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Influenza virus M1 protein binds to RNA through its nuclear localization signal

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The RNA-binding activity of influenza A virus M1 protein was studied by cross-linking the protein to viral RNA followed by sequence analysis of the oligoribonucleotide bound to the protein as well as sequence analysis of the M1 peptide bound to the RNA. M1 was found to bind to RNA without any RNA sequence specificity, as verified in a series of filter-binding experiments using a large variety of nucleic acids including DNA. The peptide sequence that bound to the RNA was the RKLKR nuclear localization signal of M1. Site-specific mutagenesis of recombinant M1 showed that most of the basic residues in that region had to be mutated in order to inhibit RNA-binding. We also constructed an M1 mutant that no longer bound to RNA but which was still able to inhibit the *in vitro* transcription activity of isolated viral ribonucleoprotein, albeit to a lower extent. Mutation of the zinc-binding sequence had no effect on RNA-binding or transcription-inhibition activity.

Introduction

Influenza virus is an enveloped virus with a lipid membrane that separates the outside of the virus (membrane-embedded glycoprotein spikes which are responsible for attachment, penetration and virus release) from the inside of the virus, which contains the nucleocapsid; this consists of eight distinct negative-strand RNA segments that are associated with nucleoprotein (NP) and an RNA polymerase complex (the complex of RNA, NP and polymerase is called RNP). In the presence of the four nucleotide triphosphates and a primer these RNPs are transcriptionally active *in vitro*. Separating the viral core from the membrane is a layer of M1 protein. M1 is thought to add to the structural integrity of the virus particle. In negatively stained virus, where stain has penetrated the lipid bilayer, M1 can be observed, aligned in rows resembling a finger-print (Ruigrok *et al.*, 1989). M1 is generally believed to make contact with the lipid membrane (Bucher *et al.*, 1980; Gregoriades & Frangione, 1981) and with the cytoplasmic tails of the two glycoproteins. However, experiments aiming to prove this interaction *in vivo*, using virus-infected cells or expression of viral proteins in eukaryotic systems, have so far led to conflicting interpretations (Enami & Enami, 1996; Jin *et al.*, 1994; Kretzchmar *et al.*, 1996; Zhang & Lamb, 1996).

Suggestions for the interaction of M1 with RNPs *in vivo* comes from two lines of research into two separate steps in the infection process. The first step where this interaction is revealed is during cell entry by the endosomal uptake process. In the acidic endosomes the viral membrane fuses with the endosomal membrane and during this process the interior of the virus is also supposed to be acidified through the action of the viral M2 membrane channel (Zebedee & Lamb, 1988; Hay, 1989; Wharton *et al.*, 1990; Pinto *et al.*, 1992). Blocking of the M2 ion channel with drugs like amantadine or rimantadine is believed to inhibit the disassembly process of M1 from RNP which inhibits subsequent virus replication (Bukrinskaya *et al.*, 1982; Martin & Helenius, 1991*b*). Further, when newly expressed M1 is present in the cytoplasm, it interferes with the nuclear uptake of the RNPs, an activity that can be overcome by the temporary acidification of the cytoplasm (Bui *et al.*, 1996). Later in infection, a second activity of M1 protein is needed in the nucleus of the infected cell in order to allow the export of newly synthesized RNPs from the nucleus to the cytoplasm for virion assembly (Martin & Helenius, 1991*a*; Rey & Nayak, 1992; Whittaker *et al.*, 1995), although it is not yet clear whether this export is mediated by a direct interaction of M1 with RNP. At this later stage of infection, newly expressed cytoplasmic M1 inhibits nuclear re-import of freshly made

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RNPs, allowing these RNPs to be taken up into budding virions (Bui *et al*., 1996; Whittaker *et al*., 1996).

Evidence for *in vitro* interaction of M1 with RNP comes from transcription-inhibition when purified M1 is added to transcribing RNPs (Zvonarjev & Ghendon, 1980; Ye *et al*., 1987, 1989; Hankins *et al*., 1990; Elster *et al*., 1994) and *in vitro* interaction of M1 with naked RNA has been shown by filter-binding assays and blotting techniques (Ye *et al*., 1989; Wakefield & Brownlee, 1989; Elster *et al*., 1994). It has been reported that M1 protein has two RNA-binding sites extending from amino acids 90 to 108 and from 135 to 164 (Wakefield & Brownlee, 1989; Ye *et al*., 1989). This first site contains an RKLKR sequence, residues 101 to 105, which was shown to be the nuclear localization signal (NLS) of M1 (Ye *et al*., 1995). Recently, using deletion mutants of recombinant M1, the residues extending from 91 to 111 were shown to be essential for RNA-binding activity and oligomerization of M1 on RNA (Watanabe *et al*., 1996).

The experiments reported here were undertaken to identify the amino acids of M1 that bind to RNA. We used two cross-linkers, *trans*-diamminedichloroplatinum(II) (*trans*-DDP) and 4-azido-phenyl-glyoxal (APG), to induce RNA–protein cross-links, and we determined both the RNA sequence(s) and the peptide sequence involved in the M1–RNA cross-link. M1 was found to bind to vRNA without any sequence specificity. However, only one peptide of M1 protein was found cross-linked to RNA, amino acids residues 95 to 108. This peptide is rich in basic residues and contains the above mentioned NLS sequence ^{101}RKLKR^{105} (Ye *et al*., 1995). Substitution of all four Arg and Lys to Ala led to a total loss of RNA-binding activity but only to a partial loss in transcription-inhibition activity.

Methods

■ **Viral M1 protein and vRNA.** Egg-grown influenza A/PR/8/34 virus was obtained from Pasteur-Mérieux, Marcy l'Etoile, France. The viral glycoproteins were removed by bromelain digestion which was stopped by addition of 100 mM iodoacetamide. Spikeless virus was purified by pelleting through 14% sucrose in PBS (150 mM NaCl; 10 mM phosphate buffer pH 7·2; 0·01% sodium azide). The virus was then disrupted with 1% Triton X-100 in PBS and centrifuged over a 10 to 30% continuous glycerol gradient in PBS (SW41 rotor, 36000 r.p.m., 4 °C, 16 h). Pure M1 was collected from the upper fractions of the gradient as described in Elster *et al*. (1994). Segment 8 viral RNA (NS gene) was produced by *in vitro* transcription run-off synthesis as described in Baudin *et al*. (1994).

■ **Recombinant M1 and mutagenesis.** The M1 protein gene of influenza A/PR/8/34 virus cloned in a pAS1 vector was kindly donated by M. Krystal, Bristol-Myers Squibb, Wallingford, Conn., USA. The gene was subcloned into pET-16b and site-directed mutagenesis was performed on this construct using PCR (Ex-Site kit, Stratagene). The synthetic oligonucleotide primers containing the mutation were placed back to back on the template, with one of the oligonucleotides being 5′ phosphorylated. The whole plasmid was amplified leading to linear double-stranded DNA. The parental DNA was digested using *Dpn*I, and the remaining PCR product was self-ligated and transformed into *E. coli*. Each construct was checked by sequencing.

E. coli (BL21/DE3/pLysS) was transformed with each mutated plasmid. A 1 litre Luria broth culture was induced with 1 mM IPTG for M1 production. After 4 h induction, the cells were harvested by centrifugation and resuspended in ice-cold buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris–HCl pH 7·9). The cells were sonicated and M1 protein purified using an Ni-chelation resin according to the manufacturer (Novagen). After protein elution, each fraction was analysed by electrophoresis on 15% SDS–PAGE. The fractions containing M1 were slowly dialysed against 20 mM Tris–HCl pH 6·5, 200 mM NaCl and 1 mM DTT.

■ **Cross-linking conditions with *trans*-DDP and vRNA fragment analysis.** Viral M1 (2 μM) was incubated with a 2-fold molar excess of viral RNA segment 8 and cross-linking was performed in 20 mM sodium phosphate buffer (pH 7·4), 1·5 mM magnesium acetate, 100 mM potassium acetate, 10% glycerol, 0·1% lubrol and 0·2 mM *trans*-DDP (Sigma) for 1 h at 20 °C in the dark. About 30 to 40% of the RNA was cross-linked, as checked by a filter-binding assay using 5′ radioactively end-labelled RNA. The excess of cross-linking agent was removed using a speedy desalting column (Pierce). The cross-linked vRNA–M1 complexes were then adjusted to 0·3 M sodium acetate and precipitated with 3 vols of ethanol. The complexes were then subjected to RNase T1 digestion (1·5 U/μg RNA, 30 min at 37 °C in 20 mM sodium phosphate pH 7·5, 1 mM EDTA and 2 M potassium acetate). The resulting covalent M1–oligoribonucleotide complexes were separated from non-cross-linked oligoribonucleotides on nitrocellulose filters (Millipore type HA, 45 μm pore size, 25 mm diameter, previously soaked in the above buffer), which were then extensively washed with 20 ml of the same buffer. After filtration, the nitrocellulose filters were soaked in 500 μl 2 M thiourea in order to reverse the cross-links. Supernatants were removed and the filters washed with 200 μl 2 M thiourea. The liberated vRNA fragments were precipitated with ethanol and then labelled at their 5′ end with T4 polynucleotide kinase and 100 μCi [γ-^{32}P]ATP according to Silberklang *et al*. (1977). The 5′-labelled fragments were fractionated by electrophoresis on a 20% polyacrylamide–8 M urea gel. After autoradiography, the fragments were excised, eluted according to Maxam & Gilbert (1977), and repurified on a 22% polyacrylamide–8 M urea gel. The fragments were then eluted, precipitated with ethanol in the presence of 10 μg tRNA as carrier, dissolved in 10 μl water, incubated for 5 min at 55 °C and sequenced using several ribonucleases. For each sequence lane between 20000 and 50000 c.p.m. was used. Digestion was done with RNase T1 (0·25 U) for G; RNase U2 (0·25 U) for A; RNase *Phy*M (0·5 U) for A and U; and *B. cereus* RNase (0·5 U) for C and U. Incubation was at 55 °C for 15 min in 20 mM citrate buffer pH 4·5, 1 mM EDTA in the presence of 8 M urea for RNases T1, U2, *Phy*M and in the absence of urea for *B. cereus* RNase. The nucleotide ladder was made in 4 μl deionized formamide at 90 °C for 30 min. Analysis of the digests was carried out by electrophoresis on 15% polyacrylamide–8 M urea gel.

■ **Cross-linking conditions with APG and M1 peptide analysis.** M1 protein (1 mg) was incubated with a 2-fold excess of an RNA oligoribonucleotide (5′ AGUAGAAACAAGGGUG 3′) in PBS, 10% glycerol, 0·1% lubrol for 1 h at room temperature and cross-linked using a final concentration of 1 mg/ml APG (Aldrich) (Sgro *et al*., 1986). APG was solubilized in PBS at 65 °C at a concentration of 10 mg/ml. After reaction of M1 with RNA and APG, excess APG was removed using a speedy desalting column. The second step of the reaction was then carried out by incubating the mixture for 90 min under UV light. The cross-linked fraction was irradiated using a 40% $Co(NO_3)_2$ solution as a filter for cutting off the light below 300 nm. The cross-linked complex was separated from non-cross-linked material on an HPLC μBondapack column, using a gradient from 0 to 50% acetonitrile in 0·1% trifluoroacetic acid. Collected peaks were dried and submitted to endoprotease Asp-N

digestion in PBS pH 7·4 at 37 °C for 12 h with an enzyme/M1 ratio of 1/200. The digest was loaded on the μBondapack column, the eluate was monitored at 220 and 260 nm and peaks were collected for amino acid sequence determination. Automated Edman degradation was performed by using an Applied Biosystems model 477A protein sequencer, and amino acid phenylthiohydantoin derivatives were identified and quantified on-line with a model 120A HPLC system, as recommended by the manufacturer.

■ **RNA-binding and transcription-inhibition assays.** The filter-binding assay was performed as described in Elster *et al.* (1994) using segment 8 viral RNA, small panhandle RNA (Baudin *et al.*, 1994) and rabies virus leader RNA as probes. All RNAs were produced by run-off transcription (Baudin *et al.*, 1994). The reaction buffer for the binding assay was 45 μl of 20 mM HEPES pH 7, 150 mM NaCl, 5 mM $MgCl_2$ and 1 mM DTT. The M1 protein was incubated with the ^{32}P-5′-labelled vRNA (about 0·5 pmol) for 20 min at room temperature and filtered through a pre-wetted nitrocellulose filter (Whatman, 0·45 μm pore size). The RNA complexed with M1 bound to the filters and could be detected by scintillation counting. The apparent K_d for RNA-binding corresponds to the M1 concentration at 50% RNA retention.

RNP transcription-inhibition assays were carried out using a fixed concentration of RNP (30 nM) and increasing concentrations of M1 protein (0, 0·33 μM, 0·67 μM, 1·35 μM and 2·7 μM) in 50 mM Tris–HCl pH 7·8, 100 mM KCl, 5 mM $MgCl_2$, 10 mM DTT, 0·4 mM APG, 1 mM ATP, 0·5 mM GTP, 0·5 mM UTP, 0·1 mM CTP and 20 μCi [α-^{32}P]CTP. After incubation for 1 h at 37 °C, the transcripts were precipitated for 30 min on ice by addition of 12·5% TCA and 1% Casamino acids, filtered on GF/A filters and then rinsed with 10 ml of 10% TCA. The filters were dried and counted in a scintillation counter. The RNP concentration was determined by measuring the absorbance at 260 nm; 1 A_{260} unit corresponds to 60 μg/ml RNP. This empirical value was checked by comparing Coomassie Blue staining of NP from an RNP preparation with known amounts of BSA. Controls for the transcription-inhibition assay were done with buffer without protein but also with buffer plus 0·1 mM Ni^{2+} since we used a nickel chelating column for purification of M1. Although bivalent cations such as Zn^{2+}, Mn^{2+}, Ca^{2+} and Co^{2+} inhibit transcription (Elster *et al.*, 1994), 0·1 mM Ni^{2+} had no effect on transcription.

Results

Trans-DDP has a square planar geometry with a 7 Å span between the two chlorines. In ribonucleoprotein complexes, platinum coordinates on the RNA side mainly to position N7 of guanines and to a lesser extent to position N1 of adenines and N3 of cytosines. On the protein side, *trans*-DDP binds to the sulphur atom of Cys and Met and to the unprotonated imidazole ring of His. M1 protein was incubated with segment 8 vRNA and then treated with increasing amounts of *trans*-DDP for 1 h, subjected to deproteinization by SDS treatment and fractionated by agarose gel electrophoresis (see Fig. 1). Increasing the concentration of *trans*-DDP led to an increase in the amount of cross-linked material unable to enter the gel. In order to avoid multiple cross-linking and RNA–RNA cross-linking, we selected for our experiments a *trans*-DDP concentration of 0·2 mM where about 50% of the RNA was cross-linked (Fig. 1, lane 3), corresponding to a *trans*-DDP/(M1–vRNA complex) molar ratio of 100, which is close to conditions described by others (Baudin *et al.*, 1989; Moine *et al.*, 1988; Tukalo *et al.*, 1987; Wikman *et al.*, 1987; Ehresmann *et al.*, 1986).

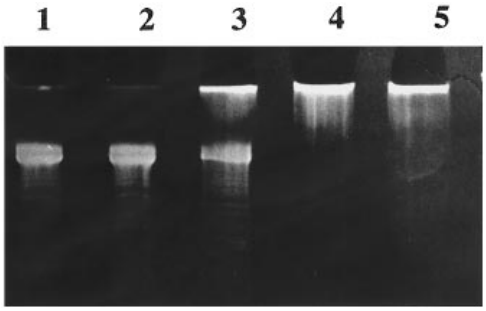
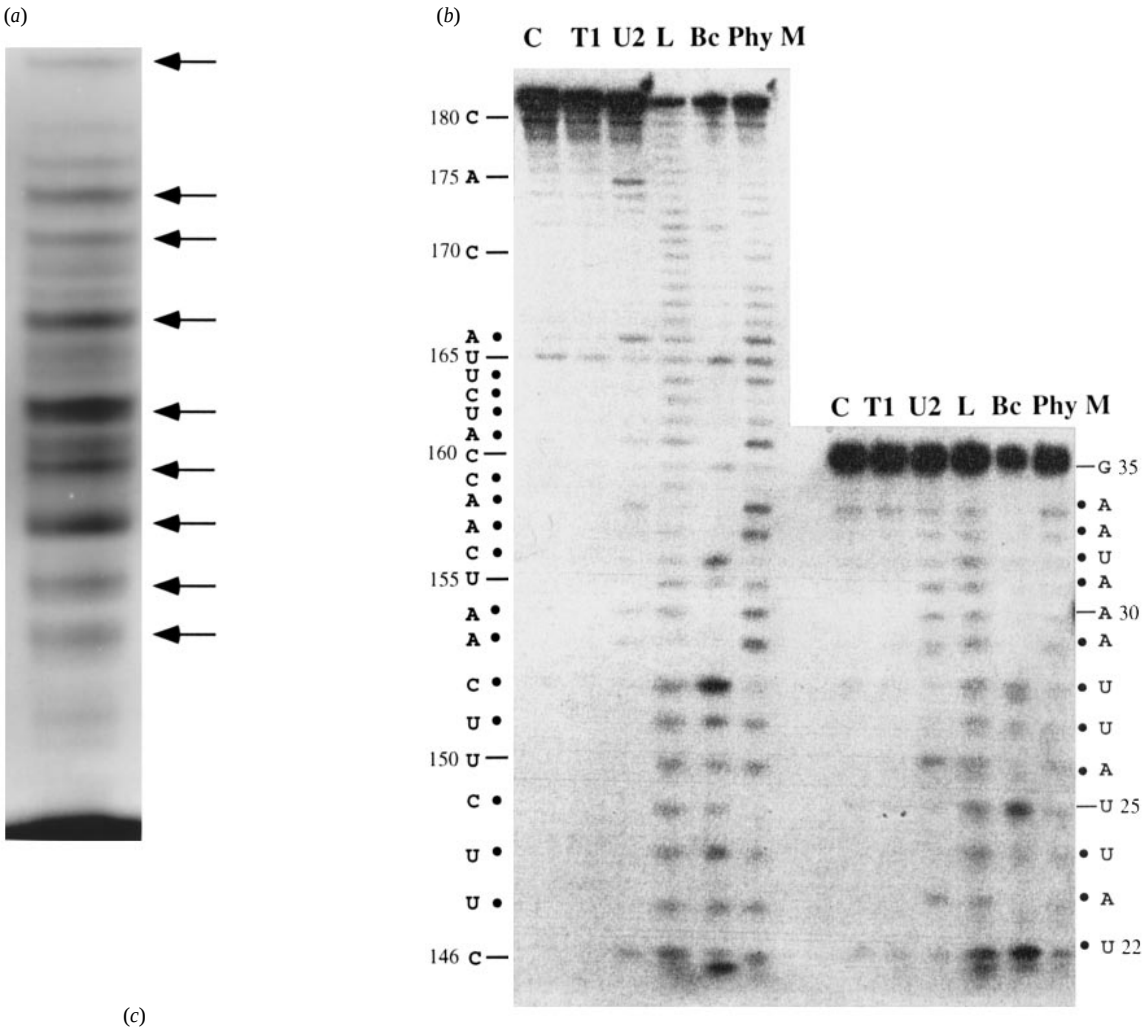


Fig. 1. Agarose gel electrophoresis of the M1–vRNA complex after *trans*-DDP cross-linking. Lane 1, vRNA control; lane 2, M1–vRNA complex without cross-linking and after deproteinization in 20 mM sodium phosphate (pH 7·5), 100 mM lithium chloride, 0·5% SDS and 1 mM EDTA for 30 min at 20 °C; lanes 3–5, before deproteinization platination was performed on 2 μM complex in the presence of 0·2, 0·3 and 0·4 mM *trans*-DDP, respectively. The RNA was stained with ethidium bromide.

In order to identify the regions of the vRNA molecule involved in the M1 interaction, the cross-linked M1–vRNA complex was treated with RNase T1. The resulting cross-linked M1–oligonucleotide complexes were separated from the non-cross-linked RNA fragments by nitrocellulose filtration in the presence of 2 M KCl. Under these conditions, only RNA fragments that are cross-linked to M1 are retained on the filter. After reversion of the cross-links by thiourea, the cross-linked oligonucleotides were 5′ end labelled and an autoradiogram of these labelled products is shown in Fig. 2(*a*). A control experiment in the absence of *trans*-DDP did not show any bands. All cross-linked fragments were repurified on a polyacrylamide–8 M urea gel and sequenced. Several examples of the sequence analysis are shown in Fig. 2(*b*). A diagram showing the sequence of the identified fragments is presented in Fig. 2(*c*). For the set of nine oligoribonucleotides sequenced, no sequence was redundant. This suggests that M1 binds to RNA without RNA sequence specificity. The absence of sequence specificity was also studied by filter-binding experiments, which showed that M1 binds to different kinds of RNA, including tRNA, with similar apparent K_d values, between 2 and 7×10^{-8} M (Fig. 3). M1 even binds to dsDNA with a K_d of 3×10^{-7} M.

In order to determine the amino acid sequence of M1 that binds to RNA, we cross-linked M1 to an oligoribonucleotide using APG, which binds covalently to guanine (Politz *et al.*, 1981). The azido group in APG is then photolysed by UV light (> 300 nm) yielding an unstable nitrene which is potentially reactive with many groups in proteins and nucleic acids. Cross-linking conditions and separation of cross-linked from non-cross-linked RNA are described in Methods. The non-cross-linked RNA was eluted from the HPLC C18 μBondapack column after 16 min retention. The cross-linked complex with a retention time of 10 min was dried and re-solubilized in PBS for Asp-N endoprotease digestion, cutting at the amino termini



(c)

AGUAGAAACA AGGGUGUUUU UUAUUAUUAA AUAAGCUGAA ACGAGAAAGU
UCUUAUCUCU UGCUCCACUU CAAGCAAUAG UUGUAAGGCU UGCAUAAAUG
UUAUUUGCUC AAAACUAUUC UCUGUUAUCU UCAGUCUGUG UCUCACUUCU
UCAAUCAACC AUCUUAUUUC UUCAAACUUC UGACCUAAUU GUUCCCGCCA
UUUUCCGUUU CUGUUUUGGA GUGAGUGGAG GUCUCCCAUU CUCAUUACUG
CUUCUCCAAG CGAAUCUCUG UAGAGUUUCA GAGACUCGAA CUGUGUUAUU
AUUCCAUUCA AGUCCUCCGA UGAGGACCCC AACUGCAUUU UUGACAUCCU
CAUCAGUAUG UCCUGGAAGA GAGGGCAGUG GUGAAAUUUC GCCAACAAUU
GUCCCCUCUU CGGUGAAGGC CCUUAGUAAU AUUAGAGUCU CCAGCCGGUC
AAAAAUCACA CUGAAGUUCG CUUUCAGUAU GAUGUUCUUA UCCAUGAUCG
CCUGGUCCAU UCUGAUACAA AGAGGGCCUG CCACUUUCUG CUUGGGCAUG
AGCAUGAACC AGUGCCUUGA CAUUUCCUCA AGAGUCAUGU CAGUUAGGUA
GCGCGAUGCA GGUACAGAGG CCAUGGUCAU UUUGAGUGCC UCAUCAGAUU
CUUCCUUCAG AAUCCGCUCC ACUAUUUGCU UUCCAGCACG GGUGGCUGUU
UCGAUGUCCA GACCGAGAGU GCUGCCUCUU CCUCUUAGGG ACUUCUGAUC
UCGGCGAAGC CGAUCAAGGA AUGGGGCAUC ACCUAGUUCU UGGUCUGCAA
CUCUUUUGCG GACAUGCCAA AGAAAGCAAU CUACCUGAAA GCUUGACACA
GUGUUUGGAU CCAUUAUGUC UUUGUCACCC UGCUUUUGCU

Fig. 2. For legend see facing page.

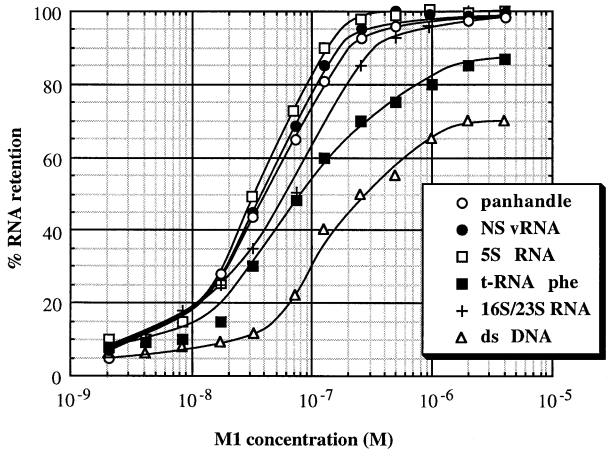


Fig. 3. Affinity of viral M1 protein for various nucleic acid species. Filter-binding experiments were done with viral M1 and a model panhandle RNA, segment 8 vRNA (NS), *E. coli* 5S ribosomal RNA, 16S/23S ribosomal RNA mixture, t-RNA^{Phe} and with double-stranded DNA. The 50% retention value corresponds to the apparent K_d for RNA-binding.

of Asp and Glu residues. The proteolysed fragments were again fractionated on HPLC using the same column and buffer systems. Compared with intact cross-linked M1–RNA, peak displacement was observed and three major displaced peaks eluted at 12, 2·2 and 1·7 min. Only the 12 and 2·2 min peaks were collected, dried and micro-sequenced. The 1·7 min peak was not further analysed because it eluted in the dead volume of the column. N-terminal sequencing of the 12 and 2·2 min peaks showed the same peptide sequence corresponding to ^{94}DKAVKLYRKLKREITFHG^{111}. The peptide sequence from the 2·2 min peak showed all amino acid residues at the same level of detection apart from R^{101}, indicating that this arginine was cross-linked to the RNA. The peptide sequence of the 12 min peak showed 2 to 3 times lower detection for K^{98}, K^{102} and K^{104} than for the other amino acid residues, suggesting cross-links between M1 and the RNA at these positions. The fact that different amino acids were cross-linked to the RNA in the two peaks might account for their difference in mobility on the C18 column.

The RNA-binding peptide of M1 contains the basic region RKLKR at position 101 to 105, which was shown to be the NLS of M1 (Ye *et al.*, 1995), but it did not contain the zinc-binding sequence. To confirm the involvement of the NLS in RNA-binding and to exclude any possible involvement of the zinc-binding sequence, we performed site-directed mutagenesis of recombinant M1. M1 was expressed in *E. coli* with an N-terminal polyhistidine tag in order to facilitate purification. The final purified M1 protein was fully soluble and migrated on an FPLC gel-filtration column as a monomeric protein with an apparent molecular mass of 38 kDa, indicating a slightly elongated shape. Circular dichroism of the wild-type and mutant recombinant proteins showed the same spectra as that of M1 protein isolated from virus (not shown). Moreover, both recombinant M1 and viral M1 proteins bound to RNA with the same affinity [K_d of about 6×10^{-8} M; compare the binding curve in Fig. 3 NS RNA for viral M1 with the RKLKR curves in Fig. 4(*a*, *b*) for wild-type recombinant M1]. Single point mutations of K and R to Q were introduced into the ^{101}RKLKR^{105} sequence for all the basic residues and the first cysteine of the zinc-binding sequence: ^{148}CATCEQIAD-SQHRSH^{162} was changed to Ser (C^{148}S). None of these single mutations had any effect on the K_d for RNA-binding (Fig. 4*a*).

Subsequently, we introduced multiple point mutations: K^{95} and K^{98} were simultaneously substituted to **A**^{95} and **A**^{98}, ^{101}RKLKR^{105} was changed to **AA**LKR and RKL**AA** and all four basic residues from the NLS were simultaneously substituted to alanines (**AALAA**). In the last mutant all basic residues present in this region were replaced by alanine: **A**AV**A**LY**AALAA**. All mutants behaved as soluble monomeric proteins. A deletion mutant of the NLS sequence was also constructed but, when overexpressed in *E. coli*, was found to be insoluble resulting in inclusion bodies, in agreement with the results of Watanabe *et al.* (1996). Filter-binding experiments of these multiple M1 mutants with segment 8 (NS) vRNA are shown in Fig. 4(*b*). Other binding experiments using short panhandle RNA (Baudin *et al.*, 1994) or rabies virus leader RNA as probes gave similar results. It appeared that all double-mutants bound to RNA with a K_d similar to that for wild-type M1 (RKLKR curve). However, the 4- and 6-fold mutations resulted in total loss of RNA-binding.

Finally, we studied the transcription-inhibition activity of the M1 mutant proteins. Fig. 5 shows the inhibition of transcription as a function of M1 concentration. Both the wild-type recombinant M1 protein and the C^{148}S mutant had a higher inhibition activity than virus-derived M1 protein (Fig. 5*a*). It is possible that these recombinant proteins had a higher activity because they were more soluble, i.e. a higher effective concentration with the same amount of protein. Similarly, both double-mutants, which still bound to RNA, inhibited transcription with an activity close to that of the wild-type M1 protein. The 6-fold mutant, which had no RNA-binding activity, still showed about half the inhibition activity of wild-type M1 protein over the entire concentration range (Fig. 5*b*).

Fig. 2. Characterization of RNA sequence cross-linked to M1 protein. (*a*) Fractionation of the *trans*-DDP cross-linked oligonucleotides on a 20% polyacrylamide–8 M urea gel. The cross-links between M1 and the vRNA fragments were reverted and the liberated oligonucleotides subjected to 5′ end labelling before fractionation. Arrows indicate the RNA oligonucleotides that were sequenced. (*b*) Two sequence analyses of the cross-linked vRNA fragments. Lane C, control in the absence of enzyme. Lanes T1, U2, Bc and PhyM, hydrolysis with RNases T1 (for G), U2 (A), *Bacillus cereus* (for C+U) and *Phy*M (for A+U) respectively. Lane L, formamide ladder. (*c*) Diagram of RNA fragments found to be cross-linked to M1 protein. The sequences of the cross-linked vRNA fragments are indicated in boxes on the sequence of influenza vRNA segment 8.

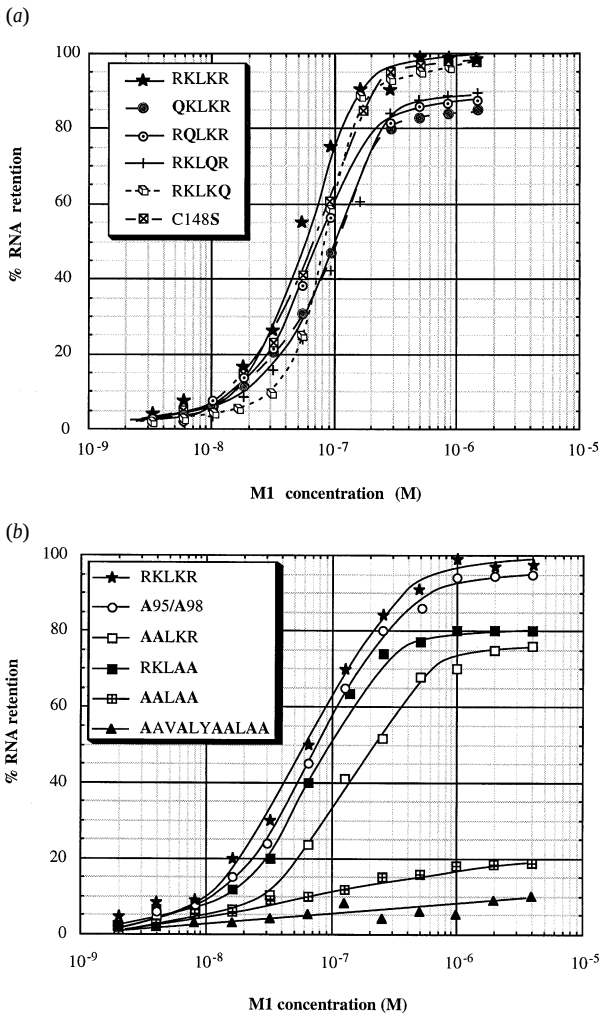


Fig. 4

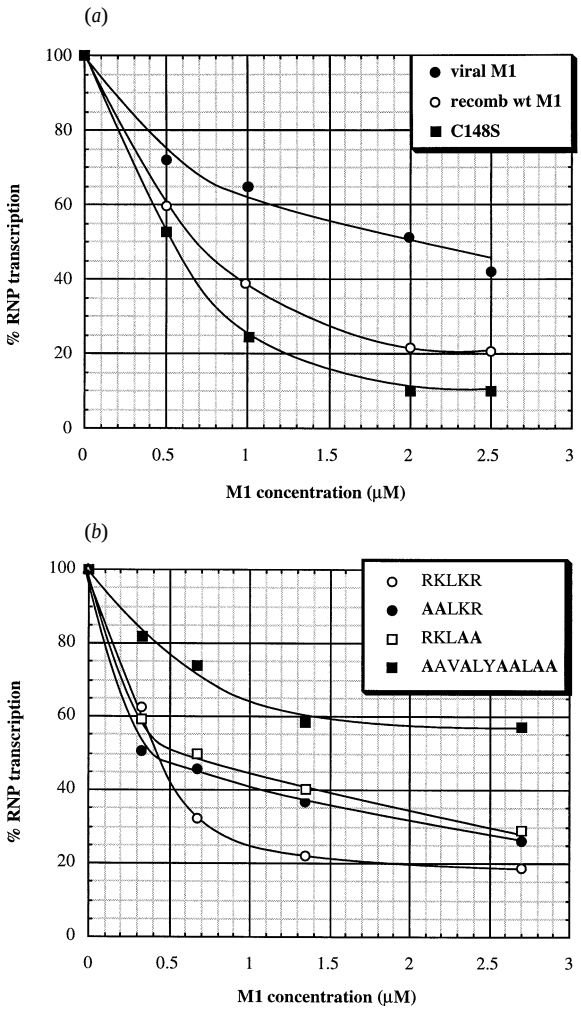


Fig. 5

Fig. 4. Filter-binding experiments of recombinant M1 protein with segment 8 vRNA. (*a*) Wild-type and single mutants; (*b*) wild-type and multiple mutants.

Fig. 5. *In vitro* transcription-inhibition experiments. *In vitro* RNP transcription in the absence and in the presence of an increasing concentration of M1 protein added to a stable concentration of RNP (30 nM). (*a*) Comparison of M1 protein isolated from virus with recombinant wild-type M1 and the C148S mutant. (*b*) Comparison of recombinant wild-type M1 (RKLKR) with double- and 6-fold mutants. (*a*) and (*b*) were independent experiments.

Discussion

The results presented here show that influenza virus M1 protein binds to RNA through its nuclear localization sequence (NLS). Our results also show that the zinc-binding sequence that had been proposed to be involved in RNA-binding (Wakefield & Brownlee, 1989) is not important for the interaction with RNA. The loss of one cysteine in this sequence is expected to lead to loss of zinc-binding, as shown for the nucleocapsid protein of Moloney murine leukaemia virus (Méric & Goff, 1989). If the zinc-binding sequence of M1 had been involved in RNA-binding, loss of zinc-binding should have led to loss of RNA-binding. This absence of an effect on RNA-binding is in agreement with our previous results (Elster *et al.*, 1994) that showed that there was no correlation between the zinc content of M1 and its RNA-binding activity. We do not know the role for the zinc-binding sequence, which is conserved among influenza A and B viruses. It is likely to be an important factor during the life-cycle of the virus since peptides containing the sequence have a strong antiviral activity (Nasser *et al.*, 1996).

That the NLS is involved in RNA-binding was also implied by the work of Watanabe *et al.* (1996) who studied deletion mutants of recombinant M1 and found that deletions between residues 91 and 111 abolished RNA-binding and transcription-inhibition. However, our results suggest that loss of RNA-binding does not correlate with loss of transcription-inhibition, since the 6-fold mutant that had lost its RNA-binding activity

could still inhibit *in vitro* transcription, albeit to a lesser extent. The difference between our results and those of Watanabe *et al.* (1996) may be due to the fact that we used point mutations whilst they used deletions. Our M1 protein preparations were completely soluble whereas theirs led to insoluble inclusion bodies that had to be solubilized with urea and subsequently stabilized with a treatment at pH 1. This drastic treatment may have had an influence on some part of the M1 protein that is not involved in RNA-binding but needed for transcription-inhibition. Results from RNA-binding and transcription-inhibition experiments using anti-idiotypic antibodies also suggested that the two activities may be related but not synonymous (Ye *et al.*, 1989).

For the transcription-inhibition experiments, we used an RNP concentration of 30 nM. If we assume an average length of vRNA of 1500 nt and an average of 20 nt per NP monomer, 30 nM RNP would correspond to 2·25 μM NP monomers. The half-maximal inhibitory concentration of M1 is around 3×10^{-7} M, which is about 5 times higher than the K_d for RNA-binding. Maximal inhibition was achieved at an M1 concentration of $1{\cdot}5 \times 10^{-6}$ M, i.e. at a M1/NP ratio of about 1. Watanabe *et al.* (1996) reported a similar ratio of 2 and in their experiments maximum inhibition was 50%, whereas in our hands it was 80%. The fact that half-maximal inhibition occurs at an M1 concentration that is only 5 times the K_d of RNA-binding could indicate that the inhibition also occurs through RNA-binding. However, this would not explain the inhibition activity of the 6-fold mutant, which could no longer bind to RNA. If M1 was bound to the phosphoribose backbone of vRNA inside RNP, another possible problem would be competition between M1 and NP, which also binds to the backbone (Baudin *et al.*, 1994). Under conditions of the same concentration of M1 and NP, M1 is not likely to displace NP, which has a 10-fold lower K_d for vRNA (Baudin *et al.*, 1994).

Most proteins that can bind to RNA or DNA and that have an NLS show a positional overlap of these two activities (LaCasse & Lefebvre, 1995). Some of the hypothetical reasons for this overlap are outlined by LaCasse & Lefebvre (1995). Both as an NLS and as an RNA-binding site, the KAVKLYRKLKR sequence falls into clear categories; K(R/K)x(R/K) is a consensus NLS (LaCasse & Lefebvre, 1995; Ye *et al.*, 1995) and the arginine-rich motif represents one of the classic RNA-binding signatures (Burd & Dreyfuss, 1994). In this RNA-binding motif, the interaction with RNA is often through the phosphoribose backbone but hydrogen bonding can also occur with the nucleotide bases (Burd & Dreyfuss, 1994).

A consequence of the overlap of RNA-binding and NLS in influenza virus M1 is that it will be difficult, even with reverse genetics methods, to test whether both activities are essential for the infectivity of the virus. M1 protein in the infected cell is supposed to exist in two separate populations, one nuclear and the other cytoplasmic (Hay & Skehel, 1975). Cytoplasmic M1 is supposed to go directly into budding virions and prevents nuclear re-import of newly made RNPs, whereas nuclear M1 aids newly formed RNPs to leave the nucleus (Martin & Helenius, 1991*b*; Bui *et al.*, 1996; Whittaker *et al.*, 1995, 1996). It is not clear if this nuclear M1 protein is exported with the RNPs and subsequently re-imported into the nucleus or whether it stays nuclear (Rey & Nayak, 1992; Whittaker *et al.*, 1995). It is also not clear how cytoplasmic M1 avoids transport into the nucleus. Somehow, its NLS must not be available for recognition by nuclear protein import factors.

In theory, the NLS could be necessary for the nuclear activity of M1 and the RNA-binding activity could be an artefact of the exposed basic residues present in the NLS (exposure of the NLS would be necessary for recognition by regulatory proteins that promote nuclear uptake). On the other hand, if the NLS is exposed, it would be hard to imagine how nucleic acid-binding can be avoided in the nucleus.

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