribonucleoprotein particles (RNPs). The RNPs are the structures responsible for transcription and replication of viral RNAs in the nuclei of infected cells, and the polymerase proteins plus NP are the minimal set of proteins required for these activities (Huang *et al.*, 1990; Kimura *et al.*, 1992; de la Luna *et al.*, 1993; Mena *et al.*, 1994). After the RNPs have entered the cell nucleus, transcription of viral mRNA starts from the 3′ ends of the vRNA templates and terminates at an oligo(U) stretch near the 5′ end of the vRNA. Later in infection, the polymerase generates full-length complementary transcripts (cRNA), which serve as templates for the production of secondary, genomic vRNAs. There is still considerable uncertainty concerning the nature of the *cis*-acting sites and the mechanisms that are involved in the regulation of the replicative processes.

The terminal sequences of the vRNA segments are highly conserved and show a partial inverted complementarity (Skehel and Hay, 1978; Robertson, 1979; Desselberger *et al.*, 1980; Stoeckle *et al.*, 1987). All necessary signals for replication and genome packaging seem to reside in these terminal sequences (Luytjes *et al.*, 1989), and several lines of evidence imply a regulatory role for a hypothetical double-stranded panhandle structure (Hsu *et al.*, 1987) for viral transcription initiation (Fodor *et al.*, 1994, 1995; Cianci *et al.*, 1995) and for transcription termination and polyadenylation (Luo *et al.*, 1991; Li and Palese, 1994). The switch from transcription to the production of full-length genomic replicates is thought to be dependent on the disruption of the panhandle structure, possibly controlled by viral and/or cellular proteins (Beaton and Krug, 1986; Shapiro and Krug, 1988).

It was assumed originally that the 3′ ends contained the promoter elements for polymerase binding and transcription initiation (Parvin *et al.*, 1989; Seong and Brownlee, 1992). However, recent studies using vaccinia virus-expressed polymerase suggest that the 5′ ends of the vRNAs are a prerequisite for both endonuclease activity and transcription initiation of influenza virus polymerase (Hagen *et al.*, 1994; Cianci *et al.*, 1995). Two distinct *in vitro* systems have been established to show an interaction of the influenza virus polymerase complex with the conserved 3′ and 5′ ends respectively, both using short RNA molecules with virus-derived sequences. The polymerase proteins could be UV cross-linked to chemically synthesized RNA oligonucleotides representing the conserved viral ends, and *in vitro* transcription assays with mutant oligonucleotides suggested that one of the structural elements recognized by the polymerase protein might be a short, base-paired RNA stretch of the panhandle stem (Fodor *et al.*, 1993, 1994). In another approach, it was shown by RNA mobility shift and modification interference assays that vaccinia virus-expressed recombinant influenza polymerase specifically binds to either of the conserved ends of viral RNAs, but most strongly to the conserved 5′ end sequence. Also, the modification interference assay suggested that the most critical sequences for polymerase binding to virus-like RNAs are located on the 5′ end (Tiley *et al.*, 1994). However, both experimental approaches do not necessarily reflect the situation in the virus or in the infected cell, where the polymerase complex is part of an RNP together with the vRNA and the NP. NP has a major structural function and the RNP structure, as seen in the electron microscope, is determined mainly by the NP polymer rather than by the RNA molecule (Pons *et al.*, 1969; Kingsbury *et al.*, 1987; Ruigrok and Baudin, 1995). We have shown previously that binding of NP to a model vRNA *in vitro* in the

absence of the polymerase results in melting of the RNA secondary structure and exposure of the bases to the outside of the complex (Baudin *et al.*, 1994). It appears that the RNA is wound around the nucleoprotein and, therefore, the interaction of the polymerase with the vRNA has now been studied using the *in vivo* assembled RNA–NP protein complex rather than the naked RNA.

In RNP the bases of the nucleotides were exposed to the solvent and accessible to reagents that modify the Watson–Crick positions except at the conserved vRNA ends, where the bases were protected. Removal of the polymerase resulted in the exposure of the 5' end bases, indicating that the vRNA ends are not base paired in the absence of the polymerase. We also studied RNPs by negative stain electron microscopy (EM). We found that RNPs are held in a circular conformation, but that the ends are no longer connected after removal of the polymerase.

Results

When influenza virus NP was reconstituted with a vRNA-like, small model RNA in the absence of the polymerase, all the nucleotide bases were exposed to the solvent and the RNA acquired a conformation that presumably improved its qualities to serve as a template for viral transcription (Baudin *et al.*, 1994). Here, we studied the accessibility of the nucleotides of the vRNA on *in vivo* assembled RNPs isolated from detergent-disrupted virus by treating the RNPs with several chemical and enzymatic probes. All the experiments were performed in a buffer in which the RNPs are biologically active. The modified bases were then identified by primer extension analysis using a radioactively labelled DNA oligonucleotide probe complementary to a sequence near the 5' end of viral RNA segment 8. Figure 1 shows the modification pattern of the 52 5'-terminal nucleotides of the vRNA using dimethylsulfate (DMS) as a probe for adenine (A) at N1 and cytosine (C) at N3. All A and C residues downstream from A23 are reactive at their Watson–Crick positions, as we found in the above-mentioned reconstitution experiments (Baudin *et al.*, 1994). This demonstrates that, also *in vivo*, NP binding to vRNA does not involve the Watson–Crick positions of the bases, but rather the phosphate backbone of the molecule. However, the residues near the top of the gel corresponding to the conserved 5' end of the vRNA, in particular A4, A6, A7, A8, C9, A10 and A11, indicated by the square bracket, were not reactive towards DMS. The complementarity of the 3' and 5' vRNA ends is not perfect, and residues A4 and A10 do not base-pair in the small model panhandle RNA (Figures 4 and 5B in Baudin *et al.*, 1994). The fact that these two adenines are not reactive in RNP suggests that the polymerase interacts with these nucleotides. Interaction of A10 with the polymerase was also suggested from modification interference experiments (Tiley *et al.*, 1994).

The polymerase was selectively removed from the RNPs by incubation with 1% sodium deoxycholate (DOC), as was originally suggested by Inglis and co-workers (1976). The detergent treatment was followed by glycerol gradient centrifugation to separate the RNPs from the released polymerase proteins and the DOC. With this procedure, the polymerase proteins could be quantitatively removed from the RNPs as determined by silver staining of gels
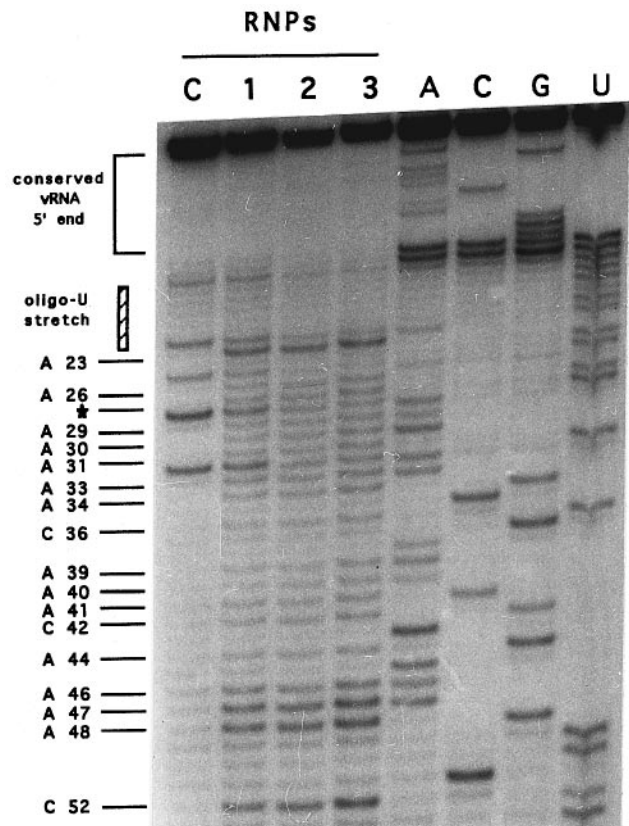

Fig. 1. A 16% PAGE autoradiogram of the cDNA fragments produced after reverse transcription of DMS-modified RNPs using a primer specific for segment 8 vRNA. The unreactive bases at the 5' end of the vRNA are indicated by a square bracket. The reactivity of all As and Cs of the vRNA sequence are shown from nucleotides 23 to 52. The star indicates nucleotide 27, where the sequence of our virus preparation deviates from the published NS vRNA sequence of influenza A/PR/8/34, see also Figure 3A. Lane C is an incubation control of unmodified RNPs. Lanes 1, 2 and 3 represent incubation of RNPs with 0.1, 0.2 and 0.6 µl of DMS respectively. Lanes A, C, G and U are vRNA dideoxy sequencing reactions of segment 8 vRNA to identify the modified positions.

(Figure 2B) and Western blotting (not shown). Figure 2 shows an SDS–PAGE analysis of glycerol gradient fractions of an RNP isolation from complete virions (Figure 2A) and of the glycerol gradient after the DOC treatment of RNPs (Figure 2B). In the following experiments, we have compared the reactivities of the vRNA nucleotides on RNPs with and without the polymerase complex (RNP-pol).

Figure 3A shows a comparison of DMS modifications on native RNP and on RNP depleted of polymerase. The residues at the 5' end that are protected on complete RNP become reactive after removal of the polymerase complex. There was no change in reactivities of the bases downstream of the conserved 5' end. Similar information, but this time on the guanines (G), was obtained by modification of RNPs with kethoxal, specific for N1 and N2 of G (Figure 3B), and RNase T1 digestion, specific for single-stranded G (Figure 3C). In intact RNPs, guanines G5, G12, G13 and G14 were not reactive to kethoxal or accessible for RNase T1. The reactivity of G16 could not be determined clearly because of unspecific stops of the reverse transcriptase in this region. However, all G residues were reactive downstream of the next G in the vRNA sequence, G35. Upon
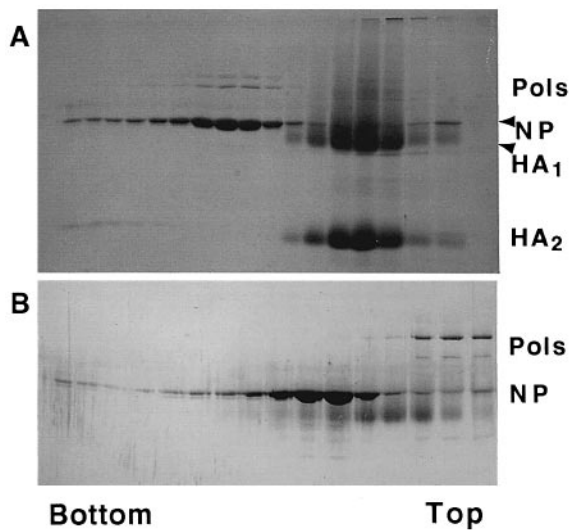
Fig. 2. A 12% SDS–PAGE of fractions from 30–60% glycerol gradients (**A**) for RNP isolation from a virus lysate and (**B**) for separation of RNP from the polymerase complex after treatment with DOC. (A) was stained with Coomassie blue and (B) was silver stained. The fraction numbers cannot be compared directly because the gradients were performed in different volumes, but in both cases RNP structures were separated from single proteins and accumulated in distinct bands of the gradient. The top and the bottom of the gradients are indicated. Pol stands for polymerase proteins, NP for nucleoprotein, HA$_1$ and HA$_2$ for haemagglutinin subunits and M for matrix protein.

removal of the polymerase, the protected G residues of the 5′ end become accessible for modification and RNase attack.

In order to analyse the reactivities of the Watson–Crick positions of bases at the 3′ end of the vRNAs, the intact RNPs were first modified with DMS before being deproteinized and 3′ end-labelled. The RNAs could then be hydrolysed selectively at the methylated cytidine moieties by successive treatment with aniline and hydrazine (Peattie and Gilbert, 1980). We observed an extensive protection of bases at the conserved 3′ end of the vRNA in native RNP (Figure 4). Note that in this figure we are looking at the total mix of viral RNAs. In particular the N3 positions of the cytosines C4′, C8′, C11′ and C12′ were not reactive to DMS on native RNPs (C2′ was not resolved on the gels). The protected region extended beyond the conserved sequence, which is indicated by the black bar in Figure 4. Significant modification of bases was detected only upstream of position 16′ of the vRNA towards the 5′ end. The signal was expected to be weak at this particular position, because only segment 6 contains a C residue as nucleotide 16′. Further upstream in the vRNA sequences many bases were modified and cleaved compared with the control reaction. The strong signals correspond to the cytosines of segment 7, suggesting that the RNP preparation was enriched with this RNA (e.g. nucleotides 21′, 25′, 28′), whereas the signals were weak when only segments 2 or 3 were involved (nucleotides 17′, 23′, 27′). We observed no bands corresponding to nucleotides 18′–20′, 22′, 24′ and 26′, because there are no C residues in any segment at these positions.

We also studied intact and polymerase-free RNPs with negative stain EM. The intact RNPs (Figure 5) resembled those imaged before using phosphotungstic acid (Schulze, 1973) or sodium silicotungstate (SST) (Ruigrok and Baudin, 1995) as negative stain. The structures are relaxed, helical

strands that are wound back on themselves, often showing a loop at one or both ends. After removal of the polymerase complex, we often observed that the strands came apart at one of the ends of the RNP, indicated by arrowheads in Figure 5 (RNP-pol, PBS panel). Otherwise the morphology of the RNPs was not changed by the DOC treatment. The alteration in the interaction at the ends became even clearer after incubation of the RNPs in either high or low salt conditions. Under both conditions, the intact RNPs were unwound and formed circular structures. The same high salt behaviour was demonstrated before by Heggeness *et al.* (1982). Polymerase-free RNPs (RNP-pol), however, unwound to linear structures under low salt conditions (30 mM NaCl), but formed very tightly packed structures in high salt (1.6 M NaCl). These observations illustrate the loss of a restrictive contact between the vRNA ends caused by the removal of the polymerase complex from the RNPs. The polymerase complex is responsible for holding the ends together by interacting with both termini of the vRNA and, in its absence, the ends are free to move and rotate.

Discussion

Figure 6 shows the compilation of the reactivities of the bases at the conserved ends of the vRNA in the RNPs with or without the polymerase. On complete RNPs we observed an extensive protection of the bases located at the 5′ and 3′ conserved ends. The non-reactivity of the nucleotides located at the 3′ end suggests that the 3′ end is part of a ternary complex together with the polymerase and the 5′ end, resulting in the protection of the Watson–Crick positions of bases until position 15′ on the 3′ end and at least position 14 on the 5′ end (Figure 6). Considering the extent of base protection at the Watson–Crick positions in intact RNPs, plus the fact that the vRNA ends on RNPs could be cross-linked by psoralen (Hsu *et al.*, 1987), it is likely that the protection results not only from direct interaction of the bases with the polymerase but that polymerase binding to the vRNA termini also induces some degree of base pairing. This would agree with *in vitro* transcription studies using mutant template RNA which suggest that the formation of a terminal RNA fork is a prerequisite for transcription initiation (Fodor *et al.*, 1995). The extent of base protection in the presence of the polymerase correlates well with the boundary of the theoretical panhandle structure that can be formed by base pairing of the vRNA ends. Depending on the virus strain and RNA segment, 12–17 nucleotides from the 3′ end could, in theory, be annealed with the corresponding 5′ ends (Skehel and Hay, 1978; Robertson, 1979; Stoeckle *et al.*, 1987). Downstream of the polymerase-binding site, all bases are exposed and highly reactive to all modifying reagents tested. These observations confirm our previous results, which showed that *in vitro* assembled RNA–NP complexes do not contain RNA secondary structures (Baudin *et al.*, 1994). The selective dissociation of the polymerase from the RNPs results in a separation of the vRNA ends as shown by the EM experiments and by the appearance of the reactivities of the Watson–Crick positions of the nucleotides located at the vRNA ends towards single strand-specific probes. These results would seem to exclude the requirement for a pre-formed panhandle structure for polymerase binding to template RNA
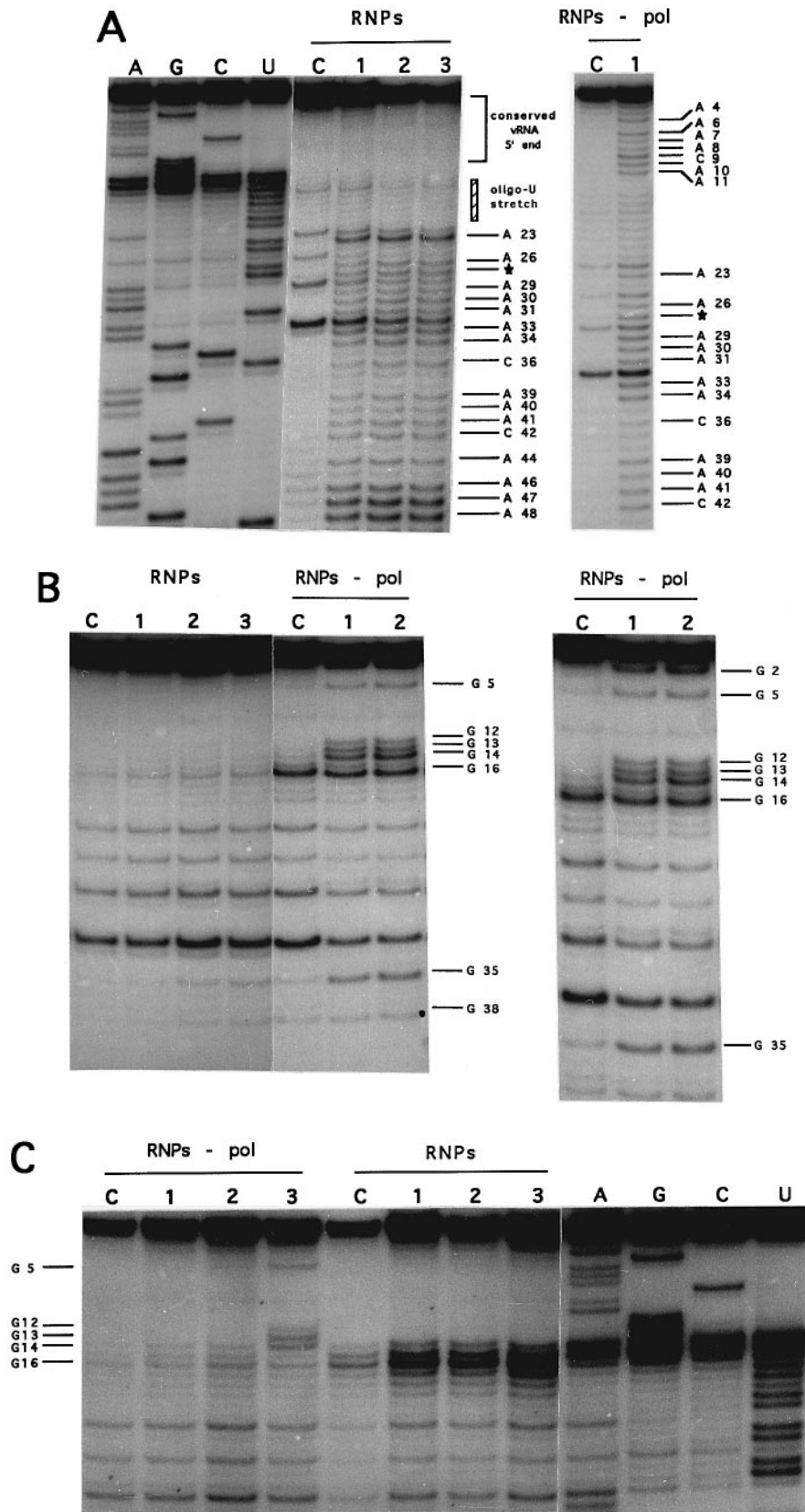
Fig. 3. The 16% PAGE autoradiograms of the segment 8-derived cDNA fragments produced after reverse transcription of RNPs and RNPs lacking the polymerase complex (RNPs-pol) modified with DMS (**A**), kethoxal (**B**) or RNase T1 (**C**). The reactive bases are indicated on the right. Lane C is an incubation control of unmodified RNPs. Lanes 1–3 are incubations of RNPs with increasing amounts of modifying reagents. Lanes A, G, C and U are vRNA dideoxy sequencing reactions of segment 8 vRNA. Base reactivity at the 5′ end of the genomic RNA is only obtained after removal of the polymerase. In (A), lanes 1–3 are incubations with 0.1, 0.2 and 0.6 μl DMS. The right panel in (B) is another gel of the same RNPs-pol experiment, which shows the reactive G2 residue more clearly. For RNPs, lanes 1–3 result from incubation with kethoxal for 5, 10 and 20 min, for RNPs-pol, lanes 1 and 2 are 20 and 60 min incubations. In (C), lanes 1–3 are digestions of RNPs with 0.1, 0.5 and 1 U of RNase T1 respectively.
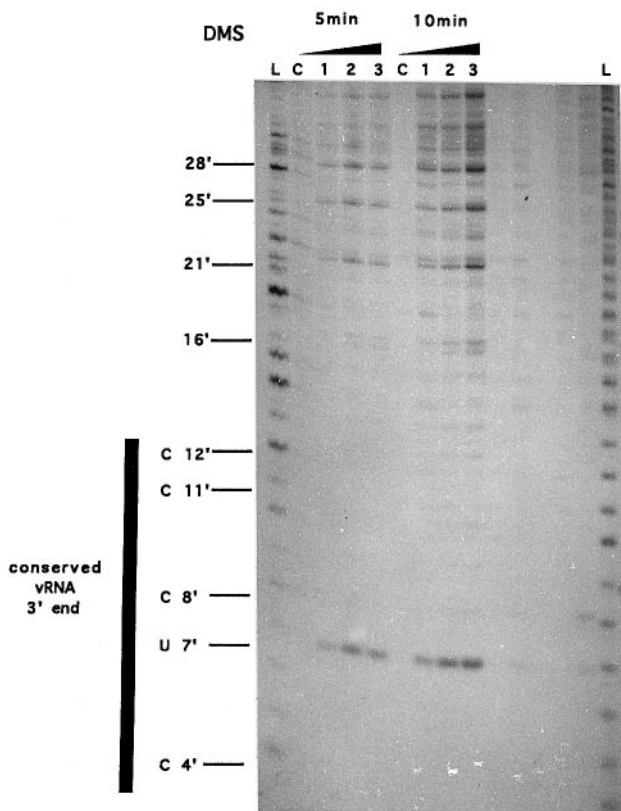
Fig. 4. Autoradiograph of chemical cleavage reactions of a mixture of all 3′ end-labelled vRNAs after DMS modification of complete RNPs. The position of the conserved 3′ end on the sequence is shown by the black bar and all C residues in this region are indicated on the left (C4′–C12′). Further upstream, the sequence of the different genome segments diverts, and strongly reactive positions have been marked by numbers according to their position on the sequence. The autoradiograph depicts two sets of DMS reactions performed on RNPs for 5 and 10 min respectively, each time with increasing amounts of DMS. Lanes L are alkaline hydrolysis ladders of vRNA, lanes C are incubation controls without DMS. Lanes 1–3 are incubations of RNPs with 0.2, 0.5 and 1 µl of DMS respectively.

and would support a model of sequential or independent polymerase binding to single vRNA ends, as has been proposed by Cianci *et al.* (1995). Such a mode would also allow newly produced polymerase to bind to the 5′ end for which it has the highest affinity, while replication is still taking place.

The fact that, in native RNPs, the nucleotides at the 5′ conserved end are protected at their Watson–Crick position but become reactive after removal of the polymerase, suggests that the polymerase is responsible for this protection. However, one could argue that DOC treatment has an influence on the stability of RNA secondary structure or that the treatment has other destabilizing effects on the structure of the RNPs. Figure 7 shows a control experiment indicating that the change in base reactivity at the vRNA 5′ end is not due to a destabilizing effect of DOC on RNA secondary structure. The naked small panhandle model RNA has its 3′ and 5′ ends base paired which protects the nucleotides at these ends against RNase T1 digestion (Baudin *et al.*, 1994). A similar experiment is shown in Figure 7, where it is clear that G12–14 are protected and do not become available for RNase T1 digestion with increasing concentrations of DOC, sug-

gesting that this detergent did not interfere with the formation or the stability of the secondary structure in the small model RNA molecule. Similar control experiments with the same results were performed using DMS and kethoxal modifications (not shown). Other possible artefacts could occur if DOC treatment did not remove all the polymerase molecules or if it removed some nucleoprotein as well. However, neither of these posibilities would lead to exposure of bases. Remaining polymerase would only lower the signal, and removal of NP would allow secondary structure to be formed. The only artefact which could lead to mistakes in our interpretation would be if NP was displaced as a result of the detergent treatment. We do not know if nucleoprotein is bound to the 3′ and/or the 5′ end of the vRNA in the intact RNPs. If it is not bound in intact RNPs, and if DOC treatment would lead to displacement of NP that then binds to one of the ends, this would lead to exposure of the bases which would not be due directly to the removal of the polymerase. However, this hypothetical situation may not be very different from the situation in the cell nucleus during transcription or replication where the polymerase must leave the 3′ end for initiation but where the nucleus contains unassembled, newly synthesized NP which may then bind to the free 3′ end.

The EM experiments show that complete RNPs unwind to circular structures under both high and low salt conditions. This may suggest that the supercoiled structures of the influenza RNPs are possibly poised to be unwound, which may be important for replicative processes. The removal of the polymerase takes away a constraint at the vRNA ends. EM of polymerase-free RNPs shows that the vRNA ends are free to rotate and unwind in the absence of the polymerase. The polymerase-free influenza virus RNPs behave as linear structures and react very similarly to the linear RNPs of other negative strand RNA viruses, such as those from rhabdo- and paramyxoviruses, that can be uncoiled reversibly in low salt and coiled to very tight structures in high salt (Heggeness *et al.*, 1980).

The influenza virus RNP appears to be assembled from two antagonistic proteins: nucleoprotein activity favours the melting of RNA secondary structures and exposes the bases to the environment, whereas the polymerase complex anneals the two vRNA ends and causes base protection. This antagonism constitutes an ideal arrangement for the regulation of a switch between a closed and an open RNP form, because in this situation such a switch only requires the manipulation of the fastening polymerase complex. An opening of the RNPs is presumably needed for the production of full-length RNAs during replication, when the vRNA 5′ end has to be freely accessible. On the other hand, during transcription, the mRNAs are incomplete transcripts terminated at the oligo(U) stretch just before the conserved 5′ end. Previously, a double-stranded RNA panhandle structure has been suggested to be important for transcription termination (Luo *et al.*, 1991; Li and Palese, 1994). Our results suggest that, in the absence of the polymerase, the vRNA ends are not base paired in RNPs, which may call into question whether the ends stay base paired once the polymerase has left after transcription initiation. Nevertheless, all structural studies on RNPs and all studies on transcriptional mechanisms are consistent with a model where the formation of a partially base-
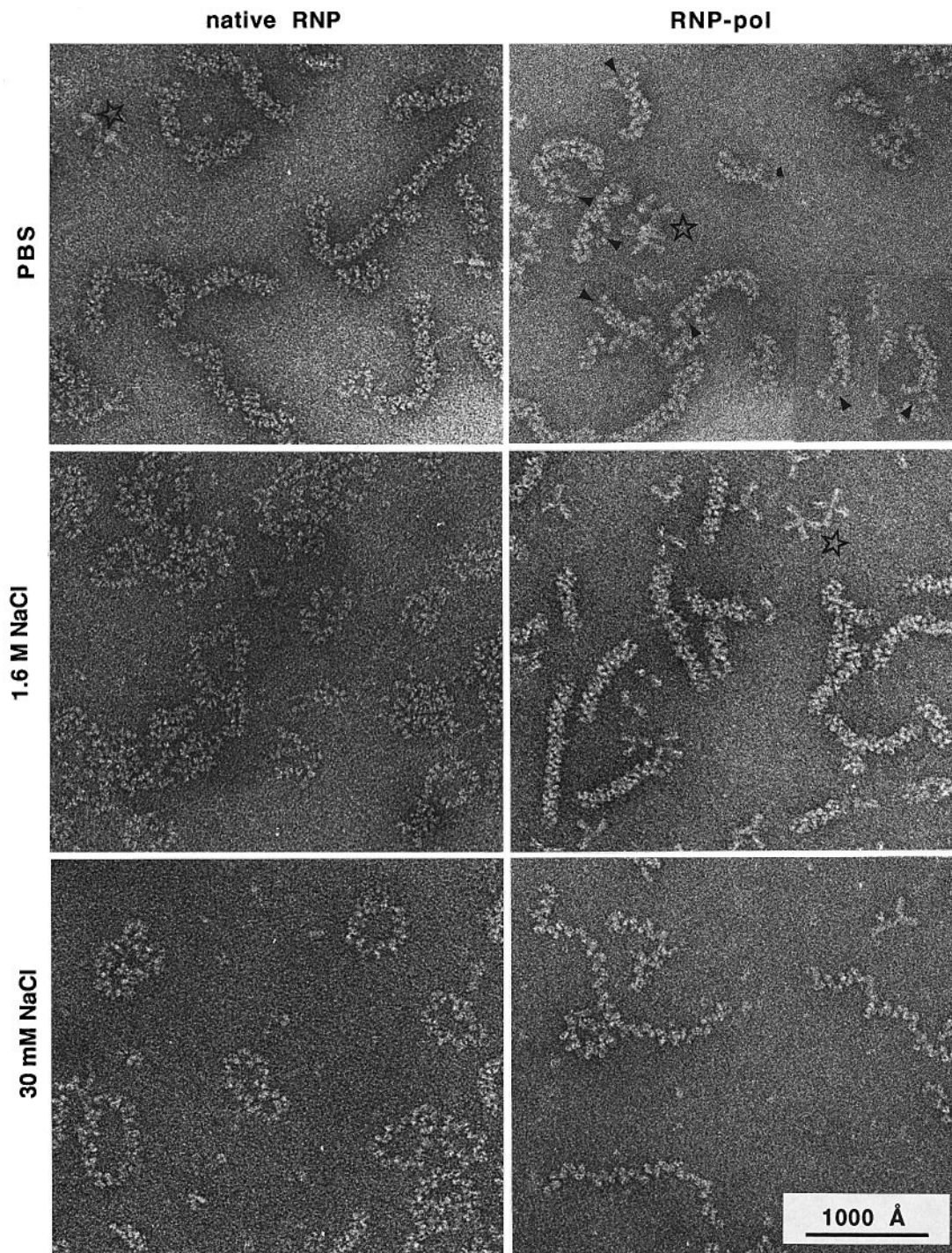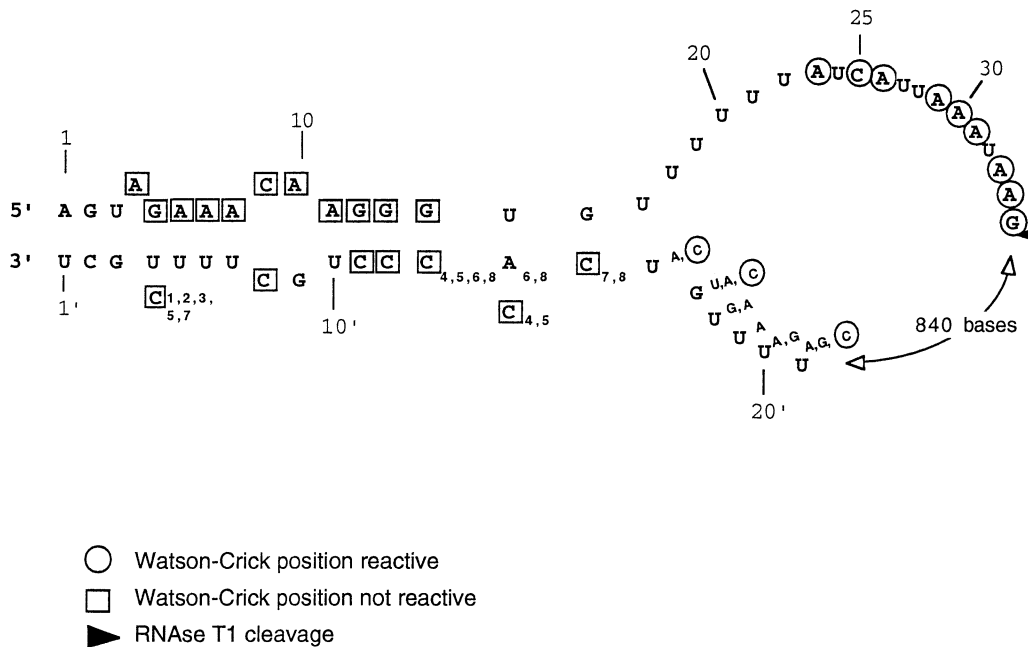
native RNP   RNP-pol

Fig. 5. Electron micrographs of negatively stained RNPs and RNPs-pol diluted and incubated with PBS, diluted five times in 2 M NaCl (1.6 M NaCl) or diluted five times in H_2O (30 mM NaCl). The stars indicate contaminating rosettes of haemagglutinin. The arrows in RNPs-pol in PBS (top right) indicate where the ends of the RNPs have come apart.

paired RNA fork (Fodor *et al.*, 1995), annealed by the polymerase complex, is necessary for transcription initiation only. Transcription termination at the oligo(U) stretch may, on the contrary, be controlled by a regulatory protein binding to the conserved 5′ end of the vRNA. This hypothetical, regulatory protein could participate in the switch between transcription and replication by determining the accessibility of the 5′ end for being copied. Because RNA modification analysis and EM show that the polymerase interacts with the 5′ end on native,

viral RNPs, the polymerase itself or one of its subunits are prime candidates to harbour this regulatory function.

Polymerase binding to the vRNA 5′ end is required for transcription initiation from the 3′ end, but both ends do not interact with each other in the absence of the polymerase. Similar genome binding patterns have been described for other multi-subunit, RNA-dependent RNA polymerases. The polymerase of brome mosaic virus, a segmented positive strand RNA virus, requires an interaction with an intercistronic region on the genome for initiation of RNA

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A. RNPs with polymerase (RNP)

○ Watson-Crick position reactive

□ Watson-Crick position not reactive

▶ RNAse T1 cleavage

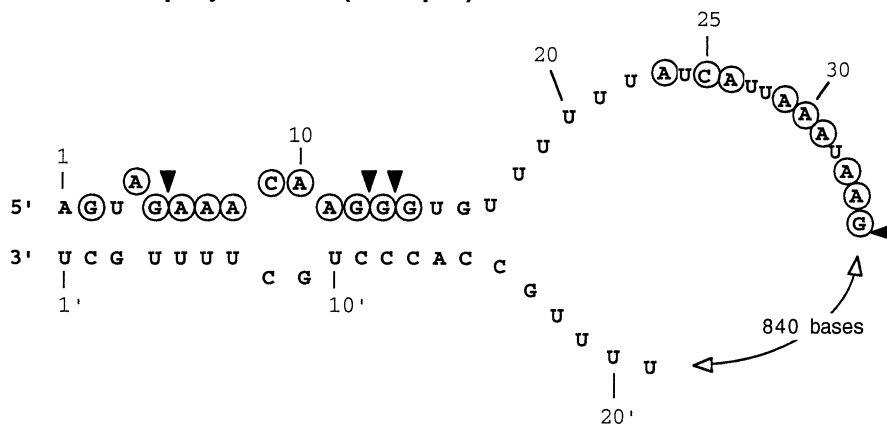## B. RNP without polymerase (RNP-pol)

Fig. 6. Compilation of modification data from complete RNPs (**A**) and polymerase-free RNPs (**B**). Circles and squares around bases indicate the reactivity of Watson–Crick positions of the bases on RNPs. The absence of circles or squares means that the reactivity could not be determined. The cleavage by RNase T1 is indicated by arrowheads. The scheme was drawn with the sequence of segment 8 vRNA in the centre. The base reactivities at the 3′ end of the vRNAs have been determined in a mixture of all segments. The sequence deviations in the different vRNA segments of influenza A/PR/8/34 are shown between nucleotides 13′ and 21′ of the 3′ end. The numbers denote the segments that carry the respective cytosine at the specific position of the sequence. The nucleotides 13′–15′ are usually, but not always, complementary to nucleotides 14–16 of the 5′ end. Another heterogeneity is observed at position 4′, which is a U in segments 4, 6 and 8 and a C in the other segments. The sequences have been extracted from the DDBJ/GenBank/EMBL database.

synthesis from the 3′ end (Quadt *et al.*, 1995). The replicase of Qβ phage binds to an internal site of the genomic (+) RNA and remains attached there, while initiating (–) strand synthesis from the 3′ end. Moreover, the binding pattern to (+) and (–) strand RNAs is different, consistent with different functions of the strands in the replication cycle (Barrera *et al.*, 1993; Schuppli *et al.*, 1994). Poliovirus RNA replication involves polymerase complex formation with both ends of the viral RNA, although in this case the complex formed at the 5′ end of (+) RNA has been proposed to catalyse *in trans* initiation of synthesis from the 3′ end of (–) RNA (Andino *et al.*,

1993; Harris *et al.*, 1994). Finally, the (+) RNA of *Saccharomyces cerevisiae* L-A virus also contains an internal binding site that binds more strongly to the L-A polymerase than the 3′ end, and which is required for *in vitro* replication (Esteban *et al.*, 1989; Fujimura and Wickner, 1992). This so-called 'action at a distance' phenomenon is common in polymerase–enhancer systems for regulation of transcription processes and to position the polymerase subunit correctly at the transcription initiation site. The polymerase–promoter interaction itself has to be relatively weak to enable easy promoter clearance after transcription initiation. The strong influenza poly-
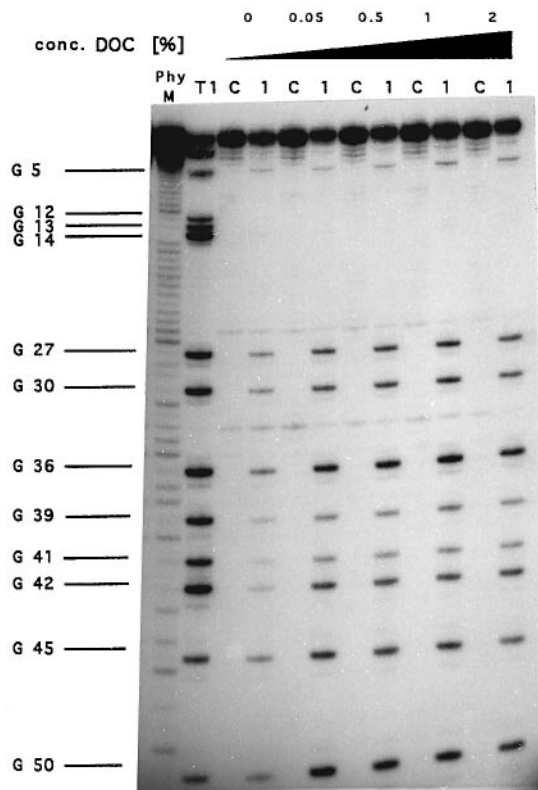
Fig. 7. RNase T1 digestion of a 3′ end-labelled, 81 nucleotide panhandle model RNA in the presence of increasing concentrations of DOC. The positions of G residues are indicated on the left. G12, G13 and G14 are protected from hydrolysis by base pairing with C residues at the 3′ end of the molecule. The base pairing interactions are not disturbed in the presence of DOC. PhyM and T1 denote sequencing reactions of the panhandle RNA with the corresponding RNases under denaturing conditions performed with 1 and 0.5 U of enzyme respectively. Lanes 'C' are incubation controls without RNase. Lanes '1' are RNase T1 digestions of panhandle RNA under native conditions with 0.5 U of enzyme.

merase binding site at the 5′ end of the vRNA assures high specificity recognition of viral RNAs and at the same time brings the polymerase into the vicinity of the low affinity 3′ end binding site to start transcription.

The studies on NP interaction with the genomic RNA underline another problem in RNA virus replication, namely the need to release the RNA replicates from the templates in order to make them available for several rounds of RNA synthesis. Many positive strand viruses presumably encode RNA helicases to solve this problem (Lain *et al.*, 1990; Gorbalenya and Koonin, 1993; Warrener and Collett, 1995), but influenza virus and negative strand viruses make use of nucleoproteins, cooperative single-stranded RNA-binding proteins, analogous to the single-stranded nucleic acid-binding proteins that are co-factors of DNA-directed RNA polymerases and DNA polymerases. It has been found that NP binding to RNA removes secondary structures and keeps the RNA single-stranded (Baudin *et al.*, 1994). Influenza replication is dependent on soluble NP produced in infected cells, which packages the newly synthesized RNAs during their synthesis. Cooperative NP binding will prevent base pairing between template and replicate from occurring and keep the template available for further rounds of replication. A very similar activity has been described recently during poliovirus replication.

The three-dimensional protein of poliovirus displays cooperative single-stranded RNA-binding activity during replication (Pata *et al.*, 1995) and it is thus able to unwind RNA duplexes of >1000 bp in length without the need to hydrolyse ATP (Cho *et al.*, 1993). On the other hand, the viral mRNAs are not dependent on NP to separate efficiently from their complementary template strands. The influenza virus polymerase uses host cell-derived, capped RNA primers for transcription initiation, which most likely results in the assembly of nuclear cap-binding and hnRNA-binding complexes on the viral mRNA and thereby prevents base pairing with the template RNA (Piñol-Roma and Dreyfuss, 1992; Matunis *et al.*, 1993; Izaurralde *et al.*, 1995).

The negative strand viruses transcribe mRNAs from their genomic RNAs after cell entry, whereas the genomes of the positive strand viruses are already in mRNA sense and can be translated directly in infected cells. This is the major reason for the differences in the genome structure optimized either for virus-specific transcription or for translation. The influenza RNPs, as packaged into virus particles, are ready to start transcription having the polymerase bound to both vRNA ends and the bases presented for transcription by the nucleoprotein.

Materials and methods

Chemicals and enzymes
DMS was obtained from Fluka; kethoxal from USB; RNAsin and AMV reverse transcriptase from Appligène (France). Radioactive nucleotides were from Amersham (UK). RNase T1, nucleotides, T4 polynucleotide kinase and T4 RNA ligase were obtained from Pharmacia.

Virus RNP preparation
Influenza virus A/PR/8/34 was grown in embryonated hen's eggs and obtained in purified form from Pasteur-Mérieux, Marcy L'Etoile, France. Viral RNPs were prepared as described in Baudin *et al.* (1994). Virus was treated with Triton X-100 (1%) and lysolecithin (1 mg/ml) in 5 mM $MgCl_2$, 100 mM KCl, 1.5 mM dithiothreitol (DTT), 5% glycerol and 10 mM Tris–HCl (pH 8) and incubated at 30°C for 15 min. This mixture was centrifuged through a linear 30–60% glycerol gradient in 100 mM NaCl, 50 mM Tris–HCl (pH 8), 1 mM DTT (SW27 rotor, 25 000 r.p.m., 16.5 h, 4°C). RNP-containing fractions were pooled, dialysed and concentrated in 100 mM NaCl, 50 mM sodium cacodylate (pH 7.5), 1 mM DTT, 10% glycerol at 4°C. This preparation was either used for modification experiments or was treated further with 1% DOC at 37°C for 15 min in 100 mM NaCl, 50 mM Tris–HCl (pH 8), 1 mM DTT. This mixture was loaded onto a second linear 30–60% glycerol gradient in 100 mM NaCl, 50 mM Tris–HCl (pH 8), 1 mM DTT and centrifuged as mentioned above for the RNP preparation. The RNP-containing fractions devoid of most of the polymerase protein were pooled, dialysed and concentrated in 100 mM NaCl, 50 mM sodium cacodylate (pH 7.5), 1 mM DTT, 10% glycerol. The dissociated polymerase proteins remained at the top of the gradient.

Chemical and enzymatical probing
The RNA modification procedure and the chemistry of the different probes have been described previously (Ehresmann *et al.*, 1987; Baudin *et al.*, 1994) and were adapted for use with intact viral RNPs.

DMS modification. DMS (0, 0.1, 0.2 or 0.6 µl; representing conditions of control, 1, 2 and 3 respectively) was added to 20–30 µg of RNP preparation in buffer A [50 mM sodium cacodylate buffer (pH 7.5), 20 mM magnesium acetate, 0.3 M KCl, 5 mM DTT] and incubated at 37°C for 5 min.

Kethoxal modification. Three µl of a 20 mg/ml solution of kethoxal in 20% ethanol were added to 20–30 µg of RNP preparation in 50 mM sodium cacodylate (pH 7.5), 5 mM $MgCl_2$, 100 mM KCl and incubated at room temperature for 0, 5, 10 or 20 min. At the end of the reaction, the solution was brought to 50 mM potassium borate (pH 7.0) to stabilize the kethoxal adduct.

RNase T1 digestion. The digestion was carried out in buffer A (see above) with 0, 0.1, 0.5 or 1 U of the enzyme in a 300 μl volume with 20–30 μg of RNP preparations. The reactions were incubated at 37°C for 15 min.

Primer extension analysis of the modified positions

Reverse transcription was carried out essentially as described before for phenol-extracted, ethanol-precipitated RNA (Baudin *et al.*, 1994) using an oligodeoxyribonucleotide complementary to nucleotides 83–100 of the segment 8 vRNA. The cDNA fragments were analysed on a 12% acrylamide/0.5% bisacrylamide, 7 M urea sequencing gel. The cDNA fragments produced from unmodified, RNP-derived vRNA served as an incubation control to detect nicks in the RNA and pauses of the reverse transcriptase due to RNA secondary structure. Dideoxy sequencing reactions were carried out in parallel on *in vitro* transcribed segment 8 vRNA (Baudin *et al.*, 1994) and run on the same gel. The reverse transcriptase stops one nucleotide before the modified base and therefore the modification band on the gel migrates to one position further than the corresponding band in the sequencing reaction.

Detection of modified positions at the 3′ end of RNAs

RNAs were purified from DMS-modified RNPs by phenol extraction, 3′ end-labeled wih [^{32}P]pCp and T4 RNA ligase according to England *et al.* (1980) and repurified on denaturing 6% polyacrylamide gels. The modified positions were analysed by chemical cleavage of the RNAs with a procedure adapted from Peattie and Gilbert (1980) (Baudin *et al.*, 1993). Briefly, the RNAs were resuspended in 10% hydrazin, 0.3 μg/μl tRNA, incubated for 5 min on ice and ethanol precipitated. This was followed by treatment with aniline (pH 4.5) for 15 min at 60°C in the dark. The cleavage products were ethanol precipitated and analysed on 20% polyacrylamide, 7 M urea gels.

Panhandle RNA and full-length segment 8 vRNA were produced by *in vitro* transcription as described (Baudin *et al.*, 1994).

Negative stain electron microscopy

Concentrated RNP samples were kept at 4°C and incubated at 37°C for 10 min before dilution. Then they were diluted with phosphate-buffered saline (PBS), pure water or with NaCl solutions as indicated, to a final protein concentration of 100–200 μg/ml and incubated further for 10 min at 37°C before adsorption onto the clean side of a carbon support film and staining with 1% SST. The experiments shown here were performed with long RNPs, predominantly segments 1–4. EM was performed with a JEOL 1200 EXII under low dose conditions. The microscope was calibrated with negatively stained crystals of catalase.

Acknowledgements

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