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Human Immunodeficiency Virus (HIV) Nef is an RNA Binding Protein in Cell-free Systems

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The function of human immunodeficiency virus *nef* gene product has been much debated but the precise activity of this protein in the HIV replication cycle remains unknown. HIV-1 Nef was obtained as a fusion protein with maltose binding protein (MBP), purified by amylose column chromatography and separated from MBP by cleavage with factor Xa. Purified HIV-1 Nef protein, but not the fusion protein MBP-Nef, binds to RNA *in vitro* as tested by three different assays, radioactive or non-radioactive Northwestern analysis, UV cross-linking or band-shift analysis. This activity was lost in a deletion mutant lacking 22 amino acids from the amino terminus of HIV-1 Nef, while a deletion of 44 residues from the carboxy terminus of the protein does not impair the RNA binding activity. Moreover, a single amino acid replacement, Arg to Gly at position 22 produces a Nef variant deficient in its ability to interact with RNA. Different Nef proteins from HIV-1, HIV-2 or SIV were fused to MBP and cleaved with factor Xa. The different Nef proteins were all endowed with RNA-binding capacity. Sequence similarities between several RNA binding proteins, including picornavirus 2C and different Nef proteins are observed. The function of Nef during the HIV replication cycle is discussed on the basis of the present findings.

Keywords: HIV; Nef; RNA binding protein

Introduction

Human immunodeficiency virus (HIV) contains the three genes typical of non-defective retroviruses: *gag*, *pol* and *env* (Wong-Staal, 1990; Levy, 1993; Ratner, 1993). In addition, HIV encodes six auxiliary proteins known as Vif, Vpr, Vpu, Tat, Rev and Nef (Subbramanian & Cohen, 1994; Trono, 1995).

The finding that simian immunodeficiency virus defective in Nef replicates more slowly in rhesus monkeys and does not induce AIDS, indicated that Nef is necessary to maintain high SIV loads in whole infected animals (Kestler *et al*., 1991). These findings may be extrapolated to HIV, since Nef also enhances virus multiplication in SCID mice transplanted with human tissues (Jamieson *et al*., 1994). These studies indicate that Nef is necessary for efficient HIV replication in some cultured cells and in animals (Cullen, 1994). Deletion of Tat or Rev, two products that regulate viral gene expression, impairs viral replication, while the activity of the other auxiliary proteins is not essential for viral growth in culture cells (Subbramanian & Cohen, 1994). Since Vif, Vpr, Vpu or Nef are not required for HIV replication, they are referred to as accessory gene products (Subbramanian & Cohen, 1994). The *nef* gene is located at the 3' end of the genome coding region, overlapping part of the LTR (Cullen, 1994). Two forms of Nef are present in HIV-infected cells, a 27 kDa species that is myristoylated and associates with membranes and the unmyristoylated 25 kDa form that is predominantly soluble in the cytoplasm (Franchini *et al*., 1986; Kaminchik *et al*., 1991; Yu & Felsted, 1992). A small fraction of Nef localizes to the nucleus in specific curvilinear tracks (Murti *et al*., 1993).

The exact function of each accessory protein during the HIV replication cycle remains poorly understood. Uncovering the activity of the *nef* gene has proven to be difficult and the requirement of Nef function for HIV replication has been much debated (Cullen, 1994). Nef is expressed early in the HIV life cycle, together with Tat and Rev. In fact, the

Abbreviations used: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; MBP, maltose binding protein; LTR, long terminal repeat; ssDNA, single stranded DNA; dsDNA, double stranded DNA.

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majority of early HIV mRNAs encode Nef (Robert-Guroff *et al*., 1990). Initial studies suggested that Nef downregulated viral multiplication (Terwilliger *et al*., 1986; Ahmad & Venkatesan, 1988; Luciw *et al*., 1987), perhaps by inhibition of transcription from the LTR promoter (Niederman *et al*., 1989; Bandres & Ratner, 1994), thus facilitating the establishment and maintenance of virus latency (Cheng-Mayer *et al*., 1989). Several findings suggested that deletion of the *nef* gene from HIV or SIV increased virus replication in culture cells (Terwilliger *et al*., 1986; Fisher *et al*., 1986; Luciw *et al*., 1987; Binninger *et al*., 1991). These results were not reproduced by others, who found no inhibition of transcription by Nef and decreased virus multiplication of Nef-deficient HIV in culture cells (Kim *et al*., 1989; Hammes *et al*., 1989; Bachelerie *et al*., 1990; De Ronde *et al*., 1992). The presence of a negative regulatory element (NRE) distinct from *nef* in the LTR could account in part for these conflicting reports (Lu *et al*., 1989). More recent findings indicate that *nef*-defective HIV-1 are less efficient in reverse transcription of their genome (Chowers *et al*., 1994; Miller *et al*., 1995; Schwartz *et al*., 1995b; Aiken & Trono, 1995). Therefore, virions formed in the absence of a functional Nef protein show defects at an early step upon infection. This step is located after virus attachment and entry, but prior to viral DNA integration (Chowers *et al*., 1994; Miller *et al*., 1995; Schwartz *et al*., 1995b; Aiken & Trono, 1995).

In addition to the genetic studies, the Nef product has been purified to test its potential biochemical activities or has been expressed in cells to analyse its effect on cellular functions (Cullen, 1994). Purified Nef showed GTPase activity and sequence comparison suggested a similarity to GTP-binding proteins (Guy *et al*., 1987). These findings were not reproduced by other groups (Backer *et al*., 1991; Harris *et al*., 1992). On the other hand, expression of Nef in $CD4^+$ cells induces internalization and degradation of the HIV receptor molecule, the CD4 glycoprotein (Garcia *et al*., 1993; Garcia & Miller, 1991). Accumulation of CD4 molecules takes place in early endosomes (Schwartz *et al*., 1995a). This degradation of CD4 presumably involves the direct interaction of Nef with the cytoplasmic moiety of the CD4 glycoprotein (Aiken *et al*., 1994). The exact biological significance of CD4 degradation remains obscure, but it has been suggested that the downregulation of CD4 would inhibit the superinfection of Nef-expressing cells by HIV (Benson *et al*., 1993). Direct interaction of Nef with β-COP (Benichou *et al*., 1994), a protein involved in vesicular traffic, may provide the molecular basis for the modification of CD4 and gp120/gp41 traffic by Nef (Cullen, 1994). HIV-1 particles from *nef* negative strains are less infectious than non-defective virus (Miller *et al*., 1995). However, the interaction between Nef and CD4 seems not to be required to increase virus replication, since the relative infectivity of HIV virions does not correlate with expression of CD4 (Miller *et al*., 1994).

Here we show that purified HIV and SIV Nef proteins have the capacity to interact with RNA in cell-free systems. This RNA-binding activity lies in an arginine-rich region present at the amino terminus of Nef. Sequence similarities between several RNA binding proteins that contain an Arg-rich motif (Lazinski *et al*., 1989), including poliovirus protein 2C (Rodriguez & Carrasco, 1995a) and HIV Nef have been noted. In addition, both proteins interact with membranes and bind RNA. These findings suggest that HIV Nef belongs to the RNA-binding family of proteins that contain an Arg-rich motif (Burd & Dreyfuss, 1994; Biamonti & Riva, 1994).

Results

RNA binding activity of isolated Nef

During the course of our studies on the NTPase and RNA binding activities of poliovirus protein 2C (Rodriguez & Carrasco, 1993, 1995a), we noticed sequence similarities between poliovirus 2C and HIV Nef. Particularly striking is the presence of the sequence MDD . . . DXXD and an Arg-rich stretch ERXRR in both proteins. Poliovirus protein 2C is an NTPase enzyme (Rodriguez & Carrasco, 1993), that binds to RNA (Rodriguez & Carrasco, 1995a) and interacts with membranes (Bienz *et al*., 1987, 1992). To assay the RNA binding capacity of HIV Nef, the gene corresponding to this protein was amplified from the cDNA corresponding to HIV-1 (BRU strain) by PCR and cloned in vector pMal-C by standard cloning techniques (Figure 1A). *Escherichia coli* clones expressing the fusion protein MBP-Nef were isolated. The fusion protein represents the major polypeptide synthesized by these clones upon induction with IPTG (Figure 1B). MBP-Nef can be easily isolated by passage through amylose columns. Genuine Nef protein is generated upon cleavage of MBP-Nef with the protease factor Xa, that specifically cleaves at the junction between MBP and Nef (Figure 1B).

Three different methods were employed to test the RNA binding activity of Nef: (1) a Northwestern method using either biotinylated or radioactive riboprobes, (2) UV cross-linking of a radioactive riboprobe to the protein and (3) a band-shift assay. Highly purified potyvirus protein CI was used as a positive control for a protein with RNA-binding capacity, while MBP served as a negative control (Lain *et al*., 1991; Rodriguez & Carrasco, 1995a). Figure 2 shows that genuine HIV-1 Nef binds RNA in each of the three different assays. When HIV-1 Nef is transferred to nitrocellulose membranes it clearly interacts with biotinylated or radioactive riboprobes. Similarly the interaction of Nef with RNA is detected by UV cross-linking or by analysis by band-shift assays (Figure 2). Notably, Nef is devoid of this RNA binding capacity when present as the fusion protein MBP-Nef. The RNA binding capacity of Nef becomes clearly apparent after its separation from MBP. There is, however, slight RNA-binding by the fusion product MBP-Nef when

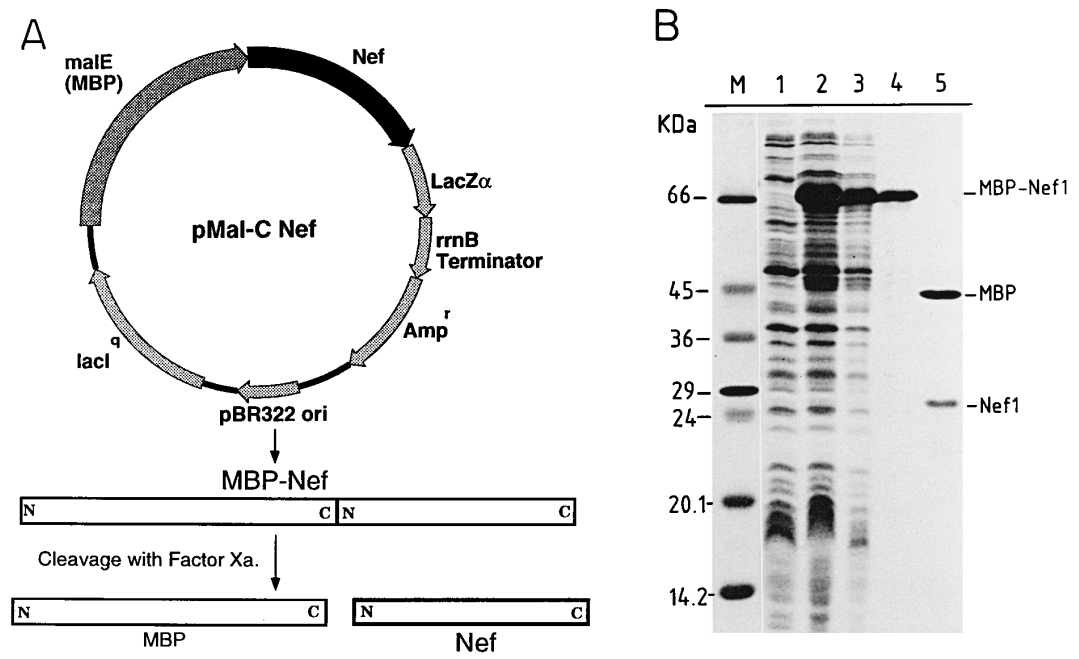


Figure 1. A, Strategy used to obtain genuine HIV-1 Nef. The sequences corresponding to *nef* were obtained from HIV-1 (Bru strain) and cloned in vector pMal-C (New England Biolabs). The fusion protein obtained MBP-Nef, is cleaved by factor Xa (Rodriguez & Carrasco, 1995b). B, Purification of Nef protein. Proteins from *E. coli* bearing plasmid pMal-C Nef were analysed by SDS-PAGE as indicated in Materials and Methods. Coomassie blue staining of total proteins from uninduced *E. coli* cells (1), or from IPTG-induced bacterial cells (2). Proteins present in the supernatant after centrifugation of total lysates (3). MBP-Nef fusion protein purified by amylose columns (4). Separated MBP and Nef proteins after factor Xa cleavage (5). M, molecular weight marker.

the UV cross-linking method is employed, but this activity is much lower than that observed with genuine Nef. This finding is not unexpected considering that the Arg-rich stretch necessary to interact with RNA is close to the amino terminus of Nef (see below). In addition, some proteins do not interact with RNA when they are attached to MBP, while shorter fragments do show this activity (Römisch *et al*., 1990; Citovsky *et al*., 1992; Rodriguez & Carrasco, 1995a). These results provide compelling evidence that Nef is endowed with RNA binding activity in cell-free systems as measured by different methods.

The majority of RNA binding proteins are able to interact with different RNA substrates. This binding is competed by a number of RNA substrates or even ssDNA depending on the protein analysed (Kiledjian & Dreyfuss, 1992; Chen *et al*., 1993; Qiu

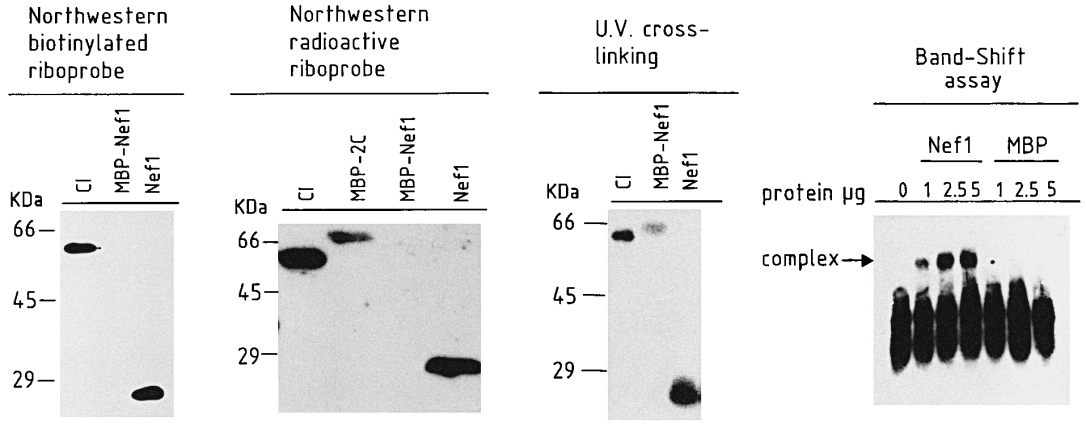


Figure 2. RNA binding activity of HIV-1 Nef protein. RNA-binding activity was assayed by Northwestern blotting using 1.1 kb BS biotinylated or radioactive riboprobes, UV cross-linking and band-shift assay. CI protein from sharka potyvirus and individually expressed MBP protein were assayed as positive and negative controls, respectively. The position of M_r markers is indicated. MBP-2C, fusion protein between MBP and poliovirus 2C. MBP-Nef1, fusion protein between MBP and Nef from HIV-1. The lanes labelled as Nef1 also contain factor Xa and MBP as a result of the digestion of MBP-Nef1, while the lanes labelled as MBP in the band-shift assay only contain this protein and factor Xa.

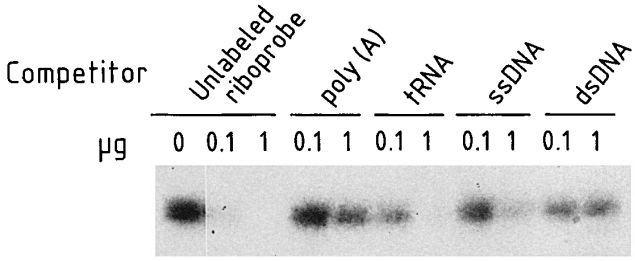


Figure 3. RNA binding competition of HIV-1 Nef with different nucleic acids. HIV-1 Nef protein was preincubated with the indicated amounts of unlabelled riboprobe, poly(A), yeast tRNA, boiled salmon sperm DNA (ssDNA) and intact salmon sperm DNA (dsDNA). Radioactive 1.1 kb BS riboprobe was then added and binding assays were carried out as indicated in Materials and Methods. The autoradiography of UV cross-linking reactions is shown.

& Krug, 1994). As an initial step to establish the specificity of the interaction between Nef and nucleic acids, the formation of the complex was competed by different substrates. Addition of a 100-fold excess of poly(A), ssDNA or dsDNA does not abolish the binding of Nef to RNA, while a 1000-fold excess of tRNA does block this interaction. Competition of RNA binding has been observed with some proteins (Chen *et al*., 1993), but not with others (Kiledjian & Dreyfuss, 1992). As a control, a 100-fold excess of cold RNA strongly diminishes complex formation between Nef and radioactive RNA (Figure 3). Partial, but not total, competition of RNA binding by Gag with cold substrate has been described (Luban & Goff, 1991).

As an attempt to identify regions of HIV-1 genome involved in RNA interaction, different RNA probes were obtained (Figure 4A). The binding in the presence of heparin was quantified by non-radioactive Northwestern assay and normalysed to the amount of biotinylated RNA probe. The fact that the RNA probes still bind to Nef in the presence of heparin indicates that it is not a simple charge interaction that is occurring. As occurs with other RNA binding proteins (Luban & Goff, 1991) interaction of the different probes to Nef occurs albeit to different extents (Figure 4B). Thus, there is consistently more binding with the 0.8 kb SS probe located close to the 5' end that encompasses a region of the HIV-1 genome that contains the ψ region and dimer linkage structure (DLS), while the 1.1 kb BS RNA probe located at the 3' end that spans most of the LTR including part of the TAR element and 2.7 kb EB that contains the RRE region, bind less efficiently. Further analysis in this direction would serve to indicate if there is a specific region in the HIV-1 genome involved in Nef interaction.

An Arg-rich stretch at the amino terminus of Nef is necessary for RNA binding activity

In order to analyse the regions of HIV-1 Nef involved in the interaction with RNA, several Nef deletion variants were generated. The corresponding proteins were purified and assayed for RNA binding activity. Deletion of 44 amino acids (NefΔ1 variant) from the carboxy terminus produces Nef protein variants that still retain their RNA-binding activity, while deletion of 22 amino acids (NefΔ2 variant) from the amino terminus produces a Nef protein incapable of binding RNA, as measured by UV cross-linking analysis (Figure 5). These findings suggest that the N-terminal region participates in the interaction with RNA molecules, while regions between residues 23 and 206 are not sufficient for this activity. Since this amino-terminal region is rich in Arg residues, it seemed plausible that they were involved in RNA interaction. Therefore, we generated and purified a number of Nef proteins in which some of the Arg residues mutated to Lys or Gly (Figure 6). Nef variants in which Arg residues 17, 19 or 21 were replaced by Lys retained their ability to bind RNA, while an Arg/Gly mutation at residue 22 generated a Nef protein with a much reduced RNA binding activity (Figure 6). These results clearly show that a single Arg replacement can produce a Nef variant deficient in its ability to interact with RNA, suggesting that Nef belongs to the RNA-binding family of proteins that contain an Arg-rich RNA-binding motif (Burd & Dreyfuss, 1994; Biamonti & Riva, 1994).

RNA binding activity of Nef from different species

The Nef protein from HIV-1 differs from the *nef* gene product encoded by HIV-2 or SIV (Shugars *et al*., 1993). The HIV-1 Nef is shorter than its counterparts in HIV-2 or SIV. In addition, the location of various motifs present in these proteins differs (Figure 7A). Curiously, the MDD sequence, found near the carboxy terminus of HIV-1 Nef, appears in a central region in both HIV-2 and SIV. HIV-2 and SIV Nef proteins share greater similarities than HIV-1 Nef with either. However, a region rich in basic amino acids is present at the amino terminus of all three proteins (Figure 7B). To determine whether RNA-binding activity is an universal feature of Nef from different immunodeficiency viruses, the Nef proteins from HIV-2 (ST strain) and SIV (MM251) were cloned and isolated by a protocol similar to that used to isolate HIV-1 Nef. Figure 7C shows that none of the proteins binds to RNA when assayed as a fusion protein with MBP, whereas, after cleavage of MBP-Nef with factor Xa, all three Nef proteins exhibited RNA-binding activity. These results indicate that the genuine Nef gene products from HIV-1, HIV-2 or SIV all possess the capacity to interact with RNA.

Discussion

Unraveling the functioning of HIV accessory proteins could provide new clues not only for a better understanding of the HIV replication cycle, but also to design new approaches to interfere

with the replication of this human pathogen (Cullen, 1994; Subbramanian & Cohen, 1994; Trono, 1995; Daniel *et al*., 1992). In this regard an intact *nef* gene is necessary to obtain high virus loads and pathogenicity in SIV-infected monkeys and HIV-1-infected mice (Kestler *et al*., 1991; Jamieson *et al*., 1994). Moreover, *nef*-defective HIV viruses have been isolated from long-term survivors, pointing to the requirement of *nef* function for the development of AIDS in humans (Huang *et al*., 1995).

The presence of a conserved Arg-rich sequence in Nef has already been noticed (Samuel *et al*., 1991). However, this sequence was interpreted as a signal for nuclear localization of Nef (Samuel *et al*., 1991). In fact, Nef is predominantly located in the cytoplasm (Cullen, 1994), although a small proportion of Nef may appear in the nucleus in some cells (Ovod *et al*., 1992; Murti *et al*., 1993). The finding that Nef interacts with RNA mediated by the Arg-rich sequence provides a function for this conserved

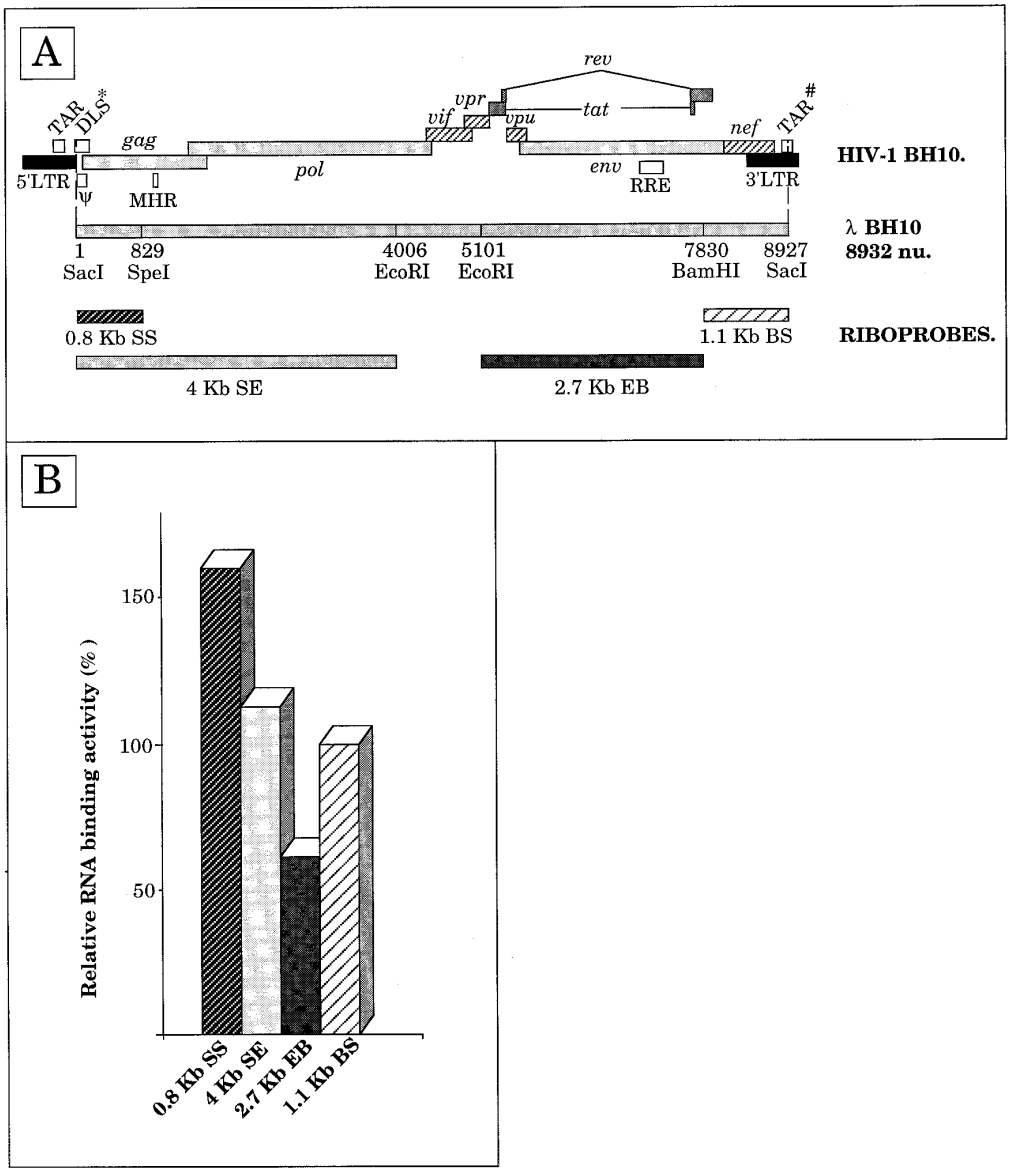


Figure 4. A, Localization of riboprobes on the HIV-1 BH10 genome. The complete HIV-1 BH10 genome is shown with all genes and regulatory or significant regions. The *Sac*I-*Sac*I fragment of HIV-1 BH10 (λ BH10 clone) with restriction sites used in cloning of the different riboprobes is shown. The numbers indicate the position of nucleotides in clone λ BH10. Four different riboprobes, its sizes, names and its localization in λ BH10 sequence are indicated. Kb, kilobases; nu, nucleotides; TAR, Tat-responsive element; DLS, dimer linkage structure; ψ, packaging signal; MHR, major homology region; LTR, long terminal repeat; RRE, Rev responsive element. *, The 5' first nucleotide of DLS is absent in probes 0.8 kb SS and 4 kb SE. #, The 54 first nucleotides of the TAR region are present in probe 1.1 kb BS. B, Binding of different HIV-1 RNA fragments to Nef. Relative RNA binding activity (represented as % of 1.1 kb BS binding) measured by Northwestern assay with four different riboprobes. The assay was performed in the presence of 3 mg/ml heparin.

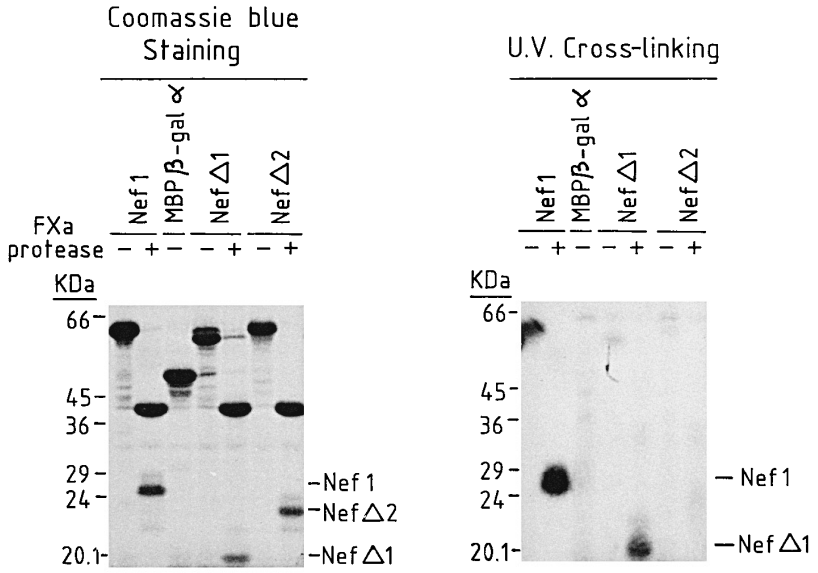


Figure 5. UV cross-linking assay of Nef size variants. Genuine and truncated Nef proteins were incubated with the 1.1 kb BS riboprobe and cross-linked as detailed in Materials and Methods. Coomassie blue staining and autoradiography of UV cross-linking assay. MBP Nef1, fusion protein between MBP and HIV-1 Nef. After factor Xa cleavage two proteins appear, MBP (40 kDa) and Nef1 (27 kDa). MBP β-gal-α was used as a control of a protein devoid of RNA binding activity. The positions of Nef1 or its variants as fusion proteins (–) or after factor Xa cleavage (+) are indicated.

sequence. This interaction is clearly observed using three different methods to assay the binding of RNA to proteins. Moreover, the Nef protein must be intact and separated from MBP to show this activity, suggesting that the folding of the fusion protein MBP-Nef does not allow the interaction of RNA with the amino terminus of Nef, where the Arg-rich sequence is located. Further arguments about the specificity of this binding are provided by the Arg/Gly variant at position 22, that possesses a greatly diminished ability to bind RNA. Therefore, a single amino acid replacement compromises the ability of Nef to interact with RNA.

Despite numerous reports on the biochemical function of Nef, little is known about the exact role it plays in HIV growth and in the infectivity of the virions formed (Subbramanian & Cohen, 1994; Trono, 1995). The initial report that Nef is a GTPase (Guy *et al*., 1987) was not supported by other groups that tested this activity (Backer *et al*., 1991; Harris *et al*., 1992; Matsuura *et al*., 1991; Nebreda *et al*., 1991). Our attempts to show NTPase activity by our purified HIV-1 Nef have also been unsuccessful (unpublished results). However, slight, but reproducible NTP-binding by highly purified Nef has been reported (Wolber *et al*., 1992; Azad *et al*., 1994), perhaps reflecting the RNA binding properties of Nef. The present finding that Nef protein has RNA binding capacity points to a novel function for this protein. Therefore, Nef is not only a membrane associated protein (Kaminchik *et al*., 1994), but also a product capable of interacting with nucleic acids.

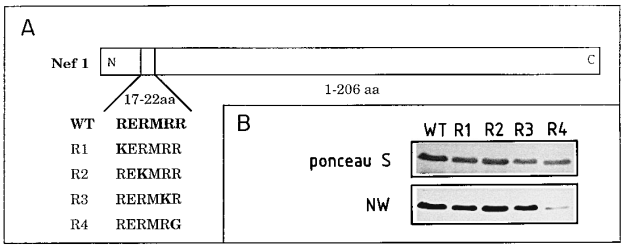
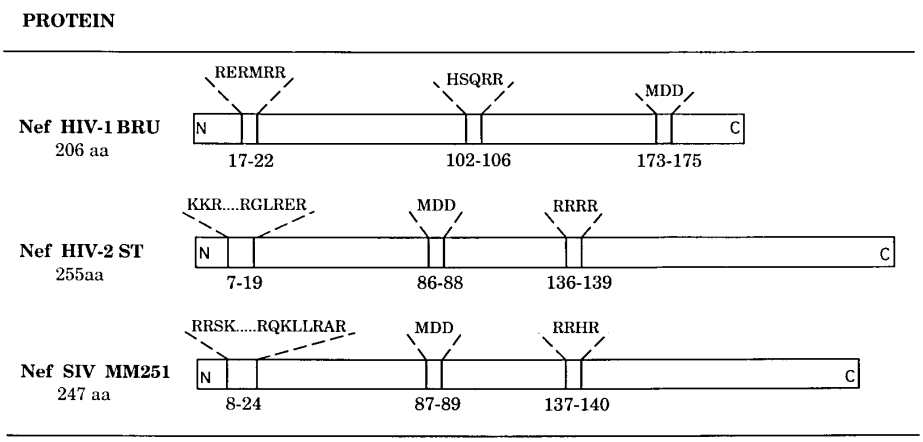


Figure 6. A, Site-directed mutagenesis of the arginine-rich region of HIV-1 Nef protein. Four site-specific variants (R1, R2, R3 and R4) were generated by PCR. Wild-type sequence and single-point mutation are indicated. B, RNA protein binding assay of site-specific Nef mutants. After cleavage of fusion proteins the Nef variants were separated by SDS-PAGE. Electrotransferred proteins were visualized by Ponceau S staining of the nitrocellulose membrane. The membrane was incubated with 1.1 kb BS biotinylated riboprobe for Northwestern blot analysis (NW).

The interaction of Nef with RNA suggests that its activity may be related to RNA functioning. HIV RNAs need to be transported through various compartments in the infected cells (Wong-Staal, 1990). This transport implies the interaction of the nucleic acid with specific proteins (Simos & Hurt, 1995). Perhaps Nef participates in viral RNA trafficking, a process already suggested for poliovirus 2C (Rodriguez & Carrasco, 1995a). Recent studies indicate that Nef enhances HIV-1 replication at an early step, that leads to viral DNA integration (Chowers *et al*., 1994; Miller *et al*., 1995; Schwartz *et al*., 1995b; Aiken & Trono, 1995). Thus, *nef*-defective HIV-1 binds, enters and *in vitro* reverse transcribes the genome as efficiently as wild-type virus (Chowers *et al*., 1995). However, mutant viruses are defective in establishing viral DNA in cells, probably due to an impairment in the intracellular reverse transcription (Chowers *et al*., 1995). Since Nef is not present in virions, it seems that the absence of this protein during assembly produces virus particles deficient in reverse transcribing upon infection. Therefore, Nef would be required for correct formation of infectious virus particles (Schwartz *et al*., 1995b; Aiken & Trono, 1995; Chowers *et al*., 1995). Thus, *nef* provided in *trans*

A



B

Nef N-Terminal BASIC REGION

	5			10			15				20				
HIV-1 BRU	M	G	G	**K**	W	S	**K**	S	S	V	V	G	W	P	T
HIV-1 BH10	M	G	G	**K**	W	S	**K**	S	S	V	V	G	W	P	A
HIV-1 ELI	M	G	G	**K**	W	S	**K**	S	S	I	V	G	W	P	A
HIV-1 MAL	M	G	G	**K**	W	S	**K**	S	S	I	V	G	W	P	**K**
HIV-2 ST	M	G	A	S	G	S	**K**	**K**	**R**	S	E	P	S	**R**	G
HIV-2 ROD	M	G	A	S	G	S	**K**	**K**	**H**	S	**R**	P	P	**R**	G
HIV-2 D194	M	G	A	S	G	S	**K**	**K**	**R**	S	E	**H**	S	Q	G
HIV-2 GHANA-1	M	G	A	S	G	S	**K**	**K**	**H**	S	**K**	**H**	S	Q	**R**
SIV MM251	M	G	G	A	I	S	M	**R**	**R**	S	**K**	P	A	G	D
SIV 1A11	M	G	G	T	I	S	M	**R**	**R**	S	R	S	T	G	D
SIV K6W	M	G	G	A	I	S	M	**R**	**R**	S	**K**	P	A	G	D
SIV GB1	M	G	S	S	Q	S	**K**	**K**	**R**	S	E	A	W	V	**R**
SIV PBJ	M	G	G	V	T	S	**K**	**K**	**Q**	**R**	**R**	**R**	G	G	N
SIV CPZ	M	G	T	**K**	W	S	**K**	S	S	L	V	G	W	P	E

	15			20				
HIV-1 BRU	L	**R**	E	**R**	M	**R**	**R**	A
HIV-1 BH10	A	**R**	E	**R**	M	**R**	**R**	A
HIV-1 ELI	I	**R**	E	**R**	I	**R**	**R**	T
HIV-1 MAL	I	**R**	E	**R**	I	**R**	**R**	T
HIV-2 ST	R	G	L	**R**	E	**R**	L	L
HIV-2 ROD	R	G	L	Q	E	**R**	L	L
HIV-2 D194	R	G	L	**R**	E	**R**	L	L
HIV-2 GHANA-1	R	G	L	**R**	E	**R**	L	L
SIV MM251	R	Q	**K**	L	L	**R**	A	**R**
SIV 1A11	R	Q	**R**	L	L	**R**	A	**R**
SIV K6W	R	Q	**K**	L	L	**R**	A	**R**
SIV GB1	R	**Q**	L	**R**	Q	L	V	
SIV PBJ	R	G	N	L	Y	**E**	**R**	L
SIV CPZ	R	**R**	I	**R**	E	A	P	

C

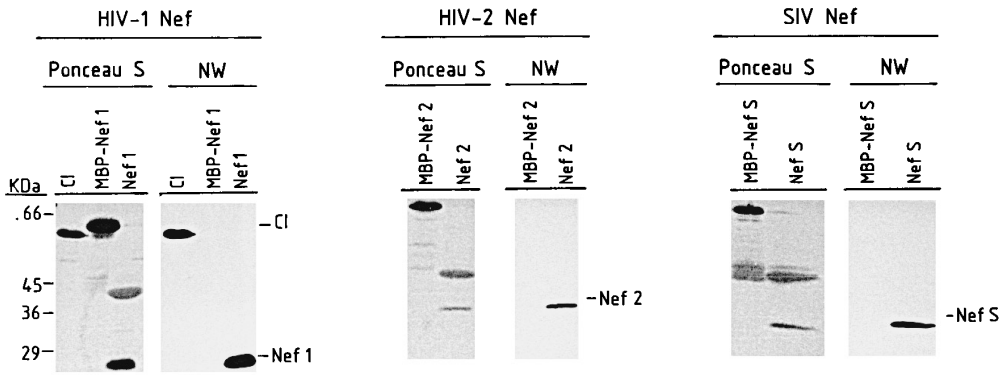


Figure 7. A, Schematic diagram of the three Nef protein molecules from HIV-1, HIV-2 and SIV. The positions of two arginine-rich regions and the MDD motifs are indicated. B, Comparison of the amino terminus Nef sequences from different strains. Basic amino acid residues are highlighted. C, RNA binding activity of the three HIV-1, HIV-2 and SIV Nef proteins. The three Nef protein species were cloned by standard cloning methods using PCR. Nef1, Nef from HIV 1; Nef2, Nef from HIV-2 ST; Nef S, Nef from SIV MM251. All three Nef proteins were obtained and purified as MBP fusion polypeptides (MBP-Nef1, MBP-Nef2 and MBP-Nef S) and cleaved with factor Xa (Nef1, Nef2 and Nef S). RNA binding was assayed by the non-radioactive Northwestern method as indicated in Materials and Methods, using the 1.1 kb BS riboprobe. Control CI protein, MBP-Nef fusion proteins as such or after cleavage with factor Xa were assayed. Each panel shows the Ponceau S staining and the autoradiography of membranes from the non-radioactive Northwestern assay (NW). The positions of M_r markers are indicated.

enhances the infectivity of *nef*-defective mutants only when the protein is present in virus producer, but not target cells (Miller *et al*., 1995; Aiken & Trono, 1995). Our findings that Nef is capable of binding RNA agree well with the idea that this Nef function may contribute to the correct formation of new infectious virions. For instance, the interaction of Nef with the genome may be involved in the assembly process of new virions. Another possibility is that Nef is encapsidated and present in virion particles, but has not been detected. If so, Nef could participate in the trafficking of the RNA genome to the nucleus.

Another well documented effect of Nef is the downregulation of CD4 in the infected cells (Cullen, 1994). This effect is mediated by accelerated CD4 endocytosis and degradation in lysosomes (Sanfridson *et al*., 1994; Aiken *et al*., 1994). However, the physiological significance of this effect remains obscure, since the diminished infectivity phenotype of *nef*-deficient variants is independent of CD4 downregulation (Saksela *et al*., 1995; Goldsmith *et al*., 1995; Miller *et al*., 1994; Aiken & Trono, 1995; Chowers *et al*., 1995). Some *nef*-deficient mutants with attenuated phenotype still retain their ability to downregulate CD4 (Saksela *et al*., 1995; Goldsmith *et al*., 1995).

The conclusion that Nef is an RNA binding protein stems not only from the biochemical evidence provided in this work, but also from sequence similarities with other proteins that interact with RNA (Lazinski *et al*., 1989; Burd & Dreyfuss, 1994). Thus, the antiterminator N proteins of λ and ϕ21 phages contain the following Arg-rich motifs: RRRERRAEKQA (residues 6 to 16) and RYKARRAELIA (residues 16 to 26) respectively, which are similar to the sequence present in HIV-1 (Bru) Nef RERMRRAEPAA (residues 17 to 27) (underlined are the matching amino acids). In addition, HIV Nef and picornavirus 2C proteins show sequence similarities, not only in the Arg-rich motif, but also in other parts of the molecules. Apart from the structural similarities between poliovirus protein 2C and HIV Nef, both proteins share similar functional properties. Thus, both 2C and Nef associate with membranes (Bienz *et al*., 1987, 1992; Cullen, 1994). The association of 2C (as its precursor 2BC) with membranes modifies membrane trafficking (Cho *et al*., 1994; Aldabe & Carrasco, 1995; Doedens & Kirkegaard, 1995; Barco & Carrasco, 1995), while Nef interferes with the transport and recycling of CD4 and gp120 (Schwartz *et al*., 1993; Cullen, 1994). This is consistent with the ability of Nef to interact with β-COP (Benichou *et al*., 1994), a protein that participates in vesicular traffic (Rothman, 1994). On the other hand, the interaction of both poliovirus 2C and HIV Nef proteins with RNA suggests that their activities are related to RNA functioning. Poliovirus 2C is required not only for RNA replication, but also for virion assembly (Li & Baltimore, 1990). Nef could be involved in the trafficking of HIV genomes through the cytoplasm to participate for instance in virus assembly. In conclusion, the finding that Nef belongs to the RNA-binding family of proteins and its potential similarities to picornavirus 2C open new avenues to further investigate the exact function of Nef.

Materials and Methods

Cloning and mutagenesis

The plasmid pTG1190 (Chenciner *et al*., 1989) containing the *nef* sequence from HIV-1 (Bru strain) was the PCR template used to amplify wild-type *nef* gene and *nef* deletion constructs (NefΔ1 and NefΔ2). Overlapping oligonucleotide primers were used to generate R1, R2, R3 and R4 point mutants by PCR. Nef protein sequence of HIV-2 (ST strain) and Nef protein sequence of SIV (MM251 strain) were obtained from pJSP4-27/H6 (Kong *et al*., 1988) and pBK28-SIV (Kornfeld *et al*., 1987) plasmids (provided by the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID.NIH). PCR amplifications of each corresponding open reading frame were performed by standard methods. The generated fragments were inserted into pMalC vector (New England Biolabs), using *Stu*I/*Hin*dIII sites in the polylinker, directly downstream of the protease factor Xa recognition site. The sequences of recombinant clones were confirmed by the dideoxy sequencing method using Sequenase (USB).

pKS Bluescript and pSK Bluescript plasmids were used to clone four regions of the HIV-1 BH10 genome, downstream T7 bacteriophage promoter. pBH10 (Hahn *et al*., 1984), containing λ BH10 clone (EMBL accession number M15654) was the source of the following constructions: pSK 0.8 kb SS, the 5' *Sac*I-*Spe*I fragment of λ BH10 was cloned in pSK to obtain the 0.8 kb SS riboprobe from 1 to 829 of λ BH10. pSK 4 kb SE, the 5' *Sac*I-*Eco*RI fragment of λ BH10 was cloned in pSK to obtain the 4 kb SE riboprobe from 1 to 4006 of λ BH10. pKS 2.7 kb EB, the *Eco*RI-*Bam*HI fragment of λ BH10 was cloned in the same sites of pKS to obtain the 2.7 kb EB riboprobe from 5101 to 7830 of λ BH10. pKS 1.1 kb BS, the 3' fragment *Bam*HI-*Sac*I of λ BH10 was cloned in pKS to obtain the 1.1 kb BS riboprobe from 7830 to 8932 of λ BH10.

Purification and cleavage of fusion proteins

Bacteria (*Escherichia coli* DH5 strain) transformed with MBP-Nef expression plasmid were grown in LB medium containing 0.2% (w/v) glucose and 100 μg/ml ampicillin to an $A_{600\,nm}$ of 0.5. Then 0.3 mM IPTG (isopropyl 1-thio-β-D-galactopyranoside) was added. After three hours at 37°C, cells were harvested by centrifugation and ground with alumina at 4°C in 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 10 mM EGTA and 1 NaCl. After centrifugation at 10,000 ***g*** for 30 minutes, the supernatant was diluted 1:10 in column buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA) containing 200 mM NaCl and loaded onto a 5 ml column of amylose resin equilibrated with the same buffer. The column was washed with 50 ml of this buffer, 30 ml of column buffer with 90 mM NaCl and 30 ml of column buffer. The protein was eluted with column buffer plus 10 mM maltose. The Nef protein was obtained by digestion of fusion protein MBP-Nef with 0.075% (w/w) factor Xa at 4°C for 24 hours.

In vitro synthesis of RNA

pSK 0.8 SS and pSK 4 kb SE plasmids were linearized at the 3' end with *Sac*I and blunt ended with T4 DNA

polymerase, while pKS 2.7 kb EB and pKS 1.1 kb BS were linearized at the 3' end with *Eco*RI and *Bam*HI, respectively. Positive sense riboprobes were obtained from digested plasmids by *in vitro* transcription. The reaction was carried out in a final volume of 50 µl containing 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 50 units of RNasin, 5 µg of linearized template, 0.5 mM ATP, GTP, CTP, 0.25 mM UTP, 0.25 mM biotin-21-UTP and 20 units of T7 RNA polymerase. The reaction was incubated at 37°C for 90 minutes. Biotinylated transcripts were purified by gel filtration through Sephadex G-50. To obtain the radioactive riboprobe, biotin-21-UTP was replaced by 50 µCi [α-^{32}P]UTP (10 mCi/ml). Radiolabelled transcripts were extracted with phenol/chloroform and were purified as the biotinylated ones. Both types of transcripts were ethanol precipitated and resuspended in distilled water. To determine the specific activity of biotinylated riboprobes, 50 ng of each riboprobe was twofold serial diluted in 20 × SSC and bound to nitrocellulose filters by aspiration with a dot blot apparatus (Bio-Rad) (SSC is 0.15 M NaCl, 0.015 M sodium acetate, pH 7.0). Filters were dried at 80°C for two hours and riboprobe signal was detected as in the band-shift assay and quantified by densitometric analysis.

Northwestern assay

Protein samples (2 to 5 µg of each protein) were electrophoresed in SDS-15% (w/v) PAGE and transferred to a nitrocellulose membrane in 50 mM Tris-HCl (pH 8.3), 380 mM glycine and 20% (v/v) methanol. The proteins were renatured for two hours in binding buffer (BB: 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% Denhardts, 0.1% Triton X-100 and 50 mM NaCl) and incubated with biotinylated riboprobe (20 to 50 ng RNA/ml BB) for one hour. When different RNA probes were screened, 3 mg/ml heparin was added and incubated for an additional 30 minutes to remove non-specific protein-RNA interactions. Unbound RNA was removed by washing with BB and blots were incubated with streptavidin-conjugated peroxidase. After washing, the nitrocellulose membrane was incubated in luminol-luciferin solution and exposed to X-ray films. The NW assay with radioactive riboprobe was carried out by incubating the membrane with radioactive probe in binding buffer (200,000 cpm/ng RNA per ml BB). The membrane was washed and exposed to X-ray films.

UV cross-linking assay

From 2 to 5 µg of each purified protein was incubated with 1 ng (200,000 cpm/ng RNA) of radioactive riboprobe in 20 mM Hepes (pH 7.5) and 10 mM KCl in a final volume of 50 µl for ten minutes at room temperature. For competition experiments samples were preincubated at room temperature for ten minutes with cold nucleic acid competitor before adding the riboprobe. The reaction mixture was irradiated using a 254 nm UV lamp placed 4 cm from the sample, for ten minutes at 4°C. Cross-linked samples were treated with 1 mg/ml RNase A at 37°C for 30 minutes. Samples were boiled for five minutes and subjected to electrophoresis in SDS-15%PAGE. [^{32}P]RNA-labelled proteins were visualized by autoradiography.

Band-shift assay

Reactions were carried out with 1, 2.5 and 5 µg of protein and 50 ng of biotinylated riboprobe under the binding conditions described for UV cross-linking and in the presence of 20 units of RNasin. After incubation at room temperature for ten minutes, glycerol was added to 10% (v/v) final concentration and the complexes were separated by electrophoresis at 30 mA on a 1% (w/v) agarose gel in 0.5 × TBE. The gel was placed in 20 × SSC for one hour, transferred to a nitrocellulose membrane and dried in a vacuum oven at 80°C for two hours. The nitrocellulose membrane was incubated in PBS/0.05% (v/v) Tween 20 (buffer P) plus 3% (w/v) BSA for 30 minutes, incubated with streptavidin-conjugated peroxidase diluted 1:10,000 in buffer P for 30 minutes, washed three times with buffer P and, after incubation in luminol-luciferin solution, the biotinylated riboprobe was visualized by autoradiography.

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