Eur. J. Biochem. 246, 38–44 (1997)
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The N-terminal Arg-rich region of human immunodeficiency virus types 1 and 2 and simian immunodeficiency virus Nef is involved in RNA binding

Asier ECHARRI, Maria Eugenia GONZÁLEZ and Luis CARRASCO

Centro de Biología Molecular CSIC-UAM, Universidad Autónoma de Madrid, Madrid, Spain

(Received 2 December 1996/5 March 1997) – EJB 96 1777/2

Comparison of the amino acid sequences of human immunodeficiency virus (HIV) Nef protein and several RNA-binding proteins shows similarities in some regions of these proteins. Thus, poliovirus protein 2C, an RNA-binding protein, shares with Nef the sequence YXQQ...MDD...DXXD. In addition, both proteins contain an Arg-rich motif that, in the case of poliovirus 2C, is involved in RNA-binding activity. Moreover, the RNA-binding, anti-terminator N proteins of λ, Φ21 and P22 phages show sequence similarities with HIV Nef at the Arg-rich motif. To assess the significance of this motif, native and deletion variants of Nef protein were assayed for RNA-binding activity. The N-terminal 35 amino acids of HIV-1 Nef that comprise the Arg-rich motif are sufficient for RNA binding. Point mutations engineered at the Arg-rich motif of HIV-1 Nef revealed that basic amino acid residues are essential for RNA-binding activity. The Nef proteins from HIV-2 and SIV can also interact with RNA, while the same proteins with the N-terminal Arg-rich domain truncated fail to interact with RNA. These findings indicate that all three Nef proteins from HIV-1, HIV-2 and simian immunodeficiency virus belong to the RNA-binding family of proteins. The three proteins contain an Arg-rich region at the N-terminus which is necessary to interact with RNA.

Keywords: human immunodeficiency virus 1; human immunodeficiency virus 2; simian immunodeficiency virus; Nef; RNA-binding protein.

In addition to the three essential genes typical of all non-defective retroviruses *gag*, *pol* and *env*, the human immunodeficiency viruses (HIV) as well as the simian immunodeficiency viruses (SIV) encode several accessory proteins that are non-essential for virus replication in tissue culture (Gibbs et al., 1994; Cullen, 1992). Despite intensive investigation (Subbramanian and Cohen, 1994; Trono, 1995), the exact function of each accessory protein during the virus-replication cycle remains poorly understood.

The *nef* gene is located at the 3′ end of the HIV genome and encodes a 27-kDa myristoylated protein that is produced as an early protein during virus infection (Ranki et al., 1994). Originally, the Nef protein was described as a negative factor (Terwilliger et al., 1986; Ahmad and Venkatesan, 1988), but the finding that SIV 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 can be extrapolated to HIV since Nef also enhances virus multiplication in SCID mice transplanted with human tissues (Jamieson et al., 1994). More recent data also showed a positive effect of Nef on viral infectivity (Chowers et al., 1994; Miller et al., 1995). Thus, HIV-1 particles from *nef*-negative strains are less infectious than their non-defective counterparts (Miller et al., 1995). Reverse transcription of *nef*-defective HIV-1 is less efficient than the non-defective virus particles, suggesting that virions assembled in the absence of Nef manifest defects at an early step of infection (Schwartz et al., 1995; Aiken and Trono, 1995; Chowers et al., 1994; Miller et al., 1995).

The biochemical activities and effects of Nef on cellular functions have also been analyzed (Cullen, 1994). Initial studies suggested that purified Nef had GTPase activity (Guy et al., 1987). However, these findings were not reproduced by other groups (Backer et al., 1991; Harris et al., 1992). However, expression of Nef in $CD4^+$ cells induces internalization and degradation of the HIV receptor molecule, the CD4 glycoprotein (Garcia et al., 1993; Garcia and Miller, 1991). The exact biological significance of CD4 degradation remains obscure, but it has been suggested that the down-regulation of CD4 would inhibit the superinfection of Nef-expressing cells by HIV (Benson et al., 1993). Direct interaction of Nef with β-coat protein (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 trafficking by Nef (Cullen, 1994). However, the interaction between Nef and CD4 seems not to be required to increase virus replication since the relative infectivity of HIV virions is not dependent on cells that express CD4 (Miller et al., 1994).

Subcellular localization of Nef protein changes during the infectious cycle and depends on N-myristoylation (Ranki et al., 1994; Kaminchik et al., 1994). Nef was found in the nucleus of infected cells (Murti et al., 1993) and in a soluble form in the cytosol, associated with intracellular membranes and with the cytoskeleton matrix (Kaminchik et al., 1994). Recently, Nef protein has been detected in association with virus particles (Welker

Correspondence to L. Carrasco, Centro de Biología Molecular CSIC-UAM, Universidad Autónoma de Madrid, Canto Blanco, E-28049 Madrid, Spain

Fax: +34 1 397 4799.

Abbreviations. HIV-1, human immunodeficiency virus type 1; HIV-2, human immunodeficiency virus type 2; SIV, simian immunodeficiency virus; β-COP, β-coat protein; MBP, maltose-binding protein; FMDV, foot-and-mouth disease virus; HRV14, human rhinovirus 14; EMC, encephalomyocarditis virus.

et al., 1996; Pandori et al., 1996). Two isoforms of Nef have been detected, the full-length 27-kDa and the 18–20-kDa. The majority of the 18–20-kDa protein was generated within the virus particle by proteolytic modification of the 27-kDa protein carried out by the viral protease (Welker et al., 1996). Nevertheless, the functional significance of the virion-associated Nef is still unknown.

We have recently shown that Nef binds to RNA in cell-free systems (Echarri et al., 1996). We now report that HIV-1 Nef shows sequence similarities with several RNA-binding proteins of viral origin. To investigate the functional importance of the Arg-rich motif in the RNA-binding capacity of HIV-1 Nef we have carried out deletion and site directed mutagenesis studies. In addition, we provide evidence that HIV-2 and SIV Nef proteins also contain an N-terminal Arg-rich motif that is critical for RNA binding.

MATERIALS AND METHODS

Construction of plasmids expressing MBP–Nef proteins. The plasmid pTG1190 (Chenciner et al., 1989) containing the *nef* sequence from HIV-1 (Bru strain) was the PCR template used to amplify Nef-encoding sequences. The Nef protein sequence of HIV-2 (ST strain) and the 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 ORF were performed by standard methods. Overlapping oligonucleotide primers were used to achieve point mutations in the Nef amino acid sequence. The fragments generated were inserted into pMalC (Nef 1) or pMalC2 (Nef S and Nef 2) vectors (New England Biolabs), using *Stu*I/*Hin*dIII or *Xmn*I/*Hin*dIII sites in the polylinker, respectively, directly downstream of the protease-factor-Xa-recognition site. pMalCNefΔ4 was obtained by digestion of pMalCNef1 with *Xho*I and blunt-ended with DNA Pol I Klenow fragment. Thus, the NefΔ4 protein contains the 35 N-terminal amino acids of native Nef 1 and additional out-of-frame tail sequence. The sequences of recombinant clones were confirmed by the dideoxynucleotide sequencing method using Sequenase (USB).

Purification and cleavage of fusion proteins. Bacteria (*Escherichia coli* DH5 strain) transformed with MBP-Nef expression plasmid were grown in Luria-Bertani medium containing 0.2% (mass/vol.) glucose and 100 μg/ml ampicillin to 0.5 A_{600}. Then 0.3 mM isopropyl 1-thio-β-D-galactopyranoside was added. After 3 h 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 M NaCl. After centrifugation at 10000 *g* for 30 min, 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 column buffer with 90 mM NaCl and 30 ml column buffer. The protein was eluted with column buffer containing 10 mM maltose. The Nef protein was obtained by digestion of fusion protein MBP-Nef with 0.075% (by mass) factor Xa at 4°C for 24 h.

***In vitro* synthesis of RNA.** Plasmid pSK0.8SS containing nucleotides 1–829 of HIV-1 (BH10 strain; Ratner et al., 1985) was linearized at the 3′ end with *Sac*I and blunt-ended with T4 DNA polymerase to obtain a positive riboprobe of 829 nucleotides by *in vitro* transcription. The transcription 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 DL-dithiothreitol, 50 U RNasin, 5 μg of linearized pSK0.8SS template, 0.5 mM ATP, GTP, CTP, 0.25 mM UTP, 0.25 mM biotin-21-UTP and 20 U T7 RNA polymerase. The reaction was incubated at 37°C for 90 min and then incubated with 1 U of RQ1 DNase/μg template for 30 min. Biotinylated transcripts were purified by gel filtration through Sephadex G-50.

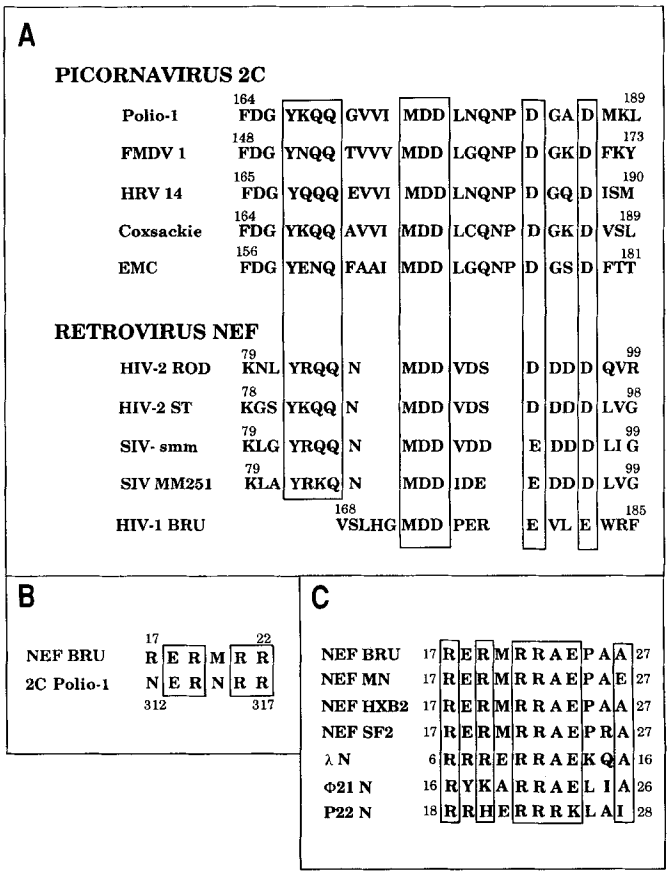

Fig. 1. Alignment of picornavirus 2C, retrovirus Nef and phage anti-terminator N protein sequences. The matching amino acid residues are boxed. The positions of amino acids in the corresponding protein are indicated by numbers. (A) Comparison of the sequences comprising the MDD motif between picornavirus 2C and Nef proteins. Foot and mouth disease virus (FMDV), Human rhinovirus (HRV14), Cocksackie virus (Cocksackie), encephalomyocarditis virus (EMC). (B) Comparison of the Arg-rich motif present in poliovirus 2C and in HIV-1 Nef. (C) Comparison of the Arg-rich sequences from phage anti-terminator N proteins and HIV-1 Nef from different strains.

Northwestern assay. Protein samples (1–5 μg of each protein) were electrophoresed in SDS/15% PAGE and transferred to a nitrocellulose membrane in 50 mM Tris/HCl, pH 8.3, 380 mM glycine and 20% methanol. The proteins were renatured for 2 h in binding buffer (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 (20–50 ng RNA/ml in binding buffer) at 22° C, for 1 h. Unbound RNA was removed by washing with binding buffer 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.

RESULTS AND DISCUSSION

Sequence similarities between Nef and other RNA-binding proteins. Recently, we found that purified poliovirus 2C and

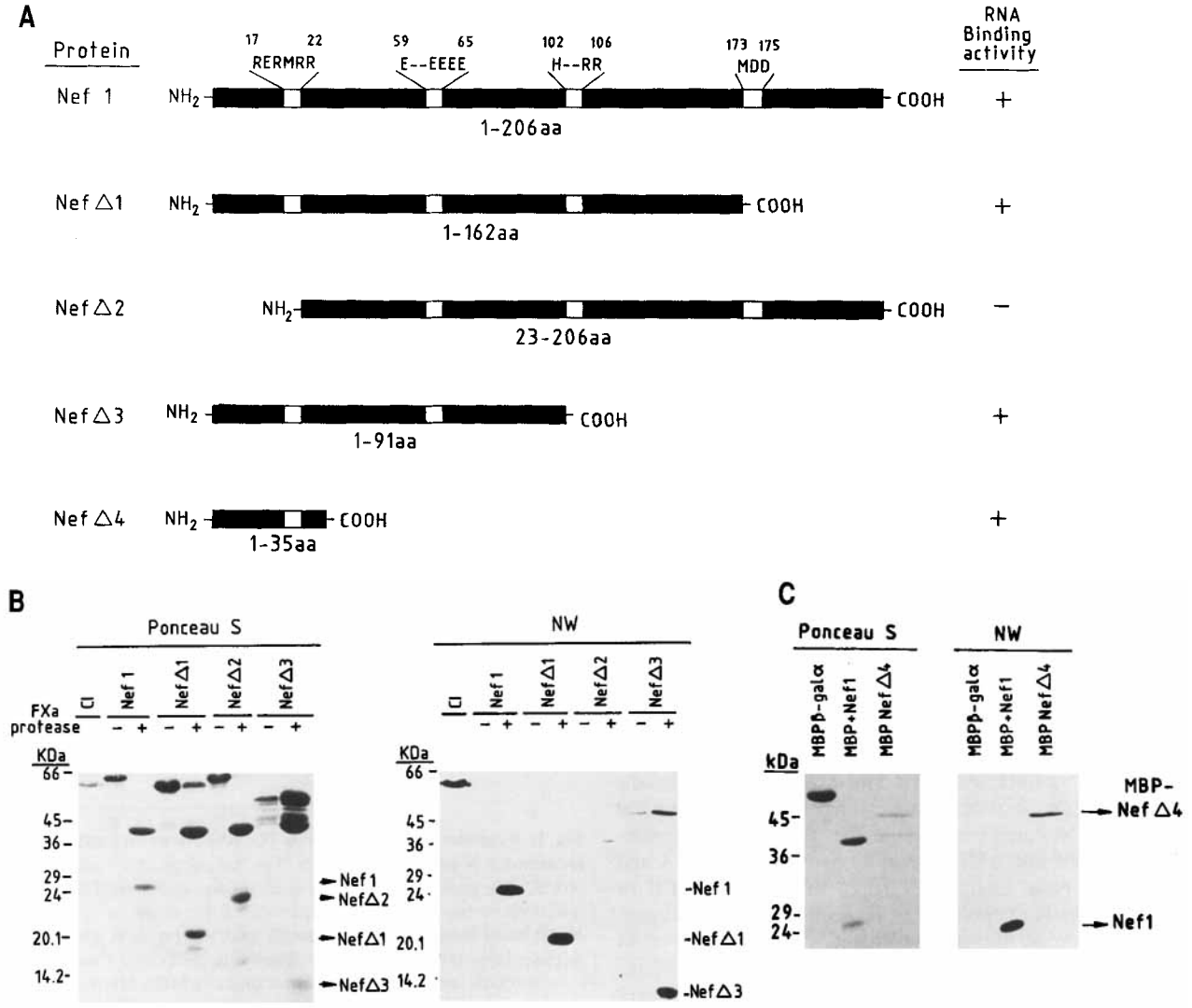

Fig. 2. Deletions of HIV-1 Nef protein. (A) Schematic representation of wild-type and truncated HIV-1_{BRU} Nef proteins. Basic and acidic regions and MDD motif are indicated in all proteins. Numbers indicate the amino acid positions. The phenotype generated by each protein is summarized to the right. (B) RNA-binding activity of Nef *Δ*1, Nef *Δ*2 and Nef *Δ*3 was tested by northwestern assay as indicated in Materials and Methods. Ponceau S staining and autoradiography (NW) of nitrocellulose membrane are shown. Potyvirus CI protein was used as a positive control. Fusion proteins between MBP and each Nef protein (−) and genuine Nef proteins separated from MBP by factor Xa protease treatment (+) were assayed. (C) RNA-binding activity of Nef *Δ*4. Ponceau S staining of electrotransferred proteins and autoradiography of northwestern assay (NW) of MBP-Nef *Δ*4 fusion protein are shown. MBP-*β*-gal *α* (47 kDa) and MBP (40 kDa) were used as negative controls.

HIV-1 Nef possessed RNA-binding activity (Rodriguez and Carrasco, 1995; Echarri et al., 1996). During our studies on poliovirus protein 2C and HIV-1 Nef (Rodríguez and Carrasco, 1993, 1995; Echarri et al., 1996), we noticed sequence similarities in two different regions of these proteins. Particularly striking is the presence of the sequence YXQQ...MDD...DXXD and an Arg-rich stretch in both proteins (Fig. 1A and B). The MDD motif is conserved both in picornavirus 2C and HIV Nef, but the exact function of this sequence has not yet been elucidated in either of these proteins. However, the Arg residues present at positions 314−317 of poliovirus 2C are crucial for RNA interaction (Rodriguez and Carrasco, 1995). Apart from the structural similarities between poliovirus protein 2C and HIV Nef, both proteins share some functional properties. Thus, poliovirus protein 2C is an NTPase enzyme (Rodríguez and Carrasco, 1993), that binds to RNA (Rodriguez and Carrasco, 1995) and interacts with membranes (Bienz et al., 1987, 1992), leading to modifications in membrane traffic (Cho et al., 1994; Aldabe and Carrasco, 1995). Similarly, Nef binds to RNA (Echarri et al., 1996) and interferes with the transport and recycling of the glycoprotein CD4 (Cullen, 1994). Nef is not only a membrane-associated protein (Kaminchik et al., 1994) but also interacts with *β*-COP (Benichou et al., 1994), a protein that participates in vesicular traffic (Rothman, 1994). Moreover, HIV-1 Nef has been implicated in signal transduction through its association with a serine-threonine kinase that recognizes histone H4 as a substrate (Nunn and Marsh, 1996).

Apart from poliovirus 2C, other typical members of the RNA-binding family of proteins bearing an Arg-rich motif are the anti-terminator N proteins of some bacteriophages (Lazinski et al., 1989; Burd and Dreyfuss, 1994). The sequences involved in RNA binding of *λ*, *Φ*21 and P22 N proteins are shown in Fig. 1C. These sequences resemble the sequence present in the Nef protein from several HIV-1 isolates (Fig. 1C). In addition to the matching of basic amino acid residues, the presence of the tetrapeptide RRAE followed by XXA, which is common to Nef and N proteins, is particularly striking. Since the Arg-rich motif is involved in RNA-binding capacity not only in the case

of protein 2C and the N proteins, but also in several other RNA-binding proteins (Burd and Dreyfuss, 1994; Biamonti and Riva, 1994; Mattaj, 1993; Tan and Frankel, 1995), we tested for this activity with the isolated native Nef proteins from HIV-1, HIV-2 and SIV and several Nef variants.

Deletions of HIV-1 Nef protein. To determine the amino acid residues of the Nef protein involved in RNA-binding activity, truncated-variants of Nef protein were generated. In designing deletion mutants, attention was focused on domains with high concentration of charged residues (Fig. 2A). The genome sequences corresponding to native *nef* gene and some fragments were amplified from the HIV-1_{BRU} cDNA by PCR. Amplified sequences were cloned in the pMalC vector downstream from the maltose-binding protein (MBP) sequence. MBP−Nef fusion proteins were purified by amylose affinity chromatography. Genuine Nef protein and its variants were generated upon cleavage of the fusion protein with the protease factor Xa that specifically cleaves at the junction between MBP and Nef. To test the RNA-binding activity a northwestern assay was performed. The specificity of the RNA-binding activity was previously shown by competition with heparin, excess of poly(A) and unlabeled probe (Echarri et al., 1996). Highly purified potyvirus protein CI was used as a positive control for a protein with RNA-binding capacity, while MBP served as a negative internal control (Lain et al., 1991; Rodriguez and Carrasco, 1995). Fig. 2B shows that genuine HIV-1 Nef binds RNA. Notably, Nef is devoid of RNA-binding capacity when present as the fusion protein MBP-Nef. This finding is not unexpected considering that the Arg-rich stretch is close to the N-terminus of Nef. 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 and Carrasco, 1995). Deletion of 44 and even 115 amino acids from the C-terminus did not impair the RNA-binding activity of Nef. However, the deletion of only 22 amino acids from the N-terminus was enough to eliminate the RNA-binding capacity of Nef. To test if the fragment present at the N-terminus of Nef was responsible for RNA-binding activity, the NefΔ4 variant was generated. Because of the small size of NefΔ4 peptide the fusion protein, but not the separated fragment was tested. Clearly, this 35-residue peptide confers RNA-binding capacity to MBP protein (Fig. 2C). These results provide evidence that Nef is endowed with RNA-binding activity and the 35 N-terminal amino acids are sufficient to confer RNA-binding activity *in vitro*. This sequence present at the N-terminal domain is likely located at the surface of the protein, as shown by proteolytic and NMR-studies (Freund et al., 1994).

Site specific mutagenesis of RNA-binding sequence. The importance of the N-terminal Arg-rich domain of Nef for RNA-binding capacity was assayed by site-specific mutagenesis of this motif. The highly conserved arginine residues, present in Nef from different HIV-1 isolates, were targeted for point mutations. Previously we demonstrated that mutations of arginine to lysine produced Nef variants with RNA-binding activity similar to that of wild-type Nef. However, a single residue substitution (R22G) dramatically decreased the RNA-binding capacity of Nef (Echarri et al., 1996) while substitution of R17 with G produces a Nef variant almost devoid of activity (Fig. 3). Furthermore, substitution of two basic residues by glycine completely abolishes the RNA-binding activity of Nef protein (Fig. 3). These results suggest that the overall charge of the Arg-rich motif may be essential for RNA binding, irrespective of residue position. Similarly, any specific arginine residue within the Arg-rich region of HIV-1 Tat protein was found to be essential for

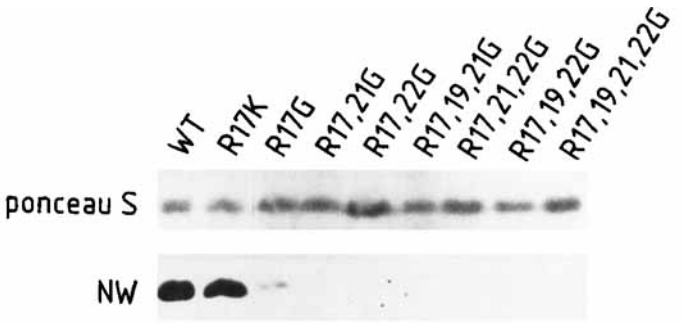

Fig. 3. Site-directed mutagenesis of the Arg-rich region of HIV-1 Nef protein. Ponceau S staining of the electrotransferred proteins (Ponceau S) and autoradiography of the nitrocellulose membrane after incubation with the riboprobe (NW) as described in Materials and Methods. Wild-type sequence and amino acid substitutions are indicated.

binding to TAR RNA (Calnan et al., 1991). Two general roles for Arginine residues in RNA binding have been proposed (Burd and Dreyfuss, 1994). First, arginine residues probe the local conformation of the RNA backbone, facilitating the search for high-affinity binding sites. Second, arginine residues make specific hydrogen-bonding networks with the RNA sugar phosphate backbone and bases (Burd and Dreyfuss, 1994). Arginine has more potential hydrogen-bonding atoms than lysine residues. Accordingly, the results shown indicate that every basic residue of the N-terminal Arg-rich motif is necessary for the RNA-binding activity of Nef. Otherwise tertiary-structure requirements may account for the loss of RNA-binding activity for Arg/Gly mutants. It has been suggested that the conformational changes undergone upon binding of HIV-1 Tat protein to TAR RNA may expose a kinase-binding site (Herrmann and Rice, 1993). Similarly binding of Nef protein through the Arg-rich domain may lead to the exposure of cellular protein-binding sites. In this context, the improvement of reverse transcription efficiency in infected cells but not in cell-free systems could be explained through activation of some cellular proteins by Nef bound to RNA (Schwartz et al., 1995).

Involvement of the N-terminal basic region of different Nef species in RNA binding. Recent studies with peptides that specifically bind to the Rev response element (RRE) demonstrated that the Arg-rich motif may be a versatile framework for recognizing RNA structures (Harada et al., 1996). Although the amino acid sequences of HIV-2 and SIV Nef diverge from that of HIV-1 Nef (Shugars et al., 1993), we observed that the three proteins share a basic N-terminal region. In addition, both HIV-2_{ST} Nef and SIV_{mm251} Nef possess an additional cluster of basic amino acids at positions 136−139 (Nef 2) and 137−140 (Nef S) (Fig. 4A). The coincidental basic charge at the N-terminal region of Nef from the three viral species could support the hypothesis that this region is involved in RNA-binding activity. To assess this possibility the 24 N-terminal amino acids from HIV-2 Nef (Nef 2) were deleted, giving rise to protein Nef 2ΔN (Fig. 4A). Similarly, the 24 N-terminal residues from SIV Nef (Nef S) were deleted to produce Nef SΔN protein (Fig. 4A). Treatment of fusion proteins with protease factor Xa produces minor cleavages inside the Nef S and Nef 2 proteins (probably at EGR and GR low-affinity cleavage sites, as suggested by the factor Xa manufacturer) apart from the major cleavage at the IEGR specific site that separates MBP from Nef protein (Fig. 4B). The native Nef S and Nef 2 proteins were able to bind RNA efficiently, but the N-terminus truncated variants of these proteins were unable to bind RNA, despite the existence of a second cluster of basic amino acids. These results support

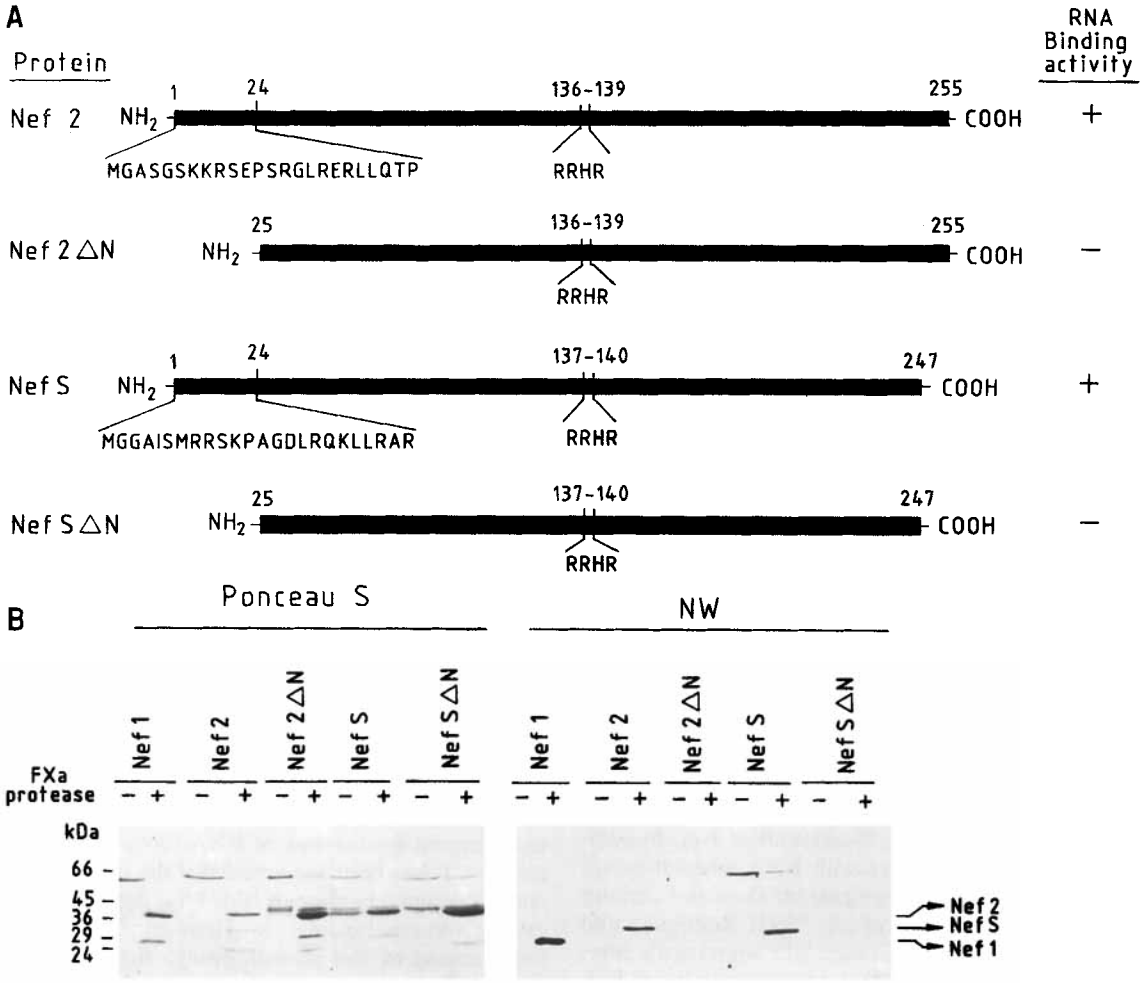

Fig. 4. Deletions of HIV-2 and SIV Nef proteins. (A) Schematic representation of the HIV-2_{ST} and SIV_{MM251} Nef proteins (Nef 2 and Nef S) and N-terminal deletion mutants of both proteins (Nef 2*Δ*N and Nef S*Δ*N). The N-terminal deleted region of 24 amino acids is indicated in Nef 2 and Nef S. The central basic region is shown in the four proteins. The numbers indicate the amino acid positions in the corresponding protein. RNA-binding capacity is indicated to the right. (B) RNA-binding activity of Nef 2, Nef S, Nef 2*Δ*N and Nef S*Δ*N. All proteins assayed were purified and electrotransferred to nitrocellulose (Ponceau S). RNA-binding activity was tested by northwestern assay (NW) as indicated in Materials and Methods. Nef 1 corresponds to HIV-1_{BRU} Nef. Fusion proteins between MBP and each Nef protein (−) and Nef proteins separated from MBP by factor Xa protease digestion (+) were analyzed.

the presence of an RNA-binding motif on all three Nef proteins located at the N-terminus. Further studies on the Arg-rich motif could take advantage of reconstituted HIV variants containing the mutations in Nef described in this work. These studies would provide the biological relevance of the Arg-rich region on virus replication.

The finding that Nef has RNA-binding capacity points to a novel function for this protein. Several possible activities of RNA-binding proteins have been suggested: RNA transport, formation of specialized protein complexes at particular sites of RNA by protein-protein interactions and modification of RNA structure (Burd and Dreyfuss, 1994). Thus, it has been suggested that poliovirus protein 2C facilitates the transport of the viral genomic RNA molecules through the cytoplasm, participating in genome replication and virion assembly (Rodriguez and Carrasco, 1995). A recent report has shown that Nef is incorporated into virus particles (Welker et al., 1996). Nef present in virions could be involved in the trafficking of HIV genomes through the cytoplasm to participate in the steps that lead to the integration of the viral genome. This might explain why virions made in the absence of Nef show defects in their infectivity (Miller et al., 1995). In the case of HIV the Nef protein would not be totally required for these steps of the virus life cycle, at least in some cell lines, but its presence would make the integration and the assembly process more efficient. In conclusion, the finding that Nef belongs to the RNA-binding family of proteins and its potential similarities to picornavirus 2C and phage N proteins opens new avenues to elucidate the exact function of Nef.

The expert technical assistance of Mr M. A. Sanz is acknowledged. We thank J. A. García and A. Fernández for kindly giving us CI protein. Bristol Myers Squibb (Spain), *Direccíon General de Investigación Científica y Técnica* project number PB94-0148 and the institutional grant to the CBM of *Fundación Ramón Areces* are acknowledged for their financial support. A. E. is a holder of a Gobierno Vasco fellowship.

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