RNA and DNA Binding Properties of HIV-1 Vif Protein

A FLUORESCENCE STUDY*

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The HIV-1 viral infectivity factor (Vif) is a small basic protein essential for viral fitness and pathogenicity. Some “non-permissive” cell lines cannot sustain replication of Vif(−) HIV-1 virions. In these cells, Vif counteracts the natural antiretroviral activity of the DNA-editing enzymes APOBEC3G/3F. Moreover, Vif is packaged into viral particles through a strong interaction with genomic RNA in viral nucleoprotein complexes. To gain insights into determinants of this binding process, we performed the first characterization of Vif/nucleic acid interactions using Vif intrinsic fluorescence. We determined the affinity of Vif for RNA fragments corresponding to various regions of the HIV-1 genome. Our results demonstrated preferential and moderately cooperative binding for RNAs corresponding to the 5’-untranslated region of HIV-1 (5’-untranslated region) and gag (cooperativity parameter $\gamma \approx 65–80$, and $K_d \approx 45–55 \text{ nM}$).

In addition, fluorescence spectroscopy allowed us to point out the TAR apical loop and a short region in gag as primary strong affinity binding sites ($K_d \approx 9.5–14 \text{ nM}$). Interestingly, beside its RNA binding properties, the Vif protein can also bind the corresponding DNA oligonucleotides and their complementary counterparts with an affinity similar to the one observed for the RNA sequences, while other DNA sequences displayed reduced affinity. Taken together, our results suggest that Vif binding to RNA and DNA offers several non-exclusive ways to counteract APOBEC3G/3F factors, in addition to the well documented Vif-induced degradation by the proteasome and to the Vif-mediated repression of transcription of these antiviral factors.

The RNA genome of all retroviruses contains gag, pol, and env genes. The genome of lentiviruses, including the human immunodeficiency virus type 1 (HIV-1), is more complex and encodes additional proteins. It is now well established that these proteins, previously considered as “accessory,” are essential for infection spreading and pathogenesis. Among them, the HIV-1 Vif is a 23-kDa basic protein required for HIV-1 replication in T-lymphocytes, macrophages, and several T-cell lines known as “non-permissive,” but dispensable in many other “permissive” cells (1–3). The non-permissive character is caused by the expression of two cellular factors, APOBEC3G and APOBEC3F, which belong to the cytidine deaminase family of nucleic acid-editing enzymes (3). In Δvif virions, APOBEC3G interacts with the nucleocapsid (NC) domain of HIV-1 Pr55$^{Gag}$ and this interaction is further stabilized by RNA recruitment (4, 5).

Once encapsidated, APOBEC3G/3F induce proviral DNA hypermutation by deaminating cytidines into uridines (6, 7). Moreover, independently from their catalytic activity APOBEC3G/3F factors also impair particle infectivity by affecting virion morphology and by destabilizing the reverse transcription complex (8–10). Vif counteracts the antiviral activity of these two HIV-1 inhibitors by promoting their degradation by the proteasome, indirectly preventing APOBEC3G/3F encapsidation (7, 11–13). In addition, Vif also negatively regulates APOBEC3G/3F translation (14). However, other studies proposed that depletion of APOBEC3G/3F from virus-producing cells is not necessary for production of infectious virions (15–17).

Despite controversial initial results, it is now usually accepted that less than 100 copies of Vif are packaged per virion (2, 18, 19). Vif and Pr55$^{Gag}$ colocalize in the cytoplasm (20) and mutations in the NC domain of Pr55$^{Gag}$ significantly reduce Vif packaging. In addition, Vif also associates with the HIV-1 genomic RNA in vitro and in vivo (21, 22). Vif binds the viral RNA in the cytoplasm of infected cells, forming a 40S mRNP complex that most likely mediates viral RNA interactions with HIV-1 Pr55$^{Gag}$ (23). Moreover, deletion of the Vif putative RNA-binding motif (approximately the first 114 residues) abolishes its packaging into virions (22). The RNA binding properties of Vif and its association with the nucleoprotein complex during virus assembly thus seems to be crucial for efficient virions assembly and subsequent optimization of viral particles morphology (24).

To gain insight into the molecular mechanisms by which Vif regulates HIV-1 replication and counteracts APOBEC3G/3F antiviral activity, we studied Vif binding to HIV-1 RNA and DNA fragments by fluorescence spectroscopy, using the natural fluorescence of the highly conserved Trp residues contained in the Vif N-terminal region (25) to detect the protein/nucleic acid interactions. Our results show that Vif preferentially and cooperatively binds to the 5’ region of HIV-1 genomic RNA, in agreement with our previous biochemical study (26). In addition, we characterized two different RNA binding modes,
whereas only one was detected by filter binding (26). Moreover, we characterized Vif binding to two high-affinity RNA binding sites and determined similar affinity for the corresponding DNA sequences. Taken together, our data suggest that both Vif/RNA and Vif/DNA interactions are important for HIV-1 replication in restrictive cells.

**EXPERIMENTAL PROCEDURES**

**Recombinant Vif Expression**—Plasmid D10Vif containing a His<sub>6</sub> tag fused at the N-terminal domain of Vif lacking the methionine initiation codon was transformed and expressed in *Escherichia coli* MC10611 cells, and protein purification was performed as previously described (27). Protein concentration was determined spectrophotometrically using an extinction coefficient of 57,535 M<sup>-1</sup> cm<sup>-1</sup> at 280 nm.

**HIV-1 RNA Fragment Synthesis and Purification**—Plasmids pHXB2-A, pHXB2-B, pHXB2-C, pHBX-E, pHBX-F, pHBX-G, and pHBX-H containing nucleotides 1–497, 499–996, 995–1495, 1998–2495, 2467–2995, 7251–7745, and 8625–9181 of HIV-1, respectively (Fig. 1), under the control of a promoter of the T7 RNA polymerase were obtained as described (26). They were linearized with SalI or PvuII and used as template for *in vitro* transcription with bacteriophage T7 RNA polymerase as described (28). After 2 h at 37 °C, the reaction mixture was incubated for 1 h at 37 °C with RNase-free DNase I (MP Biomedicals), extracted with phenol, and precipitated in ethanol. RNA was purified by FPLC (Amersham Biosciences) as described (29) and dissolved in water.

**RNA and DNA Oligonucleotides**—RNA oligonucleotides corresponding to the upper part of the TAR hairpin (TAR1 and TAR2, Fig. 2A) and to nucleotides 539–564 (B1) or 539–546 (B2) (Fig. 2B) of the HIV-1 genomic RNA (HXB2 isolate) were synthesized and purified by reverse-phase HPLC and polyacrylamide gel electrophoresis by Dharmacon (Boulder, CO). Their DNA counterpart (dTAR1, dTAR2, dB1, dB2), their complementary sequences (dTAR2c, dB2c) (Fig. 2A and B) and various DNA oligonucleotides (Table 1) were synthesized by Thermo Electron GmbH and purified by reverse-phase HPLC.

**Steady State Fluorescence Measurements**—All fluorescence experiments were recorded in quartz cells at 20 ± 0.5 °C on a Fluoromax-2 fluorometer (HORIBA Jobin-Yvon). The excitation wavelength was 295 nm, the emission wavelength was scanned from 310 to 450 nm, the integration time was 0.1 s, and the excitation and emission bandwidths were 2 and 8 nm, respectively. Fluorescence titrations were performed by adding increasing amounts of nucleic acid to 150 nM or 100 nM Vif in 30 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 2 mM or 10 mM MgCl<sub>2</sub>.

**TABLE 1**

Primary structure of short DNA single- and double-stranded DNA sequences used in this study

| Consensus sequences for cytosine deamination by APOBEC3F and APOBEC3G were found to be 5’-TTCWNT-3’ and 5’-YCCCH-3’, respectively (W = A or T, Y = C or T; H = A, C, or T) (40). |
| DNA sequences | dPPT | APOBEC3F site | APOBEC3G site | Dickerson dodecamer |
|----------------|-------|---------------|---------------|-------------------|
| 5’-AAA AAG AAA GGG GGG-3’ | 5’-TTC ACC TTT GTA A-3’ | 5’-TCC CAC CCA-3’ | 5’-GCC GCA TCC AAG GCC-3’ |

**FIGURE 1.** Viral RNA fragments used in this study and their location on the HIV-1 genome. U3 and US, unique sequences in 3’/5’-UTR; pa, polyadenylation signal; TAR, transactivation responsive element; PBS, primer binding site; DIS, dimerization initiation site; PSI, packaging signal; PPT, polypurine tract; RRE, Rev responsive element.

**FIGURE 2.** A, secondary structure of the RNA sequences TAR1 and TAR2, the DNA analog of TAR2, dTAR2, and its complementary counterpart, dTAR2c. B, secondary structure of fragment B1 encompassing nucleotides 539–564, and sequence of its single-strand 5’-part B2, its DNA analog dB2, and its complementary counterpart dB2c.

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Vif-Nucleic Acid Interactions

![Vif-Nucleic Acid Interactions](https://example.com/vif-nucleic-acid-interactions.png)

![FIGURE 1](https://example.com/figure1.png)

![FIGURE 2](https://example.com/figure2.png)
with \( I_0 \) corresponding to the protein fluorescence intensity in absence of nucleic acid. The number of consecutive nucleotides occluded upon binding of one ligand, \( n \), the observed affinity, \( K_{\text{obs}} \), and the cooperativity parameter, \( \omega \), were recovered using the model of McGhee and Von Hippel for cooperative binding (30), in Equation 2,

\[
\frac{v}{L_f} = \frac{K_{\text{obs}} \cdot (1 - n \nu)}{N} 
\]

where \( R = [(1 - (n + 1)\nu)^2 + 4\omega\nu(1 - n \nu)]^{1/2} \). Because the McGhee and Von Hippel model was derived with the simplifying assumption of an infinite lattice of binding sites, we introduced a correction factor taking into account the finite lattice size, \( N \) (31). The correction merely amounts to multiplying Equation 2 by the factor in Equation 3.

\[
Q_{\text{obs}} = \frac{I_0 - I}{I_0} \quad \text{(Eq. 1)}
\]

\[
Q_{\text{max}} = \frac{n}{(1 - n \nu)^n} \quad \text{(Eq. 5)}
\]

\[
Q_{\text{max}} = \frac{1 - (n + 1)\nu + R}{2(1 - n \nu)^2} \quad \text{(Eq. 6)}
\]

\[
K_{\text{obs}} = \frac{Q_{\text{obs}}}{Q_{\text{max}}} \quad \text{(Eq. 7)}
\]

For \( \omega = 1 \), Equation 2 simplifies to Equation 4, which corresponds to the Scatchard formulation for non-interacting ligands (32),

\[
\frac{v}{L_f} = K_{\text{obs}} \cdot (1 - n \nu) \cdot \left( \frac{1 - n \nu}{1 - (n - 1)\nu} \right)^{n-1} \quad \text{(Eq. 4)}
\]

where \( v \) corresponds to the binding density (mol of bound protein/mol of nucleotide), and \( L_f \) to the concentration of free protein. These quantities are linked by Equations 5–7 as described in Ref. 33,

\[
L_f = L_d - L_b \quad \text{(Eq. 6)}
\]

where \( D_i \) is the total nucleotide concentration, \( L_d \) and \( L_b \) are the total and the bound protein concentrations, respectively, and \( Q_{\text{max}} \) corresponds to the maximal fluorescence quenching when all protein molecules are bound to nucleic acids. Fitting of experimental data with those equations was performed with an algorithm written with Mathematica (Wolfram Research).

RESULTS

Fluorescence Properties of HIV-1 Vif Protein—HIV-1 Vif contains 8 Trp residues that confer a high intrinsic fluorescence signal to the protein. Interestingly, 7 of 8 Trp residues are located in the Vif N-terminal region, which corresponds to the RNA binding domain (22, 23). Because of their high quantum yield and their high sensitivity to changes of the physicochemical environment, Trp residues constitute very sensitive probes for investigating protein/nucleic acid interactions (34, 35). We started our study by analyzing the spectral fluorescent properties of the Vif Trp residues by stead-state fluorescence spectroscopy. The free Trp amino acid in solution displays a fluorescence emission maximum at 350 nm (36). After selective excitation of Trp residues in Vif at 295 nm, the emission spectrum revealed a maximum at 338 nm (Fig. 3). Because Vif emission spectrum constitutes an average of the different Trp residues, this large blue shift of the emission maximum revealed that Trp residues in Vif are mainly located in a nonpolar environment (36). Upon nucleic acid binding, the wavelength of maximal fluorescence emission of the protein was unchanged (Fig. 3), whereas the fluorescence intensity decreased depending on the nucleic acid sequences and concentration, as described below. Interestingly, high-affinity Vif binding to RNA and DNA was characterized by a global quenching of the fluorescence of 75–85% (Tables 2, 3, and 4, see below), likely corresponding to a quenching of 86–97% of the Trp residues located in the nucleic acid binding site, and implying that these residues behave similarly upon RNA or DNA binding.

Determination of the Binding Parameters of Vif/RNA Interactions—To identify preferential Vif binding regions on the HIV-1 genome, we synthesized a set of genomic RNA fragments of about 500 nucleotides in length by in vitro transcription (Fig. 1). RNA fragments A–F cover the 5’-third of the genome corresponding to the 5’-UTR and gag and pol coding
Vif-Nucleic Acid Interactions

TABLE 3
Characterization of high-affinity Vif binding sites on HIV-1 RNA
Experimental data were fitted with the model derived for non-interacting small ligands to finite lattice (Eq. 4) as described under “Experimental Procedures.”

| RNAs          | MgCl₂ | Kd  | Qmax |
|---------------|-------|-----|------|
| TAR           | 2     | 9.8 ± 1.8 | 0.75 |
| TAR 1         | 2     | 11.2 ± 2.3 | 0.72 |
| TAR 2         | 2     | 9.3 ± 1.6  | 0.76 |
| B1            | 2     | 9.5 ± 2.2  | 0.77 |
| B2            | 2     | 12.3 ± 2.5 | 0.81 |
| B2c           | 2     | 13.9 ± 1.2 | 0.78 |
| dTAR2         | 10    | 11.8 ± 2.0 | 0.85 |
| dT10          | 10    | 12.6 ± 1.9 | 0.82 |

TABLE 4
Characterization of Vif binding to DNA oligonucleotides
Spectroscopic data were analyzed with the model derived for non-interacting small ligands to finite lattice (Eq. 4).

| DNAs          | Kd    | Qmax |
|---------------|-------|------|
| dTAR2         | 9.5 ± 2.3 | 0.75 |
| dTAR2c        | 11.8 ± 2.6 | 0.76 |
| dB2           | 11.3 ± 1.9 | 0.74 |
| dB2c          | 9.7 ± 1.6  | 0.78 |
| dPPT          | 31.7 ± 2.7 | 0.44 |
| APOBEC3F site | 37.8 ± 2.1 | 0.50 |
| APOBEC3G site | 34.1 ± 3.8 | 0.55 |
| Dickerson dodecamer | 39.7 ± 3.5 | 0.39 |
| dG10          | 34.5 ± 2.7 | 0.58 |
| dC10          | 47.8 ± 1.7  | 0.40 |
| dT10          | 29.3 ± 2.5  | 0.53 |
| dA10          | 43.1 ± 2.9  | 0.43 |
| dA10, dT10    | 35.5 ± 2.1  | 0.40 |
| d(GT)₃       | 35.4 ± 3.3  | 0.50 |

![Graph](image)

FIGURE 4. The normalized fluorescence intensity (squares) of 150 nM of Vif was recorded as a function of the concentration of RNA C. The fluorescence intensity, I, was then converted in the fluorescence quenching parameter, Qobs (circles) according to Equation 1. The solid line corresponds to the best fit of Equation 2; values of Kd, Qmax, and ω are given in Table 2.

regions. RNA fragment G contains the Rev-responsive element, which corresponds to the binding site of the auxiliary Rev protein, whereas RNA fragment H includes the polypurine tract (PPT), which constitutes the primer for (+) strand DNA synthesis during reverse transcriptase. To characterize Vif binding to these genomic RNA fragments, reverse titrations were performed by adding small volumes of concentrated RNA solutions to a fixed amount of protein. Protein binding was monitored through the decrease of Vif intrinsic fluorescence resulting from protein/nucleic acid complex formation (Fig. 4).

Analysis of the fluorescence titration curves according to cooperative and non-cooperative binding models (Equations 2 and 4, under “Experimental Procedures”) highlighted moderate cooperativity of Vif binding to the different genomic RNA fragments, because the cooperative model (30) gave the best fit of our experimental data. Nevertheless, the McGhee and von Hippel model is based on the assumption of identical binding sites, which most likely represents a simplification of the actual situation because our previous biochemical studies revealed the existence of preferential binding sites (26). However, a more complex model including specific and nonspecific binding sites would contain too many independent parameters to obtain a reliable fit and, most likely, would even be intractable. Information about high-affinity Vif binding sites was obtained by making use of short oligonucleotides corresponding to these sites (see below).

The binding parameters of Vif deduced from our analysis clearly showed that Vif did not bind equally the different RNA fragments (Table 2). Fluorescence titrations using RNA fragments A and B revealed a decrease of the Vif fluorescence of about 80%. Similarly, the fluorescence quenching was about 70% for RNA fragments C, E, and F, while it was limited to 50% in the case of fragments G and H. Analysis of the equilibrium dissociation constants, Kd, showed that Vif had the strongest affinity for RNA fragments A, B, and C (Kd = 45–55 nM), while it had a 3-fold reduced affinity for RNAs G and H (Kd = 150 to 165 nM). Intermediate equilibrium dissociation constants were obtained with RNA fragments E and F (Kd = 70–80 nM). In addition, binding of Vif to RNAs A, B, and C was rather cooperative (ω = 60 to 80), while RNAs G to H bound Vif with limited cooperativity (ω = 17 to 30). As a negative control, fluorescence titrations were also performed on *Escherichia coli* total tRNA, which was found to bind Vif poorly in our previous biochemical study (26). This result was confirmed by spectroscopic analysis, which showed a quenching of Vif fluorescence of only 25% (data not shown).

The binding stoichiometry of Vif to the RNA fragments was estimated from the intersection of the initial slope of the titration curves with the fluorescence plateau. The number of binding sites was found to be 48 (±3) for RNAs A to F, and 20 (±2) for RNAs G and H, corresponding to 1 Vif/(10 ± 2) nucleotides and 1 Vif/(25 ± 3) nucleotides, respectively (Fig. 5, A and B).

Finally, to further characterize the Vif/nucleic acid complexes, we tested the influence of the MgCl₂ concentration on the binding of Vif to the different RNAs. Decreasing the MgCl₂ concentration from 10 to 2 mM had very little effect on the Kd values of RNAs A and B (Table 2), suggesting that these RNA/protein interactions are mainly non-electrostatic. On the contrary, the dependency of the equilibrium dissociation constant on the MgCl₂ concentration in the case of RNA H (Table 2) pointed out the contribution of electrostatic interactions.

Taken together our results demonstrate that Vif binds preferentially and cooperatively to RNA fragments A to C, which encompass the first 1500 nucleotides of the viral genome. This region overlays with the 5′-UTR of the genomic RNA, which contains numerous secondary structures essential for viral replication, and most of the *gag* gene (Fig. 1) (28, 37, 38).
Characterization of High-affinity Vif Binding Sites on HIV-1 RNA—To precisely map Vif binding sites, we previously performed ladder selection and enzymatic footprinting experiments on RNAs A and B (26). This analysis revealed the existence of a Vif binding site in the transactivation responsive region, TAR, which is a stable stem-loop structure located at 5′-end of the viral genome (38). In RNA B, protection of G541 and G545 against cleavage by RNase T1 by Vif revealed the presence of a Vif binding site in this single-stranded region. Both sites were protected at low Vif concentration, and thus most likely correspond to high-affinity binding sites (26). Here, we performed a fluorescence study to characterize Vif binding to these sites. To determine the minimal binding sites of Vif, we performed fluorescence titrations on the complete 65-nucleotide long TAR hairpin, and on the shorter TAR1 and TAR2 sequences corresponding to the apical region of TAR (Fig. 2A).

Similarly, in RNA B, we selected fragment B1 encompassing nucleotides 539–564, as well as its single-stranded 5′ part, B2 (nucleotides 539–547) (Fig. 2B).

Because our analysis of Vif binding to RNA fragments A to F revealed that the occluded binding size is about 10 ± 2 nucleotides, we expected that only one Vif molecule would bind to the shortest RNA oligonucleotides. Accordingly, experimental data on RNA oligonucleotides were best fitted with the model derived for non-interacting ligands to a finite lattice (Equation 4) (32). The best fits were obtained by decreasing the effective Vif concentration by 30–35% in comparison with the concentration of protein introduced into the quartz cell before adding nucleic acid. Because this effect was not observed with 500-nucleotide long RNA fragments, we hypothesized that binding of Vif to short oligonucleotides might generate fully neutralized poorly soluble complexes, thus decreasing the effective concentration of soluble Vif.

Data analysis revealed very similar binding parameters for TAR, TAR1, and TAR2 (Table 3). In particular, the equilibrium dissociation constants were all between 9.5 and 11 nM (Fig. 6). Because the differences between these values are within experimental errors (Table 3), we deduced that Vif binds to the apical stem-loop of TAR, which can be considered as a Vif minimal binding site. This result validates our initial hypothesis on the stoichiometry and the choice of the binding model. Moreover, decreasing MgCl2 concentration from 10 to 2 mM did not induce significant variation in these values, indicating the specificity of interactions existing between Vif and these RNA oligonucleotides. A very similar analysis could be applied to B1 and B2 oligoribonucleotides, because Vif bound to both sequences with a $K_d$ of 12.5–14 nM (Table 3). In this case, our data showed that the single-stranded B2 fragment (nucleotides 539–547) corresponds to another minimal Vif binding site.

Vif Binding to Short DNA Oligonucleotides—While the RNA binding properties of Vif have been previously documented (23, 26), the possibility that this protein may also bind DNA has not been tested. Such a property might explain how Vif is able to counteract APOBEC3G independently of its degradation and translation inhibition (15–17). Therefore, we performed fluorescence titrations on the DNA counterparts of the minimal RNA binding sites identified above (dB2 and dTAR2, Fig. 2, A and B) and on their complementary sequences (dB2c and dTAR2c, Fig. 2, A and B). Fluorescence titrations and analysis of
Vif-Nucleic Acid Interactions

the experimental data were performed as described above for the RNA oligonucleotides.

Binding of Vif to dX10, dB2, dTAR2c, and dB2c resulted in a fluorescence quenching of 75–78%. Moreover Vif equilibrium dissociation constants for these DNA oligonucleotides were very similar (K_d = 9.5–12 nm, see Table 4) and very close to those determined for the high-affinity RNA binding sites (compare Tables 3 and 4). These results could lead to the conclusion that Vif bind DNA oligonucleotides regardless of their sequence. To check this point, we extended our investigation to several DNA oligonucleotides such as the DNA equivalent of the polyuridine tract (dpPT), and tandem copies of APOBEC3G or APOBEC3F preferential editing sites (Table 1) (39, 40). Dissociation constants for these derivatives were found to be 3–4 fold higher than the values obtained for dTAR2, dB2, dTAR2c, and dB2c (Table 4). In addition, the observed fluorescence quenching was only 44–55%. Similar results were obtained with DNA oligonucleotides containing a single copy of the APOBEC3G or APOBEC3F preferential editing sites flanked by GT repeats (data not shown). In a further step, to establish whether Vif has a preference for single-stranded versus double-stranded DNA, we performed fluorescence titrations on a 12-nucleotide double-stranded DNA obtained from the self-complementary sequence dCGCGAATTCGCG commonly known as the Dickerson dodecamer (41). In this case, fluorescence quenching was about 40% and the observed affinity was 3–4 fold lower than the one observed for the Vif high-affinity binding sites (Table 4).

Furthermore, fluorescence titrations performed on dX_{10} sequences (with X = A, G, C, or T) led to binding parameters values (K_d = 29–48 nm and Q_{max} = 40–58%, Table 4) revealing a modest binding of Vif protein to dG_{10} and dT_{10} and especially to dC_{10} and dA_{10} sequences. Similar dissociation constants (K_d = 35 nm) and fluorescence quenching (Q_{max} = 50%) values were obtained for d(GT)_{5} (Table 4). Finally, annealing of dA_{10} and dT_{10} oligonucleotides yielded intermediate Vif binding parameters compared with the two corresponding single-stranded oligonucleotides (Table 4). Thus, Vif bound single and double-stranded DNA sequences with similar efficiency. However, all together our results indicated that Vif did not bind all DNA sequences with the same affinity, even though the preference for the binding sites with the higher affinity was rather modest.

DISCUSSION

HIV-1 Vif protein is required for efficient assembly, maturation, core stabilization and early steps of reverse transcription of virions produced by “non-permissive” cells (24, 42–44) expressing APOBEC3G/3F, two cytidine deaminase nucleic acid editing enzymes (3, 45). Noticeably, the antiviral activity of these proteins correlates with the inhibition of reverse transcription, rather than with their catalytic activity (9, 10). Vif is a component of cytoplasmic RNP mediating viral RNA interactions with Pr55Gag precursor and cellular factors (21–23), and Vif binds HIV-1 genomic RNA in vivo (21, 22) and in vitro (21, 26). In addition, any mutation reducing the affinity of Vif for RNA diminishes viral replication in non-permissive cell lines, suggesting that RNA plays a central role in Vif function (22, 23).

The first part of our study shows that Vif preferentially and cooperatively binds to the 5’-part of the HIV-1 genomic RNA, in keeping with our previous biochemical study (26). For RNAs A, B, and C, corresponding to the 5’-UTR and most of the gag coding region, the range of K_d values determined by fluorescence (45–55 nm) is similar to the range of values previously determined by filter binding assays (45–66 nm) (26). Both the present and our previous study (26) indicate that Vif binds the 5’ part of HIV-1 genomic RNA with moderate cooperativity (K_d ~ 65–80, Table 2), whereas binding to the RNA fragments corresponding to the 3’-part of the HIV-1 genome was weakly cooperative. With RNAs A and B, decreasing the Mg^{2+} concentration from 10-2 mM induced very small changes in the equilibrium dissociation constant, highlighting the specificity of these RNA-protein interactions (Table 2). The effect was more pronounced for RNA H, revealing an electrostatic contribution to this less cooperative binding mode.

Using fluorescence, we determined K_d values for RNAs G and H of 152 and 167 nm, respectively at 10 mM MgCl_2 (Table 2), whereas we could hardly detect any binding of Vif to these RNAs by filter binding assays (26). Thus, fluorescence allowed us to detect a second class of Vif/RNA complexes characterized by limited quenching (Q_{obs} ~ 0.5, Table 2), which is not retained on nitrocellulose filter. Both classes of Vif binding sites may exist on RNAs A, B, and C. This would explain why we observed binding of 48 ± 3 molecules of Vif per RNA by fluorescence spectroscopy, while we detected binding of only 9 and 21 Vif molecules per RNA A and B, respectively, by filter binding assays (26).

Vif binding to the 5’-part of the genomic RNA, especially to the first 500 nucleotides, most likely has important functional implications. First, this region contains all RNA signals required for efficient packaging of HIV-1 genomic RNA. The presence of large amounts of Vif in the RNA assembly complexes (at a Vif/Pr55Gag ratio of 0.5 to 0.9) (18, 20) may be crucial for proper assembly of the viral core in restrictive cells.

Indeed, the affinity of Vif for RNA A is greater than that of Pr55Gag and nucleocapsid protein (NCP) for nucleic acids (including the high-affinity NCP binding sites SL1, SL2, and SL3) at the same ionic strength (46, 47–49). Thus, the competition between Vif and Pr55Gag for specific RNA functional sites may provide fine tuning of RNA packaging and core assembly (23). In this context, our recent findings showing that Vif inhibits the NCP-induced RNA dimerization, which is a prerequisite for RNA packaging, is also significant (4).

The high-affinity of Vif for the 5’-region of HIV-1 genomic RNA is likely to be important for packaging of Vif into viral particles since deletion of the RNA packaging signal resulted in a reduced incorporation of Vif into virions (22). Incorporation of Vif into virions may contribute to counteract the antiviral activity of APOBEC3G/3F, because Vif/APOBEC3G interaction directly inhibits the enzymatic activity of the latter protein, without protein degradation (17). Finally, Vif binding to the 5’-UTR of HIV-1 genomic RNA may also participate to exclusion of APOBEC3G/3F from viri-

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4 Henriët, S., Simk, L., Bec, G., Gorelick, R. J., Marquet, R., and Paillart, J.-C. (2007) Nucleic Acids Res., in press.
ons, because packaging of these antiviral factors likely involves the SL1 motif (50) (Fig. 1).

In the second part of our work, we characterized Vif binding to two binding sites located in RNAs A and B, which have been previously identified by enzymatic footprinting (26). Comparison of Vif binding to TAR, TAR1, and TAR2 (Fig. 2A and Table 3) indicated that only one Vif molecule binds to TAR, in the upper part of the hairpin, in agreement with footprinting studies, which revealed protection of the TAR apical loop by Vif (26). The equilibrium dissociation constant of Vif for TAR was 5–6-fold lower than the $K_d$ for RNA A, confirming that analysis of our data with the model of McGhee and von Hippel yielded a mean $K_d$ value. This also suggests that some of the Vif binding sites present in RNA A have a much lower affinity for this protein. Comparison of Vif binding to oligoribonucleotides B1 and B2 (Fig. 2B and Table 3) showed that Vif binds to the 5′-CAG-ACAGG-3′ single-stranded sequence, in agreement with footprinting data (26). The $K_d$ values of Vif for B2 and TAR2 are very close, and they indicate that the region identified by enzymatic footprinting indeed constitute high-affinity Vif binding sites. Interestingly, the affinity of Vif for TAR binding site is about two orders of magnitude higher than that of NCp (51). Thus, despite the limited amount of Vif in viral particles, this protein may still bind to these high-affinity binding sites, since the Vif/NCp ratio in virions is 1/40 to 1/200 (2, 18).

Surprisingly, the DNA binding properties of Vif have not been studied previously, even though they may contribute to neutralization of the APOBEC3G/3F antiviral activity by competing to the same DNA binding sites. Fluorescence titrations on the DNA analogs of the RNA high-affinity Vif binding sites (dTAR2c and dB2) revealed that Vif bind DNA and RNA sequences with the same affinity (Table 4), indicating that the presence of the 2′-hydroxyl group and the conformation of the ribose (C2′- or C3′-endo) are not determinants for Vif binding. Surprisingly, Vif also showed high-affinity for the DNA complementary sequences dTAR2c and dB2c (Table 4). The high-affinity of Vif for the upper part of TAR and for its complementary DNA sequence may explain the positive effect of Vif on the transfer of the (-) strand strong-stop DNA to the 3′-end of the viral genome, which is an essential step in HIV-1 reverse transcription.4

The similar affinity values obtained for the DNA oligonucleotides dTAR2c and dB2 and their complementary sequences dTAR2c and dB2c raised the question of Vif binding specificity. We therefore studied Vif binding to a number of additional DNA oligonucleotides (Table 4). Vif affinity for most of these oligonucleotides was 3–4-fold lower than the values obtained for the dTAR2c, dB2, dTAR2c, and dB2c, indicating that Vif does indeed display some specificity for some DNA sequences. Interestingly, even though all RNA and DNA high-affinity Vif binding site have a looped or single-stranded stretch, Vif is not intrinsically a single-strand binding protein, as indicated by its binding to the Dickerson dodecamer (41) and to dA10−dT10. Vif does not have a strong preference for any homo-oligomeric sequence and its affinity for d(GT)$_n$, is significantly higher than that of NCp for the same sequence (52), even though this motif is considered as a high-affinity NCp binding site. Finally, our results showed that Vif binds tandem repeats of APOBEC3G or APOBEC3F editing sites with significant affinity ($K_d$ = 34–38 nM), but these oligonucleotides were not preferred Vif targets (Table 4). This result is rather surprising as dTAR2c and dB2c, which are high-affinity Vif binding sites, also contain an APOBEC3G editing site. Thus, our results suggest that Vif could protect some, but not all, APOBEC3G/3F editing sites by directly competing for these sites with the editing enzymes.

In conclusion, Vif binding to RNA and DNA may offer several non exclusive ways to counteract APOBEC3G/3F factors, in addition to their well described Vif-induced degradation by the proteasome (12, 13, 53) and to the Vif-mediated repression of translation of these antiviral factors (14).

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26368 JOURNAL OF BIOLOGICAL CHEMISTRY

Vif-Nucleic Acid Interactions

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